X-LINKED FOXP3 & OTC IN IMMUNE TOLERANCE AND AUTOIMMUNITY

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ABSTRACT

Self and non-self discrimination is one of the fundamental tenets of immunology. The main focus of this thesis is to study molecular and cellular mechanisms of immune tolerance and how its failure leads to autoimmune disease.

Antigen expression in the thymus leads to the deletion of self-reactive T cells and generation of regulatory lymphocytes, including regulatory T cells (Treg) and NKT cells. Our data presented in Chapter 5 show an Ornithine transcarbamylase (OTC) mutation causes ineffective presentation of self antigens in the thymus. As such, deletion of self reactive T cells is compromised and introthymic production of Treg and NKT cells is reduced in the OTC mutant mice. More importantly the heterozygous mice have increased susceptibility to autoimmune diseases, including the generation of autoantibodies and more severe EAE by MOG immunization.

High affinity interaction with self antigens usually induces activation induced-cell death of reactive T cells. In Chapter 4, we present evidence that the activation induced cell death by high affinity self antigens is enhanced by regulatory T cells. We demonstrate that the absence of Treg causes the accumulation of autoreactive T cells in the periphery. Treg does not directly inhibit the proliferation of autoreactive T cells, but instead induce their programmed cell death. We also demonstrate that Treg mediated
cytotoxicity is specific to the autoreactive T cells, and such specificity is due to the higher sensitivity of autoreactive T cells to apoptosis.

T cells with low affinities against self antigens are unavoidable in the periphery. In the lymphopenic environment, such low affinity interaction can drive T cell homeostatic proliferation. In Chapter 2 and 3, we provide evidence that the FoxP3 gene mutation in Scurfy mice leads to reduced thymopoiesis and subsequently less T cell output from the thymus, which causes defective T cell output from the thymus and lymphopenia in neonatal mice and leads to extensive homeostatic proliferation. The homeostatic proliferation is independent of the loss of Treg and FoxP3 mutation in the T cell lineage. We propose that the combined deficiency in T cell production and development of functional Treg may explain the severe autoimmunity associated with the FoxP3 mutation.
Dedicated to Yan and to my family
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1.1 Self/non-self discrimination

The adaptive immune system can selectively respond to foreign antigens but ignore self antigens. Under most circumstances, foreign antigens are derived from pathogens, and self antigens are those expressed by self tissues and present in the blood/lymphatic system. However, at the molecular level, no difference is found between self antigens and non-self antigens. The differences lie in when and how antigens are presented to T cells. First, self antigens are expressed in the primary lymphoid organs while most non-self antigens are only available in the periphery. Antigen expression in the primary lymphoid organs, such as in the thymus and in the bone marrow, deletes reactive immature lymphocytes. Second, self antigens are constantly available in the secondary lymphoid organs, whereas foreign antigens encounter the immune system only after pathogen invasion. Constant interaction between lymphocytes and antigens leads to clone deletion through activation-induced cell death. Finally, self antigens are presented to lymphocytes in a non-inflammatory environment, whereas non-self antigens are usually associated with the activation of the innate immune system. Activation of the innate immune system is indispensable to the function of the adaptive immune system.
Therefore, lymphocytes with high affinity against self antigens are deleted either in the thymus or in the periphery. The remaining lymphocytes in physiological conditions are not able to be activated by low affinity self antigens and remain quiescent in the body.

In the following section, I will briefly review recent advances about the immune tolerance, with the focus on how the differences between self and non-self antigens are utilized by the immune system to tolerate self constituents in vivo.

1.2 T cells are generated from the thymus

1.2.1 T cells

T lymphocytes or T cells are highly abundant and functionally diverse. Based on the expression of different T cell receptors (TCR), T cells can be categorized to two major subsets, αβ T cells and γδ T cells. αβ T cells are most abundant and the best studied. In this dissertation, unless mentioned, T cells only refer to αβ T cells. αβ T cells can be divided into two major subsets, called T helper cells and T killer cells respectively. T helper cells express co-receptor CD4 and can only be activated by peptides presented by MHC II molecules. Upon activation, helper T cells, or CD4 T cells secret various types of cytokines and upregulate CD40L, thus promote antibody responses and macrophage activities. T killer cells express co-receptor CD8 and only recognize peptides presented by MHC I molecules. The major function of CD8 T cells is to kill abnormal cells infected by virus or intracellular bacteria. T cells therefore are major effector and regulator cells in the immune system.

Despite the importance of T cells in fighting against pathogens, abnormalities in T cell function are the underlying mechanism for various autoimmune diseases. Being activated by self antigens, CD8 T cells can lyse the normal cells, and CD4 T cells can
promote the production of high affinity antibodies against self tissues; both of them can also secrete inflammatory cytokines causing the activation and recruitment of other effector cells. T cells, especially CD4 T cells, have been found both necessary and sufficient to the pathogenesis of many autoimmune diseases. For example, in Experimental Autoimmune Encephalomyelitis (EAE), an animal model of multiple sclerosis, activation of myelin specific T cells is sufficient for demyelization and transfer of CD4 T cells from the diseased animal can induce the disease in a new host.

How T cells become tolerant to self antigens is the main theme of this dissertation. As discussed later, this is achieved by various mechanisms and many of them are also fundamental to understanding how T cells’ function is regulated in vivo.

1.2.2 T cells production and autoimmune diseases

By definition, T lymphocytes are produced in the thymus. T cell development in the thymus can be divided into four stages based on the cell surface expression of CD4 and CD8 molecules. Early T cell progenitors express neither CD4 nor CD8 and are called double negative (DN) thymocytes. With the successful rearrangement of TCR β chain, thymocytes start to express both CD4 and CD8 and are called double positive (DP) thymocytes. In the DP stage, thymocytes rearrange TCR α chain. Thymocytes with rearranged TCR β and TCR α chains are then subject to the positive and negative selections. The surviving T cells normally express either CD4 or CD8 co-receptor and are called either CD4 single positive (SP) or CD8 SP thymocytes.

Surprisingly, a functional thymus is not only essential for production of naïve T cells to establish a diverse T cell pool in the periphery, but also of great importance for T cells’ immune tolerance. Premature involution of the thymus leads to less production of
 naïve T cells and are associated with various autoimmune syndromes. In the human, DiGeorge syndrome can lead to autoimmune diseases (1-5); thymoma is commonly associated with myasthenia gravis (6, 7), while thymic hypoplasia is associated with autoimmune hemolytic anemia and juvenile pemphigoid (8). Defective thymopoiesis and the export of mature T cells have also been reported in both rheumatoid arthritis (9) and multiple sclerosis (10) patients. The diabetes-prone BB rats have severe defects in thymocyte development due to mutations of the \textit{IAN} gene family members (11, 12). These observations are paradoxical as T cells are the major effectors for autoimmune diseases. However, recent studies on T cell homeostatic proliferation may reconcile the paradox. Our data presented in Chapter 2 show that in the Scurfy mice the cellularity of the thymus is greatly reduced, as a result of the diminished proliferation of immature thymocytes. Such thymic hypotrophy is intrinsic to the FoxP3 mutation on the thymic stroma and leads to the defective production of naïve T cells from the thymus. We further show in Chapter 3 that less T cell output from the thymus causes the lymphopenia in the periphery. As discussed later, in the lymphopenia environment, T cells with low affinity against self antigens are activated through homeostatic proliferation, which greatly increases the susceptibility to autoimmune diseases.

1.3 T cell tolerance in the thymus

Foreign antigens are bared access to the thymus. Therefore T cells can only encounter self antigens in the thymus. Additionally, immature T cells in the thymus are highly susceptible to apoptosis induced by TCR signaling. Therefore, the thymus plays vital roles in inducing the T cells tolerance.
1.3.1 Self antigens in the thymus eliminate autoreactive T cells through negative selection

During their development in the thymus, T cell precursors randomly rearrange their TCRs to generate functional T cell receptors (TCR). Failure of successful rearrangement leads to the death of immature thymocytes. Thymocytes with functional TCRs are then subject to the positive selection and negative selection. During the negative selection, T cells with high affinity against antigens presented in the thymus are eliminated (apoptosis) or functionally inactivated (anergy).

Although the concept of negative selection has been proposed for a long time, the molecular and cellular mechanisms have not been illustrated until recently. At least two processes are required for effective deletion of autoreactive T cells in the thymus. First, thymocytes must respond effectively to the TCR stimulation and die through apoptosis. To elicit optimal TCR signaling triggering apoptosis, thymocytes requires the signal from TCR as well as co-stimulatory B7-CD28 interaction(13). Optimal TCR signaling is transduced through adaptor protein ZAP-70; MINK, a protein kinase, is then activated after TCR stimulation and subsequently activates JNK and p38 pathways; at the distal end, Bim, a proapoptotic molecule expressed on the outer membrane of mitochondrial, is then activated to induce the apoptosis. Mutations or blockade of any of those molecules resulted in defective negative selection and autoimmune disease in mice. (13-16). Second, self antigens need to be expressed and presented effectively in the thymus. Although the identity of antigen presenting cells for negative selection is still elusive, the majority of those self antigens are expressed by thymic medulla epithelial cells (MEC). One unique property of MEC is the ability to “ectopically” express various tissue specific antigens.
Such peripheral antigen presentation by MEC is controlled by Aire(17). Loss of Aire function results in the defective negative selection and autoimmune syndromes (18). The proper development of MEC is dependent on rel-B mediated NF-κb signaling. Improper development of MEC caused by defects in NF-κb signaling was found associated with defective negative selection and autoimmune disease(19-22). Our observation in Chapter 5 indicates that the hyperammonemia associated with the OTC mutation impairs the antigen presentation of the MEC by downregulate MHC II expression. As such, OTC mutation causes the inefficient deletion of autoreactive T cells in the thymus and increased burden of autoreactive T cells in the periphery.

1.3.2. Self antigens positively selects regulatory T cells and NKT cells in the thymus

Recently, another way through which thymus purges autoimmunity has been appreciated. Some of the self-reactive T cells can be converted into specialized lineages of T cells that can dominantly repress autoimmunity in the periphery. Regulatory T cells and NKT cells are two lineages that have been extensively studied. Both of them are originated from the thymus, although the exact pathways for their intrathymic development have not been well characterized. However, as detailed later, their self reactivity seems indispensable for their development in the thymus. Our observation in Chapter 5 reveals that hyperammonemia caused by the OTC mutation impairs antigen presentation of the thymic medulla epithelial cells and thymocytes, which leads to diminished production of NKT cells and Treg.

1.4 Regulatory T cells

Even with efficient negative selection, many autoreactive T cells are still generated and exported to the periphery. Under physiological circumstances, however,
autoreactive T cells were kept in check. Various mechanisms have been found ensuring the tolerance, and regulatory T cells are particularly of importance to purge the autoreactivity in physiological conditions.

The most extensive studied Treg is the natural Treg, which is developed in the thymus, and expresses the IL-2 receptor CD25 and transcription factor FoxP3. Treg are enriched for T cell clones with autoreactivities(23). In Chapter 4, we provide evidence that regulatory T cells are enriched in the T cells against endogenous superantigen reactive T cells; in Chapter 5, we show that in the OTC mutant mice, the production of Treg in the thymus is reduced, which is correlated with the impaired antigen presentation due to hyperammonemia.

Although the significance of Treg in controlling autoimmunity has been well established(24), how they suppress autoreactive T cells \textit{in vivo} has not been fully understood. Several studies have suggested that Treg secrete inhibitory cytokines such as IL-10 and TGF $\beta$(25). A critical role of these cytokines can be demonstrated in a number of in vivo models (26-28), but not others (29), however, in vitro studies have failed to substantiate their essential role in Treg-mediated suppression (25). On the other hand, while Treg have been shown to repress proliferation of naïve T cells and the production of cytokines \textit{in vitro}, their roles in controlling T cell proliferation has not been well established \textit{in vivo}. More recently, it was demonstrated that Treg are highly cytotoxic to activated T cells \textit{in vitro} (30, 31). This result raised the intriguing possibility that Treg may kill their target cells in an \textit{in vivo} setting, although no specificity has been reported. Our data presented in Chapter 4 demonstrate that Treg is undispensable to induce the activation induced cell death of endogenous superantigen reactive T cells. Adoptive
transfer experiments reveal that the cytotoxicity of Treg is highly specific to autoreactive T cells, and such specificities is mainly due to the increased susceptibility of autoreactive T cells to apoptosis.

1.5 NKT cells, another type of regulatory lymphocytes

NKT cells recognize a different class of antigens, glycolipids. NKT cells have a very limited diversity compared to conventional T cells, with most NKT cells expressing Vα14/Jα18 paired with Vβ8 in mouse, and Vα24/Jα18 paired with Vβ11 in human(32-34). Meanwhile, NKT cells express typical NK lineage markers, such as NK1.1 and members of the Ly49 family (32-36). Much like NK cells, effector function of NKT cells can be triggered without a long priming phase. After stimulation, NKT cells can quickly produce substantial amount of cytokines, such as IL-4 and IFNγ. Such quick responsiveness qualities NKT cells to be among the first responders during infections, functioning as innate immune cells.

Similar to conventional T cells, NKT cells are positively selected by the endogenous antigens in the thymus. In the thymus, self glycolipids presented by CD1d in the DP thymocytes instruct the maturation of NKT cells. Before being presented on the cell surface, glycolipids are processed in the endosome/lysosomes. Although the exact pathway has not been illustrated, several key components of this pathway have been identified. Saposins, an endosome lipid transferase, extracts monomeric lipids from membranes and from CD1, thereby promoting the loading as well as the editing of lipids on CD1(37, 38). β-hexosaminidase b, a glycosphingolipid degrading enzyme, is required for the generation of endogenous NKT ligand, iGB3 in the lysosome(39). Cathepsin L, a protease in the endosome/lysosome, is required for the presentation of glycolipids,
although the exact function is unclear(40). Absence of any one of those molecules leads to the absence of NKT cells \textit{in vivo}, and failure of thymocytes to stimulate autoreactive NKT cells \textit{in vitro}(37-39).

NKT cells also play important roles in the immune tolerance(41). The absence of NKT cells enhances the autoimmunity in several models; whereas the enrichment of NKT cells alleviates autoimmune manifestations. Thus, NKT cells are reduced in the NOD mice(42), and the transfer of NKT cells from healthy mice protect NOD mice from type I diabetes; Similarly, stimulation of NKT cells were found able to prevent EAE induced by MOG immunization(43).

Ammonia has long been found to inhibit the functions of proteases in the lysosome and causes the defects of antigen presentation \textit{in vitro}. In Chapter 5, we demonstrate that hyperammonemia caused by a mutation of ornithine transcarbamylase (OTC) gene results in decreased production of NKT cells in the thymus. Such reduction is also associated with the reduced capacity of thymocytes to stimulate NKT hybridoma \textit{in vitro}. Therefore, OTC mutant mice provide the genetic evidence that ammonia impaires the presentation of glycolipids through CD1d \textit{in vivo} and hyperammonemia is causatively correlated with defective NKT cells production.

1.6 Homeostatic proliferation activates T cells with low reactivity against self antigens

In the peripheral lymphoid organs, the overall size of the lymphocytes is tightly regulated. When the number of T cells is reduced, remaining T cells carry out the homeostatic proliferation in an attempt to restore the T cell compartment(44). Experimentally, the homeostatic proliferation can be clearly manifested by the dividing
of transferred naïve T cells in the immune deficient host, or the expansion of residual T cells after the elimination of a large number of T cells. How T cells “sense” the abundance of T cells is unclear(44), although several molecules including CD24 and IL-7 have been found critical to the optimal homeostatic proliferation.

Low degree autoreactivity of T cells is unavoidable and is required for their survival and function in the periphery(45). In immune replete host, such autoreactivity is constrained presumably due to the competition among T cells to limited ligands. However, in immune deficient host, loss of competition may induce extensive T cell homeostatic proliferation. Several pieces of evidence suggest that such homeostatic proliferation is driven by low affinity interaction to self antigens(46). For CD4 cells, T cells selected in wild type mice failed to undergo homeostatic proliferation in H2-M deficient recipients(47); for CD8 cells, low affinity peptide but not high affinity peptide restored the homeostatic proliferation in TAP deficient host(48).

Compared to the antigen-driven proliferation, the homeostatic proliferation has several unique attributes. During the homeostatic proliferation, T cells are stimulated by low affinity self antigens rather than their high affinity cognate antigens (47). As such, homeostatic proliferation primes naïve T cells of a broad specificity, possibly including the otherwise quiescent autoreactive T cells. A recent study suggests that T cells activated through homeostatic proliferation are long-lived and resistant to activation-induced cell death (49). Additionally, the homeostatic proliferation does not rely on the co-stimulation molecules, such as B7 and CD28. Thus, the homeostatic proliferation, combined with other defects on tolerance, may provoke autoimmune disease in immune deficient host. Indeed, a recent study has demonstrated in NOD mice, a mouse model of
human type I diabetes (50, 51), the homeostatic proliferation is correlated with the development of type I diabetes.

Homeostatic proliferation can be induced by either environmental factors such as chemotherapy (52), viral infection and irradiation (48, 53, 54). Theoretically, by defective production or survival of T cells may have the same effect. Our observation in Chapter 3 demonstrates that in the Scurfy mice, the reduced thymic output is able to cause lymphopenia and extensive homeostatic proliferation. Such homeostatic proliferation, combined with the lack of regulatory T cells, leads to the deadly autoimmune disease in the Scurfy mice. Therefore, the Scurfy mice are the first genetic model which links the thymic atrophy and autoimmunity.

1.7 Genetic models for the immune tolerance

Autoimmune disease is the result of multiple factors, both genetic and environmental. Polygenetic effects are difficult to establish in an outbred population, therefore, mouse models with a single gene deficiency are valuable in illustrating the mechanisms of immune tolerance.

In this thesis, two animal models with single gene mutation are used. The first is the Scurfy mouse with a natural mutation of FoxP3 gene. The Scurfy mutation occurred spontaneously at the Oak Ridge National Laboratory in the late 1940’s. Only the male was found to be affected and the mutant male mice usually died within one month after birth due to the severe autoimmune disease characterized by lymphadenopathy, splenomegaly, and massive lymphocyte infiltrations in various organs (55). Early studies on the Scurfy mice showed that mutant CD4⁺CD8⁻ T cells were sufficient to transfer the disease to a syngeneic immune deficient host, indicating that CD4 helper T cells are the
primary cause of the deadly autoimmune disease in Scurfy mice (56). Subsequent molecular genetic studies revealed a frame shift mutation in the 8th exon of the FoxP3 gene in the Scurfy mice as the underlying genetic cause of the disease (57). Analogous mutations of human FoxP3 were found to be responsible for Immunodysregulation, polyendopathy, enteropathy, X-linked diseases (IPEX) (58-61). In both mouse and human, the FoxP3 mutation is responsible for the most aggressive autoimmune diseases result in early lethality.

One of the first established functions of FoxP3 is T cell-intrinsic. FoxP3 is found to be predominantly expressed in the CD4⁺CD25⁺ regulatory T cells (Treg), and ectopic expression of FoxP3 in CD4⁺CD25⁻ T cells is sufficient to convert them into Treg with strong suppressor activity (62-64). More importantly, targeted mutation of FoxP3 in hematopoietic cells is both necessary and sufficient to ablate Treg development (64). Thus, FoxP3 is now regarded as a master regulator for the lineage differentiation and function of Treg.

However, several lines of evidence also suggest that defective Treg development alone may be insufficient to initiate such severe autoimmune diseases as those observed in Scurfy mice and IPEX patients. First, transgenic expression of wild type FoxP3 under the lck promoter did not rescue Scurfy mice from autoimmunity, although it is unclear whether modest elevation of the FoxP3 gene in the spleen of one founder line can fully restore Treg function (65). Second, irradiation chimeras using bone marrow from Scurfy mice to reconstitute irradiated SCID mice did not lead to the development of autoimmune disease (66).
Our results presented in Chapter 2 and 3 demonstrated a T cell extrinsic function of FoxP3 gene. We found that FoxP3 gene functions in the thymic stromal to maintain the normal thymopoiesis. Therefore, mutation of FoxP3 in the Scurfy mice also leads to reduced thymic output and less T cells in the neonatal mice. Such reduced T cell compartment in the Scurfy mice causes the increased homeostatic proliferation, which is independent on the regulatory T cell defects. The importance of the T cell extrinsic function of FoxP3 is supported by the facts that depletion of FoxP3 in T cell lineage by Lck-cre does not cause the deadly autoimmune disease and Scurfy bone marrow failed to transfer the severe autoimmune disease to Rag deficient hosts.

Another model used in this thesis is the $OTC^{spf}$ mutant mice. $OTC^{spf}$ mutation was first established by irradiation induced mutagenesis. We obtained $OTC^{spf}$ mutant mice in Balb/C background during the breeding of $FoxP3^{sf/+} OTC^{spf/+}$ Balb/c mice. As reported in other background, the hermizygous males exhibit small size, paucity of hair, wrinkled skin and hyperammonemia. OTC gene is an enzyme exclusively expressed in the liver participating the urea cycle. The function of OTC gene on immune tolerance has not been characterized. Our data presented in Chapter 5 show that the OTC mutation causes the diminished MHC II expression on the thymic epithelial cells, and inefficient deletion of autoreactive T cells in the thymus; meanwhile, the production of NKT cells is reduced due to the failure of thymocytes from $OTC^{spf}$ mice to present endogenous glycolipids. The impact of OTC mutation on immune tolerance was extensively analyzed in the $OTC^{spf/+}$ mice. $OTC^{spf/+}$ mice develop more severe EAE after MOG immunization and old $OTC^{spf/+}$ mice have higher amount of autoantibodies in the serum.
CHAPTER 2
THE SCURFY MUTATION OF FOXP3 IN THE THYMUS STROMA LEADS TO DEFECTIVE THYMOPOIESIS AND OVER-EXPRESSION OF THE ERBB2 GENE

2.1 Abstract

The Scurfy mutation of the FoxP3 gene (FoxP3sf) in the mouse and analogous mutations in human result in lethal autoimmunity. The mutation of FoxP3 in the hematopoietic cells impairs the development of regulatory T cells (Treg). In addition, development of the Scurfy disease may also require mutation of the gene in non-hematopoietic cells. The T cell extrinsic function of FoxP3 has not been characterized. Here we show that the FoxP3sf mutation leads to defective thymopoiesis, which is caused by inactivation of FoxP3 in the thymic stromal cells. FoxP3 mutation also results in over-expression of ErbB2 in the thymic stroma, which may be involved in defective thymopoiesis. Our data reveal a novel T cell-extrinsic function of FoxP3. In combination, the T cell-intrinsic and extrinsic defects provide plausible explanation for the severity of the autoimmune diseases in the Scurfy mice and IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) patients.

2.2 Introduction

A long-standing but poorly understood paradox in immunology is the link between defective T-cell production in the thymus and T-cell dependent autoimmune
diseases. Thus, in the human, DiGeorge syndrome can lead to autoimmune diseases (1-5); thymoma is commonly associated with myasthenia gravis (6, 7), while thymic hypoplasia is associated with autoimmune hemolytic anemia and juvenile pemphigoid (8). The diabetes-prone BB rats have severe defects in thymocyte development due to mutations of the IAN gene family members (11, 12).

Mutations of the FoxP3 gene are responsible for the spontaneous autoimmune diseases observed in both IPEX patients and scurfy mice (57-59, 61). Elegant studies have revealed that FoxP3 gene expression in CD4 lymphocytes is essential for the development and function of CD4+CD25+ regulatory T cells (62-64). However, several lines of evidence also suggest that defective Treg development alone may be insufficient to initiate such severe autoimmune diseases as those observed in Scurfy mice and IPEX patients. Thus, irradiation chimeras using bone marrow from Scurfy mice demonstrated that the defective FoxP3 expression in the hematopoietic cells did not lead to the development of autoimmune disease(66). Second, transgenic expression of wild type FoxP3 under the lck promoter did not rescue the autoimmunity in Scurfy mice, although it is unclear whether modest elevation of the FoxP3 gene in the spleen of one founder line can fully restore Treg function (65).

T lymphocytes are produced in the thymus through an ordered process. Upon their arrival in the thymus, T cell precursors divide rapidly prior to their expression of the CD4 and CD8 co-receptors on the cell surface (67). Based on the cell surface expression of CD44 and CD25 markers, the development of DN thymocytes can be divided into 4 stages (68). DN1 expresses CD44 but not CD25. With the expression of CD25, the DN1 T cells enter into the DN2 that expresses both CD25 and CD44 and actively divide. The
DN2 cells diminish the expression of CD44 and enter into the DN3 stage. With the re-arrangement of the TCRβ gene the DN3 cells enter into the second and most active wave of division and maturate into DN4 cells, characterized by down-regulation of CD25. The DN4 T cells are the direct precursors of CD4⁺CD8⁺ immature single positive T cells (ISP) (69), which are believed to differentiate into CD4⁺CD8⁺ T cells that express functional TCRαβ genes and undergo TCR ligand-based positive and negative selection (70). Only the T cells that succeed in both selections are allowed to mature and populate the peripheral lymphoid organs. Despite the clear delineation of the developmental pathway, the molecular mechanisms by which the thymic epithelial cells control thymopoiesis remains poorly understood.

Here we report that the Scurfy mutation of FoxP3 leads to thymic atrophy, which is mainly the result of the diminished DN thymocyte proliferation. This phenomenon is due to the FoxP3 mutation in the non-hematopoietic lineages. Furthermore, defective thymopoiesis correlates with over-expression of ErbB2 in the thymic stroma, an event that is known to cause the thymic atrophy (71). FoxP3 represses expression of the ErbB2 gene in a thymic epithelial cell line. These results reveal a novel T cell-extrinsic role of FoxP3 in stromal cells for the development of T cells.

2.3 Materials and Methods

Animals

Thy1.1 BALB/c mice with mutation of FoxP3 (FoxP3sf) were produced after more than 12 generations of backcross in the University of North Carolina and maintained under specific pathogen-free conditions at the University Laboratory Animal Resources
at the Ohio State University for the duration of the study. Male Rag-2/- FoxP3sf mice were generated by breeding female FoxP3sf/+ heterozygous BALB/c mice with male Rag-2/- mice. The genotype of the FoxP3 gene was determined by allele specific PCR (72). Primers specific for mutant FoxP3 gene include forward primer (5’-TCAGGCCTCAATGGACAAAAG-3’), reverse primer (5’-AACTATTTGCCATGGCTTCC-3’) and complementary depository oligonucleotides (5’-CTTGTCCATTGAGGCTGAG-3’), and the primers specific for the wild-type FoxP3 gene were forward primer (5’-CTCAGGCCTCAATGGACAAAG-3’), reverse (5’-AACTATTTGCCATGGCTTCC-3’) and complementary depository oligonucleotides (5’-CTTTTGRCCATTGAGGC-3’). The complementary depository oligonucleotides were used to improve reliability of allele-specific PCR (73). Male FoxP3sf or FoxP3sfOtcsf mice and normal gender-matched littermates were used in some experiments.

**Thymocyte apoptosis**

Cell viability of thymocyte subsets was determined by flow cytometry with PE-conjugated anti-mouse CD4 and allophycocyanin (APC)-conjugated anti-mouse CD8 mAbs, and then fluorescein isothiocyanate (FITC)-conjugated Annexin V. Apoptotic cells were identified as Annexin V+.

**BrdU incorporation and measurement**

Mice were injected intraperitoneally (i.p.) with BrdU (1 mg/mouse in 100 μl PBS) 3 hrs before sacrifice except where indicated. The mice were sacrificed and single thymocytes and spleen cells were prepared. BrdU incorporation was detected by flow cytometry with a BrdU Flow Kit, as described by manufacturer (BD Pharmingen). Briefly, thymocytes or spleen cells were stained with various surface markers. Then the
cells were fixed, treated with DNase (Sigma) and stained with FITC-conjugated or APC-conjugated anti-BrdU mAb (clone 3D4) or FITC-conjugated mouse IgG1.

**Bone Marrow Chimera**

For the bone marrow reconstitution, 6 -8 week old Rag2−/− BALB/c mice received 500 rads of γ-irradiation one day before transplantation. Bone marrow cells from either FoxP3sf or the wild type littermates were purified and T cells were depleted using anti-CD4 (GK1.5) and anti-CD8 (2.4.3) antibodies followed by DYNAL Beads (DYNAL Biotech, NY) as described by manufacturer. The CD3+ cells within the bone marrow after the deletion were always less than 2%. Then 8x10⁶ bone marrow cells were transferred into the recipients by i.v. injection. At 10 weeks after the reconstitution, mice were sacrificed and lymphocytes were analyzed as indicated.

For the bone marrow reconstitution of FoxP3sf or FoxP3wt Rag-2−/− mice, all the mice were given 500 rads irradiation one day before reconstitution and received 5x10⁶ T-depleted bone marrow cells from BALB/c GFP transgenic mice. 6 weeks after the reconstitution, mice were sacrificed and their thymocytes and splenocytes were analyzed as indicated.

**Real-Time PCR**

Real-time PCR was done using the QuantiTect SYBR green PCR kit (Qiagen, CA) in ABI PRISM 7700 cycler (Applied Biosystems, CA) according to manufacturers’ protocols. Briefly, 1 g total RNA was pretreated with RNase-free DNase I (Invitrogen, CA) to eliminate contaminating genomic DNA and then was reverse transcribed using Superscriptase II (Invitrogen, CA) and oligo(dT) in a 20 l reaction. 1 l cDNA was
used in each 25 μl PCR reaction and all samples were run in triplicate. All PCR products were analyzed by annealing curves as well as 2% agarose gel and contained only one amplicon with the correct size. The primers were as follows: FoxP3, 5’-GGC CCT TCT CCA GGA CAG-3’ and 5’-GCT GAT CAT GGC TGG GTT GT-3’; ErbB2, 5’-TGA GAA ATG CAG CAA GCC CT-3’ and 5’-AAT GCC AGG CTC CCA AAG AT-3’; HPRT: 5’-AGC CTA AGA TGA GCG CAA GT-3’ and 5’-AAT GGC GCG CAA CT-3’ and 5’-TA AAG TTA CTA GCC AGA TGG CCA CA-3’; β-Actin 5’-GAT CTG GCA CCA CAC CTT CT-3’ and 5’-GGG GTG TTG AAG GTC TCA AA-3’.

**Purification of thymic epithelial cells**

Thymii from 4-8 weeks old BALB/C mice were cut into small pieces and stirred for 30 mins at 4°C to reduce thymocytes. The fragments left were then digested by 1 mg/ml Collagenase/Dispase (Roche, IN) + 1 mg/ml Dnase I (Sigma, St. Louis, MO) successively for three 15-minute incubations. Single-cell suspensions went through the Percoll gradient (1.07, 1.045,1.03,1.0), and the upper two layers of cells were collected. Those cells were then stained with CD45.2 APC and G8.8 (BD Biosciences) followed by FITC conjugated anti-Rat Ig G2a (BD Biosciences). Two rounds of FACS sorting were used to purify CD45+G8.8− thymocytes and CD45−G8.8+ epithelial cells.

**Transfection**

Mouse thymic epithelial cell line 6.1.7 was transfected by the pcDNA3-FoxP3 or the vector alone using Fugene 6 (Roche, IN). 24 hours after transfection, BCD was added in to deplete the untransfected cells. 72 hours after the selection, cells were harvested and expression of Her-2 was detected by real time PCR.
**Luciferase reporter assay**

$1 \times 10^4$ cells/well of thymic epithelial cells were seeded in 24-well-plates. 0.2µg of luciferase construct that contained a 500 bp ErbB2 promoter and 0.02 µg of pRL-TK (Promega) were transiently co-transfected with 0.2µg, 0.5µg, and 1µg of FoxP3 expression plasmid or the pcDNA3 vector respectively. After incubation for 48 hours, the cells were harvested with Passive Lysis Buffer (Promega), and luciferase activities of cell extracts were measured with the use of Dual luciferase assay system (Promega).

**Statistical analysis**

Unless otherwise noted, most data were analyzed with two-tailed student T-test. *, $P < 0.05$, significant; and **, $P < 0.01$, highly significant.

**2.4 Results**

**2.4.1 Defective thymopoiesis rather than accelerated death of thymocytes leads to reduced thymic cellularity of FoxP3sf mice**

The hemizygous FoxP3sf male mice usually died between 18 – 25 days of age (data not shown). We examined the mice at 1- or 2-weeks of age and found that thymii of the mutant mice were atrophic. The number of thymocytes in the FoxP3sf mice was reduced by 2 fold on day 7 and by about 3 fold on day 15 (Fig. 1a). On day 7, the major thymocyte subsets were present, although a small reduction of CD4+CD8+ (DP) thymocytes was found. Corresponding to this, the percentages of CD4+CD8-, CD4-CD8+ and CD4-CD8- populations were increased somewhat when compared to normal littermates (Fig. 1b). Since the thymic cellularity was reduced by about 3-fold, there was no increase in the number of mature T cells in the thymus.
Both proliferation and apoptosis shape thymic cellularity (74). To explore the possible contributions of apoptosis, flow cytometric analysis of Annexin V+ cells was performed on ex vivo thymocytes isolated from mice on day 7 and day 15 after birth (Supplemental Fig. S1). The percentage of Annexin V+ thymocytes in scurfy mice was comparable to that in WT littermates. These data indicate that thymic atrophy in the FoxP3sf mice is unlikely due to increased apoptosis of the thymocytes.

We then examined the proliferation of thymocytes in FoxP3sf mice. Scurfy mice and their littermates were injected with nucleotide analog bromodeoxyuridine (BrdU) at 3 hours before sacrifice and BrdU incorporation was measured by flow cytometry after surface staining for CD4 and CD8 expression and intracellular staining for BrdU incorporation. As shown in Fig. 1c &d, the percentage of BrdU+ thymocytes was significantly lower in FoxP3sf mice than that in normal littermates. Analysis of thymocyte subsets revealed that BrdU incorporation was significantly reduced in CD4+CD8+ DP (day 15 only), CD4+CD8- DN, and CD4+CD8+ SP thymocytes (Fig. 1d, e). Consistent with the previous report, the majority of the BrdU labeled CD8 SP T cells in WT mice had no or lower levels of cell surface CD3/TCR (69). The division of this immature subset was significantly repressed in the FoxP3sf mice. In contrast, only a minor reduction was observed among the more mature SP cells with high levels of TCR, regardless of whether they were CD4+SP or CD8+SP (Fig. 1d, e). Thus, the FoxP3sf mice have significant defects in the proliferation of immature T cells.

DN thymocytes differentiate sequentially through the DN1 (CD25-CD44+), DN2 (CD25-CD44+), DN3 (CD25-CD44+), and DN4 (CD25-CD44+) subsets, and the proliferation of DN thymocytes is most prominent in the DN2 and DN4 stages. The most
significant reduction of BrdU incorporation was observed in the DN4 stage, though DN2 thymocytes in the FoxP3sf mice also had less proliferation (Fig. 1f, g). Proliferation of the DN1 and DN3 thymocytes was not significantly reduced. Moreover, compared to WT littermates, the distribution of DN subsets was also greatly altered in the mutant mice. In the FoxP3sf mice, the DN1 population was expanded, while the DN4 population was significantly reduced (Fig. 1h). In addition, consistent with the reduced proliferation, the size of the DN4 thymocytes of the FoxP3sf mice was significantly smaller than those in the WT mice (Fig. 1i).

Taken together, our results revealed that defective proliferation of immature thymocytes is responsible for thymic atrophy in the FoxP3sf mice.

2.4.2 Reduced thymopoiesis in the FoxP3sf mice is independent of T cell activation in the periphery and is extrinsic to bone marrow-derived cells

Since the FoxP3sf mice have ongoing T cell activation in the periphery, it is possible that such activation can indirectly affect thymopoiesis. To address this issue, we produced Rag-2-/-FoxP3sf mice. Rag-2-/-FoxP3wt mice expressed low but detectable levels of FoxP3 mRNA, and perhaps due to RNA instability associated with the frame-shift mutation, the mutant FoxP3sf mice have further reduced levels FoxP3 mRNA (Fig. 2a, b). Since Rag-deficiency arrests thymocyte development at the DN3 stage, one can study the effect of the mutations on thymocyte development up to the DN3 stage in the absence of peripheral T cell activation. The thymocyte subsets (Fig. 2c, d) and BrdU incorporation (Fig. 2e, f) were significantly altered in the Rag-2-/-FoxP3sf mice in comparison to the Rag-2-/- mice. The alterations in the DN1-3 subsets were similar to what were found in Rag-2+/+FoxP3sf mice, particularly the expansion of DN1
thymocytes. Thus, the changes in the DN1-3 stages and diminished proliferation of DN thymocytes were independent of T cell activation in the periphery.

To study the impact of the FoxP3 mutation in the stroma cells on the development of DN thymocytes, we transferred bone marrow from BALB/c transgenic mice that express GFP into sub-lethally irradiated Rag-2\(^{-/-}\) or Rag-2\(^{-/-}\)FoxP3\(^{sf}\) mice. Six weeks after the transfer, we analyzed the proliferation and the development of GFP\(^+\) thymocytes. As demonstrated in Fig 3a, the CD4 and CD8-expressing thymocytes that developed with FoxP3\(^{sf}\) stroma were normal compared to with FoxP3\(^{wt}\) stroma. However, analysis of DN thymocytes by CD25 and CD44 markers revealed significant increases in DN2 and DN3 and a significant reduction of the DN4 (Fig. 3b, c). In addition, proliferation of DN thymocytes was also reduced in the mutant recipients. In two independent experiments, the BrdU incorporation was lower in the Rag-2\(^{-/-}\)FoxP3\(^{sf}\) mice as compared to their Rag-2\(^{-/-}\)FoxP3\(^{wt}\) littermates. Although the baseline proliferation was different in the two experiments, perhaps relating to the age of recipients and the time of analysis, the reduction was statistically significant (P=0.019) in a pair-wise comparison between WT and mutant siblings (Fig. 3d, e). Furthermore, in the mutant recipient, DN2 and DN4 thymocytes also had reduced blasting cells compared to the wild-type recipient (Fig. 3f). These results demonstrate that the mutation of FoxP3 in the thymic stroma cells is sufficient to cause defective DN development.

To test whether FoxP3 mutation in the bone marrow cells also contributes to defective thymopoiesis, we transferred T cell-depleted bone marrow from either Thy1.1\(^+\) WT or Thy1.1 FoxP3\(^{sf}\) BALB/c mice into separate Rag-2\(^{-/-}\) recipients, which received 500 Rads of \^-irradiation before reconstitution. At 10 weeks after reconstitution, the
chimera mice were sacrificed and analyzed. At this point, more than 95% of thymocytes and spleen cells were of donor origin (data not shown). The number of thymocytes in the FoxP3sf bone-marrow reconstituted mice was comparable to that in the mice receiving wild-type bone marrow (data not shown). The distribution of thymocyte subsets as revealed by CD4 and CD8 markers was also unaffected. More importantly, no appreciable effect was observed in the DN1-DN4 subsets and BrdU incorporation. Thus, both proliferation and the development of DN thymocytes were unaffected (Fig. 4a). Therefore, defective FoxP3 expression in hematopoietic lineages is not sufficient to cause defective thymopoiesis. In the periphery, an increased number of T cells was found in the spleen and lymph nodes of mice reconstituted with mutant bone marrow (data not shown). However, the activation status and rate of proliferation were comparable between the two types of chimeras (Fig. 4b).

In consistent with previous observations by one of us (VG) (66), all of the chimera consisting of mutant bone marrow and irradiated WT host survived significantly longer than the mutant scurfy mice (Fig. 5). When sacrificed for analysis at 8-10 weeks, the healthy mice show increased cellularity in the lymph nodes (data not shown), although we did not observed increased activation markers on the T cells in these chimeras (See Fig. 4b). In the C57BL/6 background, all 8 chimera survived the entire period of observation (Fig. 5a). In the BALB/c background, except those that were sacrificed for analysis, recipients of FoxP3sf bone marrow succumbed between 8-20 weeks (Fig. 5b). The difference in survival between these two strains is unclear at this point. Analysis of moribund mice, however, revealed extremely small thymii and reduced cellularity in the peripheral lymphoid organs. Thus, these moribund mice did not
have typical pathological changes observed in the Scurfy mice, i.e., an increased lymph proliferation. The T cells in the moribund mice were, however, highly activated. Since bone marrow did contain a small number of T cells, it is unclear whether the pathogenic T cells were generated in the Scurfy donor or in the WT recipients. Since the delay and/or ablation of diseases cannot be accounted for by the 3-4 weeks required for the generation of T cells in chimera mice to the level of new borne mice, it appears that the Scurfy mutation of FoxP3 in the hematopoietic cells did not result in the full-spectrum of polyclonal T cell activation and pathogenicity typical of Scurfy mice (75).

Taken together, these data demonstrate that the FoxP3 defects in non-hematopoietic tissues are both necessary and sufficient for reduced thymopoiesis. In contrast, the defects in bone marrow-derived cells are neither necessary nor sufficient for the thymopoietic defects described here in. The significant delay in onset and incidence of autoimmune diseases in the chimera mice are consistent with the notion that mutation of FoxP3 in the non-T host cells may be essential for the development of autoimmune diseases. At least, the T-cell extrinsic defects help to exacerbate autoimmune diseases in the Scurfy mice.

2.4.3 Expression of the FoxP3 gene in the thymic epithelial cells

We have shown that a defective FoxP3 gene in non-hematopoietic thymic cells mediated thymopoiesis defects. This suggests that FoxP3 must be expressed in some types of thymic stromal cells. We took two approaches to detect FoxP3 expression in the thymic stromal cells. First, we separated thymocytes into CD45+ and CD45− compartments by MACS beads. The thymic epithelial cells were isolated from the CD45− compartment based on their binding to monoclonal antibody G8.8 (76) (Fig. 6a). After
two rounds of FACS sorting of the CD45⁻G8.8⁺ epithelial cells to near 100% purity, expression of FoxP3 in the CD45⁺ thymocytes and the CD45⁻G8.8⁺ thymic epithelial cells was quantitated by real-time PCR. As shown in Fig. 6b, the highly purified CD45⁻G8.8⁺ cells have about 2-20 fold more FoxP3 mRNA compared to that in CD45⁺ cells depending on the house-keeping genes used. The FoxP3 mRNA was not due to T cell contamination, as the expression of CD3 chain was barely detectable in the epithelial population.

To locate the expression pattern of FoxP3 protein in various types of cells in the thymus, we produced affinity-purified rabbit anti-FoxP3 antibodies (Supplemental information Fig. S3). To determine whether FoxP3 was present in the thymic epithelial cells, we co-stained FoxP3 with cortical epithelial cell marker K8 and/or medullar epithelial K5. A small but significant number of epithelial cells in the cortex (Fig. 6c), but not in the medulla expressed FoxP3 protein. The specificity of the staining is confirmed, as the thymus from the FoxP3sf mice was negative for FoxP3 protein. In contrast, no K5⁺FoxP3⁺ cells can be found in the medulla (data not shown). The immunohistochemical data were corroborated by flow cytometry using an monoclonal anti-FoxP3 antibody (Fig. 6d). Interestingly, mouse thymic epithelial cells also expressed significant levels of CD4 mRNA (Fig. 6b) and protein (Fig. 6d). Thus, in addition to its expression in the lymphocyte lineages as others have reported, FoxP3 was also detected in CD45⁻ stromal cells, with high levels found among a small number of cortical epithelial cells. This expression pattern is consistent with the effect of FoxP3 mutation of development of immature thymocytes that reside primarily in the cortex.
2.4.4 *FoxP3* maintains thymopoiesis by suppressing expression of *ErbB2*

Abnormal expressions of several molecules have been found to affect thymopoiesis, including over-expression of *ErbB2* (71) and targeted mutation of *IL-7* (77). As shown in Fig. 7a, no significant difference in *IL-7* expression was observed in both 7-day old and 15-day old *FoxP3* sf thymii when compared to thymii from wild type littermates. In contrast, expression of *ErbB2* mRNA was significantly elevated in thymii of the *FoxP3* sf mice, as revealed by real-time PCR (Fig. 7b). To determine whether *FoxP3* can suppress the expression of the *ErbB2* gene in the thymic epithelial cells, we transfected the thymic epithelial cell lines 6.1.7 with *FoxP3* cDNA and analyzed *ErbB2* expression 3 days after transfection. We found that the *ErbB2* expression level in the 6.1.7 cells was reduced by 5-fold with the ectopic expression of *FoxP3* (Fig. 7c). Thus the *FoxP3* expression in the thymus epithelial cell line directly suppresses expression of the *ErbB2* gene. To determine whether *FoxP3* represses *ErbB2* promoter activity, we cloned the 500 bp *ErbB2* promoter (78-80) into a pGL-2 luciferase reporter vector and transiently transfected the reporter in conjunction with *FoxP3* or vector control into 6.1.7. As shown in Figure 7d, *FoxP3* cDNA repressed the *ErbB2* promoter activity by 2-5 fold in a dose-dependent manner.

2.5 Discussion

*FoxP3* is known as a major regulator for the development and function of CD25+/CD4+ Treg (62-64). This function requires expression of *FoxP3* in the T cell lineages. Although one of us (VG) reported that the *Scurfy* mutation in the non-T cells appears necessary for the pathogenesis of the disease in the mice (66), the immunological basis for a T-cell extrinsic function of *FoxP3* has not been identified. We show here that
FoxP3 mutation resulted in significantly reduced thymic cellularity. This reduction was due to defective proliferation of immature T cells, which was caused by FoxP3 defects in the thymic stroma, but not in the T cells. Our systematic analysis of the proliferation of thymocyte subsets revealed that FoxP3 mutation primarily affects proliferation of immature T cells, including those of DN2 and DN4, the major stages of thymocyte proliferation, and to a less extent ISP and DP. In addition, increased apoptosis of thymocytes appeared not to contribute to reduced thymic cellularity, at least at early ages (<2 weeks). This is very different from the reduced thymic cellularity associated with peripheral T cell activation, which can cause the nonspecific deletion of DP thymocytes (81). Consistent with these findings, the thymic abnormalities we observed in the Scurfy mice have not been reported in other lymphoproliferative disease models such as CTLA4 KO mice (82) (83, 84). Moreover, analysis of Rag-2<sup>-/-</sup>FoxP3<sup>sf</sup> mice demonstrated that the defects in the DN1-DN3 cannot be due to peripheral T cell activation. In addition, in chimera mice reconstituted with WT bone marrow cells, generation of DN4 and proliferation of DN was compromised in the FoxP3<sup>sf</sup>RAG-2<sup>-/-</sup> host. To our knowledge, this is the first direct demonstration of FoxP3 function in overall T cell development and a direct link between FoxP3 expressed in thymic stromal cells and proliferation of immature T cells. In addition to their diminished proliferation, we also found the subset distribution of the DN thymocytes was significantly affected by FoxP3 mutation. This is characterized by the increased DN1 and reduced DN4 population. Both changes, however, can be explained by defective proliferation at DN2 and DN4.

An important question is whether the defective thymopoiesis in the mutant mice contributes to the pathogenesis of disease in the scurfy mice and IPEX patients.
Theoretically, reduced production of new T cells may stimulate lymphoproliferation characteristic of the Scurfy mice, although additional studies are needed to establish such a link. It remains controversial whether mutation of FoxP3 in the T cell-lineage is both necessary and sufficient to cause lethal autoimmune diseases. Over 10 year ago, one of us (VG) demonstrated that adoptive transfer of bone marrow cells from nude scurfy mice into either SCID mice or irradiated syngeneic mice failed to transfer autoimmune diseases (55, 66, 85). Likewise, we have observed substantial survival irradiation chimera of the Rag-deficient mice reconstituted with FoxP3sf bone marrow. On the other hand, recent work by Fontenot demonstrated that, when CD4-promoter-driven Cre induced deletion of FoxP3 in the FoxP3\textsuperscript{flaxflox} in transgenic mice, they developed full-spectrum of Scurfy disease (42). However, it should be pointed out that although Cre under CD4 promoter has been shown to induce gene deletion in T cell lineage, its impact on the thymic epithelial cells has not been studied. Our data presented here demonstrated that CD4 gene is expressed in mouse thymic epithelial cells.

Finally, although the FoxP3 gene has features of transcription factors, its downstream targets have not been identified. Our data presented here reveals ErbB2 as one of its molecular targets, as the ErbB2 gene is over-expressed in the thymic stromal cells. Conversely, transfection of FoxP3 represses expression of ErbB2 in the thymic epithelial cell lines, at least in part by transcriptional repression. Our results are consistent with two previous studies that support a critical role for ErbB2 in thymopoiesis. First, transgenic expression of ErbB2 under the keratin promoter, which resulted in over-expression of HER-2 in both skin and thymic epithelial cells, leads to thymic atrophy (71). Therefore, over-expression of ErbB2, as a result of FoxP3 mutation, may
contribute to thymic atrophy. Secondly, a recent study revealed that mice with targeted mutation of Stat-3 gene also showed reduced thymopoiesis, increased ErbB2 expression and thymic atrophy (86).

Taken together, our results reveal the first non-T cell intrinsic function of FoxP3, as it serves is a novel checkpoint for thymopoiesis. Combined T-cell intrinsic and extrinsic defects provide a plausible explanation for the severity of autoimmune diseases in the Scurfy mice and IPEX patients.
**Fig.2.1 Abnormal thymopoiesis in the FoxP3sf mice.** (a). Progressive reduction of thymic cellularity in the BALB/c. FoxP3sf mice. The numbers represent the mean cell number±SEM per thymus. The number of mice examined: Day 7-9, FoxP3sf n= 5, wild type n=8; days15-18, FoxP3sf n=10, wild type n=12. (b) Thymocyte subsets based on expression of CD4 and CD8 markers. Thymi were isolated in day 7-9 or day 15-18 after birth and analyzed by flow cytometry. Data shown were representative profiles of day 15 mice. (c). Proliferation of thymocyte subsets. Representative histograms of thymocytes and their subpopulations of mice at day 15 of age are shown. The numbers in the histogram represent the percentages of BrdU+ cells in each subpopulation. Mice at day 7 or 15 of age were injected i.p. with BrdU (1 mg/mouse) 3 hrs before sacrifice. Thymocytes were stained with PE- or PerCP-Cy5-conjugated anti-mouse CD4 and APC-conjugated anti-mouse CD8 mAbs, then intracellularly with FITC-conjugated anti-BrdU antibody, and were analyzed by flow cytometry. (d). Summary of BrdU incorporation data of major thymocyte subsets. Data shown are mean percentages ± SEM. The upper panel shows the data from 7-day old mice (FoxP3sf mice, n=5, WT littermates n=7) and the lower panel depicts the data of 15-day old mice (FoxP3sf mice n =10, normal littermates n=12). (e). As in (d), except that the CD8CD4 thymocytes were further divided into CD3+ and CD3− subsets. (f). BrdU incorporation of DN thymocytes at four stages. DN1: CD44CD25; DN2: CD44CD25; DN3: CD44 CD25; DN4: CD44CD25-. (g). Summary of BrdU+ % among DN1-4. Data represent mean± S.E.M. FoxP3sf mice: n=10; wild type n=12. (h). FACS profiles of DN thymocytes of 15-day old FoxP3sf mice. Thymocytes from FoxP3sf or wild type littermates were stained by CD4, CD8, CD25 and CD44. The DN were further divided based on expression of CD44 and CD25. (i). Cell size of the mutant thymocytes at different DN stages compared to wild-type littermates. Data are representative of 4 independent experiments.
Figure 2.1 (continued)
Figure 2.1
Figure 2.2 Abnormal thymopoiesis in the FoxP3sf Rag2^{-/-} mice. a & b. Expression of the FoxP3 gene in the BALB/c. Rag-2^{-/-} and BALB/c. Rag-2^{-/-}FoxP3sf thymus, as determined by real-time PCR, a typical PCR profile is shown in a, and the summary data including 3 mice per group is shown in b, each samples were analyzed in triplicates. c - e. The impact of FoxP3 mutation on the DN development. Five-week old BALB/c.Rag 2^{-/-} FoxP3sf mice received an injection of BrdU (1mg/mouse) six hours before sacrifice. (c). FACS profiles of the thymocytes from either FoxP3sf Rag2^{-/-} or FoxP3wt Rag2^{-/-} recipients; (d). BrdU incorporation at different stages. Data are representative of 2 independent experiments involving 3 Rag2^{-/-} FoxP3sf mice and 5 Rag 2^{-/-} FoxP3wt mice. e. Summary of BrdU incorporation data from two independent experiments involving 3 mutant and 5 WT mice.
Figure 2.2
Figure 2.3  *FoxP3* defect in thymic stroma cells leads to defective thymopoiesis.

5x10⁶ T cell depleted bone marrow cells from GFP transgenic mice were transferred into 3 or 4 week old *Rag-2⁻/⁻ FoxP3sf* mice or *Rag-2⁻/⁻ FoxP3wt* littermates, which received sublethal irradiation (500 rads) 1 day before transplantation. Five or six weeks after the transplantation, the donor thymocytes were gated based on GFP expression. Thymocyte subsets based on expression of CD4/CD8 (top panels), and DN subsets based on CD25/CD44 markers (lower panels). Representative FACS profile (a) and summary data from two independent experiments (b) are shown. (c and d). BrdU incorporation of DN of donor (GFP⁺) origin. Representative FACS profile (c) and summary data (d) from two independent experiments are presented. In experiment 1 (open symbol), the recipients are 4-week old at the time of reconstitution and were analyzed at 6 weeks after reconstitution; in experiment 2 (solid symbols), the recipients were 3 week-old at the time of reconstitution and were analyzed 5 weeks later. The two-related sample Wilcoxon test was used to examine the statistical significance of the difference between Wt and mutant littermate recipients. (e). Cell size of GFP⁺ DN thymocytes at different stages. All data have been repeated in 2 independent experiments involving 3 *FoxP3sf Rag-2⁻/⁻* and 5 *FoxP3wt Rag-2⁻/⁻* mice.
Figure 2.3
Figure 2.4 Normal thymopoiesis of WT and FoxP3sf bone marrow reconstituted Rag-2⁻/⁻ mice. T-depleted bone marrow cells from either Thy1.1⁺FoxP3sf mice or wild type littermates were transferred into sublethally (500 Rad) irradiated Thy1.2⁺Rag-2⁻/⁻ mice. Ten weeks after the transfer, thymocytes were harvested and their cellularity, proliferation and subset distribution were analyzed. (a). Subsets of total thymocytes (top), DN (middle) and BrdU incorporation of DN thymocytes (bottom) over a 3-hour period. (b). Activation and proliferation of spleen T cells. Top, distribution of CD4 and CD8 T cells subsets. Note an increase in the proportion of T cells when bone marrow is from the FoxP3sf mice. Middle panels, activation status as revealed by CD44 and CD62L. Lower panels, incorporation of BrdU over a 3-hour period. Data shown are profiles of Thy1.1⁺ cells, which account for >95% of both spleen T cells and thymocytes and are from a representative mouse in an experiment involving two mice per group. Essentially identical results were obtained when C57BL/6.FoxP3sf bone marrow cells were used to reconstitute the irradiated C57BL/6.Rag-1⁻/⁻ mice and when BALB/cFoxP3sfOTcspf mice bone marrow was used to reconstitute the BALB/cRag-2⁻/⁻ mice.
Figure 2.4
Figure 2.5 Bone marrow cells from the FoxP3sf mice either fail or have diminished ability to transfer autoimmune diseases to the Rag-deficient syngeneic host. Data shown are the survival of either Scurfy mice or syngeneic Rag-deficient mice that received bone marrow cells from the Scurfy mice in either C57BL/6j (a) or BALB/c (b) background. Kaplan-Meier analyses were used to compare the two groups. P values of the log rank test are presented. One accidental death at 3 week in the BALB/c group was excluded from the analysis. Some mice have not been observed throughout the experiments either because the experiments are ongoing or because they were used to analyze T cell development. These samples were marked as censored (+) in the Kaplan-Meier curves.
Figure 2.6 FoxP3 expression in thymic epithelial cells. Thymii from 4-6 week old wild type mice were digested with collagenase/Dispase to prepare single-cell suspensions. The cells were stained with anti-CD45.2-PE and unlabeled G8.8. The CD45+ cells (99% pure) were first isolated with anti-PE-MACS beads. After extensive depletion of MACS+ cells, we added FITC-conjugated anti-Rat IgG2a to the CD45− population. The CD45− G8.8+ cells were sorted twice using the FACS Vantage. a. FACS profiles that show expression of CD45 and G8.8 of total cell preparation (upper left), purified CD45+ cells (lower left), remaining cells after depletion of CD45+ cells (upper right) and the sorted CD45−G8.8+ epithelial cells (lower right). b. FoxP3 and CD4 expression in the thymic epithelial cells (CD45−G8.8+) compared to the CD45+ population as measured by real time PCR. To normalize the cell numbers, the ratios of FoxP3 or CD4 mRNA and that of house keeping gene Hprt or -actin are presented. Expression of the CD3 chain was used to mark potential T cell contamination. Data shown are means and SEM of triplicates from two independent experiments. c. Immunofluorescence staining of the thymus sections for cortical epithelial marker Cytokeratin 8 (K8, left panels) and for FoxP3 protein (as determined by Rhodamine conjugated goat anti-rabbit IgG, middle panels), merged images are presented in the right panels. The cortex regions are shown. Medulla epithelial cells show no FoxP3 staining (data not shown). Top panels are specificity controls that show an absence of FoxP3 protein in the thymus of FoxP3sf mice. d. Expression of FoxP3 and CD4 as revealed by flow cytometry.
Figure 2.6
Figure 2.7 *FoxP3* maintains thymopoiesis by repressing expression of *ErbB2*. a & b. Real time PCR analyses of *IL-7* (a) or *ErbB2* (b) expression in the wild-type and *FoxP3*~sf~ thymii at different ages. Data shown are the means ± SEM. Day 7, *FoxP3*~sf~ n=5, wild type n=4; Day 15, *FoxP3*~sf~ n=3, wild type n=3. c. Real time PCR analysis of *ErbB2* expression in the thymic epithelial cell line 6.1.7 after transient transfection of *FoxP3* cDNA. The transfectants were selected with blasticidin (5ug/ml) to remove untransfected cells. After removing non-adherent dead cells, mRNA was isolated and quantitated by real-time PCR. Data are representative of two independent experiments, using either *Hprt* or β-actin as house-keeping gene. d. *FoxP3* represses *Her-2* promoter. Data shown are summary of two independent experiments, depicting relative promoter activity with vector control arbitrarily defined as 1.0.
CHAPTER 3
HOMEOSTATIC T CELL PROLIFERATION AS THE MISSING LINK BETWEEN DEFECTIVE THYMOPOIESIS AND AUTOIMMUNE DISEASES

3.1 Abstract

A long-standing but still poorly understood phenomenon is the association between defective T cell production in the thymus and autoimmune diseases. We have recently reported that the Scurfy mutation of FoxP3 (FoxP3<sup>sf</sup>), which leads to rapid onset of lethal autoimmune diseases, also causes defective thymopoiesis. Here we show that reduced thymic output of CD4 T cells cause lymphopenia in newborn FoxP3<sup>sf/y</sup> mice, which was accompanied by a massive but transient homeostatic proliferation, independent of the defect in regulatory T cells. Moreover, in mice with T lineage-specific ablation of FoxP3 gene, induced lymphopenia exacerbates T-cell activation. Taken together, our data revealed homeostatic proliferation as a missing link between defective thymopoiesis and autoimmune diseases.

3.2 Introduction

Paradoxically, autoimmune disease and immune deficiency, which are usually regarded as two opposite extremes of immune responses, coexist in the same patient (6, 8). This paradox can be resolved by the homeostatic proliferation associated with lymphopenia, as is best illustrated by the spontaneous proliferation and expansion of naïve T cells after transferred into a T cell deficient host (46). Compared to other
mechanisms of T cell proliferation, homeostatic proliferation has some unique attributes which could facilitate destructive autoimmunity. During this process, T cells are stimulated by low affinity self antigens rather than cognate antigen (47). As such, homeostatic proliferation primes naïve T cells of a broad specificity, possibly including the otherwise quiescent autoreactive T cells. More recent studies suggest that T cells activated through homeostatic proliferation are long-lived and resistant to activation-induced cell death (49). A causal connection between homeostatic proliferation and autoimmunity in the NOD mice has been proposed recently (50). However, it remains mysterious how the homeostatic proliferation synergizes with other defects of self tolerance to cause autoimmune diseases.

Regulatory T cells have recently emerged as a major regulator of autoimmune disease. Defective development and function of regulatory T cells have been observed in various animal models and human patients (24). Nonetheless, ablation of regulatory T cells alone was not sufficient to cause autoimmune disease (87). Most of autoimmune readouts in vivo for Treg defects involve lymphopenia.

Scurfy mice, with a natural mutation of the FoxP3 gene, spontaneously develop lethal autoimmune disease. The autoimmune disease in the scurfy mice is due to the aberrant activation of CD4 T cells (56). FoxP3 gene is essential for the development and/or function of regulatory T cells (62-64) and the complete absence of regulatory T cells in the scurfy mice has been proposed as the sole cause of the deadly autoimmune disease (64). However, it is unclear how the autoreactive T cells are rapidly activated in the Scurfy mice; nor is it known why the autoimmune syndromes caused by the Foxp3 mutation are much more severe than those caused by defective Treg in other models.
We have demonstrated that the Foxp3 mutation in the Scurfy mice results in diminished thymopoiesis and thymic atrophy (88). Given the importance of thymic output in establishing the peripheral T cell pool in neonates (89), we have proposed that the defective thymopoiesis could cause reduced lymphopenia in the scurfy mice, which in turn causes homeostatic proliferation and acts in concert with Treg defects to cause rapid onset of autoimmune diseases. The supporting data for this hypothesis is presented herein.

3.3 Material and Methods:

Animals

Thy1.1 BALB/c mice with mutation of FoxP3 (FoxP3sf) were produced after more than 12 generations of backcross in the University of North Carolina. B6 FoxP3sf/+ mice were purchased from Jackson Laboratory, and were bred with wild type B6. The genotype of scurfy mice were determined through allele specific PCR as described (90). B6 OT-1 mice were purchased from Jackson Laboratory. All the mice were maintained under specific pathogen-free conditions at the University Laboratory Animal Resources at the Ohio State University for the duration of the study.

BrdU incorporation and measurement.

Mice were injected i.p. with nucleotide analog bromodeoxyuridine (BrdU; 1 mg/mouse in 100 µl PBS) 3 h before sacrifice. Splenocytes and lymph node cells were prepared and BrdU incorporation was detected by flow cytometry with a BrdU Flow Kit in conjunction with other cell surface markers, as described by the manufacturer (BD Biosciences, La Jolla, CA).
Antibodies and flow cytometry

Single cell suspension of thymus, spleen or lymph nodes were prepared and first blocked with anti-FcR (2.4G2) to eliminate Fc-mediated non-specific bindings. For cell surface staining, samples were stained with antibodies on ice for 30 minutes in staining buffer and were fixed by 1% PFA. Intracellular staining of the FoxP3 was performed as described by the manufacturer (eBiosciences, La Jolla, CA). The following antibodies were used: Perp cy5.5 conjugated anti-CD4 and anti-CD8 (BD Biosciences), APC-conjugated anti-CD4,anti-CD8 and anti-Thy1.2 (eBiosciences), PE-conjugated anti-CD25 (PC61) and anti-Foxp3 (FJK-16) (eBiosciences). All samples were analyzed by a four color FACS Caliber (BD biosciences).

Cell purification and adoptive transfer

To purify OT-1 T cells, spleen and lymph node cells from 6-8 weeks old B6 OT-1 mice were first incubated with anti-FcR (2.4 G2), anti-CD4 (GK1.5), anti-CD11b (MAC-1), anti-B220, and N418 (anti-CD11c) antibodies. The antibody-coated cells were then depleted with anti-Rat IgG-coated magnetic beads (Dynal, Invitrogen). The purified cells were then labeled with 2.5uM CFSE at 37 degree in the presence of 0.1% FBS. Unlabeled CFSE was then washed away using 5% RPMI for three times.

To purify CD4+CD25+ cells, CD4+ T cells were first purified using the Dynal beads to remove non-CD4 cells and then CD25+CD4+ T cells were further purified using the MACS beads. Briefly, spleen and lymph node cells from 6-8 weeks old BALB/C or B6 Thy1.1 mice were first incubated with anti-FcR (2.4 G2), anti-CD8 (2.4.3), anti-CD11b (MAC-1), anti-B220, and N418 (anti-CD11c) antibodies. The antibody-coated cells were then depleted with anti-Rat IgG-coated magnetic beads (Dynal, Invitrogen).
Purified CD4 T-cells were stained with anti-CD25 PE followed by anti-PE MACS beads (Milteny Biotec, Auburn, CA), CD4⁺CD25⁺ cells were then positively selected using MACS LS columns. Then purity of CD4⁺CD25⁺ cells was routinely around 92% to 95%. 1 million purified CD4⁺CD25⁺ cells were resuspended in serum free RPMI and i.v injected into 2-3 days old Thy1.1 BALB/c scurfy mice and their wild type littermates.

**TREC assay**

To determine the sjTREC, CD4 or CD8 T cells were purified using Dynal Beads as described before. The total DNA was then purified with DNA easy kit (Qiagen). Real time PCR was done using Quantitative PCR kit (invitrogen, CA). Following primers were used(91): m Rec primer (5’-GGGCACACACAGCTGTG), Jα primer (5’-GCAGGTTTTTGTAAAGGTGCTCA), and mRec-Jα fluorescent probe (5’-FAM-CACAAGCACCTGCACGCCTGTGCA-TAMRA-3’). CD8 β forward primer (5’-CAGGACCCCAAGGACAAGTACT-3’), reverse primer (5’-CACTTTCCAACCATAAAAACCTCCTTTG-3’), and probe (5’-FAM-TGAGTCTCCTGGCCTCTGAGTCTTTC-TAMRA-3’).

Number of TREC/million cells were calculated by: 2x (number of TREC/number of CD8β) x 10⁶.

**Cytokine staining**

Lymph nodes cells with transferred OT-1 T cells were incubated in 5% RPMI with or without 2ug/ml OVA peptides (SIINFEKL) for 6 hours. Intracellular IFN γ stained was then performed following manufacture’s instruction. (B.D.Bioscience, CA)
Depletion of regulatory T cells in neonatal mice

All the male mice from the breeding of Thy1.1 B/c Foxp3^{sf/+} x Thy1.1 B/C were i.p. injected with 100 ug anti-CD25(PC61) antibody at day 2-3 after the birth. The mice were then pulsed with BrdU as described and sacrificed at day 10.

Bone Marrow Chimera

Bone marrow cells were isolated from 15 day old either CD45.1^{+}Foxp3^{sf} mice or CD45.1^{+}Foxp3^{wt} mice, and mixed with the bone marrow of 10 weeks old wild type B6 mice at 1:1 ratio. The mixed bone marrow cells were then i.v. transferred to B6.Rag-1^{-/-} mice, which received 500 rad γ-irradiation one day before the transplantation.

3.4. Results

3.4.1 Defective thymic output and lymphopenia precedes lymphoproliferation in the Scurfy mice

We have recently reported in this page that the Scurfy mutation of Foxp3 resulted in defective thymopoiesis (88). To determine whether the defective thymopoiesis leads to reduced thymic output to the periphery, we purified CD4 and CD8 T cells from pooled spleen and lymph node cells isolated from 10-day-old WT and scurfy mice and measured the frequency of thymic migrants based on the number of the T cell receptor excision circles (TREC) (92). As shown in Fig 1a, a 3-4 fold reduction of TREC frequency is observed in the CD4 cells of the scurfy mice than of the wild type mice. Interestingly, frequency of TREC in CD8 T cells was not significantly changed (Fig 3.1a). Since CD4 T cells constitute the majority of T cells in the peripheral, total number of T cell output is also lower in the scurfy mice.
We next compared the percentages and absolute numbers of both CD4 and CD8 T cells in the spleen of the scurfy mice to those in the wild type littermates. In the 2-3 day-old mice, the number of CD4 and CD8 T cells are similar between the two groups (Fig. 3.1b, c). However, from day 6 to day 16, the scurfy mice have less CD4 T cells than WT mice. The difference reached the maximum at days 8 after birth, when the number of CD4 T cells in the scurfy spleen was less than 1/3 of their WT counterpart. By the third week, the number of CD4 cells in the scurfy spleen surpasses their WT counterpart. Corresponding to the apparently normal thymic output (Fig. 3.1a), the number and frequency of CD8 T cells were similar in the two groups in the first 10 days. By day 16, the number of CD8 T cells was substantially higher in the scurfy spleen. Our analysis of the T cell numbers during the perinatal period revealed that lymphopenia precedes lymphoproliferation in the scurfy mice.

In addition, mathematical modeling of T cell number revealed distinction patterns between scurfy and wild type mice. In the wild type mice, T cells linearly increased in the spleen in the first three weeks (CD4: $y = 118962x - 390557$; CD8: $y = 62256x - 214431$). However, in the scurfy mice, the T cell numbers increased exponentially (CD4: $y = 22567e^{0.2701x}$; CD8: $y = 8727.9e^{0.3733x}$) (Figure 3.1c). The distinct patterns suggest that the T cell compartment was formed by distinct mechanisms in WT and scurfy mice.

### 3.4.2 Homeostatic proliferation precedes antigen driven proliferation in the scurfy mice

Next we tested whether the lymphopenia in the scurfy mice can promote homeostatic proliferation. First, we carried out kinetic analysis for T cell proliferation in
WT and Scurfy mice. The mice were pulsed with BrdU for 3 hour prior to flow cytometric analysis. A representative profile and summary data were shown in Fig. 3.2a and b, respectively. In WT mice, the % of BrdU$^+$ cells decline during the first three weeks. In contrast, the rate of T cell division in the scurfy mice accelerates during the same period, even though T cells in the two groups have the same division rate on day 3. It is particularly worth noting that a dramatic increase in the T cell division was found on day 10 in the scurfy mice, when the scurfy mice have least T cells compared to the wild type mice.

T cells undergoing homeostatic proliferation can be distinguished from those undergoing antigen-driven proliferation by lack of acute T cell activation marker, such as CD69 and CD25, which allowed us to probe the nature of stimuli for the proliferating T cells in the scurfy mice. As shown in Fig. 3.3, in the 7-day-old scurfy mice, most dividing T cells do not express CD69. In contrast, by day 15, the majority of the BrdU$^+$ T cells acquired the CD69 expression (Fig 3.3a). All the dividing cells, either in day 7 or day 15, highly express memory T cell marker CD44( Figure 3.3a). At all the time points tested, in the wild type mice dividing T cells only express CD44, but not CD69 (data not shown). These data suggest that the stimuli that drive T cell proliferation in the scurfy mice switched before and after second week.

As an alternative approach, we labeled naïve T cells with CFSE and transferred them into 5 and 16 day old mice. Four days later, the spleens and lymph nodes are harvested and the numbers of T cells undergoing proliferation were determined by the dilution of CFSE. As shown in Fig. 3.3b, the % of T cells undergoing homeostatic proliferation is drastically reduced in day 16 recipients, in comparison to day 5 recipients.
To rule out the possibility that cognate antigen may be involved in the process, we labeled the transgenic OT-1 T cells, which recognize a peptide from chicken ovalbumin and thus lack cognate antigen in the mice, and transferred them into day 5 and day 16 recipients. Again, in the 5-day old recipients, nearly 20% of OT-T cells have undergone proliferation, while less than 2% of the OT-1 cells divided in the 16 day old recipients. Few cells divided in the WT recipients in the 4-day period regardless of the donor and recipient combination.

Autoimmune disease in the Scurfy mice may cause tissue destruction and the release of “de novo” epitopes, which may cross-react with the transgenic T cells. To exclude that possibility, we tested if FoxP3<sup>sf</sup> splenocytes can stimulate transgenic T cells without the peptide stimulation. As shown in Figure 3.9, neither FoxP3<sup>sf</sup> nor FoxP3<sup>wt</sup> splenocytes could stimulate the transgenic T cells in vitro without the cognate peptide. Therefore, the T cell proliferation in the Scurfy mice is not the result of cross-reactivity of de novo epitopes.

Taken together, the data presented so far revealed a strong correlation between lymphopenia and homeostatic proliferation of T cells in the scurfy mice.

### 3.4.3 Homeostatic proliferation in the neonatal scurfy mice primes naïve T cells

Because homeostatic proliferation in the lymphopenic host can prime naïve T cells and convert them to memory T cells, we tested whether the homeostatic proliferation in the scurfy mice can also affect naïve T cells similarly.

We adoptively transferred CFSE-labeled OT-1 cells into 4-day-old scurfy mice or their WT littermates. At day 7 after the transfer, donor cells were analyzed for their division and cell surface phenotypes. All the donor cells did not express CD69 (Figure
4a, upper panel), but had increased CD44 expression with the increase of the dividing cycles (Figure 4a, lower panel). We then stimulated those divided OT-1 cells with OVA peptide. As shown in Fig, 4b, nearly twice as many cells in the Scurfy recipients produced IFNγ in the short-term recall response. Thus, homeostatic proliferation primes naïve T cells in the neonatal scurfy mice and converts them to memory T cells both phenotypically and functionally.

Interestingly, after the homeostatic proliferation, TCR expression of the OT-1 cells is increased greatly in the Scurfy mice, indicative of the increased affinity after the homeostatic proliferation.

3.4.4 No intrinsic role of FoxP3 in response to homeostatic cue

Besides constitutively expressed in regulatory T cells, FoxP3 can also be induced in non-regulatory T cells during homeostatic proliferation in the periphery (93). Any intrinsic role of Foxp3 in homeostatic proliferation has not been studied. We compared the homeostatic proliferation of naïve scurfy T cells and wild type T cells in response to lymphopenic environment. In order to use T cells with the same activation status, we constructed the mixed bone marrow chimera using scurfy bone marrow (CD45.1⁺Foxp3sf) and congenic wild type bone marrow (CD45.2⁺ Foxp3 w) to reconstitute Rag1 KO mice. The chimera mice were healthy and no autoimmunity as reported(64). Moreover, the activation status of the two groups of T cells was comparable (data not shown). T cells from the chimera mice were then purified and transferred to new Rag1 c− mice after CFSE labeling. CFSE dilution of T cells with two genotypes was compared at four days and seven days after the transfer. As shown in Fig 3.5b, T cells with scurfy mutation (CD45.1⁺) have the same pace of division as wild type
T cells (CD45.2⁺). Additionally, the ratio between CD45.1+ and CD45.2+ T cells after the homeostatic proliferation remained the same as before the transfer.

The data in this part demonstrate that scurfy mutation in T cells per se does not increase the lymphopenia driven homeostatic proliferation.

3.4.5 Enhanced homeostatic proliferation in the scurfy mice is independent of Treg defects

We took two approaches to determine whether enhanced homeostatic proliferation in the scurfy mice is a consequence of the Treg defect. First, we evaluated whether depletion of CD25⁺ cells in neonatal wild type mice can increase the homeostatic proliferation. As shown in Figure 4a, one dose anti-CD25 antibody injection of 2 or 3 day old mice almost completely depleted CD25⁺CD62L⁺ T cells in the periphery when analyzed at day 10. However, the proliferation of neither CD4 nor CD8 T cells was increased after the antibody treatment (Figure 3.5). To test whether CD25 is required for T cell homeostatic proliferation, we injected anti-CD25 antibodies into the scurfy mice. As shown in the Fig. 3.5a, the scurfy T cells continued to proliferate. Thus, absence of regulatory T cells is not sufficient causing the enhanced homeostatic proliferation in the neonates.

Second, we tested if wild type Treg can inhibit the increased homeostatic proliferation in the scurfy mice. We transferred purified CD4⁺CD25⁺ T cells to the new born scurfy mice, and compared the proliferation of endogenous T cells at ten days after the transfer. The donor Treg expanded and accumulated in the lymph nodes of the scurfy mice (Fig 3.5b). However, BrdU incorporation experiments found no inhibition of the endogenous T cells’ proliferation in the presence of regulatory T cells (Figure 3.5b).
To further confirm that Treg is not sufficient to inhibit homeostatic proliferation in the scurfy mice, we co-transferred purified Treg with CFSE labeled OT-1 cell. At day seven after the transfer, the CFSE dilution of OT-1 cells were examined. Even in the presence of regulatory T cells, OT-1 cells divide as much as without Treg (Figure 3.6c).

3.4.6 Bulk T cells inhibits the homeostatic proliferation in the Scurfy mice

Next, we tried to determine whether the enhanced homeostatic proliferation in the Scurfy mice is caused by the less T cells. To avoid transferring Foxp3+ regulatory T cells, we transferred 20 million unlabelled OT-1 transgenic CD8 T cells from OT-1 mice together with 2 million CFSE labeled OT-1 T cells into 2-3 day old Scurfy mice. Seven days after the transfer, the CFSE labeled donor cells were analyzed. As shown in Figure 3.6c, majority of the OT-1 T cells only divide one to two times, as compared to 5-6 time division observed without bulk OT-1 cell co-transfer. Similarly, OT-1 cell division was also inhibited by the co-transfer of bulk polyclonal T cells (data not shown). Therefore, the enhanced homeostatic proliferation is due to reduced T cell compartment in the scurfy mice.

3.4.7 Synergy between homeostatic proliferation and Treg defects in the pathogenesis of autoimmune diseases

A recent study from Fontenot et al (94) demonstrated that mice with CD4-cre driven ablation of FoxP3 gene developed autoimmune disease with a severity comparable to those with germ-line mutation of FoxP3. Since we have demonstrated that CD4 promoter is active in the thymic stromal cells, in which FoxP3 mutation caused defective thymopoiesis, we chose another line of transgenic mice, Lck-cre, to carry out T-cell lineage-specific ablation of the FoxP3 gene. As shown in Fig. 3.7a, at one week, the
Cre+ mice had barely detectable FoxP3+ cells in the thymus and in the spleen, which suggests that the FoxP3 gene was effectively ablated in T cell lineage. Normal thymopoeisis and no increased homeostatic proliferation were observed in Cre+ mice (Fig. 3.7b). Interestingly, despite of the efficient Treg ablation, all but one of the Cre+ mice showed long-term survival (Fig. 3.7c), although a small number of FoxP3+ cells were observed in 2 month old Cre+ mice. These data suggest that Treg ablation alone is insufficient to cause rapid onset of lethal autoimmune diseases.

In order to test whether lymphopenia can exaggerate the autoimmunity caused by the Treg deficiency, we treated the FoxP3loxP CreYck+ mice with anti-CD4 and anti-CD8 antibodies to create a transient lymphopenia. As shown in Fig. 3.8, the transient lymphopenia leads to the enhanced activation of T cells in the peripheral blood in the FoxP3loxP CreYck+ mice, whereas it has little effect on the FoxP3loxP CreYck- mice.

3.5 Discussion

The purpose of this study is to study how the diminished thymopoiesis contributes to deadly autoimmune disease in the Scurfy mice. We show that reduced thymic output leads to lymphopenia in neonatal Scurfy mice and enhanced homeostatic proliferation. The nature of the T cell hyperproliferation in the neonatal Scurfy mice is homeostatic, in that most dividing cells do not express activation marker and the division can be independent on the cognate antigens of transgenic T cells.

We conclude that the neonatal scurfy mice suffer lymphopenia based on that the scurfy mice have both less percentage and less number of T cells in the neonatal stages. Unlike other models with chronic lymphopenia, the lymphopenia in the scurfy mice is quickly obscured by the subsequent T cell proliferation and expansion. This point is
underlined by the distinct patterns of T cells expansion between scurfy and wild type mice. In the wild type mice, T cells in the periphery expand linearly; whereas in the scurfy mice, they expand exponentially. Consequently, despite the initial lymphopenia, the scurfy mice have much more T cells than the wild type mice at the late stage.

What is the cause for the lymphopenia in the neonatal scurfy mice? Given the importance of thymic output in establishing peripheral T cell compartment in neonates, we propose that the reduced thymopoeisis in the scurfy mice may cause the reduced thymic output and the lymphopenia. This is supported by the reduction of recent thymic output in the scurfy mice (Fig 3.1a). Besides that, the lymphopenia in the scurfy mice could also be caused by the increased apoptosis of T cells in the periphery at the neonatal stage. Another possibility is that T cells in the neonatal scurfy mice preferentially migrate to the peripheral organs, thus causing the lymphopenia in the secondary lymphoid organs. Those possibilities obviously are not mutually exclusive.

The homeostatic proliferation in the scurfy mice is not secondary to the autoimmune disease and massive T cell activation. If the homeostatic proliferation was a consequence of the autoimmune disease in the Scurfy mice, one should expect it was correlated with the severity of the disease. Indeed, homeostatic proliferation of either endogenous or transgenic T cells is only observed in the Scurfy mice at early age, but not the later stage with more severe disease. In addition, transfer of the regulatory T cells ameliorated the disease of the Scurfy mice in the short term(64), but has no effect on the homeostatic proliferation. Thus, the homeostatic proliferation in the scurfy mice is independent to the autoimmune disease.
The homeostatic proliferation is rather a provision for the lethal autoimmune disease in the Scurfy mice. This point is strengthened by two pieces of evidence. First, only transfer of large amount of T cells, but not small number of regulatory T cells alone, was capable of fully rescuing the Scurfy mice (95). Second, depletion of FoxP3 in the T cell lineage by Lck-Cre did not result in homeostatic proliferation and lethal autoimmune disease as in the Scurfy mice. This also strongly suggests that the increased homeostatic proliferation is independent on the FoxP3 mutation in the T cell lineage, most likely caused by the reduced thymic output due to FoxP3 mutation in the thymic stroma.

How the homeostatic proliferation exaggerates the autoimmune disease in the Scurfy mice? After the homeostatic proliferation, naïve T cells are converted to “memory T cells” phenotype, which may decrease their activation threshold. In addition, TCR expression is greatly increased accompanied with cell cycle, thus their affinity to those self-antigens is increased greatly and potentially become autoreactive.

Our data also argue against the notion that the failure to generate Treg in the thymus due to the FoxP3 mutation could result in a more autoreactive TCR repertoire (96). As our data revealed in Figure 3.5, the FoxP3 deficient T cells showed equal pace of division as the wild type T cells in the lymphopenia host, indicative of a similar self reactivity between two types of T cells. Instead, our data indicate that in the FoxP3 mutant mice, it is the homeostatic proliferation that increases the autoreactivity of the T cells.

Absence of regulatory T cells in the Scurfy mice exaggerates the autoimmunity triggered by homeostatic proliferation. Homeostatic proliferation alone in most circumstances is not able to cause autoimmune disease; this may be due to the restoration
of T cell compartment and subsequent cessation of T cell proliferation. However, in the scurfy mice, due to the absence of regulatory T cells, T cells are still hyperproliferative even with extra amount of T cells. Exactly how the absence of Treg synergerate autoimmunity remains to be determined.
Figure 3.1 Reduced thymic output leads to lymphopenia in the Scurfy mice. (a). TREC analysis were carried in purified either CD4 or CD8 T cells from pooled spleen and lymph nodes cells of 10-day-old Scurfy mice. Data are summary of 6 scurfy mice and 6 wild type littermates from three independent experiments. Error bars indicate standard error of the mean. (b,c) Scurfy mutant mice or their wild type littermates in the C57BL/C background were analyzed for the percentages (b) and absolute numbers (c) of CD4 and CD8 T cells in the spleen at different ages. The percentages of CD4 and CD8 T cells were determined with CD4 and CD8 staining of the splenocytes, and the absolute numbers were calculated based on total number of splenocytesx CD4 % or CD8%. Data are summary of at least 3 mice of each age and error bars indicate standard error of the mean. Fig 1b, upper panel, $R^2=0.97$ (scurfy) and 0.94(wt); lower panel, $R^2=0.92$ (scurfy) and 0.97( wt). Fig 1c, upper panel, $R^2=0.99$ (scurfy) and 0.97(wt); lower panel, $R^2=0.96$ (scurfy) and 0.97( wt).
**Figure 3.2 T cell hyperproliferation in the Scurfy mice.** B6 Scurfy mice and their wild type littermates were pulsed with 1mg BrdU e.a. for three hours and dividing cells were accessed based on the BrdU+ cells. a. Representative FACS profile and b. Summary of BrdU+% cells among CD4 or CD8 T cells at different ages. Data are summary of at least 3 mice for each age and error bars indicate standard error of the mean.
Figure 3.2
Fig.3.3  **Homeostatic proliferation precedes antigen driven proliferation in the scurfy mice.** a. Phenotypic change of the dividing T cells between 7-day and 15-day old scurfy mice. Thy1.1 Balb/c Scurfy mice at either 7-day or 15- day-old were pulsed with BrdU for three hours, and lymph node cells were stained with anti-BrdU and anti-CD69(upper panel) or anti-CD44(lower panel). Data are repeated three times with similar results. The similar results were also obtained from the splenocytes (data not shown). b. Naïve T cells only divide in 7-day-old scurfy mice, but not in wild type mice or 15-day-old scurfy mice. 3~4x10^6 purified OT-1 CD8 T cells, polyclonal CD4 T cells or polyclonal CD8 T cells were labeled with CFSE and transferred into B6 Scurfy mice or their wild type littermates at different ages. Four days after the transfer, donor cells were analyzed for CFSE dilution.
Figure 3.3
Figure 3.4 Homeostatic proliferation in the neonatal scurfy mice primes naïve T cells. a. Divided T cells express memory T cell marker CD44 (upper panel), but not activation marker CD69 (middle panel) and enhance TCR expression. Purified CD8 T cells from naïve OT-1 transgenic mice were labeled with CFSE and transferred to 4 to 5-day-old scurfy mice and donor T cells in the lymph nodes were analyzed for the CD44 or CD69 expression at 7 days after the transfer. Donor cells were gated as CFSE⁺ CD8⁺. Data are representative of two independent experiments. b. Higher proportion of divided OT-1 T cells produce cytokine in response to peptide stimulation. As in a, total lymph node cells were then stimulated with or without OVA peptide (2μg/ml) in vitro for 6 hours in the presence of Golgi-stop for the last four hours. Donor cells were gated as above and IFN γ production was determined using intracellular staining. Data are representative of three mice from two independent experiments.
Figure 3.4
Figure 3.5 No intrinsic function of Foxp3 inhibiting homeostatic proliferation. Bone marrow cells from FoxP3sfCD45.1+ and FoxP3wtCD45.2+ mice were mixed with 1:1 ratio and used to reconstitute Rag1−/− mice. Total T cells purified from chimera mice were labeled with CFSE and transferred into new Rag1−/− mice. At either day 4 or day 7 after the transfer, donor cells were analyzed for their division based on the CFSE dilution. a. ratio of two genotypes in either CD4 or CD8 compartment. b. dividing of donor cells in Rag1−/− mice. Note CD45.1+ represents FoxP3sf T cells, and CD45.1- represents Foxp3wt T cells. Data are representative of two independent experiments.
Figure 3.6 Enhanced homeostatic proliferation is not due to absence of Treg, but because of the less T cells. a. In vivo depletion of Treg does not cause exacerbated homeostatic T cell proliferation in young WT mice, nor did it abrogate T cell hyperproliferation in the Scurfy mice. Newborn BALB/c WT mice and their FoxP3^{sf} littermates were treated with 100 micrograms of anti-CD25 antibody at birth. On day 10, the mice received a BrdU pulse for 3 hours and were then sacrificed for analysis. Data shown are representative of 2-3 mice per group. Age matched WT mice were left untreated and used as control. b. CD4^{+}CD25^{+} T cells from WT mice had no effect on homeostatic proliferation of lymph node cells in the Scurfy mice. The FoxP3^{sf} and FoxP3^{wt} mice were either left untreated or treated with 10^{6}/mouse of CD4^{+}CD25^{+} T cells from WT mice on day 2 of birth. Seven days later, the mice were analyzed for BrdU incorporation and expression of CD69, CD4 and CD8. Data shown are BrdU and CD69 profiles of gated CD4 (top panels) and CD8 (middle panels) cells from lymph node, where the majority of Treg migrated to after adoptive transfer. The numbers of donor T cells were identified by expression of high levels of CD25 and Thy1.2 marker. Recipients are Thy1.1 strain. c. Bulk T cells, but not Tregs inhibit the homeostatic proliferation of OT-1 cells. 2-3 days old Scurfy mice were transferred with 2~3 x10^{6} OT-1 T cells alone, with 1x10^{6} CD4^{+}CD25^{+} T cells from B6.Thy1.1 mice or 20x10^{6} unlabeled OT-1 CD8 T cells. Seven days after the transfer, the donor OT-1 T cells were identified as CFSE^{−}CD8^{−}Vβ5^{+} and analyzed for CFSE dilution. Donor Treg was identified as Thy1.1^{+}FoxP3^{+} (left panel). Data are representative of 2~3 mice from at least two independent experiments.
Figure 3.6
Figure 3.7 Deletion of FoxP3 in the T cell lineage is not sufficient causing T cells homeostatic proliferation or lethal autoimmune disease. (a). Deletion of FoxP3 in T cell lineages with $\text{Cre}^{\text{lox}}$. $\text{FoxP3}^{\text{floox}}$ mice were crossed with $\text{Cre}^{\text{lox}}$ mice and CD4SP thymocytes or CD4 splenocytes from 7-day-old mice were analyzed for FoxP3 and CD25 expression. (b). Normal thymopoiesis in $\text{FoxP3}^{\text{floox}\text{Cre}^{\text{lox}}}$ mice. 8-week old mice were pulsed with BrdU for 3 hours and cell division of DN thymocytes (upper panel) and CD4 splenocytes (lower panel) were accessed by measuring BrdU incorporation. (c). Long term survival of the mice with FoxP3 deletion in T cells.
Figure 3.8. Transient T cell depletion exaggerates autoimmune disease in FoxP3<sup>loxp</sup> Cre<sup>lck</sup> mice. Adult FoxP3<sup>loxp</sup> Cre<sup>lck+</sup> or FoxP3<sup>loxp</sup> Cre<sup>lck-</sup> mice were treated with anti-CD4 and anti-CD8 antibody and 2 months after the treatment, CD4 in the peripheral blood were stained for activation marker CD62L and CD44. Numbers in the figure indicate the percentage of activated T cells (CD62L<sub>low</sub> CD44<sub>high</sub>).
Figure 3.9 The proliferation of transgenic T cells in the neonatal Scurfy mice is not caused by de novo epitopes. (a) Transgenic T cells specific to tumor antigen P1A undergo homeostatic proliferation in neonatal Scurfy mice. 3–4 million P1CTL CD8 T cells were transferred to either day 4 or day 14 old Balb/C Thy1.1 Scurfy mice and wild type littermates. Three days later, donor cells were analyzed for CFSE dilution. Data are representative at least three independent experiments. (b) Scurfy splenocytes fail to stimulate P1CTL transgenic T cells in vitro. Fresh P1CTL cells were labeled with CFSE and co-cultured with fresh splenocytes from either day 7 old Balb/C Thy1.1 scurfy mice or wild type mice. Three days later, transgenic T cells were gated as Thy1.2^CD8^+ and analyzed for the CFSE dilution. Similar results were obtained with 15- day old Scurfy mice.
CHAPTER 4

TARGET SUSCEPTIBILITY VS SHARED SPECIFICITY IN REGULATORY T CELL MEDIATED CYTOLYSIS OF AUTOREACTIVE T CELLS

4.1 Abstract

The specificity and mechanism of Treg in their control of autoreactive T cells have not been analyzed in animals with a normal T cell repertoire. Using endogenous viral superantigens (VSAg) as the primary self antigens and mice with the Scurfy mutation of FoxP3, we show here that although genetic ablation of the Treg lineage does not affect thymic clonal deletion of VSAg-reactive T cells, the Treg defect causes preferential accumulation of VSAg-reactive T cells. In the Scurfy mice, the proliferation of VSAg-reactive T cells was comparable to that of non-VSAg-reactive T cells. In contrast, preferential proliferation of VSAg cells was observed in wild-type mice with normal Treg. Adoptive transfer of Treg did not inhibit T cell proliferation of VSAg-reactive T cells in vivo. Thus, selective expansion of autoreactive T cells cannot be explained by selective inhibition of their proliferation by Treg. Surprisingly, Treg selectively kill self-reactive T cells. Since Treg is significantly enriched among VSAg-reactive cells, we tested whether the selective elimination was due to shared specificity of Treg with their autoreactive target cells or increased susceptibility of the autoreactive T-cell to Treg. Our data demonstrate significantly higher susceptibility of autoreactive T cells to Treg.
4.2 Introduction

It is well established that Treg play a critical role in controlling autoimmune diseases (24). However, the specificity and mechanism by which Treg control autoreactive T cells are largely unclear (25) The specificity of Treg is inferred by a variety of studies including the substantially higher activity of Treg isolated from draining lymph nodes of the affected organ (97) and those isolated from animal that have received the same infection (98). In vitro studies however, suggest that while the activation of Treg requires TCR engagement, its effector function is not antigen-specific (99).

An elegant study by Rudensky’s group has demonstrated that Treg are enriched for self-reactivity (100). This enrichment can be due to two different mechanisms. First, data from two groups suggest that thymic expression of antigen positively select Treg (23, 101). Second, although van Santen et al (102) showed that thymic expression of autoantigen does not increase the number of Treg (102), it increases the frequency of Treg by preferential removal of autoreactive effector T cells with the same specificity. Thus, on a per-cell basis, self reactive T cells are likely matched with a higher number of Treg with potentially the same specificity in comparison to T cells specific for a non-self antigen. An intriguing issue is whether the function of Treg in controlling autoimmunity relates to their shared specificity with their target cells.

Another largely unresolved issue is mechanisms by which Treg control autoreactive T cells in vivo. Several studies have suggested that Treg secrete inhibitory cytokines such as IL-10 and TGF β. A critical role of these cytokines can be demonstrated in a number of in vivo models (26-28), but not others (29). However, in
vitro studies have failed to substantiate their essential role in Treg-mediated suppression (25). On the other hand, while Treg have been shown to repress proliferation of naïve T cells and the production of cytokines in vitro, their roles in controlling T cell proliferation has not been well established in vivo. More recently, it was demonstrated that Treg are highly cytotoxic to activated T cells in vitro (30, 31). This result raised the intriguing possibility that Treg may kill their target cells in an in vivo setting, although no specificity has been reported.

The two issues have not been adequately addressed largely due to difficulties in studying the behavior of self-reactive Treg and effector cells in models other than TCR transgenic mice. An exception to this rule is VSAg, which are encoded by murine mammary tumor viruses (MMTV) (103). In strains that have genomic insertion by endogenous MMTV, the VSAg has served as model antigens to provide the first demonstration of clonal deletion, as the specificity is encoded by the Vβ region (104). In this study, we compared the fate of VSAg reactive T cells in mice with a mutation of FoxP3, a master regulator for the development and function of Treg (62-64), to those in normal mice. We found that although thymic clonal deletion of VSAg-reactive T cells was equally efficient in the two strains, accumulation of VSAg-reactive T cells was observed only in the Scurfy mice. In addition, in the Scurfy mice, the VSAg-reactive and nonreactive T cells divided at a comparable rate and that their proliferation was not affected by adoptive transfer of functional Treg. Furthermore, we showed that Treg selective killed VSAg-reactive T cells and that such selective killing was due to increased target susceptibility rather than shared specificity between the Treg and the autoreactive T cells.
4.3 Material and Method

Animals

Thy1.1 BALB/c mice with mutation of FoxP3 (FoxP3sf) were produced after more than 12 generations of backcross in the University of North Carolina. The genotype of scurfy mice were determined through allele specific PCR as described (90). Wild type BALB/c mice were obtained through a subcontract of National Cancer Institute. B10.BR mice were purchased from Jackson Laboratories. All the mice were maintained under specific pathogen-free conditions at the University Laboratory Animal Resources at the Ohio State University for the duration of the study.

BrdU incorporation and measurements

Mice were injected i.p. with nucleotide analog bromodeoxyuridine (BrdU; 1 mg/mouse in 100 µl PBS) 3 h before sacrifice. Splenocytes and lymph node cells were prepared and BrdU incorporation was detected by flow cytometry with a BrdU Flow Kit in conjunction with other cell surface markers, as described by the manufacturer (BD Biosciences, La Jolla, CA).

Antibodies and flow cytometry

Single cell suspension of thymus, spleen or lymph nodes were prepared and first blocked with anti-FcR (2.4G2) to eliminate Fc-mediated non-specific bindings. For cell surface staining, samples were stained with antibodies on ice for 30 minutes in staining buffer and were fixed by 1% PFA. Intracellular staining of the FoxP3 was performed as described by the manufacturer (eBiosciences, La Jolla, CA). The following antibodies were used: FITC or PE conjugated antibodies against TCR Vβ3, Vβ5, Vβ8, Vβ11, Vβ12 (BD biosciences), Perpe cy5.5 conjugated anti-CD4 and anti-CD8 (BD Biosciences),
APC-conjugated anti-CD4, anti-CD8 and anti-Thy1.2 (eBiosciences), PE-conjugated anti-CD25 (PC61) and anti-Foxp3 (FJK-16) (eBiosciences). All samples were analyzed by a four color FACS Caliber (BD biosciences).

For the Annexin V staining, cells were first stained with cell surface antibodies and then were incubated with PE conjugated Annexin V (BD Biosciences) at room temperature for 15 min and were analyzed by FACS immediately after the staining.

**Cell purification and adoptive transfer**

To purify CD4⁺CD25⁺ cells, CD4⁺ T cells were first purified using the Dynal beads to remove non-CD4 cells and then CD25⁺CD4⁺ T cells were further purified using the MACS beads. Briefly, spleen and lymph node cells from 6-8 weeks old BALB/C mice were first incubated with anti-FcR (2.4 G2), anti-CD8 (2.4.3), anti-CD11b (MAC-1), anti-B220, and N418 (anti-CD11c) antibodies. The antibody-coated cells were then depleted with anti-Rat IgG-coated magnetic beads (Dynal, Invitrogen) were used to deplete. Purified CD4 T-cells were stained with anti-CD25 PE followed by anti-PE MACS beads (Miltenyi Biotec, Auburn, CA), CD4⁺CD25⁺ cells were then positively selected using MACS LS columns. Then purity of CD4⁺CD25⁺ cells was routinely around 92% to 95%. 1 million purified CD4⁺CD25⁺ cells were resuspended in serum free RPMI and i.v injected into 2-3 days old Thy1.1 BALB/c scurfy mice and their wild type littermates.

**In vitro cytotoxicity of regulatory T cells**

CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells were stimulated with 10 μg/ml of plate-bound anti-CD3 and 100U/ml of IL-2 for 72 hours. These pre-activated T cells were then mixed with fresh lymph nodes cell from 8-10 days old scurfy mice in 1:1 ratio for 4 hours. The cells
were then surface-stained with APC conjugated anti-Thy1.1, PE-conjugated anti-CD4 and FITC-conjugated anti-TCR V\(_{\beta}\)5 or V\(_{\beta}\)8. After the surface staining, 7-AAD was added to each sample which was then analyzed by FACS immediately. Thy1.1\(^+\)CD4\(^+\) V\(_{\beta}\)5\(^+\) or Thy1.1\(^+\)CD4\(^+\) V\(_{\beta}\)8\(^+\) were gated as target cells respectively. Death of the target cells was determined by the % of 7-AAD\(^+\) cells. Specific lysis was calculated by % of 7-AAD\(^+\) cells with effector cells minus % of 7-AAD\(^+\) cells in cultures without any effector cells.

**Statistic analysis**

All the data are shown in Mean±SEM. Two tail student T test were employed and statistic significance is **, P<0.01; *, P< 0.05.

4.4 Results

4.4.1. Normal clonal deletion of VSAg-reactive T cells in the Scurfy mice

The genome of the BALB/c mice has insertions of mouse mammary tumor provirus (MMTV) type 6, 8 and 9 as well as H-2I-E, which in conjunction formed viral superantigens recognized by T cells expressing V\(_{\beta}\)3, 5, 11 and 12 (103). The VSAg-reactivity of these T cells allows us to follow the fate of the autoreactive T cells by flow cytometry in a polyclonal TCR level manner. We first determined whether mutation of FoxP3 affects clonal deletion of the thymocytes. We stained the thymocytes from the Scurfy mice and their WT littermates with anti-CD4 and CD8 mAbs in conjunction with the mAbs specific for V\(_{\beta}\)3, 5, 8, 11 and 12. Representative profiles of CD4 and CD8 single positive thymocytes are shown in Fig. 1a and the summary data are presented in Fig. 1b. Among both CD4 and CD8 T cells, V\(_{\beta}\)3, 5, and 12-expressing cells were less
than 2% in both WT and the Scurfy mice. In our experience, Vβ11+ T cells were not completely deleted in the thymus at this time point and the frequency of Vβ11+ T cells was also comparable between WT and the FoxP3 mutant mice. These results demonstrate that the FoxP3 mutation does not affect clonal deletion in the thymus.

4.4.2. Enrichment of Treg in VSAg-reactive T cells

In the WT mice, clonal deletion of VSAg reactive T cells also resulted in the enrichment of regulatory T cells in those undeleted autoreactive T cells. Thus, the proportion of CD25+CD4+ T cells was not uniformly distributed among different Vβ-expressing T cells. As shown in Fig. 2, almost 25% of the Vβ3, 5, 11 and 12-expressing T cells were CD25+, while less than 10% of the Vβ8+ T cells expressed CD25. Similarly, more than 35% of VSAg-reactive T cells expressed transcription factor Foxp3, a lineage marker of regulatory T cells, whereas less than 15% of non-VSAg reactive T cells had FoxP3 expression. Since the total number of VSAg-reactive Treg was not higher than the B6 mice that lack the I-E for presentation of superantigens (data not shown), the increase was likely due to the elimination of CD25- T cells rather than positive selection of VSAg-reactive Treg.

4.4.3 Increased accumulation of VSAg-reactive T cells in the scurfy mice does not associate with hyper-proliferation of VSAg-reactive T cells

Interestingly, in the scurfy mutant mice a substantial increase in the % of VSAg-reactive T cells was observed in the spleen and lymph nodes in comparison to what was observed in the thymus. As shown in Fig. 3a, b, at 15 days of age, while WT littermates had less than 1% of Vβ3 and Vβ5-expressing CD4 T cells in the spleen, the Scurfy mice had almost 10-fold more Vβ3+ or Vβ5+ CD4 T cells. Likewise, we also observed a
significantly higher number of $V_{\beta}11^+$ or $V_{\beta}12^+$ CD4 T cells in the Scurfy spleen, although the fold of expansion was not as striking as what was observed for the $V_{\beta}3^+$ and $V_{\beta}5^+$ CD4 T cells, perhaps due to higher baseline levels in the WT mice. A similar expansion was also observed for the CD4 and CD8 T cells (Fig. 3b) and can be found in the lymph nodes (data not shown).

Selective expansion of the VSAg-reactive T cells in the scurfy mice can be due to either their enhanced proliferation or survival of the autoreactive T cells. To test if the increased proliferation was responsible for their accumulation, we pulsed the scurfy mice and the WT littermates with BrdU for 3 hours and analyzed the % of cells that had undergone proliferation. As shown in Fig. 3c &d, in WT mice, the $V_{\beta}3$, 5 and 11$^+$ CD4 T cells divided substantially faster than the $V_{\beta}8^+$ T cells, which are not VSAg-reactive in the BALB/c mice. The proliferation did not lead to expansion of the VSAg-reactive T cells as their frequencies were no higher than what was found in the thymus. In contrast, in the Scurfy mice, equally rapid BrdU incorporation was observed in the VSAg-reactive and nonreactive subsets. Nevertheless, preferential accumulation of the VSAg-reactive T cells was found in the Scurfy mice. Our data demonstrate that increased proliferation is neither necessary nor sufficient for preferential accumulation of VSAg-reactive T cells.

4.4.4. Elimination of autoreactive T cells by Treg after their proliferation in the periphery.

Since Scurfy mice lack Treg, we tested whether the expansion of VSAg-reactive T cells can be suppressed by Treg. We adoptively transferred Treg isolated from WT mice into 2 or 3 day old Scurfy mice. On day 15, the recipients were pulsed for 3 hours with BrdU prior to sacrifice and analyzed for the frequency and proliferation of both
VSAg-reactive and non-reactive T cells  Since the Scurfy mice are of Thy1.1 genotype and the donor Treg were from Thy1.2 BALB/c mice, we were able to follow the fate of both Treg and endogenous VSAg-reactive T cells. As shown in Fig. 4a, the Treg expanded substantially in the Scurfy host in comparison to the WT host, although higher number of Treg. Treg treatment caused reduction in Vβ3+ and Vβ5+ T cells in the lymph node and spleen (Fig. 4b, c). The reduction was not due to Treg-mediated suppression of proliferation of VSAg-reactive T cells, as this proliferation was unaffected by adoptive transfer of Treg (Fig. 4d, e). Interestingly, significant reduction of Vβ11+ and Vβ12+ T cells was not observed. Lack of effect on Vβ11+ T cells may relate to lack of clonal deletion of this subset, although the lack of effect on Vβ12+ T cells suggests that other yet unknown factors may be responsible for the discrepancy.

We tested if the donor Treg in scurfy mice enhanced the cell death of VSAg-reactive T cells. We used the cell surface staining of Annexin V to determine the early apoptotic cells among VSAg-reactive T cells (Vβ3, 5, 11 or 12+) or non VSAg-reactive T cells (Vβ8). In the scurfy mice treated with Treg, the Annexin V+ T cells in VSAg-reactive T cells were much more than non-VSAg-reactive T cells (Fig 4f). In untreated scurfy mice, no difference in the Annexin V+ cells was observed between VSAg-reactive and non-reactive T cells. These data demonstrate that Treg preferentially kill VSAg-reactive T cells in vivo.

4.4.5. Higher susceptibility rather than shared specificity explain preferential elimination of VSAg-reactive T cells

We developed an in vitro co-culture system to test if Treg can specifically target VSAg-reactive T cells for their apoptosis. CD4+CD25+ T cells purified from wild type
Balb/C mice were first activated for 72 hours by plate bound anti-CD3 and IL-2. Then, lymph node cells from 8-10 days old scurfy mice were incubated with pre-activated Treg with 1:1 ratio in 37°C. Four hours after the incubation, dead cells were determined by 7-AAD staining. We compared the death of Vβ5+ and Vβ8+ CD4 T cells. As shown in Fig. 5a, Treg triggered the death of Vβ5+ but not Vβ8+ T cells.

Since negative selection enriched VSAg-reactive T cells among Treg, we tested the possibility that higher ratio of Treg/target among Vβ5+ T cells may explain its preferential elimination. Vβ5+ T cells were depleted from the CD25+ CD4 T cells prior to pre-activation and co-culture. As shown in Fig. 5b, depletion of Vβ5-depleted Treg still preferentially killed Vβ5+ CD4 T cells. Likewise, depletion of Vβ3, 5, 11, and 12-expressing cells all together in the Treg also failed to abolish the selective killing of the Vβ5+ cells (data not shown).

The above data indicate that VSAg-reactive T cells are more susceptible to apoptosis. To substantiate these observations, we generated alloreactive T cells by stimulating the B10BR spleen cells with irradiated BALB/c spleen cells. Five days later, we carried out co-culture experiments and evaluated killing of Vβ5+ and Vβ8+ T cells. As shown in Fig. 5c, Vβ5+ T cells were more effectively lysed by alloreactive T cells. Thus, higher susceptibility of VSAg-reactive T cells is not limited to Treg.

4.5 Discussion

We used VSAg as the model self-antigen to study the specificity and mechanism of Treg-mediated suppression of autoreactive T cells and showed that Treg preferentially kills autoreactive T cells and that the specificity is based on increased susceptibility of
VSAg-reactive T cells to T cell-mediated cytolysis. Our conclusion is based on several lines of evidence.

First, we demonstrated that despite equal efficiency in clonal deletion, VSAg-reactive T cells have a very different fate in the mice with or without Treg. In mice with mutation of the Foxp3 gene, VSAg-reactive T cells expanded in the periphery. In contrast, in mice with normal Treg, the representation of VSAg-reactive T cells was further diminished in the periphery. Interestingly, in the Treg-sufficient WT-mice, the disappearance of the VSAg reactive T cells occurred despite their preferential proliferation in vivo. The expansion and disappearance of the VSAg-reactive T cells was demonstrated more than 15 years ago(105). Our data provided direct evidence that the disappearance of the VSAg-reactive T cells is mediated by Treg.

Second, we showed that Treg are cytotoxic to VSAg-reactive T cells that are expanded in the absence of Treg. Thus, when Treg was transferred into the Scurfy host, higher proportion of apoptotic T cells was observed among T cells expressing VSAg-reactive TCR. In addition, co-culture of total activated Treg with the lymph node cells from the Scurfy mice led to preferential killing of VSAg-reactive T cells. These data provide direct evidence that VSAg-reactive T cells are more susceptible to lysis by T cells, including both Treg and conventional alloreactive T cells. The cellular difference between the autoreactive T cells and other T cells remains to be elucidated. We have not observed any difference in expression of Fas (data not shown). In addition, since the non-VSAg-reactive T cells in the Scurfy mice proliferated equally well, it is clear that proliferation alone does not confer higher susceptibility. Thus, Treg discriminates self-reactive vs nonreactive proliferating T cells in vivo. This feature allows Treg to control
autoreactivity while at the same time preserving significant response to foreign antigens, even though the latter is also subject to Treg control (98).

Thirdly, several studies have demonstrated that Treg are enriched for self-reactivity (23, 100-102). In some models, the enrichment was associated with increase in absolute number of Treg (23, 101). In the others, however, this was attributed to preferential elimination of non-Treg (102). Our data show that although there is no increase in the number of VSAg-reactive T cells in mice that express in the thymus, the ratio of Treg/non-Treg among the VSAg-reactive T cells is increased by 2-3 fold. To test if this enrichment explains the preferential elimination of self-reactive T cells by the Treg, we tested the effect of depleting VSAg-reactive Treg on the selective elimination of Treg-reactive T cells. Since this depletion has no effect on selective elimination of VSAg-reactive T cells, Treg and their effector need not to share TCR specificity. Nevertheless, it is possible that Treg and their targets are brought together by antigen-expressing cells in vivo. In this scenario, it is plausible that co-localization of Treg and their targets can be achieved if the Treg and their target recognize antigens expressed on the same cells. Thus, our data did not rule out the possibility that shared specificity facilitate Treg function in vivo. In this regard, it is worth noting that Mathis et al. showed that Treg that shared the same receptor as the autoreactive T cells are more efficient suppressors of type I diabetes, primarily at the target organ (106).
Figure 4.1 Endogenous VSAg-specific T cells are effectively deleted in the thymus of scurfy mice. Thymocytes from 15 days old BALB/c scurfy mice or their wild type littermates were stained with anti-CD4, anti-CD8 and various anti-TCR V\(\beta\) antibodies, and analyzed by flow cytometry. a. Representative FACS profiles showing the percentage of CD4\(^{+}\)CD8\(^{-}\) or CD4\(^{-}\)CD8\(^{+}\) thymocytes expressing different TCR V\(\beta\) segments. Numbers in each panel represent the percentage of cells expressing the given TCR V\(\beta\) segments. b. Summary of percentage of CD4 SP (upper panel) and CD8 SP (lower panel) thymocytes expressing the corresponding TCR V\(\beta\) segments. Data are a summary of at least 8 mice per group from 5 independent experiments.
Figure 4.2. Clonal deletion enriches VSAG-reactive regulatory T cells. Thymocytes and splenocytes from 5-6 weeks old wild type Balb/c mice were stained with CD4, CD8, respective TCR Vβ antibodies and CD25 or FoxP3. VSAG-reactive CD4 T cells were defined as T cells expressing at least one of the following Vβ: Vβ3, Vβ5, Vβ11, or Vβ12, and Vβ8+ T cells were used as non-VSAG-reactive T cell control. a & b. Comparison of regulatory T cells in VSAG-reactive T cells and non-VSAG reactive T cells in the thymus (a) and in the spleen (b). c. Summary of percentage of CD25+ (upper panel) and FoxP3+ (lower panel) T cells in CD4+ VSAG-reactive and CD4+ VSAG-non-reactive population of the thymus and spleen.
Figure 4.3. Selective accumulation of VSAg-reactive T cells in the periphery.

Splenocytes and lymph node cells from 15-day old scurfy mice or their wild-type littermates were stained by CD4, CD8 and respective TCR Vβ antibodies and analyzed by flow cytometry. a. Representative profile of TCR Vβ expression in the spleen. The numbers on each panel represent the percentage of cells expressing the given TCR Vβ segments. b. Summary of the distributions of various TCR Vβ in the spleen. Upper panel shows the distribution in CD4+ population; lower panel shows those in CD8+ population. Number of mice examined, scurfy n=12; wild type n=9. Similar data were observed in the lymph node (data not shown). c. Proliferation of VSAg-reactive T cells in the lymph nodes. Scurfy mice or their wild type littermates were pulsed by BrdU (1mg/mouse) for three hours. Lymph node cells were surface-stained with CD4, CD8, and Vβ3, 5, 11 or Vβ8 antibodies, then intracellular staining for BrdU+ cells. The numbers on the upper right quadrant represent the percentage of BrdU+ cells in the given TCR Vβ+ CD4 cells. d. Summary of proliferation of VSAg-reactive T cells in the lymph nodes. Data are summary of 8 mice per group from 3 to 4 independent experiments. Similar data were observed in the spleen (data not shown).
Figure 4.3
Figure 4.4 Regulatory T cells eliminate VSAg-reactive T cells in scurfy mice. $10^6$ CD4$^+$CD25$^+$ T cells isolated from wild type BALB/c mice (Thy1.2$^+$) were i.p. injected into 2-3-days old scurfy mice. When the mice reached 15-16 days of age, spleen and lymph node cells were analyzed. a. Accumulation of donor T cells in the lymph nodes of scurfy mice. Live cells from spleen and lymph nodes were analyzed by flow cytometry using antibodies against CD4 and Thy1.2. The numbers indicate the percentage of Thy1.2$^+$CD4$^+$ or Thy1.2$^-$CD4$^+$ cells in either lymph nodes or spleen. b. Reduction of VSAg-reactive T cells caused by adoptive transfer of Treg. Data shown are representative profile of treated- and untreated-spleen and lymph node cells. Numbers indicate the percentages of CD4 T cells expressing corresponding TCR V$\beta$. c. Summary of the alteration of frequencies of the VSAg-reactive T cells in scurfy mice after the Treg transfer. Data are from three Treg treated scurfy mice and four wild type littermates in two independent experiments. d. Proliferation of VSAg-reactive T cells after the neonatal Treg transfer. Mice were pulsed with BrdU as detailed in Figure 3 legends. CD4$^+$ cells from the lymph nodes were analyzed by flow cytometry after the staining of BrdU and corresponding TCR V$\beta$ antibodies. The numbers indicate the percentage of BrdU$^+$ cells in CD4 T cells expressing corresponding TCR V$\beta$ chains. e. Summary of BrdU$^+$ cells in V$\beta$ 3, 5, 11$^+$ CD4$^+$ cells after the regulatory T cells transfer. f. Apoptosis of VSAg-reactive (left) and non-reactive (right) T cells in scurfy mice with or without the Treg transfer. Histograms show the representative Annexin V staining from two independent experiments.
Figure 4.4 (continued)
Figure 4.4
Figure 4. 5. Increased susceptibility of VSAg-reactive T cells to cell-mediated cytolysis. a. Representative profiles of Treg-induced apoptosis of VSAg-specific T cells in vitro. CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells from syngeneic wild-type mice were stimulated with plate bound anti-CD3 and IL-2 for 72 hours. The pre-activated Treg were then mixed with fresh Scurfy lymph node cells at 1:1 ratio for 4 hours. Thy1.1⁺CD4⁺ Vβ5⁺ or Thy1.1⁺CD4⁺ Vβ8⁺ were gated and analyzed as target cells. Death of the target cells was determined by the % of 7-AAD⁻ cells. b. Summary of Treg induced apoptosis of VSAg-reactive T cells. Top panels show efficacy of depletion, while the lower panel show specific lysis, as calculated by % of 7-AAD⁺ cells with effector cells minus % of 7-AAD⁻ cells without effectors. Each sample was in triplicate and data are representative of three independent experiments. c. As in b, except that alloreactive T cells were used as effector cells. The experiments have been repeated twice with similar results. Data in b and c are presented as Mean±SE.
Figure 4.5
5.1 Abstract

Ornithine transcarbamylase (OTC) plays an essential role in the urea cycle for clearance of nitrogen waste associated with amino acid metabolism. Mutation of OTC results in hyperammonemia with early lethality. Here we show that deficiency in OTC attenuates clonal deletion of autoreactive T cells and production of CD1d restricted NK T cells in the thymus. More importantly, mice heterozygous for the OTC mutation have high titers of autoantibodies and have dramatically higher susceptibility to the experimental autoimmune encephalomyelitis (EAE). Our data provide the first genetic link between dysregulation of amino acid metabolism and autoimmune disease.

5.2 Introduction

Ammonia generated during metabolism poses a great threat to most animals; therefore different species have evolved various mechanisms to clear nitrogen waste accumulated in the body. In mammals, nitrogen waste from nuclear acids is fixed in the uric acid; and nitrogen waste generated during amino acid metabolism is fixed in the urea through the urea cycle in the liver. Recently, it has been demonstrated that uric acid crystals can function as an endogenous “danger signal” to activate the DC cells and then
trigger the adaptive immune responses (107). Whether or not the dysfunction of the urea cycle plays any role in the immune system has not been characterized.

**OTC** is an X-linked gene encoding a critical enzyme in the urea cycle. Inactivating mutations lead to early lethality in males, while less severe mutations cause various degrees of health defects. Interestingly, although germline mutations are carried by heterozygous females, a significant number of carriers show clinical symptoms including lethality. Most of the clinical symptoms can be attributed to hyperammonemia. Ammonia has long been known as a lysosomotropic inhibitor *in vitro*. It can prevent the fusion of the endosomes and lysosomes. Proper function of the lysosome is essential to the presentation of antigens of APCs to T cells through the MHC II and CD1d pathways. *In vitro*, it has been shown that ammonia inhibits MHC II presentation (108). It is therefore of great interest to determine whether OTC mutation affects immune function.

*Spare fur* (*spf*) mice have a spontaneous mutation (His117>Asn) of the Otc gene, which results in a marked decrease in the enzyme activity under physiological condition(109). While mutant hemizygous male mice have significantly shortened life spans, the female carriers have a fairly normal life span and are fertile. Since the *spf* mutation results in hyperammonemia(110), it offered a valuable model to explore potential immune defects associated with abnormal nitrogen waste clearance. Here we show that despite absence of Otc expression in the thymus, the mutation of OTC selectively impairs MHC class II expression in thymic epithelial cells, which causes inefficient deletion of autoreactive T cells and production of regulatory T cells in the thymus; the mutation also impairs the presentation of endogenous NKT ligand on thymocytes, which leads to less NKT cells produced in the thymus. In comparison to
wild-type littermates, female carrier mice had a higher titer of autoantibodies and showed a dramatic increase in susceptibility to EAE. Our results provide the first genetic link between abnormal amino acid metabolism and risk for autoimmune diseases.

5.3 Material and Methods

Animals

Balb/C OTC<sup>spf</sup>+ mice were isolated from the breeding of Balb/C FoxP3<sup>sf/+</sup>OTC<sup>spf</sup>-/+ with wild type Balb/C male mice, and were then crossed with wild type Balb/C for more than 8 generations. The genotype of OTC<sup>spf</sup>+ mice were determined as described (111). Briefly, PCR was carried out using primers 5’-CTA ACC CAT CAG AGT TTG AAA TAA AC-3’ and 5’-CCC CTC TCA ATA CAT TCA CTG TCT-3’. PCR products were digested by the restriction enzyme Mse I and analyzed with 4% agarose gel. Wild type allele gave one fragment around 200 base pairs; OTC<sup>spf</sup> allele mice gave rise to one 100 bp fragment. Wild type BALB/c mice were obtained through a subcontract of the National Cancer Institute. All mice were maintained under specific pathogen-free conditions at the University Laboratory Animal Resources at the Ohio State University for the duration of the study.

Antibodies and flow cytometry

Single cell suspensions of thymus, spleen or lymph nodes were prepared and first blocked with anti-FcR (2.4G2) to eliminate Fc-mediated non-specific binding. For cell surface staining, samples were stained with antibodies on ice for 30 minutes in staining buffer and were fixed with 1% paraformaldehyde. Intracellular staining of FoxP3 was performed as described by the manufacturer (E-Biosciences, La Jolla, CA). The following antibodies were used: FITC or PE conjugated antibodies against TCR V<sub>β</sub>3,
Vβ5, Vβ8, Vβ11, Vβ12 (BD biosciences), Percepy5.5 conjugated anti-CD4 and anti-CD8 (BD Biosciences), APC-conjugated anti-CD4,anti-CD8 and anti-Thy1.2 (E-Biosciences), PE-conjugated anti-CD25 (PC61) and anti-Foxp3 (FJK-16)(E-Biosciences). All samples were analyzed by a four color FACS Caliber (BD biosciences).

α-Galcer-CD1d tetramer staining was done as described(112). Briefly, single cell suspension were incubated with 2.4G2 soup and unlabelled strepavidin on ice for 15 minutes, and were then stained with PE labeled α-Galcer-CD1d tetramer at room temperature for 20 minutes.

**Detecting ant-double strand DNA autoantibodies in the serum**

For the ELISA assay used to detect anti-double strand DNA in the serum, plates were first coated with double strand DNA (Sigma) in 0.1M NaHCO₃ overnight at 4 °C. Wells were then blocked with 1% BSA at room temperature for 1 hour. Serum samples were collected by eye bleeding and diluted using 1% BSA. 100 ul diluted serum was then incubated in a pre-coated plate for 2 hours at room temperature. After washing, the plates were then incubated with HRP labeled anti-mouse IgG F (ab’)_2 (Pierce, IL) for 1 hour. Following the incubation and wash, the substrate (Fast OPD tablet, Sigma) was added into each well and was then stopped with 2M HCl.

**NKT hybridoma stimulation**

Fresh thymocytes from 3-week-old OTC<sup>opf</sup> (Balb/c x B6 F1) mice or their wild type littermates were incubated with NKT hybridoma DN32-D3 in 5% DMEM at 37 °C for 20-24 hours. Production of IL-2 was then measured by standard sandwich ELISA assay. Briefly, plates were coated overnight with 2 ug/ml affinity purified anti-mouse interleukin-2 (clone JES6-1A12, E-Bioscience, CA). Supernant from the co-culture was
incubated with precoated plate at 4 °C overnight, and 1ug/ml biotin anti-mouse IL-2 (clone JES6-5H4, E-bioscience, CA) was used as the detecting antibody. Recombinant mouse IL-2 was used as standard.

**Immunofluorescence staining**

Frozen sections of the thymus were fixed in ice-old acetone at room temperature for 10 mins. Biotin UEA-1(Molecular probe) and rabbit anti-mouse K5 were incubated with sections for 1 hour at room temperature. Alexa 488 Strepavidin and alexa- 518 anti-rabbit IgG (molecular probe) were used as secondary antibodies. Con-focal images were acquired using the Zeiss 510 META Laser Scanning Confocal microscope.

**Isolation of thymic epithelial cells**

Thymii from 3-4 week old BALB/C OTC spf mice or their wild type littermates were cut into small pieces and stirred for 30 mins at 4°C to remove most thymocytes. The fragments left were digested using 1 mg/ml Collagenase/ Dispase (Roche, IN) + 1 mg/ml DNase I (Sigma, St. Louis, MO) in successive three 15-minute incubations. Cells from the last two digestion were stained with CD45.2 APC and G8.8 (BD Biosciences) followed by FITC conjugated anti-Rat Ig G2a (BD Biosciences).

### 5.4 Results

#### 5.4.1 OTC spf mutation results in selective defects in MHC class II expression in thymic epithelial cells and in defective negative selection

Given the documented effect of ammonium on MHC class II presentation *in vitro* (108), we compared wt and spf mutant littermates for MHC class II expression on antigen presenting cells. As shown in Fig. 1a, cell surface MHC class II levels in the
dendritic cells (DC) were comparable between wt and mutant littermates, regardless of whether the DC from spleen, thymus or lymph nodes were analyzed. Likewise, MHC class II levels on B cells and macrophages were also unaffected. Interestingly, a drastic reduction in MHC class II levels was observed in thymic epithelial cells.

Further analysis indicated that defective class II expression was limited to the medullar epithelial cells, as no difference was found in the cortical epithelial cells (Figure 1). The number of medullar epithelial cells, as revealed by flow cytometry, was largely unaffected. Histological analysis revealed that in the mutant thymus, the boundary between the medulla and cortex was less clear-cut (Figure 1b). Moreover, as revealed by immunofluorescence staining, there were less UEA-1⁺ thymic epithelial cells in the mutant thymus, indicating that the thymic epithelial cells were less mature (Figure 1b). Taken together, our data revealed that the thymic medullar cells are exquisitely sensitive to the effect of OTC mutation, even though the OTC gene is not expressed in the thymus.

Given the importance of thymic medullar epithelial cells on negative selection, we tested whether negative selection is affected by the OTC mutation. The genome of the BALB/c mice has insertion of mouse mammary tumor provirus (MMTV) type 6, 8 and 9 and H-2I-E, which in conjunction causes clonal deletion of T cells expressing Vβ3, 5, 11 and 12 (103). As shown in Fig. 2a, with exception of Vβ3, the frequencies of all other VSAg-reactive T cells are more than doubled in the mutant thymus. Correspondingly, the frequency of non-VSAg-reactive thymocytes (Vβ8⁻) is reduced in the OTC⁺/+ mice. Consistent with the defect in clonal deletion, the % of most VSAg-reactive T cells (Vβ5,11, and 12⁺) were more than doubled in the Otc⁺/+ mice. A similar increase of VSAg-reactive T cells was also observed in the spleen of the OTC⁺/+ mice (Figure 2b).
Thus our data in this part demonstrate that the OTC mutation affects MHC II expression of thymic medulla epithelial cells and subsequently the negative selection in the thymus. In support of this idea, Balb/c Rag−/− mice reconstituted with OTCspf bone marrow had normal deletion of the superantigen reactive T cells in the thymus (data not shown), indicating that the OTC mutation in non-hematopoietic lineages contributes to the defect in negative selection.

5.4.2 Reduced NKT maturation in the Otcspf/y thymus

Presentation of glycolipids through CD1d also requires the endosomal/lysosomal compartment(113), and proper presentation of endogenous glycolipids is essential for the introthymic development of NKT cells *in vivo*. Therefore, we also tested whether the development of CD1d dependent NKT cells is affected by the hyperammonemia associated with the OTCspf mutation. CD1d expression on the OTCspf/y thymocytes was normal (Figure 3a); however, the frequency of NKT cells in either thymus or spleen was significantly reduced in the OTC mutant mice. As shown in Fig. 3b, α-Galcer-CD1d tetramer staining revealed about a 40% reduction of NKT cells in the thymus and in the spleen of the OTCspf mice compared to wild type littermates (Figure 3b). Non-CD1d restricted NKT cells, however, were almost unaffected by the OTC mutation (Figure 3b). In addition, the binding affinity of NKT cells to CD1d-tetramer was also reduced in the OTCspf mice. Thus, mutation of the OTC gene decreases the production of CD1d-restricted NKT cells and results in less NKT cells in the periphery.

To study whether the defects on NKT cell production was due to defective presentation of endogenous glycolipids in the thymus, we took advantage of the fact that some NKT cell hybridomas are self-reactive and can be stimulated by thymocytes.
through CD1d(32). Thus, we tested the capacity of thymocytes from Otc<sup>spf/y</sup> mice to stimulate the self reactive NKT clone DN32-D3(32). As shown in Fig. 3d, the NKT hybridoma stimulated by OTC<sup>spf/y</sup> thymocytes produce much less IL-2 compared to wild type thymocytes, suggesting that the endogenous glycolipids are either less presented or differently presented on the OTC<sup>spf</sup> thymocytes, suggesting that the endogenous glycolipids were either presented less or presented differently on the OTC<sup>spf</sup> thymocytes.

5.4.3 Reduced production of FoxP3<sup>+</sup> regulatory T cells in the OTC<sup>spf/y</sup> thymus

Selection of regulatory T cells in the thymus also requires the presentation of self antigens in the thymus. Therefore, we also investigated whether the OTC mutation affects the production of regulatory T cells in the thymus.

Since CD4<sup>+</sup>CD25<sup>+</sup> T cells expressing the transcription factor FoxP3 are the best characterized regulatory T cells to date, we compared the frequency of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in the thymus as well as in the spleen. As shown in Figure 4, frequency of Foxp3<sup>+</sup> cells in CD4SP cells of the OTC<sup>spf</sup> thymus was about 1% less than in wild type thymus. In addition, FoxP3<sup>+</sup> T cells in the mutant mice had less CD25 expression, indicative of an immature phenotype.

The reduction of Foxp3 expressing cells in the thymus, however, did not persist in the secondary lymphoid organs. As shown in Figure 4, an even higher frequency of Foxp3<sup>+</sup> cells was found in the mutant mice than in the wild type mice. This result was probably due to the expansion or de novo induction of regulatory T cells in the periphery. Therefore, it was of interest to determine if those peripherally expanded Treg in the OTC<sup>spf</sup> mice is of the same function as Treg in wild type mice. Preliminary analysis of
Treg function in vitro, however, found they had same capacity to inhibit T cell proliferation in vitro (data not shown).

5.4.4 The \textit{OTC}^{spf} mutation increases the susceptibility to autoimmune disease

Corresponding to an increased burden of autoreactive T cells and reduction of NKT cells, we found that T cells in OTC$^{spf/y}$ mice of the second lymphoid organs are more activated than their wt counterpart (Fig. 5a).

Due to the early lethality of the \textit{OTC}^{spf} mice, we sought to study the impact of the \textit{OTC}^{spf} mutation on autoimmunity by testing the susceptibility of \textit{OTC}^{spf/+} mice to autoimmune disease. \textit{OTC}^{spf} mutation has been found to be “semi dominant”, thus, only partial enzyme activity exists in the liver of the \textit{OTC}^{spf/+} mice compared to OTC$^{+/+}$ mice. \textit{OTC}^{spf/+} mice are also affected by hyperammonemia, although the syndromes are much less severe compared to \textit{OTC}^{spf} mice.

We first tested the autoantibodies in the serum of \textit{OTC}^{spf/+} mice. Young \textit{OTC}^{spf/+} mice do not have detectable autoantibodies, (Data not shown). When \textit{OTC}^{spf/+} mice reach 15 month old, however, by ELISA analysis, we were able to detect significant amounts of anti-double strand DNA antibody in the \textit{OTC}^{spf/+} mice in comparison to age-matched wild type (Figure 5b). Additionally, some of the \textit{OTC}^{spf/+} mice (3 out of 5) at the age of 15 months developed autoantibodies against various tissue specific antigens, but none of the age matched wild type mice did (0 out of 3)(Figure 5a).

We further tested whether the OTC mutation increases the susceptibility to induced experimental autoimmune diseases. We immunized the \textit{OTC}^{spf/+} mice (Balb/c x B6, F1) and their wild type littermates with MOG peptide and monitored the EAE. As shown in Figure 5c, much more severe EAE was observed in the \textit{OTC}^{spf/+} mice, actually
about one third of mice (2 out of 6) died after the immunization. Both the peak disease score and incidence of EAE in OTC spf/+ mice were also much higher than the wild type littermates.

5.5 Discussion

Taken together, we have demonstrated that the OTC mutation selectively attenuated expression of MHC class II in thymic medulla epithelial cells and CD1d antigen-presentation in thymocytes. Consequently, the load of self-reactive T cells was increased, while NKT cell, which are known as a negative regulator of autoimmune diseases was reduced. Since both defects have been shown to render increased susceptibility to autoimmune diseases, it is conceivable that these defects are responsible for the increased autoimmune diseases in mice, although other mechanisms may also be involved in the autoimmune diseases described herein. The significance of our observation in the mouse is further demonstrated by the strong association between genetically encoded variations in OTC activity and risk of multiple sclerosis in humans (data not shown).

The attenuated negative selection in the OTC spf mice was demonstrated by less deletion of endogenous superantigen reactive T cells in the thymus. VSAg mediated deletion of reactive T cells has been deemed as the first evidence of negative selection. However, TCR affinities to superantigens are usually much higher than to most peptide antigens, thus whether negative selection induced by conventional peptide antigens is also affected by the OTC mutation remains to be determined.
Given the importance of thymic epithelial cells on negative selection, it is conceivable that the impaired negative selection is due to the decreased MHC II expression on thymic epithelial cells, although other mechanisms may also be involved.

How does the OTC mutation attenuate antigen presentation of medulla thymic epithelial cells and thymocytes? The most significant phenotype caused by the OTC mutation is the built-up of ammonia. Ammonia has been long known as an inhibitor of lysosome/endosome function, where many enzymes participate in antigen presentation, including peptides generation and processing. More importantly, most of the enzymes required an acidic pH optimum, thus the higher pH caused by the ammonia may impair the functions of several key enzymes in the antigen presentation pathway.

The reason that the OTC mutation selectively impairs MHC II expression on thymic epithelial cells is unknown. Interestingly, expression and activity of many proteases in the lysosome/endosome is tissue specific. For example, cathepsin L has been shown to play an essential role in processing Ii in the cortical thymic epithelium, but not for spleen APCs (114). Thus, it is possible that some proteases expressed by the thymic epithelial cells are in particular sensitive to the OTC mutation. Another reason may be that MHC II expression in many APCs in the periphery is more dynamically regulated and affected the immune status. Indeed MHC II and B7 expression in dentritic cells of OTC<sup>spf</sup> mice is even higher than in wild type mice, indicative of the activated phenotype, which may obscure the defects on MHC II expression. Besides lower MHC II expression, other aspects of MHC presentation may be also affected by the OTC mutation, such as the antigens/epitopes presented.
From the data presented here, it is hard to conclude if the defective production of Treg contributes to the higher susceptibility to autoimmune diseases. In vitro, the function of Tregs in the $OTC^{spf}$ mice is the same as in wild type mice. However, given that less Treg are produced in the thymus, the Treg in the OTC spf mice presumably are less diverse than the wild type. Interestingly, a similar expansion of Treg was also observed in Cathepsin L deficient mice, which provides protection to diabetes in the NOD background. Over-expansion of Treg in the periphery may also explain the absence of severe autoimmunity in the $OTC^{spf}$ mice.
Figure 5.1. *OTC*<sup>spf</sup> mutation causes selective downregulation of MHC II in the thymic medulla epithelial cells. (a). Comparison of MHC II expression between *OTC*<sup>spf</sup> mutant mice and wild type littermates. Thymus was digested and thymic MEC was gated as CD45<sup>G8.8^+</sup>Ly51<sup>−</sup>, and CEC was gated as CD45<sup>G8.8^+</sup>Ly51<sup>+</sup>. (b). Representative profile of thymocyte of *OTC*<sup>spf</sup> mice. (c). Thymic medulla cells in the *OTC*<sup>spf</sup> mice are less mature. Upper panel, H&E staining of the thymus sections. Note the blurred border between medulla and cortex in the *OTC*<sup>spf</sup> mice; Lower panel, immunofluorescence staining of the thymus section with UEA-1 and anti-K5 antibody. Note the less UEA-1+ cells in *OTC*<sup>spf</sup> mice.
Figure 5.2. Defective deletion of endogenous superantigen reactive T cells in the OTC spf mutant mice. (a) Inefficient deletion of vSAG reactive T cells in the thymus of OTC spf mutant mice. Thymocytes from 15-day-old Balb/C OTC spf mutant mice and wild type littermates were stained with anti-CD4, anti-CD8 and corresponding antibodies against different TCR Vβ segments. Frequencies of different Vβ+ cells in either CD4SP (left) or CD8 SP (right) population were analyzed. The number in each panel represents the frequency of T cells expressing indicated TCR Vβ segments. (b) Summary of data from A. (c) As in A, frequencies of vSAG reactive T cells and non-reactive T cells in CD4+ or CD8+ splenocytes were compared between OTCspf and OTC wt mice. (d) Summary of data of c. Error bars indicate standard error of the mean.
Figure 5.2
Figure 5.3. *OTC*<sup>spf</sup> mice have reduced NKT cells and diminished presentation of endogenous glycolipids in the thymus. (a) CD1d expression is conserved in the *OTC*<sup>spf</sup> thymocytes. Fresh thymocytes were stained with CD1d, CD4 and CD8 antibodies, and CD1d expression on CD4<sup>+</sup>CD8<sup>+</sup> cells was compared between *OTC*<sup>spf</sup> and *OTC*<sup>wt</sup> mice. (b) CD1d restricted NKT cells are reduced in the thymus. Thymocytes from 3-week old *OTC*<sup>spf</sup> (B6×Balb/c F1) mice were stained with α-Galcer-CD1d tetramer, anti-NK1.1 and anti-TCR β. Left panel is a representative profile and the right panel is a summary. (c) As in (b), TCR β<sup>+</sup> splenocytes were gated and analyzed for α-Galcer-CD1d tetramer staining and NK1.1 expression. (d). *OTC*<sup>spf</sup> thymocytes partially lose their capacity to stimulate NKT cells *in vitro*. Fresh thymocytes were co-cultured with NKT cell hybridoma DN32-D3 for 20-24 hours and IL-2 in the supernatant was determined by standard sandwich ELISA. Data are representative of three independent experiments.
Figure 5.3
Figure 5.4. Regulatory T cells are produced less in the thymus, but expanded more in the periphery of the \textit{OTC}^{spf} mice. (a,b) Thymocytes and splenocytes from 3-week-old \textit{OTC}^{spf} mice were stained with CD4, CD8, CD25 and intracellular FoxP3. (a) Representative profile and (b) summary of FoxP3$^+$ regulatory T cells in the \textit{OTC}^{spf} mice.
Figure 5.5. OTC<sup>opf</sup> mutation increases the susceptibility to autoimmune diseases. (a). More T cells in the OTC<sup>opf</sup> mice have activated/memory phenotype. Splenocytes from 3-week-old OTC<sup>opf</sup> mice were stained with CD4, CD8, CD44 and CD62L. Data shown were gated on CD4 T cells. Numbers on the plot indicate the percentage of CD62L<sub>low</sub> CD44<sub>high</sub> cells. Data are representative of at least 3 experiments. Similar results were obtained for CD8 T cells. (b). Development of anti-tissue autoantibodies in aged OTC<sup>opf/+</sup> mice. Serums from 15-month-old Balb/C OTC<sup>opf/+</sup> mice or age matched wild type Balb/C mice were used to stain tissue sections from Balb/C Rag2<sup>−/−</sup> mice followed by the staining with Alexa 488 labeled anti-mouse IgG and DAPI. (c) Increased anti-double strand DNA antibody in the serum of OTC<sup>opf/+</sup> mice. As in b, anti-double strand antibodies in the serum were analyzed by ELISA. (d). OTC<sup>opf/+</sup> mice develop more severe EAE after MOG immunization. Adult OTC<sup>opf/+</sup> (Balb/C X B6, F1) mice were immunized with MOG peptide and the development of EAE was monitored. Data are representative of two experiments, each with at least 5 mice for each genotype.
Figure 5.5
CHAPTER 6
CONCLUDING REMARKS

Immune tolerance is mainly achieved by self/non-self discrimination. Unique properties about self antigens, including their availability in the thymus and constant presence in the periphery, assure that they are ignored or tolerated by the immune system. Multiple mechanisms are then utilized by the immune system to tolerize self antigens while remaining highly responsive to foreign antigens. The findings presented here further the understanding of the molecular and cellular mechanisms of self/non-self discrimination and immune tolerance.

The thymus anatomically separates self antigens from foreign antigens. Thus, in the thymus self antigens need to be expressed and effectively presented to immature T cells, which either induce apoptosis of reactive T cells or convert them to some other specialized subsets. Our data show that hyperammonemia caused by the OTC gene mutation selectively inhibits antigen presentation in the thymus, including downregulation of MHC II on thymic medulla epithelial cells and reducing the presentation of endogenous glycolipids by thymocytes. As such, the deletion of endogenous superantigen reactive T cells and production of regulatory T cells as well as CD1d restricted NKT cells are attenuated in the OTC mutant thymus. Importantly, the
increased burden of autoreactive T cells in the periphery greatly increases the susceptibility to autoimmune diseases in human and in mice.

Further studies on $OTC^{spf}$ mutant mice should be devoted to investigate how this mutation selectively affects MHC II expression on medulla epithelial cells. Many proteases in the lysosome are tissue specific and redundant. Thus one possibility is that it is due to the differential sensitivities of proteases in lysosomes to higher pH caused by the hyperammonemia. Another possibility is that MHC II expression on the other APCs is also affected, but is obscured by the ongoing autoreactive T cell immune responses, which may upregulate the MHC expression. Analysis of the OTC mutant mice in a RAG null background may help to address this possibility.

Another interesting area to explore is how this mutation affect the presentation endogenous glycolipids by thymocytes. One of the endogenous NKT ligands identified is iGB-3, thus it is of great interest to determine the level of iGB-3 in the $OTC^{spf}$ thymus.

Besides abnormalities of metabolism, hyperammonemia is also observed during some bacterial infections. In this context, how the immune responses against pathogens are affected by the hyperammonemia will be of great interest to study. Two pieces of evidence presented here may suggest that hyperammonia may be employed by pathogens to avoid attack from the host immune system. First, NKT cells are reduced and presentation of endogenous glycolipids is impaired in the $OTC^{spf}$ mice. NKT cells function as a “bridge” between the innate immune system and the adaptive immune system. Moreover, NKT cells in vivo are activated at least in some cases by the endogenous glycolipids during pathogen infections. Therefore, the hyperammonemia associated with pathogen infections may curtail the activity of NKT cells. Second,
regulatory T cells are expanded in the periphery of OTC\textsuperscript{\textit{norm}} mice, although the exact mechanism for this expansion is unclear. One explanation may be directly caused by the hyperammonemia, which may also affect the function of peripheral APCs. Regulatory T cells have been demonstrated clearly to be able to suppress the effective T cell responses against bacteria. Thus, over-expanded Treg may also help pathogens avoid the attack from the immune system.

Even with efficient thymic selection, some self reactive T cells are still exported to the periphery, where they are further deleted through activation-induced cell death (AICD). At the molecular level, Fas-FasL interaction is required for the AICD; in addition, defects of IL-2 signaling in vivo have also been associated with failure of activation induced cell death. AICD can be clearly exemplified by tracking the superantigen reactive T cells, which are further deleted in the periphery. In chapter 3, we demonstrate that activation induced cell death is dependent on the FoxP3\textsuperscript{+} regulatory T cells. In the FoxP3 deficient mice, superantigen reactive T cells are expanded in the periphery, although they are effectively deleted in the thymus. Such expansion is due to the lack of AICD, instead of hyperproliferation in vivo. Further adoptive transfer experiments reveal that regulatory T cells can induce the apoptosis of vSAG reactive T cells. More interestingly, Treg mediated cell death is specific to superantigen reactive T cells, which is due to the higher susceptibility of autoreactive T cells to activation induced cell death at least in vitro. An area of further study is how Treg facilitates the activation induced cell death in vivo at the molecular level.

Various models have been proposed about the mechanisms of Treg mediated suppression. Most of those studies are limited because they are either in vitro or similarly
based on transgenic T cells. In addition, elimination of Treg using antibodies also causes the deletion of activated T cells. In our study, Treg is eliminated all together by the genetic mutation of FoxP3, and autoreactive T cells are tracked at a polyclonal level. Our findings suggest that Treg functions in vivo by eliminating autoreactive T cells through AICD. Our data also suggest that the specificity of Treg is mainly determined by the targets vulnerability to apoptosis.

Low degree self reactivity is essential for the survival and function of naïve T cells and thus is unavoidable under physiological conditions. Such self reactivity can be provoked to immune responses against self antigens under some conditions, including viral infection and lymphocyte depletion. In particular, in a lymphopenic environment, naïve T cells can be activated by self antigens through homeostatic proliferation. This suggests that under lymph-replete conditions, intraclonal competition among T cells may be the main mechanism to maintain their unresponsiveness to self antigens. Our data presented in Chapter 2 and 3 demonstrate that homeostatic proliferation plays vital roles in the lymphoproliferative disease found in Scurfy mice. The thymus of the Scurfy mice is prematurely atrophic, which is caused by the reduced proliferation of immature thymocytes. Such thymic involution is intrinsic to the FoxP3 mutation in the thymic epithelial cells. The importance of FoxP3 function in non-T cells is further supported by our observation that depletion of FoxP3 in the T cells lineage is not sufficient for severe autoimmune disease in the Scurfy mice. Associated with the thymus involution, output of naïve T cells from the Scurfy thymus is reduced, and the neonatal Scurfy mice are lymphopenic. The lymphopenia in the Scurfy mice elicits extensive homeostatic proliferation, which primes naïve T cells with low-reactivity against self antigens. Such
homeostatic proliferation is independent of the loss of Treg and is not due to the FoxP3 mutation in T cells. An area of further study is to illustrate how the homeostatic proliferation is synergized with the loss of Treg in the Scurfy mice.

Taken together, our work reveals multiple mechanisms of immune tolerance. Two genes are studied for their impact on immune tolerance to self antigens. Hopefully, this work will advance our understanding of the immune system and ultimately provide novel approaches to manipulate immune responses.


