2012-08-21

The Role of IL-2/IL-2R Signaling for Treg Cell Development, Homeostasis and Function

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THE ROLE OF IL-2-/IL-2R SIGNALING FOR TREG CELL DEVELOPMENT, HOMEOSTASIS AND FUNCTION

By

Guoyan Cheng

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

June 2012
A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

THE ROLE OF IL-2/IL-2R SIGNALING FOR TREG CELL DEVELOPMENT, HOMEOSTASIS AND FUNCTION

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CD4^+ Foxp3^+ regulatory T (Treg) cells belong to a distinct T cell lineage which develops in the thymus and is essential for the prevention of self-reactivity by suppressing peripheral auto-reactive T cells that escape thymic negative selection. IL-2/IL-2R signaling is crucial and non-redundant for the development of thymic Treg cells, as well as the homeostasis and competitive fitness of peripheral Treg cells. The central role of IL-2 in Treg biology is exemplified by the uncontrolled massive lymphoproliferation associated with IL-2^-/-, IL-2Ralpha^-/- and IL-2Rbeta^-/- mice which typically die by 4-12 week of age. It is noteworthy that a restored normal percentage and number of peripheral Treg cells in Bim^-/- IL-2^-/- mice did not rescue these mice from severe autoimmunity. Instead, additional IL-2 was still required for the proper functioning of peripheral Bim^-/- IL-2^-/- Treg cells. Consistently, in the current studies, we found that the development of thymic Treg cells was blocked with mostly CD4^+ CD25^- Foxp3^- T cells in the IL-2Rbeta^-/- mice, and these cells were mainly defective for functional maturation. However, weak IL-2Rbeta signaling associated with IL-2Rbeta mutant mice largely supported normal Treg development and function in the thymus. Further examination indicated that the contribution of this weak IL-2Rbeta signaling was associated with pSTAT5 activity.

Although low IL-2 signaling is sufficient for programming mature and functional thymic Treg cells prior to their populating the periphery, aged IL-2Rbeta mutant mice
bearing weak and transient pSTAT5 signal develop autoimmunity by involving T cell infiltration into tissue sites. In addition, gene array analysis of peripheral Treg cells with lower IL-2R signaling suggested that the expression of some molecules is IL-2 dependent, such as KlrG1. We found that KlrG1 marked a small fraction of peripheral Treg cells. They were highly activated, antigen-responsive and short-lived. KlrG1+ Treg cells were also potentiated with superior suppressive function and represented a terminally differentiated subset derived from KlrG1- Treg precursors. Importantly, the development of KlrG1+ Treg subset required extensive IL-2R signaling. This activity of IL-2 is distinct from its contribution to Treg homeostasis and competitive fitness. Overall, these properties are analogous to KlrG1 marked terminally differentiated short-lived CD8+ T effector cells. Thus, a pathway driving antigen activated conventional T lymphocytes is also similarly co-operated by Treg cells.
DEDICATION

I’d like to dedicate this work to my parents, parents-in-law and my husband for their
great love, support and encouragement, as well as to my lovely daughter Elyshia. I could
not have accomplished this without all of you.
ACKNOWLEDGEMENTS

First, I would like to sincerely express my appreciation for my mentor, Dr. Thomas R. Malek, who has inspired, aided and guided me in scientific research for the past four years, during which I was able to gain experience and credentials as a scientist.

At the same time, I would also like to thank everyone on the committee: Dr. Richard Riley, Dr. Robert Levy, Dr. Alberto Pugliese and Dr. Jacob McCauley. Thanks to all of you for spending your precious time reviewing my proposal and progress reports as well as annually attending these meetings.

To Dr. Abul K. Abbas, thank you for being my external examiner at this dissertation defense.

Thank you everyone who works or worked in Dr. Malek’s lab: Aixin Yu, Xiaomei Yuan, Iris Castro, Michael Dee and Jing Yang. All your support and assistance during these years are very, very appreciated. Thank you to the people working in the Diabetes Research Institute: Allison Bayer, Cecilia Cabello and Oliver Umland; and the Flow Lab: Jim Philips, Jay Enten and Shannon Opiela for all your patience and help. Also, thank you, Dr. Eckhard Podack and Matthew Tsai, for your cooperation related to this work.

Last but not least, thank you to our graduate program in the Department of Microbiology and Immunology, especially Michelle M. Perez and Dr. Mathias G. Lichtenheld, and all the friends I have made here. You make my graduate life wonderful!
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<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>B6</td>
<td>C56BL/6</td>
</tr>
<tr>
<td>Blimp-1</td>
<td>B lymphocyte induced maturation protein 1</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CNS</td>
<td>Conserved non-coding DNA sequence</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>γc</td>
<td>Common gamma chain</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-2R</td>
<td>Interleukin -2 receptor</td>
</tr>
<tr>
<td>IL2-IC</td>
<td>IL-2/JES61A12 complex</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>INFγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>Idd</td>
<td>Insulin-dependent diabetes</td>
</tr>
<tr>
<td>IDD3</td>
<td>NOD&lt;sup&gt;86IDD3&lt;/sup&gt;</td>
</tr>
<tr>
<td>iTreg</td>
<td>Induced Treg</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>Klrg1</td>
<td>Killer cell lectin like receptor G1</td>
</tr>
<tr>
<td>LN</td>
<td>Lymph node</td>
</tr>
<tr>
<td>LP</td>
<td>Lamina propria</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>Nrp-1</td>
<td>Neuropilin-1</td>
</tr>
<tr>
<td>nTreg</td>
<td>Natural Treg</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2, 3-dioxygenase</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>PLN</td>
<td>Pancreatic lymph node</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer's patches</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>SOCS1</td>
<td>Suppressor of cytokine signaling 1</td>
</tr>
<tr>
<td>STAT5</td>
<td>Signaling transducer and activator of transcription 5</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Teff</td>
<td>T effector</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Treg cell</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSDR</td>
<td>Treg-specific demethylated region</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Y0</td>
<td>IL-2Rβ&lt;sup&gt;WT&lt;/sup&gt;</td>
</tr>
<tr>
<td>Y1</td>
<td>IL-2Rβ&lt;sup&gt;Y341&lt;/sup&gt;</td>
</tr>
<tr>
<td>Y2</td>
<td>IL-2Rβ&lt;sup&gt;Y395,498&lt;/sup&gt;</td>
</tr>
<tr>
<td>Y3</td>
<td>IL-2Rβ&lt;sup&gt;Y341,395,498&lt;/sup&gt;</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
Chapter 1
INTRODUCTION

1.1 The biological relevance of IL-2/IL-2R signaling in T cells

Interleukin-2 (IL-2), a soluble hormone-like cytokine, is mainly produced by activated CD4^+ T cells after sensing the signals from the T cell receptor (TCR) and co-stimulatory receptors. The biological functions of IL-2 are manifested in multiple aspects, e.g. the development and homeostasis of regulatory T cells, the generation of effector memory T cells, all of which are achieved by IL-2 interacting with its high affinity IL-2 receptor (IL-2R). IL-2R is a trimeric molecule, containing IL-2Rα (also known as CD25), IL-2Rβ (i.e. CD122 and shared with IL-15R) and the common cytokine receptor γ-chain (γc, i.e. CD132 and shared by the receptors of all members of the γc cytokine family). Initial antigen activation via TCR leads to the up-regulation of IL-2Rα on T cells and the interaction between IL-2 and IL-2Rα promotes the association of this binary complex to IL-2Rβ. The ternary complex IL-2Rα/IL-2/IL-2Rβ then recruits γc through a weak interaction with IL-2 and a stronger interaction with IL-2Rβ to produce a stable quaternary high-affinity IL-2R [1, 2].

Tyrosine kinases, including Janus kinase 1 (JAK1) and JAK3, are then recruited and closely bind to the cytoplasmic tails of the IL-2Rβ and γc chains, leading to the phosphorylation of JAKs. These activated JAKs further phosphorylate the tyrosines located on the cytoplasmic tail of the IL-2Rβ chain, and this eventually activates three main intracellular signaling pathways (Figure 1.1). They are MAPK (mitogen-activated protein kinase), PI3K/AKT (phosphatidylinositol 3-kinase/AKT) and STAT5 (signal
transducer and activator of transcription 5) pathways. MAPK and PI3K/AKT pathways are primarily activated through the adapter protein Shc, which associates with the phosphorylated tyrosine residue (Tyr-338 in human and Tyr-341 in mouse) within the A-region of the IL-2Rβ chain. STAT5 pathway is related to the phosphorylated tyrosine 392, 510 in human or tyrosine 395, 498 in mouse located on the H region of the IL-2Rβ chain, which recruit and further phosphorylate the transcriptional factor STAT5 for activating STAT5 pathway. Collectively, these key tyrosines involved pathways contribute to IL-2 dependent T cell cycle entry, growth, survival, proliferation, differentiation, etc [3].

Figure 1.1
*IL-2/IL-2R associated signaling pathways in mouse T cells*

Although all T cells similarly utilize IL-2 for further signaling transduction, CD4⁺ Foxp3⁺ regulatory T (Treg) cells exhibit many fundamental properties that are different
from the activated CD4+ Foxp3− effector T (Teff) cells (Table 1.1). First, unlike the IL-2 producing Teff cells, Treg cells are not able to make IL-2 due to the repressive activity by the interaction between the Foxp3, Runx1, NF-AT and IL-2 transcriptional regulatory sites [4, 5]. However, the ability of Teff cells to produce IL-2 is also under control by turning off the transcription of IL-2 via two negative regulators which are the Tbet and Blimp-1 transcriptional factors [1, 3]. Second, IL-2Ra expression on Teff cells is very transient and at a lower level, while on Treg cells, it is highly apparently expressed probably due to the Foxp3 expression in Treg cells where it acts as a directly positive activator for the IL-2Ra gene [6]. Third, since a higher level of PTEN (phosphatase and tensin homolog) (Figure 1.1) is expressed in Treg cells, they poorly activate the PI3K/AKT pathway [7, 8]. Therefore, the STAT5 pathway is actually predominant for Treg cells. Fourth, even with regard to the same pathway, the biological effects may differ in Treg cells vs. Teff cells. For example, after translocation into the nucleus, the phosphorylated dimerized STAT5 binds to the promoter of Foxp3 gene to initiate the Foxp3 expression in Treg cells [9]. However, in Teff cells, STAT5 promotes the production of INFγ or IL-4 for the development of Th1 or Th2 [10, 11].

Table 1.1
Comparison of CD4+ Treg and CD4+ Teff cells

<table>
<thead>
<tr>
<th>Property</th>
<th>Treg</th>
<th>Teff</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 production</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-2 repression</td>
<td>Foxp3/Runx/NF-AT</td>
<td>Tbet, Blimp-1</td>
</tr>
<tr>
<td>High affinity IL-2R</td>
<td>Constitutive</td>
<td>Transient</td>
</tr>
<tr>
<td>IL-2R signaling</td>
<td>STAT5</td>
<td>MAPK, PI3K/AKT, STAT5</td>
</tr>
<tr>
<td>Activity for development</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Peripheral homeostasis</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Growth and survival</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Function</td>
<td>Suppression</td>
<td>Th1, Th2</td>
</tr>
</tbody>
</table>
1.2 Treg cell and its lineage stability and plasticity

1.2.1 Recognition of Treg cells

In 1995, Sakaguchi and his colleagues reported that a small population of CD4 T cells marked with high CD25 expression exhibited a dose dependent regulation of autoimmunity which was induced by an inoculation of CD4+ CD25− T cells in nu/nu mice [12]. However, a unique marker to distinguish Treg cells from activated CD4+ CD25+ Teff cells was not identified until early this century. In 2001, several groups noticed that, similar to the IL-2/IL-2R deficient mice, severe autoimmunity was also observed in the naturally occurring X chromosome-linked scurfy mutant mouse [13] as well as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) patients [14, 15]. Further analysis found that both species contained the same mutated target gene called Foxp3. Thus, studies from either selective deletion of Foxp3 in CD4+ Foxp3+ T cells or enforced expression of Foxp3 in CD4+ CD25− T cells indicated that Foxp3, a X-chromosome-linked intracellular transcriptional factor, is required for programming a normal profile of Treg cells and is a reliable hallmark for Treg cells, especially in the mouse [16-18].

1.2.2 Treg cells exhibit instability and plasticity

Although CD4+ Foxp3 expressing Treg cells belong to an independent cell lineage, the fate of these cells is not fixed but plastic. This is illustrated by the observation of gain and loss of Foxp3 on Treg cells under certain circumstances. One piece of evidence was from the study of crossing transgenic Foxp3-GFP-Cre mice with Rosa26-loxP-Stop-loxP-YFP mice, where the expression of a green fluorescent protein (GFP)-Cre recombinase
fusion protein was controlled by Foxp3 promoter and a yellow fluorescent protein (YFP) under the Rosa26 promoter expressed only after excision of a loxp-flanked stop cassette by Cre. Thus, this model enabled monitoring the up- and down- regulation of Foxp3 concurrently by examining three defined Treg populations, including GFP+ YFP− marked recently developed Foxp3+ Treg cells, GFP+ YFP+ marked stable Treg cells, and GFP− YFP+ marked ‘exTreg’ cells which lost the GFP linked Foxp3 expression after the GFP+ YFP+ stage [19]. Moreover, these unstable exTreg cells (~20% in total Treg cells) were plastic by shifting their initial regulatory identity into an activated and memory phenotype with a lower expression of Treg signature markers like CTLA4 (Cytotoxic T-lymphocyte antigen 4), but a higher expression of CD44 and enhanced production of inflammatory cytokine like INFγ. By further crossing Foxp3-GFP-Cre mice x Rosa26-YFP mice with BDC2.5 TCR transgenic mice where pancreatic islet auto-antigen can be recognized, around 2-fold more ‘exTreg’ cells were found in the pancreatic lymph nodes [19]. Therefore, the autoimmune environment favors the generation of ‘exTreg’ cells.

Additional evidence showing the unstable and plastic property of Treg cells was obtained from an adoptive transfer experiment where a small population enriched in CD25− Foxp3+ Treg cells lost the expression of Foxp3 after transferring into either lymphopenic or lymphoreplete mice. These de-differentiated Treg cells also exhibited Teff functions by producing INFγ and IL-17. Interestingly, some of these de-differentiated unstable Treg cells re-upregulated the expression of Foxp3 upon TCR stimulation and re-differentiated into Foxp3+ Treg cells. However, when similar experiments were performed by using CD25high Foxp3+ Treg cells, no conversion was
observed after multiple cell divisions and they were much more stable than their counterparts. [20].

Not only the CD25\(^{-}\) Foxp3\(^{+}\) cells potentially undergo conversion from Treg lineage into Teff cells, Foxp3 attenuated Treg cells also exhibit certain plasticity. By knocking in a gene cassette encoding IRES-luciferase-IRES-eGFP into the 3'-UTR (un-translated region) of the endogenous Foxp3 locus, a Foxp3-IRES-luciferase-IRES-eGFP (FILIG) mouse was generated. Lower Foxp3 expression was detected in these GFP linked Treg cells of the heterozygous FILIG female mice and hemizygous FILIG male mice. Furthermore, these Foxp3\(^{lo}\) Treg cells exhibited impaired immunosuppressive activity followed by decreased expression of Treg signature genes. Eventually, by producing IL-4, these low Foxp3 expressing Treg cells converted into a Th2 lineage [21].

1.2.3 Regulation of Treg cells stability and plasticity

Foxp3 on/off and the conversion between Treg and Teff is actually common. However, the stability and plasticity of Treg cells are not just correlated with phenotypically lower CD25 or Foxp3 expression, but also regulated under several levels. Analysis at the gene expression and transcriptional regulation levels provided some hints of the transition between one lineage to another. For example, methylation of CpG islands in the Treg cell–specific demethylated region (TSDR) of Foxp3 is over 90% in naive CD4\(^{+}\) T cells but both natural Treg (nTreg) cells and in vivo generated induced Treg (iTreg) cells exhibit fully unmethylated status of the same region [22-24]. ‘exTreg’ cells from the Foxp3-GFP-Cre x R-26-YFP transgenic mice exhibited a varied pattern of methylation status where 74% of the DNA strands had over 85% of methylated CpG sites
in the TSDR and 11% had over 85% of CpG islands unmethylated, moreover, 13% had partial methylation with 15–85% of the CpG sites being unmethylated [19]. Thus, the observation of unstable Treg cells is not just reflected to the phenotype or functional changes but also to the altered regulation at gene level.

MicroRNAs are small non-coding RNAs, regulating the gene expression involved with development, homeostasis, differentiation, etc [25]. The contribution of microRNA to Treg cells stability has been recently examined by analyzing the Foxp3-specific Dicer-knockout mice where ‘exTreg’ cells were accumulated with loss of proper suppressive activity in vivo [26]. By further examining microRNA155-deficient mice, mechanistic studies suggested that Foxp3-driven microRNA155 limited the expression of SOCS1 (suppressor of cytokine signaling 1) which is known to repress the IL-2 dependent STAT5 activity [27]. Consequently, lack of miRNA155 releases the inhibitory factor of SOCS1 to block the normal IL-2R signaling in Treg cells [28].

In addition to the above mentioned mechanisms, cytokine signals also play a crucial role to impact the polarization of activated T cells and conversion of alternative cell fates. Antigen experienced CD4+ T cells co-expressed RORγt and Foxp3 transcription factors during an intermediate stage. However, in vitro analysis showed that IL-6 combined with TGFβ favored the generation of Th17 cells, while IL-2 with TGFβ promoted the induction of Treg cells [29]. Moreover, IL-2 supplement prevented these iTreg cells from de-differentiating into Teff cells [30, 31], probably due to the repression of RORγt transcription under a strong IL-2 signaling. Overall, Treg cells are not fixed but flexible in their lineage status. Instability and plasticity of these cells can be regulated and
controlled at multiple levels, such as environmental cues, transcriptional regulation and modification.

1.3 Heterogeneity of Treg cells

1.3.1 Treg subsets distinguished by anatomic sites where they develop

Analogous to the conventional CD4$^+$ T cells which are divided into Th1, Th2 and Th17, Treg cells are also heterogeneous with regard to several aspects. First, depending on the anatomic sites where Treg cells arise, they have been divided into nTreg originally from the thymus and iTreg cells converted from peripheral activated CD4$^+$ T cells. Both subsets somewhat vary in the pattern they develop and the specificity they exhibit [32, 33]. Based on TCR repertoire analysis, it is estimated that 5-20% of total peripheral Treg cells may be iTreg cells [34, 35]. However, iTreg cells seem to possess a much higher frequency in the gut where environmental TGFβ and retinoid acid as well as commensal bacteria derived local antigens constitutively elicit the conversion from Teff to iTreg cells [36, 37]. For example, after retrovirally transducing colonic Treg TCRs into immature Foxp3$^{GFP}$ Rag1$^{−/−}$ thymocytes, very poor thymic Treg cell development was initiated. In contrast, Treg TCRs normally found in other peripheral locations readily developed in the thymus. Therefore, TCRs from many colonic Treg cells were shaped extrathymically by encountering local antigenic and environmental milieu. Noticeably, if these colonic unique TCR failed to instruct colonic conventional T cells to convert into iTreg cells, potential colitis was induced in the adoptive transfer model [37]. Thus, nTreg cells are developed to recognize self-antigen while iTreg cells can respond to both self and foreign
antigens, consequently nTreg and iTreg cells are equally required for the full protection from immune challenge [38, 39].

However, it remains quite challenging to study iTreg and nTreg cells since no specific hallmark has been identified yet to clearly distinguish these two subsets. Helios, an Ikaros family transcription factor, and Nrp-1 (neuropilin-1), a type 1 trans-membrane protein, have been suggested as markers for iTreg cells [33, 40, 41], which are up-regulated on nTreg cells but down-regulated on iTreg cells. A recent study argues that the expression of Helios is more closely associated with cell activation and division instead of peripheral conversion from Teff to iTreg cells. Both in vitro and in vivo analysis indicated the correlation between Helios up-regulation and Treg cell proliferation, thereby making it a less useful marker to separate nTreg from iTreg cells [42]. Moreover, Helios deficient mice only showed minimal impact on T cell development and no significant impairment in Foxp3+ Treg cells [43]. Therefore, up to now, there is no common marker available to easily separate nTreg from iTreg cells, as well as to further understand the suppressive mechanisms which are similarly or differentially operated in both Treg subsets.

1.32 Treg subsets distinguished by surface activation markers

Utilizing surface activation or memory markers analogous to conventional T cells, several distinct Treg subsets have been reported. One subset is marked by CD103, expressed by 20-30% of splenic Treg cells. However, a much higher proportion of CD103+ Treg cells were found in the lamina propria of small intestine and some other tissue sites [44, 45]. The superior suppressive function of CD103+ Treg cells over CD103- Treg cells was suggested due to these cells accessibility and retention in the
places where infection or inflammation existed [46, 47]. Moreover, by co-culturing WT CD103$^+$ or CD103$^-$ Treg subset with HA-peptide specific TCR-transgenic CD4$^+$ CD25$^-$ T cells ex vivo, both Treg subsets suppressed the proliferation of responder T cells when soluble anti-CD3 antibodies were added as a polyclonal stimulator. However, when HA peptide was used to specifically stimulate CD4$^+$ CD25$^-$ responder T cells, only the CD103$^+$ subset was capable of suppressing proliferation directly ex vivo. Thus, CD103$^+$ Treg cells also exhibit an antigen non-specific suppression [48]. Just recently, another activation molecule named Klrg1 (Killer cell lectin-like receptor subfamily G, member 1) was also identified in a small population of CD4 T cells where Klrg1 marked CD4$^+$ CD25$^+$ cells were more potent than Klrg1$^-$ CD4$^+$ CD25$^+$ cells in suppressing responder T cells proliferation [49]. Klrg1 expression was initially identified in NK cells and virus activated CD8$^+$ T cells [50, 51]. The expression of Klrg1 on CD8$^+$ T cells exhibited an effector/memorphenotype and linked to a proliferative history after repetitive antigenic stimulation [52, 53]. Both CD103$^+$ and Klrg1$^+$ Treg subsets will be covered in later chapters of my thesis.

1.3 Treg subsets distinguished by chemokine receptors

Chemokine receptors have been utilized to identify specific Treg subsets since some Treg cells prefer to home and localize to distinct peripheral tissue sites. For example, CCR4$^+$ Treg cells are devoid in the thymic Treg compartment but contain a higher proportion in the skin. Consistently, Ccr4$^{-/-}$ Treg cells were not able to control of inflammation in skin [45]. In addition, CCR6 expressed Treg subset is also distinct from CCR6$^+$ Treg cells. The former is enriched in peripheral blood and exhibits an activated
effector-memory like phenotype by rapidly responding to antigen stimulation. An in vitro assay showed that, compared to the CCR6− Treg cells, CCR6+ Treg cells rapidly up-regulated the production of IL-10, a functional suppressive cytokine to control Teff cells. In an induced EAE (experimental autoimmune encephalomyelitis) mouse model, accumulated CCR6+ Treg cells were attracted to the central nervous system [54].

1.3.4 Treg subsets distinguished by transcriptional factors

Just like the association between specific transcriptional factor and specific T helper (Th) response, Treg cells similarly use these factors and co-opt these pathways to control the Teff cells. For example, a subset of Treg cells expressing Tbet is essential for suppressing the Th1 reaction [55]. And Treg cells expressing Irf-4 and Gata-3 are required for controlling the Th2 responses [56, 57]. Similarly, Stat3 expressed Treg cells efficiently control the inflammation caused by Th17 cells [58]. Over all, based on distinct phenotype, activity, homing and functional regulation, the heterogeneous property of Treg cells has been broadly examined. However, the relationship of different Treg subsets and the potential developmental progression of Treg cells have not been studied in detail. Studies in this dissertation research investigate interrelationship between Treg subsets which are separated by surface activation markers.

1.4 Mechanisms of Treg suppressive function

1.4.1 Direct suppressive action on responder T cells

Suppressive function of Foxp3+ Treg cells has been proposed to carry out via multiple mechanisms by directly or indirectly acting with responder T cells. One well defined
mechanism is through the inhibitory cytokines TGFβ and IL-10. This is supported by the mice where IBD (inflammatory bowel disease) like autoimmunity is resulted due to impaired production or processing of TGF-β in T cells [59, 60]. Further evidence is manifested by reversing the detrimental Teff response after adoptive transferring of TGF-β produced Treg cells [59]. Interestingly, the production of IL-10 by Treg cells is associated with TGF-β because anti-TGFβ antibody treatment failed to generate IL-10 producing Treg cells in the intestine-associated lymphoid compartment [61] where IL-10 is an essential immuno-regulator to inhibit pro-inflammatory cytokines. Most recently, by specifically knocking out IL-10R in Foxp3+ Treg cells, a severe intestinal inflammation resulted due to selective dysregulation of Th17 responses [62]. In addition to TGFβ and IL-10, other inhibitory cytokines like IL-35 and galectin-1 have also been suggested to function in suppressing responder T cells. IL-35 was reported to directly act on responders [63], and galectin-1 potentially interferes with Treg cell-DC (dendritic cell) or Treg cell-T cell interactions [64]. Overall, these effects eventually lead to cell cycle arrest and apoptosis of T responders, as well as inhibition of pro-inflammatory cytokines production [65].

Another mechanism of suppression by Treg cells is via granzyme B induced cytolysis. It has been shown that granzyme B is up-regulated in activated Treg cells and granzyme B deficient Treg cells are not competent for suppression in vitro [66]. A study in tumor microenvironment has recently found that Treg cells could attack CTLs (cytotoxic T lymphocytes) in a granzyme B- and perforin-dependent manner [67].
1.42 Indirect suppressive action by antigen presenting cells on responders

Along with direct suppressive action on responder T cells, several other mechanisms are mediated by affecting the APC (antigen presenting cell). First, interaction between CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) on Treg cells and CD80 and CD86 on DC transmits an inhibitory signal in DC by generating IDO (indoleamine 2,3-dioxygenase), a potent regulatory molecule inducing the catabolism of tryptophan into proapoptotic metabolites and thus suppressing the activation of Teff cells [65]. Second, accumulation of extracellular ATP due to cell damage and tissue destruction is suggested to stimulate and activate DC. Therefore, catalytic digestion of ATP to ADP to AMP by CD39 expressed on Treg cells is another potential anti-inflammatory mechanism by blocking the activation of DC [68]. Moreover, by examining CD39<sup>+</sup> Treg cells, it has been shown that they were anergic and less efficient in suppressing responder T cells [69]. Indeed, this immuno-regulatory effect by CD39 through ATP clearance can even be amplified by another ecto-enzyme called CD73, which further dephosphorylates AMP into adenosine [69] to inhibit the functions of DC and/or to act on activated T cells via the A2A adenosine receptor to suppress the subsequent response. Some other surface molecules expressed on Treg cells, like LAG3 (CD223) and Nrp-1, may also contribute to the regulatory mechanisms applied by Treg cells to decrease antigen presentation via DC [65].

1.43 Suppressive action via transcriptional regulation

More recent studies, at a transcriptional level, further illustrated how Treg cells specifically inhibit distinct Teff responses (Figure 1.2). For example, the key
transcription factors T-bet, Irf-4, Gata-3 and Stat3 respectively orchestrating Th1, Th2 and Th17 cells differentiation are indispensable for Treg cells to suppress the correspondent Teff response [55-58]. Foxp3, enforcing the Treg suppressive program, up-regulates these distinct transcriptional factors in Treg cells, and ablation of these factors selectively in Treg cells leads to severe inflammation which is sometimes fatal. Nevertheless, regulated via multiple mechanisms, Treg cells are crucial suppressors in the adaptive immune system.

**Figure 1.2**  
*Treg suppression mediated by transcriptional factors for Teff cells*

### 1.44 Suppressive action in innate immune system

Treg cells exhibit their regulatory activities not only in the adaptive immune system but also in the innate immune system where they can target macrophages, mast cells, natural killer (NK) cells, etc [65]. For example, Treg cells inhibited degranulation of mast cells by co-culturing both cell types in vitro. Immunohistochemical staining further indicated the co-localization of Treg cells and mast cells in vivo. By utilizing the FceRI mediated acute systemic anaphylaxis model, interaction between OX40 receptor expressed on Treg cells and OX40 ligand expressed on mast cells was suggested to cause the inhibition of histamine release from the mast cells. Consistently, in vivo depletion or inactivation of Treg cells deteriorated the anaphylactic reaction by mast cells [70]. Thus,
the regulatory function of Treg cells is not limited in response to adaptive immune challenges, but also plays a non-redundant role in the innate immune reactions.

1.5 Role of IL-2/IL-2R signaling for Treg cells

For a long period of time, IL-2 was considered as a key cytokine for T cell clonal expansion. However, IL-2−/− mice [71], as well as IL-2Rα−/− and IL-2Rβ−/− mice [72, 73], all exhibit autoimmune symptoms with massive lympho-proliferation. Thus, impaired IL-2/IL-2R signaling clearly did not cause the lack of T cell expansion in these mice. One explanation may be an altered balance between the expansion and activation-induced deletion of auto-reactive T cells, and another possibility is the lack of negative control of those activated and expanded T cells. Followed by an adoptive transfer of CD4+ CD25+ T cells, a dose dependent immunoregulatory function by these donor cells was exhibited in mice bearing rapid T cell proliferation and autoimmune response [12]. Therefore, the latter idea turns to be quite possible. More direct supporting evidence is from adoptive transfer of wild type (WT) CD4+ CD25+ T cells into those neonatal IL-2Rβ−/− mice [74]. Strikingly, only after a singular transfer of this particular T cell subset, IL-2Rβ−/− mice can live a healthy life and as long as normal mice. This study also initiated the exploration of the role of IL-2/IL-2R signaling for regulatory CD4+ CD25+ T cells since these regulatory cells were greatly diminished in all of the IL-2−/−, IL-2Rα−/− and IL-2Rβ−/− mice.

1.5.1 Role of IL-2/IL-2R signaling for thymic Treg cells

Treg cells mainly develop in the thymus during the CD4 single positive stage. Few of them are also found in the double positive population but flow cytometry analysis
(FACS) indicated that these are mostly doublets including CD4+ CD8+ Foxp3− and CD4+ Foxp3+ cells [75]. Anatomically, Treg cells are preferentially detected in the thymic medulla since IL-2 is readily produced in the cortical medullary junction and medulla [1]. Although the production of IL-2 is very rapid and transient in vivo, the role of IL-2/IL-2R signaling for Foxp3+ Treg cell development in thymus has been supported by different mouse models and experiment settings. First, the frequency and number of thymic CD4 single positive Foxp3+ Treg cells are reduced around 50% in the IL-2−/− and IL-2Rα−/− mice [6]. Second, IL-2Rβ−/− mice, but not IL-15−/− or IL-15Rα−/− mice [76, 77] which also utilize IL-2Rβ chain for signaling, are devoid of a normal compartment of thymic Treg cells. Third, abrogation of IL-2 and IL-7 signaling almost completely blocks the development of thymic Treg cells [78, 79]. However, IL-7 functions early during the thymopoiesis [2], probably in the double negative stage while generation of thymic Treg cells is relatively delayed, around day 3 after birth [80]. Therefore, IL-2/IL-2R signaling is superior in controlling the proper thymic Treg cells development. Fourth, transgenic expression of WT IL-2Rβ in the background of IL-2Rβ−/− mice successfully rescues the Treg compartment in thymus [81]. Similarly, when IL-2 was restored in the IL-2−/− mice, Treg cells were reconstituted [82]. Furthermore, IL-2 neutralizing antibody reduced the pool of thymic Treg cells in neonatal WT mice [83]. Fifth, IL-2 associated STAT5 signaling is involved in the induction of Foxp3 expression, and a high level of Foxp3 expression is required for Treg lineage commitment during the process of thymic Treg development [17, 18, 21, 84, 85].

Several years ago, one potential mechanism was proposed for thymic Treg cells development [86, 87], which was named ‘two-step’ model. Briefly, TCR signaling first
triggers the up-regulation of CD25 on CD4⁺ CD25⁻ Foxp3⁻ thymocytes, and then IL-2 dependent STAT5 signaling induces CD25⁺ Foxp3⁻ precursor cells to differentiate into CD25⁺ Foxp3⁺ Treg cells. More recently, based on our current understanding of thymic Treg cells, an alternative but similar developmental model was also hypothesized where, at the single CD4 stage, it is the proper IL-2 signaling that determines the differentiation of thymic CD4⁺ CD25lo Foxp3lo immature Treg cells into CD4⁺ CD25hi Foxp3hi mature Treg cells. However, the role of TGFβ in thymus is not Treg developmental related but more associated with anti-apoptosis of those self-antigen experienced Treg precursor cells by up-regulating Bcl-2 and down-regulating Bim [2]. All together, these studies support an idea that IL-2/IL-2R signaling plays an important and non-redundant role in the development of thymic Treg cells.

1.52 Role of IL-2/IL-2R signaling for peripheral Treg cells

IL-2/IL-2R signaling is essential for homeostasis and competitive fitness of Treg cells in the periphery. Direct evidence was obtained from the WT mice treated with IL-2 neutralizing antibody, IL-2⁻/⁻, IL-2Rα⁻/⁻ and IL-2Rβ⁻/⁻ mice where the number and proportion of Foxp3⁺ Treg cells were decreased in peripheral lymphoid compartments [6, 81, 83]. In contrast, recombinant IL-2 supplement to IL-2⁻/⁻ mice potentiated the expression of CD25 and Foxp3 on Treg cells [6]. Gene array analysis by comparing Treg cells from untreated and IL-2 treated IL-2⁻/⁻ mice indicated that genes associated with cell cycle control, cell growth, proliferative self-renewal and metabolic fitness of peripheral Treg cells were IL-2 dependent [6]. Previous studies from our lab also demonstrated that bone marrow cells from mice bearing thymic restricted expression of IL-2Rβ in the
background of IL-2Rβ−/− could normally reconstitute the thymic compartment but the reconstitution was very poor in the periphery where IL-2Rβ signaling was substantially impaired [88].

Since aged mice accumulated a relatively higher amount of Treg cells in the periphery, recent studies indicated that this observation was due to prolonged life span of peripheral Treg cells by down-regulating the expression of proapoptotic molecule Bim [89]. Bim deficient IL-2−/− or IL-2Rα−/− mice were most recently generated. Further examination of the Treg compartment in these double knockout mice indicated that the amount of Treg cells was successfully restored [90]. However, by abrogating the expression of Bim, rescued Treg cells in periphery were not competent to prevent the autoimmune diseases associated with IL-2−/− or IL-2Rα−/− mice. Therefore, IL-2/IL-2R signaling is required for not only proper development and homeostasis of Treg cells but also their efficient suppressive function. This idea is further illustrated by IL-2 treated IL-2−/− Bim−/− mice where a progressing autoimmune disease was prevented and even reversed after IL-2 provision [90]. Additional evidence for supporting the functional role of IL-2 in peripheral Treg cells also comes from another transgenic mouse model where IL-2Rβ signaling is greatly dampened in the periphery. Gene array analysis of splenic Treg cells from these mice indicated that they are defected in the expression of Il-10 and Gzmb [91] which participate in the suppressive activity to Treg cells.

1.53 Role of IL-2/IL-2R signaling for iTreg cells

In addition to nTreg cells, iTreg cells also depend on optimal IL-2 signaling for proper development. First, CD4+ Foxp3+ T cells from IL-2−/− mice were not able to
convert into induced CD4⁺ Foxp3⁺ Treg cells after anti-CD3 and anti-CD28 stimulation [92]. Second, in vitro IL-2 supplement in combination with TGFβ solidified the commitment of iTreg cells. Moreover, addition of IL-2 prevented in vitro induced Treg cells from de-differentiating into Teff cells [30, 31]. Third, conventional CD4⁺ T cells with lower IL-2Rβ signaling were also not able to convert into iTreg cells after adoptive transferring into lymphopenic mice (recent observation from Malek’s lab).

Collectively, IL-2/IL-2R interactions provide essential signals for Treg cells in terms of the thymic development, peripheral homeostasis, competitive fitness, regulatory function as well as the conversion from the non-Treg cells to iTreg cells.

1.6 Sub-optimal IL-2/IL-2R signaling and Treg cells

Normal development, homeostasis and function of Treg cells require IL-2/IL-2R interaction to provide proper signaling. Abrogation of either IL-2 or IL-2R substantially impairs the Treg compartment and eventually leads to massive systemic autoimmune responses in these knock-out mice. However, recent studies from our lab indicated that sub-optimal IL-2R signaling is largely able to support Treg cells in thymus and periphery [88, 91], which is indicated by proper cellularity of Treg populations in thymus and peripheral lymphoid compartments as well as largely normal phenotype of these low IL-2 signal bearing mice.

The initial evidence is derived from the transgenic IL-2RβWT/Thymus mice where the expression of transgenic WT IL-2Rβ chain is controlled by the proximal lck promoter and preferentially expressed in the thymus of IL-2Rβ⁻/⁻ mice. IL-2RβWT/Thymus mice are able to live a healthy life although T cells in the periphery are theoretically non-
responsive to IL-2. Moreover, after thymectomy of these adult mice followed with the loss of consistent thymic output, no severe autoimmune symptoms were shown and a normal frequency and amount of peripheral Treg cells were retained [88]. Therefore, a normal peripheral Treg compartment may not be completely a result of thymic output in these IL-2Rβ\textsuperscript{WT/Thymus} mice. Further analysis of these Treg cells from the periphery indicated that STAT5 signaling was not completely blocked, instead, a weak and transient STAT5 activity was detected. This result is consistent that low IL-2 signaling within peripheral Treg cells from IL-2Rβ\textsuperscript{WT/Thymus} mice may support normal homeostasis in periphery [91].

Further evidence that low IL-2R signaling supports Treg cells has been recently shown by another transgenic mouse model created in our lab (Table 1.2). These mice, in the background of IL-2Rβ\textsuperscript{-/-}, were constructed to contain either WT IL-2Rβ chain or 1 (Y341), 2 (Y395 and Y498) and 3 key tyrosine (Y341, Y395 and Y498) mutation(s) [91] in cytoplasmic tail of the IL-2Rβ chain. Tyrosine 341, 395 and 498 are phosphorylated sites for optimal IL-2/IL-2R signaling. The mutations from tyrosine to phenylalanine (F) retain the structure of IL-2Rβ chain but interrupt the normal IL-2R signal transduction. Consequently, all of these transgenic IL-2Rβ subunits were specifically expressed in T cell lineage, and respectively named Y0, Y1, Y2 and Y3 mice in the following studies.
These IL-2Rβ mutant mice with titrated IL-2Rβ signaling are largely able to maintain a normal Treg compartment with a comparable proportion and number of Treg cells expressing high level of Foxp3 in both thymus and peripheral lymph nodes. Although Y2 and Y3 mice are impaired in STAT5 signaling and only contain weak and transient STAT5 activity, just like Y0 and Y1 mice, they are phenotypically normal and healthy. However, it is noteworthy that as Y2 and Y3 but not Y1 mice age, they exhibit autoimmune symptoms manifested by local T cell infiltration in peripheral tissue sites [91]. Gene array analysis by comparing the Treg cells bearing normal and low IL-2R signaling has suggested that, unlike the data from IL-2−/− Treg cells where targeted genes are more related to homeostasis and competitive fitness [6], striking difference within these Treg cells, at the mRNA level, is reflected in the varied expression of several molecules, e.g. Klrg1 and CD103 [91].

Therefore, the low IL-2 signaling largely maintains the thymic and peripheral Treg population with normal Foxp3 expression. However, some specific properties of Treg cells may require strong IL-2 signaling. For example, diminished mRNA levels of Klrg1

### Table 1.2
*Mutant IL-2Rβ transgenic mice*

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Extracellular TM</th>
<th>Cytoplasmic</th>
<th>Pathway affected</th>
<th>STAT5 activation</th>
<th>Treg cells</th>
<th>Lethal disease</th>
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<td>Yes</td>
<td>No</td>
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<tr>
<td>IL-2Rβ&lt;sup&gt;341&lt;/sup&gt; (Y1)</td>
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<td>Yes</td>
<td>No</td>
<td>No</td>
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<tr>
<td>IL-2Rβ&lt;sup&gt;395,498&lt;/sup&gt; (Y2)</td>
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<td>FY Y</td>
<td>STAT5</td>
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<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>IL-2Rβ&lt;sup&gt;341,395,498&lt;/sup&gt; (Y3)</td>
<td>S A H</td>
<td>FY F</td>
<td>She/STAT5</td>
<td>Weak</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
or CD103 in Treg cells with low IL2-Rβ signaling raises the possibility that specific Treg subsets marked by these molecules may depend on strong IL-2 signaling. Moreover, impaired development of these IL-2 signaling dependent Treg subsets may further account for the autoimmunity seen in aged Y2 and Y3 mice.

1.7 IL-2/IL-2R signaling and autoimmune diseases

Type 1 diabetes (T1D) is the most common autoimmune disorder in childhood, but it may manifest at any age, even in senior people [93]. The disease results from the autoimmune destruction of insulin-producing β cells in the pancreatic islets of Langerhans, and is characterized by a loss of blood glucose homeostasis accompanied by inflammatory infiltrates in the islets. Insulin-dependent diabetes (Idd) loci are linked to disease susceptibility [94] in both humans and Non-Obese Diabetic (NOD) mice which develop T1D spontaneously and become a very useful animal model for dissecting the pathogenesis of T1D. As one of the loci contributing to breakdown the tolerance in NOD mice, Idd3 locus is located on chromosome 3 and harbors five known genes, Tenr, Iil2, Il21, Cetn4, and Fgf2, and two predicted genes of unknown function [95].

Among these, Iil2 and Il21 are strong candidates for the contribution to this disease in NOD mice. The main effect of IL-21 is to drive the pathogenic Teff cells to fuel the impaired T cell compartment [96] and to counteract the suppressive function of Treg cells [97]. However, IL-2 may have stronger association to T1D. First, based on the correlation between suppressive potential of peripheral Treg cells and IL-2 signaling [90], Treg cells from 16-week-old NOD mice were less suppressive in vitro and in vivo experiments than those from 8-week-old mice, and this decline in Treg function is a crucial step in
determining the fate of the disease process in T1D development [98]. This similar trend was also shown in the studies from human T1D [99, 100]. Accordingly, a single copy of Idd3 allele from diabetes-free WT B6 mice (Idd3<sup>WT</sup>) in the NOD background strikingly reduced the incidence of T1D [101, 102] and helped to transcribe 2-fold more of IL-2 at the mRNA level while demonstrating no effect on IL-21. Thus, the increased production of IL-2 in vivo facilitates the Treg activity in inflamed sites [103]. In addition, the effect of Idd3<sup>WT</sup> allele prolongs the islet allograft survival [104]. Recently, two groups have shown that diabetes can be prevented or even reversed after IL-2 therapy [105, 106].

Large-scale case-control studies suggested that single nucleotide polymorphisms in human CD25 (IL-2Rα) gene are associated with T1D [107], and severe autoimmunity has been observed in patients with mutations in CD25. Moreover, owing to fewer Treg cells with less suppressive activity, IL-2 gene polymorphisms also contribute to autoimmune susceptibility [1]. Polymorphisms in Il-2<sup>ra</sup>, Il-2<sup>rb</sup> and Il-2 are potential risk factors for other autoimmune diseases, like Grave’s disease, multiple sclerosis, rheumatoid arthritis and celiac disease [108]. Therefore, how weak IL-2/IL-2R signaling regulates Treg cells and to what extent low IL-2 signal contributes to autoimmune responses is still not fully understood.

1.8 Statement of research purpose

IL-2/IL-2R signaling is non-redundant for thymic Treg cells development as well as peripheral Treg cells homeostasis. However, our recent findings suggest that low IL-2/IL-2R signaling also largely supports Treg cells in these common aspects [91]. Thus, besides these well-defined activities of IL-2/IL-2R signaling for Treg cells, the following
hypothesis is that some un-defined properties of Treg cells and/or some distinct sub-populations of Treg cells might differ in their requirement of the IL-2 signal.

Impaired expression of some interested target genes, like Klrg1 and CD103, in peripheral Treg cells bearing sub-optimal IL-2Rβ signaling suggests that these molecules may mark specific Treg subsets under the control of strong IL-2 signaling. In addition, both Klrg1⁺ and CD103⁺ Treg cells were recently reported [46-49] by exhibiting some distinct properties varied from Klrg1⁻ and CD103⁻ Treg cells. Therefore, it is worthy of further examining how IL-2R signaling regulates the development of particular Treg subsets and how these subsets potentially respond in vivo. Moreover, IL-2 and IL-2R polymorphisms are closely associated with multiple autoimmune diseases [108]. IL-2 is also currently administered in clinical trials for the treatment of cancers, viral infections, etc. A complete understanding of IL-2 and its interrelationship with other players, like Treg cells, in the immune system is of great importance for a successful IL-2 mediated immunotherapy.

Although the IL-2 signal is a key growth/survival factor for developing thymic Treg cells, studies have not yet indicated whether the thymic Treg cells can be activated and functional under an environment of no or low IL-2 signaling. By utilizing IL-2Rβ⁻/⁻ and IL-2Rβ mutant mice where IL-2 signal is weak, I want to better understand IL-2 and to what extent this signal programs the profile of thymic Treg cells. The results presented in chapter 2 of this dissertation address the following issues: 1. What are the defects related to the absence of IL-2Rβ signaling for thymic Treg cells? 2. Are any defects observed in IL-2Rβ⁻/⁻ Treg cells intrinsic to the abrogated IL-2R signaling or indirectly caused by the autoimmune induced micro-environment in IL-2Rβ⁻/⁻ mice? 3. What is the property of
mature vs. immature thymic Treg cells under normal IL-2 signaling? 4. How does low IL-2 signaling determine and program the development of thymic Treg cells?

In addition, because the expression of several targeted genes were impaired under the lower IL-2R signaling [91], and Treg cells consistently exhibit heterogeneous property with respect to phenotype, function and transcriptional regulation, the study of distinct Treg subsets and the interrelationship between these subsets are subsequently essential for further understanding the role of IL-2R signaling for Treg cells, as well as the correlation between a low IL-2R signal and some unique Treg subsets. This part of the work has been summarized in chapter 3 of this dissertation and addressed the following issues: 1. Are surface activation markers useful for peripheral Treg subsets study and what is the relationship among these subsets? 2. What types of Treg cells are impaired under low IL-2R signaling and what are their properties? 3. How does IL-2 regulate and control the development of IL-2 dependent Klrg1+ Treg cells? 4. What is the potential contribution of Klrg1+ Treg cells to autoimmune T1D?
Chapter 2

ROLE OF IL-2 SIGNALING FOR THYMIC TREG CELLS

2.1 IL-2 influences the growth and maturation of thymic Treg cells

IL-2Rβ\(^{-/-}\) mice are not able to develop a normal compartment of Treg cells, and massive autoimmune responses eventually cause pre-mature death in these mice [74]. However, there are two interpretations for the impaired Treg cells in this model. One is intrinsically due to the abrogated IL-2R signaling, and another possibility is due to inflammatory environment followed by polyclonal T cell activation and autoimmunity. Previously, we have shown that IL-2Rβ\(^{-/-}\) mice were able to avoid massive autoimmune responses and maintain a normal life-span after adoptive transfer of a small amount of congenic CD45.1\(^{+}\) WT splenic Treg cells during the neonatal stage [74]. Therefore, the “cured” IL-2Rβ\(^{-/-}\) mice were helpful for clarifying the internal relationship between Treg cells and IL-2 signaling.

By analyzing the autoimmune-prone IL-2Rβ\(^{-/-}\) and adoptively “cured” autoimmune-free IL-2Rβ\(^{-/-}\) mice in comparison to the WT C57BL/6 (WT) strain, we found that the proportion and distribution of double negative (DN), double positive (DP), CD4 single positive (SP), and CD8 SP thymocytes were largely in a normal range among these mice (Figure 2.1A). However, thymic cellularity was somewhat reduced in both IL-2Rβ\(^{-/-}\) and “cured” IL-2Rβ\(^{-/-}\) mice (Figure 2.1B). This effect might reflect some inefficiency related to lack of expression of IL-2Rβ on lymphoid precursor cells. Moreover, the proportion of CD4\(^{+}\) Foxp3\(^{+}\) T cell compartment either in IL-2Rβ\(^{-/-}\) mice or “cured” IL-2Rβ\(^{-/-}\) mice was significantly diminished by 2-3 fold in comparison to the WT (Figure 2.1C-D). As IL-
2Rβ−/− and “cured” IL-2Rβ−/− mice contain similar numbers of CD4 SP (Figure 2.1A-B), this difference also represents the decrease of absolute numbers in CD4+ Foxp3+ T cells. Noticeably, in the “cured” IL-2Rβ−/− mice, a substantial fraction of the CD4+ Foxp3+ cells were CD45.1+ donor derived (Figure 2.1D), reflecting either competitive disadvantage of IL-2Rβ−/− Treg cells compared to WT Treg cells or an increased number of IL-2Rβ−/− Treg cells favored by the autoimmune environment in untreated IL-2Rβ−/− mice.

![Diagram](image)

**Figure 2.1 Impaired compartment of thymic Treg cells from IL-2Rβ−/− mice (A-B).** Strategy of gating thymocytes and total thymic cellularity in WT, IL-2Rβ−/− and “cured” IL-2Rβ−/− mice. (C-D). Strategy of gating thymic Foxp3+ Treg cells in SP CD4 T cells (C) and the proportion (D) of thymic Treg in WT, IL-2Rβ−/− and “cured” IL-2Rβ−/− mice. Data are means ± SD from 3-8 mice/group.

Thus, the lower frequency and amount of CD4+ Foxp3+ thymocytes in IL-2Rβ−/− and “cured” IL-2Rβ−/− mice might reflect impaired proliferation and/or survival ability. By utilizing Ki67, a marker of cells in the cell cycle [109], and Bcl-2 which is an anti-apoptotic marker preventing cell death in various conditions [110], we found that the
CD4⁺ Foxp3⁺ thymocytes from WT and untreated IL-2Rβ⁻/⁻ mice or “cured” IL-2Rβ⁻/⁻ mice expressed a similar amount of Bcl-2 (Figure 2.2A-B). However, in comparison to the Foxp3⁺ thymocytes from WT mice, host-derived IL-2Rβ⁻/⁻ CD4⁺ Foxp3⁺ cells from “cured” IL-2Rβ⁻/⁻ mice exhibited a slightly lower fraction of Ki67⁺ cells, further supporting the notion that inflammatory environment associated with autoimmunity in untreated IL-2Rβ⁻/⁻ mice somewhat increased the overall numbers of CD4⁺ Foxp3⁺ thymocytes by enhancing their proliferation.

![Figure 2.2 Proliferation and survival of thymic Treg cells (A-B). Expression of Ki67 and Bcl-2 was compared in total thymic Treg cells from WT and IL-2Rβ⁻/⁻ mice, as well as host thymic Treg cells (Host) from “cured” IL-2Rβ⁻/⁻ mice. Shown is the representative histogram and the data are means ± SD from 5-8 mice/group.](image)

For the phenotypic analysis of IL-2Rβ⁻/⁻ Treg cells, we found that Foxp3 expression was lower (Figure 2.3A) in these cells no matter whether they were from autoimmune-prone or adoptively transferred “cured” autoimmune-free IL-2Rβ⁻/⁻ mice. CD25 was also correspondingly down-regulated in IL-2Rβ⁻/⁻ Treg cells (Figure 2.3B). Since higher Foxp3 expression is a determinant check-point for Treg lineage commitment [111], the abrogated IL-2Rβ signaling by impairing the optimal expression of Foxp3 and CD25 is closely associated with the diminished population of Treg cells in the thymus.
Genome-wide expression profiling previously suggested that there were two molecules, Klrg1 and CD103, potentially dependent on strong IL-2 signaling. Therefore, we also examined these two markers expression in thymic IL-2R$\beta^{-/-}$ Treg cells. Consistent to findings from other labs, even in the thymus of WT mice, the expression of Klrg1 was rarely detected. However, CD103$^+$ thymic Treg cells were significantly diminished (<5%) in the IL-2R$\beta^{-/-}$ Treg population in comparison to those from the WT control (~ 20%) (Figure 2.3C). Since CD103 was considered as a Foxp3 induced marker and CD103$^+$ Treg cells exhibited superior suppressive function over CD103$^-$ Treg cells [112], absence of IL-2R$\beta$ signaling therefore may potentially affect the suppressive function of thymic Treg cells.

**Figure 2.3 Phenotype of thymic Treg cells**

(A-B). Mean fluorescent intensity (MFI) of Foxp3 (A) and CD25 (B) on thymic Treg cells from WT, IL-2R$\beta^{-/-}$ mice and host compartment (Host) of "cured" IL-2R$\beta^{-/-}$ mice. (C). Representative expression of Klrg1 and CD103 on indicated thymic Treg cells and data are means ± SD from 3-8 mice/group.
In order to evaluate the functionality of thymic Treg cells in a simple and straightforward way, the expression of Treg functional molecules CD39, CD73 and CTLA4 [65] was compared between the WT, IL-2Rβ<sup>−/−</sup> and “cured” IL-2Rβ<sup>−/−</sup> mice. Clearly and consistently, all of the IL-2Rβ<sup>−/−</sup> Treg cells from autoimmune-prone or autoimmune-free IL-2Rβ<sup>−/−</sup> mice expressed a significantly lower level of all 3 markers compared to the WT control. No obvious difference was detected between autoimmune-prone and autoimmune-free IL-2Rβ<sup>−/−</sup> mice (Figure 2.4). Overall, impaired quantity and quality of thymic Treg cells are intrinsic properties related to the impaired IL-2Rβ signaling. Thus, IL-2/IL-2R signaling is essential for proper programming of thymic Treg cells and for their activity and functionality.

**Figure 2.4 Expression of function associated molecules**

Expression of CD39, CD73 and CTLA4 was examined on thymic Treg cells from WT, IL-2Rβ<sup>−/−</sup> mice and host compartment (Host) of “cured” IL-2Rβ<sup>−/−</sup> mice. Shown is the representative histogram and the data are means ± SD from 3-8 mice/group.
2.2 Properties of WT mature vs. immature thymic Treg cells

The IL-2Rβ⁻/⁻ mice mostly harbor CD4⁺ Foxp3<sup>lo</sup> CD25⁻ thymocytes with impaired expression of activation and function associated molecules. Thus, we asked if these abnormalities are also similarly reflected in CD25⁻ and Foxp3<sup>lo</sup> Treg cells (~30%) from the WT mice where CD25 and Foxp3 are heterogeneously expressed in Treg cells. We found that, like the IL-2Rβ⁻/⁻ Treg cells, WT CD25⁻ Foxp3<sup>+</sup> thymic Treg cells expressed a significantly lower level of Foxp3, CD103, CTLA4, CD39 and CD73 than those from the CD25<sup>+</sup> Foxp3<sup>+</sup> counterpart (Figure 2.5). In addition, a similar trend was observed in the Treg population when they were separated into Foxp3<sup>lo</sup> vs. Foxp3<sup>hi</sup> Treg cells (Figure 2.6) where WT Foxp3<sup>lo</sup> cells were defective in the expression of CD25, CD103, CD39, CD73 and CTLA4.

Figure 2.5 Properties of WT CD25⁻ vs. CD25<sup>+</sup> thymic Treg cells
Expression of Foxp3, CTLA4, Bcl-2, CD103, CD39, CD73 and Ki67 in CD25⁻ vs. CD25<sup>+</sup> thymic Treg cells from WT mice. Data are means ± SD from 5-6 mice/group.
However, the expression levels of Ki67 and Bcl-2 in CD25⁻ vs. CD25⁺ Treg cells or Foxp3<sub>lo</sub> vs. Foxp3<sub>hi</sub> Treg cells were similar (Figure 2.5 and Figure 2.6). Overall, these results suggested that there is a positive correlation between the key markers CD25 and Foxp3 of Treg cells and their status of activity and functionality. Moreover, those CD25⁻ and Foxp3<sub>lo</sub> Treg cells are not an aberrant cell population only present in IL-2Rβ<sup>−/−</sup> mice. Their presence in WT mice may represent a stage during Treg development when the IL-2 signal is unavailable.

2.3 Sub-optimal IL-2Rβ signaling and thymic Treg cell development

We recently developed transgenic mice (Y3) on the IL-2Rβ<sup>−/−</sup> background whose T cells express Y→F mutations in 3 tyrosine residues (Y341, Y395, Y498) of IL-2Rβ that
are required for normal recruitment of the adaptor Shc and Stat5 as a consequence of the IL-2/IL-2R interaction. Even though Y3 mutation leads to weak transient IL-2-induced pStat5, Y3 mice have no outward problems in Treg thymic development or peripheral homeostasis as reflected in Treg number, frequency, and Foxp3 levels [91].

To further define the efficiency of this weak IL-2R signaling during thymic Treg development, CD4^+ Foxp3^{hi} vs. Foxp3^{lo} thymocytes from WT, Y3, and IL-2Rβ^{−/−} mice were examined. First of all, we assessed the expression of CD24 (Figure 2.7). This is a T cell differentiation antigen, highly expressed on both DN and DP thymocytes but down-regulated at the SP stage [113]. We found that thymocytes from either Y3 or IL-2Rβ^{−/−} mice were able to successfully develop into SP T cells. Second, in comparison to the WT control, there was nearly absent expression of CD25 on IL-2Rβ^{−/−} Treg cells but it was only slightly lower in Y3 mice vs. WT either in the Foxp3^{lo} or Foxp3^{hi} subset (Figure 2.7). Third, the expression of CD103 was severely blocked in the IL-2Rβ^{−/−} mice but moderately reduced in the Foxp3^{lo} Treg subset of Y3 mice, and its expression was recovered in the Foxp3^{hi} Treg subset (Figure 2.7). Lastly, although Ki67 is slightly lower in Y3 Treg cells, no dramatic difference was detected in respect to CTLA4 and Bcl-2 in either Foxp3^{lo} or Foxp3^{hi} Treg cells, comparing WT and Y3 mice (Figure 2.7). Overall, these data suggest that weak IL-2R signaling does not fully support proliferation of developing Treg cells in the thymus. However, activation and regulatory function of Treg cells are readily programmed by Y3 IL-2Rβ signaling.
Furthermore, in vitro stimulation of the Foxp3 expression by IL-2 resulted in most thymocytes by immediately evaluating pStat5 expression without addition of IL-2. In comparison to IL-2Rβ− CD4+ Foxp3+ T cells, Y3 Treg cells showed substantial pStat5 activity which was nearly comparable to that associated with WT Treg cells (Figure 2.8). The lack of pStat5 by Foxp3+ cells from IL-2Rβ− mice indicates that this ex vivo pStat5 expression by WT and Y3 Treg cells is primarily the result of IL-2R signaling. Furthermore, in vitro stimulation of the Foxp3+ thymocytes with IL-2 resulted in most

**Figure 2.7 Properties of thymic Treg cells with low IL-2R signaling**

Thymic Treg cells from the WT, Y3 mice and IL-2Rβ− mice were separated into Foxp3lo vs. Foxp3hi populations and the expression of CD24, CD25, CD103, CTLa4, Ki67 and Bcl-2 was examined. Data are means ± SD from 2 mice/group.
WT and Y3 Treg cells to activate pStat5, although the percentage and level of pStat5+ cells was somewhat reduced for Y3 Treg population (Figure 2.8A).

Collectively, the nearly normal thymic development, as assessed by CTLA4, CD103, and CD25 expression, associated with Y3 Treg cells is accounted by pStat5 signaling approximately equivalent to that induced by WT Treg cells.

Figure 2.8 pSTAT5 activity of thymic Treg cells
(A.) Thymic Treg cells from the WT, Y3 mice and IL-2Rβ−/− mice were evaluated for the pSTAT5 activity both ex vivo without IL-2 stimulation and in vitro 15 minutes after IL-2 treatment. (B) Pooled result from ex vivo analysis. Data are means ± SD from 2 mice/group.

Although several properties associated with Y3 Treg cells appeared outwardly normal, Y3 Treg cells showed somewhat lower proliferation as assessed by Ki67 expression (Figure 2.7). To more broadly evaluate the efficiency of IL-2R signaling associated with Y3 IL-2Rβ, mixed bone marrow chimeras were prepared to compare the development of Y3 vs. WT Treg cells in the same environment. In one set of experiments, development by CD45.1 B6 Treg cells was compared to Treg cells expressing WT (Y0) or Y3 transgenic IL-2Rβ (on the IL-2Rβ−/- genetic background) after
a 1:1 mixture of the respective T cell-depleted bone marrow was transferred into lethally irradiated Thy1.1 recipients. In another set of experiments, development by CD45.1 B6 Treg cells was compared to WT B6 Treg cells or Y3 Treg cells after a 1:1 mixture of the respective T cell-depleted bone marrow was transferred into sublethally irradiated TCRα−/− recipients (Figure 2.9A). The gating strategy to follow donor-derived CD4⁺ Foxp3⁻ SP thymocytes and CD4⁺ Foxp3⁺ Treg cells is shown in Figure 2.9B.

Thymic reconstitution was largely comparable (60-100 x10⁶ cells) when recipients were examined 4-8 weeks post-bone marrow transfer. The relative representation of CD4 SP T cells and Treg cells from donor Y0 or B6 and Y3 was normalized to that from the CD45.1 B6 control (Figure 2.9 C-D). We expected that the ratios of Y0:B6CD45.1 and B6:B6CD45.1 to be 1:1 in the thymus, if the number of precursor cells in bone marrow were equivalent. However, these ratios were each lower when Thy1.1 or TCRα−/− recipients were analyzed 4 and 8 weeks post-transfer, respectively, suggesting that the numbers of Y0 or B6 progenitors were lower vs. B6CD45.1. Nevertheless, a generally similar representation of Y3 CD4⁺ SP T cells and Treg cells in relation to the B6CD45.1 cells was noted in the thymus for the Thy1.1 (Figure 2.9 C) and TCRα−/− (Figure 2.9 D) recipients. In marked contrast, this analysis revealed that Y3 Treg cells, but not conventional Y3 CD4⁺ T cells, were highly underrepresented in the spleen. These overall trends were also seen when the data were normalized to reflect the efficiency of the Treg reconstitution from the thymus and spleen, for example, by individually comparing for the fraction of Y0 Treg cells to Y0 CD4⁺ T cells, B6 Treg cells to B6 CD4⁺ T cells or Y3 Treg cells to Y3 CD4⁺ T cells. Collectively, the data from these competitive experiments
indicate that signaling associated with Y3 IL-2Rβ is much more effective in supporting Treg thymic development than peripheral homeostasis.

**Figure 2.9 Reconstitution of Y3 bone marrow in the thymus and periphery** (A). T cell depleted BM from B6 CD45.1 mouse was mixed at 1:1 ratio with that from the transgenic Y0 or Y3 mouse and i.v. injected into the lethal dose (900 Rads) irradiated Thy1.1 B6 mouse. T cell depleted BM from B6 CD45.1 mouse was also similarly mixed with that from WT B6 or Y3 mouse and i.v. injected into the sub-lethal dose (600 Rads) irradiated TCRα−/− mouse. Reconstitution of mixed bone marrows was analyzed respectively 4 and 8 weeks later. (B). Shown is the representative histogram to follow donor-derived CD4+ Foxp3− SP thymocytes and CD4+ Foxp3+ Treg cells. (C-D). The contribution of SP CD4 T cells and Treg cells from the thymus and spleen of Y3 or Y0 or B6 bone marrow was normalized to that from the B6 CD45.1 internal control and presented as a relative value of ratio. The efficiency of Treg cells reconstitution was further normalized to the reconstitution of CD4 T cells. Data are means ± SD from 3-5 mice/group.
Chapter 3

ROLE OF IL-2 SIGNALING FOR PERIPHERAL TREG SUBSETS

3.1 Characteristics of peripheral Treg sub-populations

Current studies of Treg cells have indicated that these cells are heterogeneous in many aspects. For example, Treg cells show extensive phenotypic heterogeneity in the expression of T cell activation molecules (CD69, CD62L, CD103) and chemokine receptors (CCR7 and CCR6) [46, 54, 114]. Treg cells also exhibit functional heterogeneity at the level of transcriptional regulation. By utilizing the transcription factor Tbet which is essential for Th1 development in conventional CD4⁺ T cells, Treg cells are able to successfully inhibit Th1 response [55]. This is similar for Irf-4 expressing Treg cells to control Th2 response and Stat3 expressing Treg cells to control Th17 [56, 58]. Here, we questioned to what extent this phenotypic and functional heterogeneity defines unique vs. interrelated Treg sub-populations.

Based on the expression of CD69, CD62L and Klrg1, all of which have been utilized to discriminate the conventional T cells with properties of resting naïve, activated effector and memory cells [115, 116], we isolated splenic Treg cells from Foxp3/RFP reporter mice and separated them into several sub-populations (Figure 3.1), including Klrg1 depleted CD69⁻ CD62Lʰⁱ (Fr1 Klrg1ⁿᵉᵍ), CD69⁻ CD62Lʰᵒ (Fr2 Klrg1ⁿᵉᵍ) and CD69⁺ CD62Lʰᵒ (Fr3 Klrg1ⁿᵉᵍ) Treg cells, as well as Klrg1⁺ included CD69⁺ CD62Lʰᵒ Treg cells (Fr3 Klrg1⁺). For further understanding the characteristics of these Treg cells and the correlation between distinct subsets, total RNA was extracted and gene array analysis of
each of these Treg subsets was performed by using Affymetrix Mouse Gene ST 1.0 arrays.

**Figure 3.1 Separated Treg subsets for gene array analysis.** Splenic Treg cells from Foxp3/RFP reporter mice were enriched with CD4 beads and FACS purified to yield 4 subsets which were Klrγ1 depleted CD69CD62L<sup>hi</sup> (Fr1 Klrγ1<sup>neg</sup>), CD69CD62L<sup>lo</sup> (Fr2 Klrγ1<sup>neg</sup>) and CD69<sup>+</sup>CD62L<sup>lo</sup> (Fr3 Klrγ1<sup>neg</sup>) Treg subsets, as well as Klrγ1 included CD69<sup>+</sup>CD62L<sup>lo</sup> (Fr3 Klrγ1<sup>+</sup>) Treg cells.

Comparing the genome-wide expression profile of these 4 Treg subsets to that of the non-Treg conventional CD4<sup>+</sup> T cells, we found 1,136 genes were differentially expressed by ≥2-fold (Figure 3.2). Additionally, around half of the total Treg signature genes [117] were overlapped in this array profile. When we compared the genes that were differentially expressed by ≥2-fold only among these 4 Treg subsets, 745 unique targets were identified. To verify this list, several selected molecules exhibited expression largely in accordance with their mRNA levels from the gene array profile (Figure 3.3).

**Figure 3.2 Overlap of gene profile from Treg signature and Treg subsets** The overlap between Treg signature genes and unique gene profile expressed in 4 Treg subsets. Samples (n=2, except Fr3 Klrγ1neg, n=4) represent independent biological replicates.
Among the selected genes expressed by Treg subsets, Klrg1 was one that varied and highly expressed by Fr3 Klrg1+ since these cells were purified on the basis of its expression (Figure 3.4). However, mRNAs for IL-2R subunits (Il2ra, Il2rb and Il2rg) were similarly expressed in each of these 4 subsets, consistent with their similar ability to respond to IL-2. Gene enrichment analyses (Figure 3.4) implied that Fr1 Klrg1− Treg cells were a naive resting-like Treg sub-population with preference to residing in peripheral lymphoid compartments. First of all, these cells expressed lower levels of many Treg suppressive molecules, like Gzmb, Fgl2, Il10, Lag3, Ebi3 (a component of IL-35), and Entpd1 (CD39). Second, Fr1 Klrg1− Treg cells also expressed lower levels of cytokines, chemokines (Il10, Il18,Ccl1, Ccl5) and chemokine receptors targeting tissue migration (Cxcr6, Ccr5, Ccr3, Ccr2, Ccr6), but increased level of the lymphoid homing receptor Ccr7. Third, these cells expressed lower levels of activation markers (Cd38, Cd81) and pro-apoptotic molecules (Casp4, Casp1), but a higher level of anti-apoptotic Bcl2. Lastly, they also showed increased expression of genes related to hemopoiesis [Ikrz1 (Ikaros), Foxp1] and Wnt signaling (Lef1, Tcf7, Frat1, Sox4, Kremen1, Wnt3), suggesting that this Treg sub-population might be enriched in cells capable of self-renewal.

**Figure 3.3 Verification of gene array profile**

(A). Shown are the expression levels (log2) of selected genes from Treg subsets chosen for FACS analysis. (B). FACS expression of selected genes by Treg subsets from WT B6 mice. Data are mean ± SD for 4 mice per group.
However, unlike Fr1 Klrg1– Treg cells, Fr2 Klrg1–, Fr3 Klrg1–, and Fr3 Klrg1+ Treg cells exhibited properties of activated highly functional Treg cells with tissue seeking properties, often with a gradient of increased expression from Fr2 Klrg1– to Fr3 Klrg1+ (highest) for genes mentioned above related to Treg function, chemokines, cytokines, tissues migration and cell death. Moreover, at the transcriptional regulation level, these 3 subsets also gradually increased the expression of Tbx21 (Tbet) and especially Prdm1 (Blimp-1), both of which have been related to efficient Treg suppressive activity [55, 118]. Reciprocally, these 3 subsets expressed a reduced level of targets related to lymphoid homing, survival, hemopoiesis, and Wnt signaling, with the lowest expression often associated with Fr3 Klrg1+. Therefore, the range of expression from extremely high or low often associated with Fr3 Klrg1+ raises one possibility that this specific Treg sub-

**Figure 3.4 Distinct gene array profile of specific Treg subset**

*The heat map represents the differentially expressed target genes in 4 Treg subsets. These genes were further separated into several categories which were related to cell activation, suppressive function, cytokine/chemokine, turnover/self-renewal and transcriptional regulation. The expression level of these listed genes is shown in Log2 scale.*
population may predominantly function as a terminally differentiated, short-lived Treg cells. These results indicate that, analogous to conventional T cells, CD62L, CD69, and KlrG1 also represent useful phenotypic markers to distinguish specific but interrelated Treg sub-populations.

Furthermore, euclidian clustering of differentially expressed genes between Treg sub-populations revealed two clades, with Fr1 KlrG1− and Fr2 KlrG1− vs. Fr3 KlrG1− and Fr3 KlrG1+ more related to each other (Figure 3.5).

![Figure 3.5 Correlation between Treg subsets](image)

Euclidean clustering of sample relatedness for genes varied by ≥ 2-fold between these 4 Treg subsets.

To further analyze the function of these 4 separated Treg subsets, we measured the level of CTLA4, an inhibitory molecule involved with inactivation of DC [65]. We noticed that CTLA4 expression was substantially increased in KlrG1+ Treg cells even in comparison to activated Fr3 KlrG1− Treg cells (Figure 3.6A). As CTLA4 was not detected as a differentially expressed gene (Figure 3.4), this increase may represent a post-transcriptional mechanism or reflect an inability to detect mRNA difference due to the high levels of CTLA4 mRNA in Treg cells. Moreover, CD39 which hydrolyzes ATP to ADP to AMP and mediate an anti-inflammatory effect [65] was also significantly increased by KlrG1+ Treg cells, however, this statistical difference was only observed in comparison to “resting” Fr1 KlrG1− Treg cells. In vitro suppression assays (Figure 3.6B)
consistently showed the greatest inhibition from Klrg1⁺ Treg cells, but significance was only present when compared to F1 Klrg1⁻ Treg cells. Together, with our gene array analysis, these data support the notion that Klrg1⁺ Treg cells are distinct in the total population of Treg cells and armed to optimally deliver a number of molecules associated with Treg suppressive function.

3.2 Klrg1⁺ Treg cells are unique and antigen-responsive in situ

Klrg1 is minimally expressed on conventional CD4⁺ T cells but is found at a relatively high level on a small population of Treg cells in peripheral lymphoid compartments (Figure 3.7A) such as the spleen and mesenteric LN (MLN). Klrg1⁺ Treg cells from the spleen expressed a more activated phenotype (CD69⁺, CD62Llo, CD103⁺, and CD44hi) when compared to Klrg1⁻ Treg cells (Figure 3.7B). Blimp-1, which marks

Figure 3.6 Suppressive activity of Treg subsets

(A). The expression of CTLA4 and CD39 was measured in indicated splenic Treg subsets from WT B6 mice, and the MFI expressed in each indicated splenic Treg subset was normalized to the MFI of total Treg cells. (B). Purified splenic Treg subsets from Foxp3/RFP reporter mice were co-cultured with responder CD4⁺ T cells at the indicated Treg:Teff ratios. The % of inhibition was determined in comparison to [³H]thymidine incorporation by responder CD4⁺ T cells without co-culture of Treg cells. Results (mean ± SD) are from 3 experiments.
high IL-10-producing activated Treg cells [118], was also highly expressed by Klrg1\(^+\) Treg cells. Additionally, Klrg1\(^+\) Treg cells expressed higher levels of Foxp3 and several other important Treg function related molecules (CTLA4, CD39, CD73).

**Figure 3.7 Unique specificity of splenic Klrg1\(^+\) Treg subset**

(A). Expression of Klrg1 was examined in CD4\(^+\) conventional T cells and Foxp3\(^+\) Treg cells from WT spleen and MLN. (B). Expression of indicated markers was compared between Klrg1\(^+\) and Klrg1\(^-\) WT splenic Treg cells. Expression of Blimp-1 was analyzed in splenic Treg cells from WT Blimp/GFP Foxp3/RFP dual reporter mice. Data (mean ± SD) are representative of 3-8 mice/group and the relative MFI of indicated markers was normalized to the total Treg cells.
Thus, considering the specific gene profile and phenotype of Klrg1+ Treg cells as well as our recent observation of a high proportion of Klrg1+ Treg cells in the LP (lamina propria) of small intestine at a steady state [recent findings from Malek’s lab], we propose that these cells may preferentially home and/or be activated within tissue sites. To test this notion, Klrg1 expression by Treg cells in the spleen, draining bronchial LN (dLN) and lung was examined after mice were either sensitized with ovalbumin (OVA) and alum or after aerosol challenge to induce allergic hypersensitivity (Figure 3.8).

![Figure 3.8 mouse model of acute asthma](image)

*WT B6 mice were immunized on day 0 by i.p. injection of OVA+Alum with a further sensitization on day 5. On day 12, mice were either sacrificed for study or aerosol challenged with OVA for analysis 3 days after.*

We found that, after sensitization, the number of total Treg cells and Klrg1+ Treg cells was increased in the bronchial dLN and lung, but not the spleen (Figure 3.9A). However, the relative proportion of Klrg1+ Treg cells (Figure 3.9B) did not parallel increase in the lung but did in the dLN, suggesting that Klrg1+ Treg cells do not preferentially migrate to the lung. Noticeably, three days after the aerosol challenge with OVA, both the number and proportion of total Treg cells and Klrg1+ Treg cells were increased in the lung but not the dLN when they were compared to the mice that were only sensitized without further challenge (Figure 3.9B). Therefore, most of the accumulated Klrg1+ Treg cells in the lung are probably a result of a response in situ rather than migration from periphery into the air-way mucosal tissue site.
Figure 3.9 Klrg1+ Treg cells are antigen responsive in situ

(A). The proportion and total number of Treg cells from indicated tissue sites were analyzed and compared in naive, OVA sensitized (Sensitized) and OVA sensitized with aerosol challenged (Aerosol) WT B6 mice. (B). The proportion and total number of Klrg1+ Treg cells were also examined and compared in these mice (C). Accumulated Treg cells were correlated with the increased potential in proliferation as assessed by Ki67. Data (mean ± SD) are representative of 3-8
In addition, after OVA sensitization and aerosol challenge, the increased number of Treg cells were mainly achieved by enhancing the proliferation of responding cells since the expression of Ki67 was significantly higher in the Treg cells from the dLN and lung but not spleen (Figure 3.9C). Collectively, these data indicate that Klrg1⁺ Treg cells are a highly activated Treg subpopulation with enhanced expression of several Treg functional molecules and they are ready to respond to antigenic challenge in situ.

3.3 IL-2 is essential for Klrg1⁺ Treg cells development in periphery

We previously showed that mutations of Y341 (Y1), Y395 and Y498 (Y2), or Y341, Y395 and Y498 (Y3) to phenylalanine in the cytoplasmic tail of IL-2Rβ resulted in a dose-dependent reduction of IL-2-dependent tyrosine Stat5 phosphorylation (pStat5) with lowest, but detectable, pStat5 associated with Y3 [91]. Notably, unlike IL-2Rβ⁻/⁻ mice which develop rapid lethal systemic autoimmunity due to failed maturation of Treg cells, mice expressing each mutant IL-2Rβ were overtly healthy and contained a normal ratio and number of Treg cells and a normal level of Foxp3 expression. Thus, nTreg development and homeostasis are readily supported by a low level of IL-2R signaling. However, symptoms of autoimmune diseases do develop in aged Y2 and Y3 mice and gene expression profile from Treg cells with impaired IL-2R signaling revealed a substantial number of genes that were IL-2-dependent. Two such genes are Klrg1 and Itgae (CD103).

Accordingly, we found fewer Klrg1⁺ Treg cells in the spleen as well as peripheral MLN of mice bearing mutant IL-2Rβ. Moreover, this decrease was generally proportional to increased mutations of Y→F on the IL-2Rβ chain (Figure 3.10A). As a
transgenic control, a normal level of Klrg1+ Treg cells was found in the spleen of Y0 mice where transgenic WT IL-2Rβ chain is expressed in the background of IL-2Rβ−/−. In addition, comparable amounts of Klrg1+ Treg cells were also found in IL-15−/− mice although IL-15 shares with IL-2 the IL-2Rβ chain for signaling transduction (Figure 3.10B). However, a decrease in CD103+ Treg cells was only seen in the Y1 and Y3 mice, suggesting that it correlates primarily with the Y341 mutation (Figure 3.10C). Thus, the reliance upon IL-2R signaling is not quite the same for CD103+ and Klrg1+ Treg cells, and the normal compartment of Klrg1+ Treg cells in periphery needs strong IL-2 signaling involved with all of these three key tyrosines on the IL-2Rβ chain.

![Diagram of Figure 3.10 Diminished Klrg1+ Treg cells under low IL-2 signaling]

Figure 3.10 Diminished Klrg1+ Treg cells under low IL-2 signaling

(A). The expression of Klrg1 was analyzed in total Treg cells from the spleen and MLN. The comparison was between WT B6 and transgenic Y1, Y2 and Y3 mice. (B). The expression of Klrg1 was also examined in splenic Treg cells from the IL-15−/− and transgenic Y0 mice and statistical study was performed between B6, IL-15−/−, Y0, Y1 and Y3 mice. (C). The expression of CD103 was analyzed in total Treg cells from the spleen and MLN. The comparison was between WT B6 and transgenic Y1, Y2 and Y3 mice. Data (mean ± SD) are representative of 3-8 mice/group.
The paucity of Treg cells expressing Klrg1 or CD103 in Y3 mice may simply reflect the decreased expression of these molecules by common related Treg cells or it may indicate the absence of a unique IL-2-dependent Treg subset. In order to address this question, we examined whether WT Klrg1+ or CD103+ Treg cells were selectively repopulated in Y3 mice where Klrg1 or CD103 marked Treg cells were endogenously impaired. Although WT donor Treg cells bearing normal IL-2/IL-2R signaling are expected to have a competitive advantage over the recipient Y3 Treg cells, limited number (5×10^5) of adoptively transferred donor cells should preferentially favor the generation of specific Treg cells that are selectively diminished in Y3 mice. Thus, GFP+ WT Treg cells from Foxp3/GFP reporter mice were adoptively transferred into either B6 or Y3 recipient mice and analyzed 4 weeks later (Figure 3.11A).

A.

B.

Figure 3.11 Competitive advantage of WT Treg cells in Y3 mice
(A). Splenic Treg cells from Foxp3/GFP reporter mice were injected into WT B6 or IL-2Rβ transgenic Y3 mice. The engraftment of donor Treg cells was examined 4 weeks later. (B). The percent of total splenic Treg cells in CD4+ T cells and the absolute number of Treg cells either from GFP+ donor or GFP- host were compared between B6 and Y3 recipients. Data (mean ± SD) are representative of 3 mice/group.
The results indicated that the frequency and total number (Figure 3.11B) of splenic Treg cells were comparable in both of these recipients. However, donor GFP\(^+\) Treg cells comprised a substantial fraction (~20%) of the total Treg cells in Y3 recipients while GFP\(^+\) Treg cells represented <1% of the total Treg cells in B6 recipients, demonstrating the competitive advantage of WT Treg cells in the Y3 recipients.

Strikingly, the donor GFP\(^+\) Treg cells in Y3 recipients were highly enriched in Klrg1\(^+\) Treg cells (~50% of total GFP\(^+\) Treg cells). In contrast, the GFP\(^-\) endogenous Treg cells only expressed a minimal level of Klrg1, such that over 90% of Klrg1\(^+\) Treg cells detected in recipient Y3 mice were actually GFP\(^+\) donor derived (Figure 3.12A). However, Klrg1\(^+\) Treg cells in B6 recipients were mostly from the GFP\(^-\) endogenous compartment and its expression on donor GFP\(^+\) Treg cells was under represented. Except for Klrg1, the expression pattern of CD103 was also similarly reflected in this model where CD103\(^+\) Treg cells were preferentially developed from the donor GFP\(^+\) Treg cells in Y3 mice comparing to B6 recipients.

The expression of other markers like CD69 and CD62L on donor GFP\(^+\) Treg cells was largely unaltered between the Y3 recipients and B6 recipients (Figure 3.12B), although Klrg1\(^+\) Treg cells are mostly CD69\(^+\) CD62L\(^{lo}\) in steady state. These data support the notion that Klrg1 and CD103 marked Treg subsets are defective in Y3 mice where the Treg niche is not fully filled with them, and therefore are selectively favored when WT Treg cells are engrafted in Y3.
Despite the lower expression of Klrg1 and CD103, peripheral Treg cells from Y3 mice were relatively more activated (Figure 3.13A). Thus, the proportion of Fr1 CD69\(^{hi}\) CD62L\(^{hi}\) Treg cells was decreased while Fr3 CD69\(^{hi}\) CD62L\(^{lo}\) was increased in the spleen of Y3 mice (Figure 3.13B). Moreover, Klrg1\(^{+}\) Treg cells from Y3 mice were significantly underrepresented in Fr2 and Fr3 while CD103\(^{+}\) Treg cells were decreased in Fr2 (Figure 3.13B).

Figure 3.12 Selective expansion of WT Treg cells in Y3 mice
(A). Shown is the representative histograms of the composition of engrafted GFP\(^{+}\) cells in total Treg population and the expression of Klrg1 or CD103 in either GFP\(^{+}\) donor cells, GFP\(^{-}\) host cells or total Treg cells from the spleen of recipient B6 or Y3 mice. (B). Expression of CD69 and CD62L in indicated splenic Treg cells from B6 or Y3 recipients. Data are representative of 3 mice/group.
Therefore, the regulation of Klrg1+ and CD103+ Treg cells is quite different under the impaired IL-2R signaling as supported by Y3, but general Treg activation is not dampened due to altered distribution of Treg composition. Moreover, these changes do not reflect the differences in proliferative or survival potential as assessed by Ki67 and Bcl-2 expression in Fr1-3 subsets (Figure 3.13C).

Collectively, although low IL-2R signaling abrogates the proper development of Klrg1+ and CD103+ Treg cells, yet it readily supports the homeostatic proliferation of...
Treg cells. The increase in Fr3 may reflect some compensatory mechanism related in part to recent TCR engagement to override impaired IL-2/IL-2R signaling.

The near lack of Klrg1+ and CD103+ Treg cells in Y3 mice indicates that these cells probably depend on more extensive IL-2R signaling for their development. In order to further test this notion, we evaluated the effect of extra extensive IL-2R signaling on Treg sub-populations. Since the half-life of IL-2 in vivo is very short, recent study indicated that IL-2 combined with anti-IL-2 antibody prolongs the effect of IL-2 treatment. Moreover, the antibody clone JES6-1A12 has been shown to preferentially drive the expansion of Treg cells [119, 120]. Thus WT B6 mice were treated with IL-2/JES6-1A12 (IL2-IC) and examined in a time course study at 3 days, 5 days, and 7 days after IL2-IC treatment (Figure 3.14).

We noticed that there was no obvious change in the relative proportion of total CD4+ and CD8+ T cells (data not shown) but a selective expansion of Treg cells accompanied with increased CD25 expression on day3 (Figure 3.15A). Strikingly, the increase was ~2.5-fold for total Treg cells, but almost 4-fold for Klrg1+ Treg cells (Figure 3.15A). However, the expression of other molecules, like CD103, CD69, and CD62L, was
minimally differed in Treg cells after the treatment (Figure 3.15B). Further analysis indicated that there is a close correlation between the number of Treg cells and proliferation potential since at the peak of the response, nearly all Treg cells, especially Klrg1+ cells, were recently in cell cycle as evidenced by the enhanced expression of Ki67 (Figure 3.15C) but minimally changed Bcl-2 expression (Figure 3.15B).

Figure 3.15 Klrg1+ Treg cells are preferentially responsive to IL2-IC (A). Shown is the representative FACS from the IL-2/IC treated WT B6 mice on day 3. Splenic Treg cells, including Klrg1+ Treg cells were expanded with increased CD25 expression. (B-C). Shown is the representative FACS of the expression of CD103, CD69, CD62L, Ki67 and Bcl-2 in splenic Treg cells on day 3.

Based on the time course analysis of all these markers (Figure 3.16), it was observed that the expansion of Treg cells, including Klrg1+ Treg cells reached the peak on day 3 but gradually reduced from day 5 to day 7, probably reflecting the consumption of supplemented IL-2, and this is also correlated with decreased proliferation. In addition,
the other subpopulations marked by either CD103 or CD69 and CD62L are stably maintained and not selectively or preferentially responsive to IL-2. Altogether, the findings suggest that extensive IL-2R signaling through IL2-IC in lympho-replete mice is particularly and preferentially efficient in driving the development of Klrg1⁺ Treg cells although this effect is relatively transient.

![Figure 3.16 Time course study of IL2-IC treatment](image)

**Figure 3.16 Time course study of IL2-IC treatment**

Enumerated number of splenic Treg cells, including Klrg1⁺ Treg cells, and the expression of indicated markers on splenic Treg cells during a time course study. Data (mean ± SD) are representative of 3 mice/group.

Although Klrg1⁺ Treg cells were diminished in the lack of sufficient IL-2R signaling and selectively expanded after IL2-IC treatment, it is not quite clear at which step IL-2 is required. For example, IL-2 may function in driving the expansion of Klrg1⁺ Treg cells and/or it may also play a role in promoting the differentiation of Klrg⁻ Treg cells to Klrg1⁺ Treg cells. To directly examine these possibilities, splenic Klrg1⁺ or Klrg1⁻ Treg cells from Foxp3/RFP reporter mice were FACS purified and transferred into TCRα⁻ mice with splenic conventional Treg-depleted CD4⁺ T cells from Foxp3/ GFP reporter mice. The reason to co-inject conventional T cells in this study is to provide potential growth factors generated by these cells and also to avoid the tendency of de-differentiation of injected Treg cells. After co-transfer, on the next day, the recipient mice
were administered with IL2-IC treatment once a day constitutively for 3 days and then analyzed 2 days later (Figure 3.17A).

A. TCRα<sup>-/-</sup>

[Day0 Day1 Day2 Day3 Day6]

Treg subsets transfer

1ug IL-2 + 5ug IL2-Ab i.p.

analyze

B. % RFP<sup>+</sup> in CD4<sup>+</sup> % Klrg1<sup>+</sup>

<table>
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<tr>
<td>Control</td>
<td>IL2-IC</td>
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<tr>
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Figure 3.17 Response of Klrg1<sup>+</sup> and Klrg1<sup>-</sup> Treg subsets to IL2-IC treatment (A). The model of IL2-IC treatment of lymphopenic TCRα<sup>-/-</sup> recipient mice which were co-injected with splenic Klrg1<sup>+</sup> or Klrg1<sup>-</sup> Treg subsets from Foxp3/RFP reporter mice and splenic conventional CD4<sup>+</sup> T cells from Foxp3/GFP reporter mice. (B). The contribution of IL2-IC to Treg subsets engraftment and the expression of Klrg1 on engrafted donor Treg cells in spleen. Data (mean ± SD) are representative of 2-4 mice/group.

The results indicated that engrafted Klrg1<sup>-</sup> Treg cells in the spleen expanded in response to IL-2 (Figure 3.17B). However, for recipients receiving Klrg1<sup>+</sup> Treg cells, only few donor cells were detected in the spleen and no significant difference was noted between the control PBS treated and IL-2 treated mice. In addition, we noted that Klrg1<sup>+</sup> Treg cells were derived from the Klrg1<sup>-</sup> input and IL2-IC treatment promoted their differentiation by around 3-fold (from ~10% to ~30%). Although few Klrg1<sup>+</sup> Treg cells
engrafted in the recipients, almost all retained the expression of Klrg1. Therefore, the expansion of Klrg1+ Treg cells by IL2-IC in lymphoreplete mice likely reflects the proliferation of Klrg1− precursors that are driven by IL-2 to differentiate into Klrg1+ Treg cells.

### 3.4 Klrg1+ Treg subset is short-lived

Since Klrg1+ Treg subset was diminished in Y3 mice, we then further compared the proliferation and turnover of Klrg1+ vs. Klrg1− Treg cells in the spleen of WT and Y3 mice. Proliferation, as assessed by Ki67, and survival, as assessed by Bcl-2, of Klrg1+ and Klrg1− Treg cells from the spleen were comparable between B6 and Y3 mice (Figure 3.18), suggesting that homeostatic proliferation of Treg cells is maintained by low IL-2R signaling associated with mutant IL-2Rβ in Y3 mice. In both types of mice, higher Ki67 expression was associated with Klrg1+ Treg cells in the spleen and Bcl-2 expression was equivalently lower for Klrg1+ Treg cells in B6 and Y3 spleen (Figure 3.18).

![Figure 3.18 Proliferation and survival of Klrg1+ and Klrg1− Treg subsets](image)

*Figure 3.18 Proliferation and survival of Klrg1+ and Klrg1− Treg subsets*

*Splenic Treg cells from B6 and Y3 mice were examined for the expression of Ki67 and Bcl-2 in either Klrg+ or Klrg− subsets. Data (mean ± SD) are representative of 6-8 mice/group.*
Further, we tracked the proliferating cells during a period of time instead of a snapshot of cells undergoing proliferation, BrdU (bromodeoxyuridine) incorporation analysis was performed where cohorts of WT and Y3 mice received BrdU in their drinking water for 5 days and then BrdU was chased by providing normal drinking water. Immediately after the 5 day labeling period (Day 0), BrdU incorporation was measured and this largely agreed with the expression of Ki67 (Figure 3.19). Upon BrdU withdrawal, the rate of loss BrdU was identical when directly comparing each subset individually between B6 and Y3 mice (Figure 3.19A). This indicates that weak IL-2R signaling is largely effective in supporting homeostasis for splenic Tregs. In addition, splenic B6 and Y3 Klrg1+ Treg cells showed greater turnover than Klrg1- counterpart, as reflected by a more rapidly reduced proportion of BrdU+ cells (Figure 3.19B).

**Figure 3.19 BrdU incorporation by splenic Treg subsets**

(A). BrdU incorporation in Klrg1+ or Klrg1- Treg cells was examined and compared between B6 and Y3 mice. (B). BrdU incorporation was examined and compared between Klrg1+ and Klrg1- Treg cells from either WT B6 mice or IL-2Rβ transgenic Y3 mice. Data (mean ± SD) are representative of 3 mice/group.

Thus, the rapid loss of BrdU and higher Ki67 expression associated with Klrg1+ Treg cells suggest they may be potentiated to fast proliferate. However, lower Bcl2 and lack of expansion after IL2-IC treatment (Figure 3.17B, Figure 3.18 respectively) alternatively
indicate that Klrg1+ Treg cells may be more susceptible to apoptosis and consequently lose initially BrdU labeled cells.

Consistent with the latter view, enriched splenic WT CD4+ T cells including Klrg1+ and Klrg1− Treg cells were co-cultured with 5% FCS complete media in 24-well plate where each well contained $2 \times 10^6$ cells. The recovery of Treg subsets was evaluated 1-3 day(s) after. We found that the Klrg1+ Treg subset was around 50% less capable of recovering and more susceptible to cell death in comparison to those Klrg1− Treg cells on day 1 and day 2 (Figure 3.20). However, on day 3, no significant difference was detected for both Klrg1+ and Klrg1− Treg subsets because almost all the Treg cells were dead in the vitro condition.

Collectively, based on the rapid loss of BrdU, minimal level of Bcl-2 expression and poor recovery in vitro, Klrg1+ Treg cells are mainly shorter-lived in contrast to the Klrg1− Treg cells. However, the analysis between WT B6 and IL-2Rβ mutant Y3 mice indicates that there is no significant difference for either Klrg1+ or Klrg1− Treg subset in terms of the proliferation and survival. Thus, the relative lack of Klrg1+ Treg cells within the
spleen of Y3 mice most likely reflects a requirement for a high IL-2R signaling to promote the development from their precursor cells.

3.5 Klrg1+ Treg subset is terminally differentiated

Recent studies indicated that Klrg1 expression on CD8+ T cells is associated with replicative senescence [121, 122] and Klrg1hi CD8+ T cells behave as terminally differentiated cells [116, 123]. Thus, given the notion that Klrg1+ Treg cells are activated effector/memory-like and short-lived, as well as they are preferentially responsive to antigen in situ, we hypothesize that Klrg1+ Treg cells may represent a sub-population of terminally differentiated effector Treg cells.

In order to study the lack of further differentiation of Klrg1+ Treg cells, we co-transferred RFP+ Klrg1+ or RFP+ Klrg1− nTreg cells purified from the Foxp3/RFP reporter mice with GFP+ CD4+ conventional T cells from the Foxp3/GFP reporter mice into TCRα− recipients (Figure 3.21). The input Treg subset and T conventional cells were mixed at around 1:10 ratio in total amount of 1.1×10^6 and the composition and phenotype of injected Treg subset were confirmed before transfer (Figure 3.21).

A.

![Input donors](image)

B.

![Input donors](image)

**Figure 3.21 Verification of adoptively transferred Klrg1+ or Klrg1− Treg subset (A-B).** Purified splenic RFP+ Klrg1+ (A) or RFP+ Klrg1− (B) Treg subset from RFP/Foxp3 reporter mice was co-transferred with splenic GFP+ CD4+ conventional T cells from GFP/Foxp3 reporter mice into TCRα− recipients. Shown is the representative FACS of the proportion of injected Treg cells and the confirmation of distinct Treg subsets.
Considering the low efficiency of cell engraftment after adoptive transfer (~ 10%), in order to let donor cells grow, expand and further differentiate, we analyzed these mice 4 weeks later. We found that donor Treg cells were readily detected in the spleen of recipients receiving RFP\(^+\) Klrg1\(^-\) nTreg cells but not RFP\(^+\) Klrg1\(^+\) cells (Figure 3.22A). Thus, very few Klrg1\(^+\) donor Treg cells engrafted in the spleen of mice receiving RFP\(^+\) Klrg1\(^+\) Treg cells which may suggest another possibility, that these cells might preferentially home to tissue sites instead of locating in the spleen, for example the LP of the small intestine where a higher frequency of Klrg1\(^+\) Treg cells were observed in a steady state study [recent findings in Malek’s lab]. Thus, we also examined the Treg cells in the LP of the small intestine, and similarly, very few RFP\(^+\) Klrg1\(^+\) nTreg cells were detected in the mice receiving Klrg1\(^+\) Treg cells (Figure 3.22B). Moreover, limited number of donor Klrg1\(^+\) nTreg cells found either in the spleen or LP retained the expression of Klrg1, while the donor Klrg1\(^-\) nTreg cells readily gave the rise to Klrg1\(^+\) cells after engraftment (Figure 3.22A-B).

Interestingly, in this adoptive transfer model, not only the RFP\(^+\) nTreg cells but also the GFP\(^+\) iTreg cells derived from co-transferred GFP\(^-\) conventional CD4\(^+\) T cells expressed Klrg1 (Figure 3.22A-B). Given the high purity of GFP\(^-\) conventional CD4\(^+\) T cells (>99.7%) and a substantial proportion (30%-60%) of Klrg1 expressed in GFP\(^+\) Treg cells, it is unlikely that GFP\(^+\) Treg cells found after adoptive transfer are solely resulted from a few contaminating GFP\(^+\) nTregs in the donor conventional CD4\(^+\) T cells inoculum. Therefore, although no Klrg1\(^+\) Treg cells detected in the thymus (Figure 2.3C) raises the possibility that Klrg1 marked Treg cells may be related to iTreg cells in the
periphery, our current finding actually indicates that Klrg1 is not a common marker for iTreg cells.

Figure 3.22 Lack of differentiation by Klrg1+ Treg cells (A-B). The engraftment of donor Klrg1+ or Klrg1− Treg subsets and GFP+ conventional CD4+ T cells were evaluated 4 weeks after transfer. Shown is the representative FACS of the engrafted T cells from the spleen (A) and LP of small intestine (B). The expression of Klrg1 on RFP+ nTreg cells or GFP+ iTreg cells were also examined (A-B). Data (mean ± SD) are representative of 4 mice/group.

It is noteworthy that the potential for generating Klrg1+ Treg cells from Klrg1− Treg precursor cells is not just a consequence resulted from a lymphopenic pressure of the TCRα−/− recipients. When purified splenic CD45.2+ RFP+ Klrg1− Treg cells from
Foxp3/RFP reporter mice were adoptively transferred into the lymphoreplete congenic CD45.1\(^+\) WT B6 mice (1\(\times\)10\(^5\) cells/mouse), 4 weeks post transfer, CD45.2\(^+\) Klrg1\(^+\) Treg cells were also derived from the donor Klrg1\(^-\) cells (Figure 3.23), yet the engraftment was very limited in the WT B6 recipient mice in comparison to the lymphopenic mice.

![Figure 3.23 Generation of Klrg1\(^+\) Treg cells in lymphoreplete mice](image)

**Figure 3.23 Generation of Klrg1\(^+\) Treg cells in lymphoreplete mice**

*Shown is the representative FACS (2 independent experiments) of the engraftment of donor CD45.2\(^+\) Klrg1\(^-\) Treg cells from Foxp3/RFP reporter mice into the congenic CD45.1\(^+\) WT B6 recipient mice and the expression of Klrg1 on host and donor Treg cells.*

Because of the instability of adoptively transferred Treg cells in the lymphopenic condition [20], the nearly undetectable RFP\(^+\) Klrg1\(^+\) donor Treg cells in TCR\(\alpha\)^- recipients may also result from loss of Foxp3 and further conversion to non-Treg cells. Thus, we transferred similar amounts of 1\(\times\)10\(^5\) splenic Thy1.1\(^+\) RFP\(^+\) Klrg1\(^-\) or Thy1.1\(^+\) RFP\(^+\) Klrg1\(^+\) donor Treg cells without conventional T cells to favor the dedifferentiation of Treg cells in Thy1.2\(^+\) TCR\(\alpha\)^- mice, 2 weeks post-transfer, Thy1.1\(^+\) donor cells were examined for the expression of RFP reporter which marks Treg cells. We found that around 50% of engrafted donor cells from RFP\(^+\) Klrg1\(^+\) resource were converted into RFP\(^-\) T cells, while around 70% of engrafted donor cells from RFP\(^+\) Klrg1\(^-\) resource were converted into RFP\(^-\) T cells (Figure 3.24A).
More importantly, remaining Treg cells in TCRα−/− mice receiving Klrg1+ Treg cells maintained the expression of Klrg1, and the total number of Thy1.1+ T cells, including RFP+ and RFP−, was dramatically underrepresented compared to the total number of injected cells. Moreover, these numbers were significantly lower in recipients receiving Klrg1+ Treg cells in comparison to those receiving Klrg1− Treg cells (Figure 3.24B). This observation was not only found in spleen, but also in the MLN (Figure 3.24A-B) which drains lymphocytes to the gut. Overall, the poor engraftment of Klrg1+ Treg cells is not a result of tissue seeking migration or dedifferentiation, but is more likely derived from lack of further expansion and being senescent.

**Figure 3.24 Stability of transferred Treg subsets in lymphopenic mice** (A). Shown is the representative FACS of engrafted donor cells in spleen and MLN of TCRα−/− mice receiving RFP+ Klrg1+ or RFP+ Klrg1− Treg cells. (B). The number of engrafted RFP+ or converted RFP− cells in spleen and MLN, and the comparison between mice receiving Klrg1+ and Klrg1− Treg cells. Data (mean ± SD) are from 2 mice/group.
Furthermore, we also transferred splenic Thy1.1⁺ RFP⁺ Klrg1⁺ or Thy1.1⁺ RFP⁺ Klrg1⁻ Treg cells (1 × 10⁵/mouse) into IL-2Rβ mutant Y3 mice which are lymphoreplete but specifically lack of Klrg1⁺ Treg cells (Figure 3.10, 3.12). Two weeks post-transfer, we found that, unlike the engrafted Treg cells in lymphopenic recipients where they underwent de-differentiation, adoptively transferred Treg cells in Y3 recipients were largely stable in lineage, and that fewer engrafted Klrg1⁺ donor cells retained their expression of Klrg1, instead, Klrg1⁻ donor cells rapidly expanded and generated a substantial amount of Klrg1⁺ Treg cells (Figure 3.25). Collectively, Klrg1⁺ Treg cells are ready to develop from the Klrg1⁻ Treg cells, and the lack of expansion and further differentiation of Klrg1⁺ Treg cells indicate that they are terminally differentiated.

**Figure 3.25 Stability of transferred Treg subsets in lymphoreplete mice**

*Spleenic Thy1.1⁺ RFP⁺ Klrg1⁺ or Thy1.1⁺ RFP⁺ Klrg1⁻ Treg cells were purified from the Foxp3/RFP reporter mice and i.v. injected into the Y3 mice. 2 weeks later, the stability evaluated by RFP maintenance and the differentiation evaluated by Klrg1 expression of injected Treg subsets were examined. Data (mean ± SD) are from 3 mice/group.*

We then wanted to explain the higher Ki67 expression and BrdU incorporation of Klrg1⁺ Treg cells in the face of the impaired ability to proliferate in lymphopenic mice. It is possible that the high proliferation by Klrg1⁺ Treg cells actually reflects the proliferating experience of precursor Klrg1⁻ Treg cells. In order to examine this
hypothesis, we purified CD69\(^{-}\) CD62L\(^{hi}\) CD103\(^{-}\) Klrg1\(^{-}\) “resting” splenic nTregs from the Foxp3/RFP reporter mice and labeled them with CFSE (carboxyfluorescein diacetate succinimidyl ester) prior to transfer into Y3 mice (Figure 3.26A). The reasons why we chose CD69\(^{-}\) CD62L\(^{hi}\) CD103\(^{-}\) Klrg1\(^{-}\) Treg cells to transfer is because these cells are most naive like and should go through developmental progression and differentiation. The reason why we chose non-lymphopenic Y3 mice as recipients is because the lymphopenic pressure drives very rapid proliferation of donor cells in a short time while lymphoreplete mice do not provide enough space for the engraftment of donor cells (Figure 3.11-12, 3.23), thus both of these two kinds of recipients are not feasible for tracking cell division and differentiation in vivo. Moreover, Y3 mice are impaired with the development of Klrg1\(^{+}\) Treg subset, therefore, there is a specific niche available in Y3 mice for the generation of Klrg1\(^{+}\) Treg cells from the Klrg1\(^{-}\) precursor cells.

Three days after adoptive transfer, most nTreg cells in Y3 mice did not start dividing and retained their initial naïve like phenotype. However, on day 6, a large proportion of engrafted Treg cells have fully diluted CFSE and only around 30% of total Treg cells were CFSE labeled. The gradually diluted CFSE peaks indicated multiple cell divisions after transfer and the phenotypes of cells in each division were analyzed. We noticed that engrafted cells were getting more and more activated after repeated divisions, as implicated by the decreased proportion of naïve like Treg cells but increased CD69\(^{-}\) CD62L\(^{lo}\) and CD69\(^{+}\) CD62L\(^{lo}\) Treg cells. Additionally, unlike the expression of CD103 which was exhibited very early after cells started dividing, Klrg1\(^{+}\) Treg cells were hardly detected in the CFSE labeled cells and only observed after multiple cell divisions in the CFSE\(^{-}\) Treg population (Figure 3.26B). Meanwhile, when we analyzed these injected
mice 12 days after transfer, almost all of the engrafted Treg cells lost the CFSE and a higher frequency of Klrg1⁺ Treg cells was observed. Thus, these data once again support the idea that Klrg1⁺ Treg subset is terminally differentiated and the generation of Klrg1⁺ Treg cells is a result of extensive proliferation from Klrg1⁻ Treg cells.

Figure 3.26 Developmental progression of CFSE labeled Klrg1⁻ Treg subset
(A). CFSE labeled splenic CD69⁻ CD62Lhi CD103⁻ Klrg1⁻ RFP⁺ Treg cells were transferred into Y3 mice. The engraftment and phenotype of donor Treg cells from the spleen were analyzed on indicated days. (B). Expression of distinct markers on CFSE⁺ RFP⁺ Treg cells were examined peak by peak. Data represent 3 experiments.
Given that the expression of Klrg1+ Treg cells is not accomplished until the naive precursor Treg cells have undergone extensive proliferation and become activated, we found that adoptively transferred activated Treg subset, like Fr2 Klrg1− or Fr3 Klrg1−, also gave rise to the Klrg1+ Treg cells (Figure 3.27A-B).

Collectively, Klrg1+ Treg cells lack further expansion in both lymphopenic and lymphoreplete conditions, and the generation of Klrg1+ Treg cells from Klrg1− Treg precursor cells are not only seen in lymphopenic mice but also in lymphoreplete mice. Moreover, after experiencing multiple rounds of proliferation from those precursor cells, terminally differentiated Klrg1+ Treg cells are mostly short-lived.

Figure 3.27 Development of Klrg1+ Treg cells from activated Treg subsets (A-B) Splenic Treg subsets CD69−CD62Lhi Klrg1− (Fr1 Klrg1−), CD69−CD62Llo Klrg1− (Fr2 Klrg1−) and CD69+CD62Llo Klrg1− (Fr3 Klrg1−) cells were purified from the Foxp3/RFP reporter mice and respectively co-transferred with conventional CD4+ T cells from the Foxp3/GFP reporter mice into TCRα−/− recipients at the ratio of 1:10 in total amount of 1.1×10⁶ cells. 4 weeks post transfer, cells from the spleen and MLN were analyzed for the proportion of engrafted RFP+ Treg cells in CD4+ T cells (A) and the expression of Klrg1 derived from the input Treg cells (B). Data (mean ± SD) are from 3 mice/group.
3.6 Treg subset in NOD mice

The difference in Treg subsets seen in Y3 mice suggested a potential relationship between altered Treg subsets and autoimmunity. To explore this hypothesis, we utilized the NOD model of autoimmune diabetes, in which auto-reactive T cells destroy the insulin producing β cells in the pancreatic islets. Although autoimmune process starts early in NOD mice, the urine glucose level is usually in the normal range until these mice are over 12 weeks old. Typically, mice with confirmed blood glucose levels >250mg/dl are diagnosed as diabetic. Thus, we compared WT B6, pre-diabetic NOD (6-10 weeks old) and new onset diabetic NOD (>12 weeks) mice for further analysis.

The results indicated that the expression of CD103 and Klrg1 was reduced in splenic Treg cells of the NOD mice compared to B6 mice (Figure 3.28A). However, no significant difference was detected between the pre-diabetic NOD and new onset diabetic NOD mice. Additionally, IL-2 associated pSTAT5 activity was also similarly expressed among the WT, pre-diabetic NOD and diabetic NOD mice (Figure 3.28B). In contrast, Treg cells from the diabetic NOD mice were relatively more activated than pre-diabetic NOD mice by showing more CD69⁻CD62L⁻ cells than CD69⁻CD62L⁺ cells (Figure 3.28C).
It is possible, however, that potential difference may be more closely associated with the local tissue site where the disease is manifested. Thus, we examined the pancreatic LN (PLN) which is the draining LN to pancreas. In comparison to the WT B6 mice, we found more CD4⁺ T cells but fewer Treg cells as well as Klrg1⁺ and CD103⁺ Treg cells in NOD mice. However, the reduction of Klrg1 and CD103 expression is not very striking by comparing pre-diabetic NOD mice to diabetic NOD mice (Figure 3.29). Assessing the expression of Ki67 and Bcl-2 on Treg cells from the PLN indicated that cells from the diabetic NOD mice were reduced in proliferation when compared to pre-diabetic NOD mice, which may contribute to the decreased number of Treg cells during the progression of disease and eventually lose the control of massive auto-reactive T cells in pancreas.
Collectively, although there are fewer Klrg1\(^+\) Treg cells in NOD mice than in the WT B6 mice, no dramatic difference was detected in the peripheral lymphoid compartments by comparing the pre-diabetic to diabetic NOD mice. This leads to the further examination of lymphocytes in pancreas where autoimmune destruction takes place.

Since there is no inflammation or T cell infiltration in the pancreas of a normal WT B6 mouse, for the following studies we chose NOD\(^{B6idd3}\) (IDD3) as control. These congenic mice are largely protected from T1D [101] as they have increased IL-2 production which is considered as a main contributor for the prevention of diabetes. Comparing the pancreas of new onset diabetic NOD mice and age matched IDD3 mice, we found there

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**Figure 3.29 Properties of Treg cells from PLN of NOD mice**

The frequency of total CD4\(^+\) T cells and Foxp3\(^+\) Treg cells in PLN was examined in NOD mice. In addition, the expression of Klrg1, CD103, Ki67 and Bcl-2 was also evaluated. Data (mean ± SD) are from 4-11 mice/group.
were more lymphocytes infiltration in the pancreas of NOD mice than that in IDD3 mice. The frequency of conventional CD4\(^+\) T cells was increased with relatively decreased proportion of Treg cells in diabetic NOD mice. Besides, CD25 expression on pancreatic Treg cells was lower in diabetic NOD mice. However, unlike the other tissue sites we have examined (lung under hypersensitivity or LP of small intestine), the frequency of Klrg1\(^+\) Treg cells in the pancreas was not very high and not significantly different between diabetic NOD mice and IDD3 mice (Figure 3.3).

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Figure 3.30 Comparison of pancreatic Treg cells from IDD3 and NOD mice**

(A). Cellularity of total lymphocytes from the pancreas of age matched non-diabetic IDD3 mice and new onset diabetic NOD mice. (B-C). The frequency of total CD4\(^+\) T cells and Treg cells (B), as well as the expression of CD25 and Klrg1(C) on total pancreatic Treg cells were also evaluated and compared. Data (mean ± SD) are from 4-5 mice/group.

In order to investigate whether Klrg1\(^+\) Treg cells are anergic to IL-2 in NOD mice, we treated 8-week old pre-diabetic NOD mice with IL2-IC, similarly as we did for the WT B6 mice. Two days after the last injection of IL2-IC, the pancreas of NOD mice was dissected and analyzed. We found that the total number of lymphocytes was increased after IL-2IC treatment, and the percent of total Treg cells, including the Klrg1\(^+\) Treg cells was selectively expanded (Figure 3.31A-B) without obvious changes of CD4 and CD8 T cells proportion. The increased number of Treg cells is also correspondent to the enhanced expression of Ki67. However, the expression of CD103 and Bcl-2 was not
dramatically changed after this treatment (Figure 3.31B). Therefore, pancreatic Klrg1⁻ Treg cells in NOD mice are able to respond to IL-2 and have the potential to generate a substantial amount of Klrg1⁺ Treg cells.

Figure 3.31 IL2-IC treated pre-diabetic NOD mice

The cellularity of pancreas, the frequency of total Treg cells and Klrg1⁺ Treg cells, as well as the expression of Ki67, CD103 and Bcl-2 were compared between IL2-IC treated and PBS (Control) treated pre-diabetic NOD mice. Data (mean ± SD) are from 3 mice/group.

To address whether Klrg1⁺ Treg cells have defective migration, beads enriched splenic CD4⁺ T cells (10×10⁶) from the IL2-IC treated pre-diabetic NOD mice were CFSE labeled and adoptively transferred into another un-manipulated pre-diabetic NOD mice. Two days later, the migration of Klrg1⁺ Treg cells were examined by following those CFSE⁺ cells. Since a quite higher amount (20-30%) of splenic Treg cells expressed Klrg1 in IL2-IC treated NOD mice, we hypothesized that if this unique Treg subset has the preferential to migrate into specific tissue sites where autoimmune reactions are undergoing, we should be able to find a relatively higher proportion of Klrg1⁺ Treg cells
in the pancreas. We analyzed different tissue sites, including spleen, MLN, LP of small intestine, PLN and pancreas, yet we did not detect any dramatic difference for the engraftment of CFSE labeled Treg cells as well as the distribution of Klrg1⁺ Treg cells (Figure 3.32). These results actually are consistent with our previous finding that, in the acute asthma mouse model where hypersensitivity is induced in the lung, accumulated Klrg1⁺ Treg cells are responsive to local antigen and generated in situ, instead of migrating from the periphery.

![Figure 3.32 Engraftment of IL2-IC expanded Treg cells](image)

**Figure 3.32 Engraftment of IL2-IC expanded Treg cells**

CFSE labeled IL2-IC expanded splenic CD4⁺ T cells were transferred into pre-diabetic NOD recipients and analyzed 2 days later. Shown is the representative FACS of the engraftment of donor cells and the expression of Klrg1 on CFSE⁺ Treg cells from the spleen or pancreas of. Data represent 3 mice/group.

Overall, the pancreatic Klrg1⁺ Treg subset was not dramatically altered between NOD mice and IDD3 mice. However, we cannot rule out the potential function of this specific Treg subset, since IDD3 mice are not 100% protected from the T1D and also, except the Idd3 locus, there are more than 20 susceptibility loci contributing to the disease of NOD mice [94]. Moreover, Klrg1⁻ Treg cells in the pancreas were responsive to the provision of IL-2 which drove the development of Klrg1⁺ Treg cells. IL-2 treatment has been
shown to be effective in preventing or even reversing diabetes in NOD mice [105, 106]. Therefore, the correlation between IL-2 therapy, the expansion of KlrG1+ Treg subset and prevention of diabetes needs to be further deciphered, i.e. suppressive function in vivo and their antigen specificities, etc.
Chapter 4
DISCUSSION

IL-2/IL-2R signaling is broadly related to the development, homeostasis and function of Treg cells. In the current studies, I furth ered our understanding by demonstrating that low IL-2 signaling is required and sufficient for programming functional Treg cells in the thymus prior to populating the periphery. In contrast, optimal proliferation of developing Treg cells in the thymus may still depend on a strong IL-2 signal. In addition, low IL-2 signaling readily supports the homeostasis of Treg cells in periphery and these cells are not impaired in getting activation in vivo. However, it is noteworthy that Treg cells are not homogeneous in their requirement of IL-2 signaling. A small population of Klrg1 marked Treg subset is diminished under weak IL-2 signaling, but selectively responsive to IL2-IC. Moreover, Klrg1+ Treg cells are highly activated and mainly short-lived. They also exhibit a lack of expansion either with or without IL-2 addition. The development progression experiment indicates Klrg1+ Treg cells are derived from Klrg1− Treg cells after extensive proliferation. These properties are similar to terminally differentiated Klrg1+ CD8+ CTL. This work defined an important and previously unknown activity of IL-2 signaling for supporting Klrg1+ Treg subset, a terminally differentiated effector Treg population potentially for effective suppression of auto-reactive T cells in periphery.
4.1 Impaired thymic Treg compartment by abrogating IL-2Rβ signaling

IL-2/IL-2R signaling is considered to function as a growth/survival signal for the development of thymic Treg cells. This conclusion is primarily based on the finding that the thymus of IL-2- or IL-2R-deficient mice harbor somewhat lower numbers of CD4⁺ Foxp3⁺ Treg cells [3]. However, recent work suggested that IL-2⁻/⁻ Bim⁻/⁻ mice readily restored a normal proportion and number of Treg cells in the periphery, but these cells are not able to rescue their suppressive function [90]. Therefore, it raises one potential question that, beyond the growth/survival activity, some other undefined properties of developing thymic Treg cells may also require IL-2 signal.

Although the IL-2Rβ chain is also related to IL-15 signaling, only modest defects were exhibited in IL-15 knockout mice without obvious autoimmune symptoms [77]. Thus, in contrast to any other cytokines, IL-2 is critical to the Treg population [1]. In Chapter 2, I directly show that thymic development of Treg cells is highly dysregulated in IL-2Rβ⁻/⁻ mice and blocked by yielding mostly CD4⁺ Foxp3lo CD25⁻ T cells. Moreover, these immature thymic Treg cells dramatically reduced the level of expression for some key molecules associated with regulatory function, i.e. CTLA, CD39, and CD73. Thus, functional profile of Treg cells is programmed in advance of exiting the thymus, and systemic autoimmunity in the periphery either from IL-2⁻/⁻, IL-2R⁻/⁻, or even IL-2⁻/⁻ Bim⁻/⁻ mice is the result of impaired functional maturation of Treg cells in the thymus.

In addition, the detection of primarily CD4⁺ Foxp3lo CD25⁻ Treg cells in the thymus of IL-2Rβ⁻/⁻ mice does not represent some aberrant cell populations due to high cytokine production associated autoimmunity. Indeed, CD4⁺ Foxp3lo CD25⁻ Treg cells were also observed from “cured” IL-2Rβ⁻/⁻ mice which were rendered autoimmune-free by
providing them WT Treg cells at birth [74]. Moreover, the thymus of WT mice also harbors a substantial proportion of CD4\(^+\) Foxp3\(^{lo}\) CD25\(^{lo}\) cells where the expression of CTLA4, CD39, and CD73 is largely reduced. Thus, the immature Treg cells in the thymus of IL-2R\(\beta^+/\) mice likely reflect some blockade during Treg cell development.

A simple explanation of the impaired expression of Treg functional molecules by immature Treg cells is that their lower levels of Foxp3 is not sufficient to properly induce and reinforce the Treg suppressive program [21]. It is important to note that we also detected a small fraction of Foxp3\(^{hi}\) Treg cells in the thymus of IL-2R\(\beta^+/\) mice. These cells, however, still expressed a low level of CTLA4 even though CTLA4 is a direct target of Foxp3 regulation. This finding indicates that normal levels of Foxp3 are not sufficient to induce the Treg suppressive program without IL-2R signaling. However, low IL-2R\(\beta\) signaling manifested in Y3 mice readily supports functional thymic Treg cells. Thus, a weak IL-2 signal through activation of STAT5 is required and sufficient for furthering Treg development and programming functional activity, such that they are ready to suppress auto-reactive response once trafficking into the periphery.

4.2 The activity of IL-2 signal in proliferation of thymic Treg cells

Our findings also indicate a role for IL-2 in supporting the expansion of developing Treg cells in the thymus. This conclusion is based on the reduced level of Ki67 on IL-2R\(\beta^+/\) Treg cells in the thymus from the “cured” autoimmune free IL-2R\(\beta^+/\) mice. In contrast, the immature Treg cells from autoimmune-prone, untreated IL-2R\(\beta^+/\) mice showed similar Ki67 expression to WT, suggesting that some factors associated with the concomitant autoimmunity due to IL-2R\(\beta\) deficiency may somewhat increase the
numbers of immature Treg cells in response to auto-reactive T cells. Of course, we cannot rule out that the reduced proliferation and diminished compartment of immature Treg cells from the “cured” IL-2Rβ−/− mice might actually reflect competition of resources or niche due to donor WT Treg cells used to prevent autoimmunity that also populate the thymus. Furthermore, we also noted that Foxp3lo and Foxp3hi Y3 thymic Treg cells expressed lower Ki67. This is consistent with a role for IL-2 in supporting proliferation of developing Treg cells in the thymus.

However, the expression of anti-apoptotic Bcl-2 is largely normal in developing thymic Treg cells from the untreated IL-2Rβ−/−, “cured” IL-2Rβ−/− and Y3 mice in comparison to the WT. Thus, low IL-2 signal is required and sufficient for a normal level of Bcl-2 expression by thymic Treg cells. Overall, IL-2 signaling is probably not crucial for the survival of developing Treg cells, but is necessary for their optimal proliferation.

4.3 Developmental pathway of thymic Treg cells

Past work indicates that CD4+ CD25+ Foxp3− thymocytes contain precursors for the Treg lineage [86, 87]. Our findings raise the possibility that the CD4+ Foxp3lo immature Treg cells might also represent a developmental intermediate for mature CD4+ Foxp3hi CD25+ Treg cells (Figure 4.1). However, these immature Treg cells are largely CD25−, a phenotype unlike the reported precursor with a high level of CD25. Thus, if Foxp3lo CD25− immature Treg cells can further differentiate into mature Treg cells, we would expect them to appear in the thymus before the mature Treg cells. Instead, careful ontogeny studies from previous work do not support this notion because when Treg cells first appear in the thymus, on day 2 of neonatal life, the large majority (~80%) already
exhibits a mature CD4⁺ Foxp³⁺ CD25⁺ phenotype with very few CD4⁺ Foxp³⁻ CD25⁻ cells [80].

Therefore, an alternative scenario is that these immature Treg cells may represent aborted products of the Treg development. In this model, IL-2 would directly drive CD4⁺ Foxp⁻ CD25⁻ thymocytes into mature CD4⁺ Foxp³⁺ CD25⁺ Treg cells. However, some thymocytes through the selection process are also destined to become Foxp⁺ Treg cells but without an IL-2 signal their development is arrested at a CD4⁺ Foxp³⁻ CD25⁻ stage. Due to their high Bcl-2 levels these cells survive and seed the periphery but cannot suppress peripheral autoreactive T cells due to impaired expression of Treg function related to the lack of IL-2 signal.

However, findings from Y3 mice demonstrate that developing Treg cells are exquisitely sensitive to IL-2 signal in the thymus. First, Y3 Treg compartment is qualitatively and quantitatively normal in the thymus under a steady state. Second, in competitive bone marrow chimeras, weak IL-2Rβ signaling in Y3 is much more efficient for thymic Treg development than peripheral Treg homeostasis. Thus, this increased

**Figure 4.1 Thymic Treg development model and the role of IL-2**

*Shown is the schematic model of the development of thymic Treg cells from immature to mature phenotype and the role of IL-2 signaling in this step.*
sensitivity may be intrinsically related to a lower threshold for IL-2 in the thymus. However, an alternative but not mutually exclusive possibility is that IL-2 concentration is relatively greater in the thymus than in the periphery. Regardless, increased IL-2 sensitivity achieved by pStat5 activation is very effective in driving Treg development in the thymus.

To further our understanding at the gene regulation level (Figure 4.2), we predict that, under the condition of low IL-2 signaling, STAT5 binds to the promoter of Foxp3 gene and de novo Treg generation is favored by the interaction between C-Rel and CNS3 (conserved non-coding DNA sequence 3). Physiologically, the association between weak STAT5 activity and CNS2 [9] may also support a normal level of Foxp3 expression on peripheral Treg cells after several rounds of proliferation. However, weak STAT5 activation associated low IL-2 signaling might be not sufficient for the heritable maintenance of Foxp3 expression especially after extensive proliferation as in the competitive bone marrow chimeras. Nevertheless, our work indicates that weak IL-2R signaling is very efficient in promoting thymic Treg development and programming suppressive function prior to populating the periphery.

![Figure 4.2 FoXP3 locus and its interaction with the transcription factor STAT5](image)

**Figure 4.2 FoXP3 locus and its interaction with the transcription factor STAT5**

*Shown is the interaction between distinct transcription factors and CNSs on FoXP3 locus as well as the potential role of specific CNS.*

### 4.4 Peripheral Treg subsets and their interrelationship

Current understanding of Teff cell responses and the development of memory T cells has been greatly facilitated by defining phenotypic and molecular properties that
delineate important cell subsets and by establishing extrinsic and intrinsic factors favoring the development of one subset over another. Two lines of recent work indicate that this paradigm also holds for Treg cells. First, transcription factors important for Th development also function in Treg cells in a manner to distinctively coordinate Treg suppressive programs toward the effector subtype that they inhibit [3]. Second, the gene profiles of Treg subtypes, particularly as they relate to nTreg vs. iTreg, were distinguished from each other by their transcriptional profile [124]. Our study extends this notion by showing that phenotypically distinct nTreg cells express distinctive functional and gene expression profiles, and that IL-2R signaling represents a critical developmental signal for one such subset, i.e. Klrg1+ Tregs.

In a manner analogous to conventional T cells, expression of CD62L, CD69, and Klrg1 delineates nTreg subsets. Gene expression analyses give a picture of Treg subsets that uniquely function within the immune system that is largely defined based on varied activation, growth, death, self-renewal, and migratory properties. Some of these properties were also found to define ex vivo nTreg and iTreg cells [124]. Gene expression and immunological analysis indicated that these subsets generally exhibited a progressively greater trend (Fr1 Klrg1−→Fr2 Klrg1−→Fr3 Klrg1−→Fr3 Klrg1+) for increased proliferation, apoptosis, expression of chemokine receptors targeting inflammatory sites and molecules associated with suppressive function. Fr1 Klrg1+ Treg cells exhibited properties akin to “resting” lymphoid tissue residing long-lived Tregs. Precursor-product studies indicate that at least some Fr1 Treg cells readily support the development of these other more highly activated Treg subsets culminating in production of Klrg1+ Tregs.
4.5 Klrg1 marked Treg subset is distinct

Klrg1 has been shown to mark conventional T cells with a highly proliferative history [53]. Expression of Klrg1 by CD8+ T cells delineates important steps during an immune response [116, 123]. Expression of a high level of Klrg1 by CD8+ T cells only occurs after extensive antigen-dependent proliferation. The CD8+ Klrg1hi cells are associated with Blimp-1-dependent terminally differentiated CD62Llo tissue-seeking effector cells. Consistently, Klrg1+ Treg cells, mainly Klrg1hi, express a higher level of Ki67 (~40%) but a much lower level of Bcl-2 (~5%) and CD62L (~10%). In addition, key genes associated with Treg function, particularly Il10, Fgl2, and Entpd1 (CD39), as well as chemokine receptors associated with cells within inflammatory sites were most highly expressed in the Klrg1+ subset. Blimp-1 expression was also highly enriched (~100%) in Klrg1+ Treg cells. By evaluating naïve and antigen-challenged airway hypersensitive mice and the patterns of Ki67 expression and BrdU incorporation, most Klrg1+ Tregs behave as recent antigen-stimulated, highly proliferative, but short-lived cells. In marked contrast to Klrg1− Tregs, Klrg1+ Tregs did not undergo homeostatic expansion in lymphopenic TCRα−/−, lymphoreplete WT B6 and Y3 recipients. For the few donor Klrg1+ Treg cells found, the expression of Klrg1 was retained. Overall, these properties are similar to short-lived terminally differentiated CD8+ CTL. This characteristic and their high prevalence in the LP and the lung of allergic hypersensitive mice are consistent with the notion that most Klrg1+ Tregs are terminally differentiated tissue-residing suppressor cells. However, we cannot exclude that a small fraction of Klrg1+ Tregs are long-lived and might be analogous to memory-precursor cells.
4.6 Role of IL-2/IL-2R signaling for the development of Krlg1⁺ Treg subset

IL-2 is well known to provide essential homeostatic signals for nTreg cells. IL-2 also shapes the competitive fitness of Treg cells through its ability to maintain Foxp3 levels. Our previous work showed that these key activities of IL-2 readily occur under conditions where suboptimal IL-2R signaling is conducted in peripheral Treg cells [91]. As shown here, weak IL-2R signaling failed to support Klr1⁺ and CD103⁺ Treg cells in the IL-2Rβ mutant Y3 mice. For the Klr1⁺ Treg subset, IL-2 appears to drive the development of this subset from Klr1⁻ Tregs rather than to directly support their proliferation and/or survival. Klr1⁻ Tregs readily proliferate in lymphopenic recipients and develop into Klr1⁺ Treg cells in both lymphopenic and lymphoreplete mice. Moreover, agonist IL2-IC preferentially supported the differentiation of Klr1⁻ Treg cells to Klr1⁺ Treg cells. In marked contrast, purified Klr1⁺ Treg did not undergo homeostatic proliferation or expand to IL2-IC. Thus, the high expression of Ki67 or BrdU associated with Klr1⁺ Tregs mostly likely reflects the extensive proliferation of those Klr1⁻ Treg cells which give rise to Klr1⁺ Treg cells.

An important new finding from this study is that we have defined another function for IL-2R signaling in the periphery, i.e. the development of terminally differentiated Klr1⁺ Tregs. This IL-2-dependent activity on Treg cells is also highly analogous to the contribution of IL-2 for CD8⁺ T effector cells [125-127]. Thus, Treg cells use a similar strategy as effector CD8⁺ T cells to promote their development into cells with heightened suppressive effector function. Additionally, weak IL-2R signaling readily supported Treg homeostasis as Ki67 expression as well as BrdU uptake and loss were very similar for WT B6 and Y3 Treg cells.
Based on our gene profiling and direct analysis of Klrg1+ and Klrg1− Treg cells, we favor a model where distinct expression of CD62L, CD69, and Klrg1 represents distinct activation states (Figure 4.3). The heterogeneity in expression of these and other cells surface molecules has been appreciated for some time [46-49, 54, 114, 128-130], but little is known concerning their potential inter-relationships. This study establishes one such relationship by showing that high IL-2R signaling supports the proliferation of Klrg1− Treg cells and promotes them to develop into short-lived Klrg1+ Treg population with heightened expression of a number of molecules associated with suppressive function. These Klrg1+ Treg cells are also poorly proliferative, lack of further differentiation after adoptive transfer and unresponsive to exogenous IL-2 supply. These properties are highly analogous to the association of Klrg1 expression with CD8+ T cells and NK cells that exhibit replicative senescence [121, 122, 131, 132], and behave as terminally differentiated cells [116, 123].

**Figure 4.3 Interrelationship between Treg subsets and distinct properties**

*Shown is the schematic model of Treg differentiation in the periphery by encountering autoantigen and distinct properties of Treg subsets in terms of their activation, proliferation and survival.*
As illustrated for Treg suppressive activity, our findings indicate that Treg cells also co-opt strategies used by conventional antigen-activated T cells to drive the development of Treg cells of distinct activation states. The linkage of high IL-2R signaling for the production of Klrg1+ Treg cells suggests that this subset is favored under conditions where a stronger or more persistent autoreactive T cell response occurs leading to greater levels of IL-2. High IL-2 levels by the autoreactive T cells may then be counterbalanced by the development of Klrg1+ Treg cells that over-express Treg suppressive molecules. This may occur in peripheral lymphoid tissues but is likely important in inflamed tissues which contain pathogenic effector cells. The short-lived nature of most Klrg1+ Treg cells leads to a diminished contribution by these Treg cells, if the autoreactive response is restrained. However, chronic immune stimulation, as occurs within the gut mucosa, appears to favor continued development of Klrg1+ Tregs. Thus, lower levels of Klrg1+ Treg cells may represent a risk factor for autoimmunity. Indeed, the autoimmune symptoms associated with older Y3 mice may be due in part to the lower level of Klrg1+ Treg cells in these mice. In any case this model provides a frame-work for future studies to more fully understand the inter-relationship and development of Treg subpopulations that encounter self or foreign antigens.

4.7 Correlation between Klrg1+ Treg subset and autoimmune T1D

An important contributing factor to T1D in NOD mice is reduced IL-2 levels, which does not impair Treg development or homeostasis, but leads to a reduction in the proportion of Treg cells within the inflamed islets [103, 106, 133]. Moreover, polymorphisms linked closely to Il2ra, Il2rb and Il2 have been associated with a number
of human autoimmune diseases [108]. Such a genetic contribution may likely manifest itself as a quantitative effect on IL-2R signaling over a period of time. Thus, it is intriguing to speculate that impaired development of Klrg1$^+$ Treg cells may represent a contributing factor in T1D since Klrg1$^+$ Treg cells require strong IL-2 signaling for their development and lower IL-2 production and CD25 expression were observed in pancreatic Treg cells of NOD mice. Thus, we examined the Klrg1 marked Treg cells in NOD mice and found this subset was diminished in peripheral lymphoid organs when compared to WT B6 mice. However, similar expression pattern of Klrg1$^+$ Treg cells in periphery between NOD and IDD3 mice leads us to further analysis of tissue specific T cell compartment where we found more T cell infiltration in the pancreas of NOD mice but relatively lower proportions of Treg cells expressing lower CD25 levels when compared to IDD3 mice. Interestingly, unlike the other tissue sites we’ve examined, for example the small intestine and lung, the frequency of Klrg1$^+$ Treg cells was low in the pancreas and no dramatic difference was detected between NOD and IDD3 mice. Given the notion that IDD3 mice are not fully protected from T1D and IL-2 therapy is protective for NOD mice [105, 106], as well as pancreatic Klrg1$^+$ Treg cells did respond to IL2-IC treatment, we would expect that other Idd loci except for Idd3 in NOD mice may also be involved in diminishing the Klrg1$^+$ Treg subset. The therapeutic role of IL-2 for NOD mice and its correlation with Klrg1$^+$ Treg cells will need to be further clarified in future studies.
Chapter 5

MATERIALS AND METHODS

Mice

C57BL/6, TCRα−/− mice (B6.129S2-Tcra<sup>tm1Mom</sup>/J), NOD mice (NOD/ShiLtJ), IDD3 mice (NOD.B6-<i>Idd3</i><sup>C57BL/6</sup>), were obtained from Jackson laboratory (Bar Harbor, Maine). Congenic C57BL/6 mice (CD45.1, Thy1.1), IL-15−/− mice (C57BL/6NTac-<i>IL15</i><sup>tm1lmxN5</sup>) were obtained from Taconic (German Town, NY). The reporter mice, Foxp3/GFP (kindly provided by A. Rudensky), Foxp3/RFP (kindly provided by R.A. Flavell), and Blimp-1/GFP (kindly provided by S.L. Nutt) were previously described [21, 84, 118]. IL-2Rβ transgenic mice, including IL-2Rβ<sup>−/−</sup>, IL-2Rβ<sup>WT</sup> (Y0), IL-2Rβ<sup>Y341</sup> (Y1), IL-2Rβ<sup>Y395,498</sup> (Y2), IL-2Rβ<sup>Y341,395,498</sup> (Y3) mice, were generated on the IL-2Rβ<sup>−/−</sup> genetic background and previously described [74, 91]. Blimp-1/GFP mice and Y3 mice were each crossed to the Foxp3/RFP mice in order to mark their Treg cells with the RFP reporter. All mice were maintained in animal facility under VAF conditions at the University of Miami, Miller School of Medicine. Animal studies were approved by the Institutional Animal Care and Use Committee at the University of Miami.

Cure Injection

Cured IL-2Rβ<sup>−/−</sup> mice were previously described [74]. Here bead (Miltenyi Biotec) enriched total splenic CD4<sup>+</sup> T cells from congenic CD45.1 B6 mice were used for adoptive transfer (2-3×10<sup>6</sup> cells/neonatal mouse), so that it was possible to distinguish the Treg cells either from donor CD45.1<sup>+</sup> or host CD45.2<sup>+</sup> population.
**Cell preparation and purification**

Single cell suspensions from thymus, spleen, axillary LN, Mesenteric LN, pancreatic LN and pancreas were prepared by mechanic disruption. LP cells were prepared as previously described [134]. In brief, the small intestine was cut at 0.5 cm below the stomach (from the duodenum) to 1 cm above the cecum, and flushed with HBSS containing 5% FBS. After dissecting the PP, the intestines were cut into pieces (2–5 mm) and treated (shaking at 200 rpm for 20 minutes at 37°C) with Ca^{++}-Mg^{++}-free HBSS containing 5% FBS and 1.3 mM EDTA. Sections of gut were further treated (shaking at 200 rpm for 60 minutes at 37°C) with RPMI1640 containing 5% FBS, 100 U/ml collagenase VIII and 100 U/ml trypsin inhibitor. LP lymphocytes were then purified on a 44/67% Percoll gradient (800 g for 20 minutes at 20°C).

For adoptive transfer and microarray analyses of total Treg cells or Treg subset cells, splenic cells were first enriched in CD4 T cells by positive selection using anti-CD4 magnetic-beads (Miltenyi Biotec), and after staining with CD69, CD62L and KlrG1, distinct Treg cell populations were sorted using a FACS Aria IIu cell sorter (Becton Dickenson). The purity of sorted populations was typically over 99% and never less than 97%.

**Antibodies and Flow Cytometry**

Conjugated antibodies CD4 (clone RM4-5), CD8 (clone 53-6.7), CD25 (clone PC61), CD69 (clone H1.2F3), CD62L (MEL-14), CD103 (clone 2E7), KlrG1 (clone 2F1/KLRG1), CD45.2 (clone 104) and Thy1.2 (clone 30-H12) were purchased from Biolegend (San Diego, CA.). Conjugated antibodies Foxp3 (clone FJK-16S), CTLA4
(clone UC10-4B9), CD39 (clone 24DMS1) and CD73 (clone TY/11.8) were purchased from eBioscience (San Diego, CA.). Ki67 (clone B56) and Bcl-2 (clone Bcl-2/100) were purchased from BD Pharmingen (San Jose, CA.). Intracellular staining of Ki67, Bcl-2 and Foxp3 was performed together according to the manufacturer’s instructions for Foxp3 (eBioscience). Flow cytometry analysis was performed on an LSRII or Fortessa flow cytometer with FACS Diva software (Becton Dickenson). Typically at least 300,000 events were obtained for each sample.

**pStat5 activity**

Intracellular staining of pStat5 was performed as previously described [91]. In brief, IL-2-stimulated cells were harvested and fixed with 1.5% PFA at 37°C for 10 minutes and further incubated with 0.5ml methanol on ice for 30 minutes. After washing twice with PBS containing 0.02% NaN₃ and 0.5% BSA, cells were stained with antibodies to surface and intracellular markers for 1 hour at room temperature in the dark. To assess pStat5 in vitro, thymocytes were cultures with IL-2 (10 ng/ml) for 15 min prior to paraformaldehyde fixation. To assess pStat5 activity in vivo, thymus cell suspensions were immediately prepared in ice cold RPMI 1640 containing 5% FCS and a 100 µl aliquot (~2% of the suspension) was transferred into a test tube and the cells were fixed by addition of 900 µl of 1.5% paraformaldehyde.

**Bone marrow chimeras**

Bone marrow chimeras were generated as previously described [88]. Briefly, host congenic Thy1.1 B6 mice or TCRα⁻/⁻ mice received a single dose of total body irradiation
(900Rads or 600Rads) and after 24 hours, these mice received a 1:1 mixture of the indicated T cell-depleted bone marrow (5 × 10⁶ total cells) by i.v. injection into the tail vein. The mice were then maintained with gentamycin (1mg/dl) containing drinking water.

**Microarray analysis**

Total RNA was isolated using TRizol and further purified with RNAeasy Minikit (QIAGEN, Valencia, CA). RNA quantity and quality was assessed by analysis with an Agilent 2100 BioAnalyzer. 20 to 50 ng RNA was used in a single round of linear RNA probe amplification and labeling using NuGEN Ovation Pico WTA system, WT-Ovation Exon module and Encore Biotin Module (NuGEN, San Carlos, CA.). Probe preparation and microarray analyses by using Affymetrix Mouse Gene ST 1.0 arrays were performed at the Microarray and Gene Expression Core within the John P. Hussman Institute for Human Genomics at the University of Miami. Image analysis was performed using the Affymetrix Command Console Software (AGCC). Data were normalized with the RMA method using software at GeneSifter (Seattle, WA). Multi-group comparisons of the transformed data of at least 2 independent biological replicates were performed using ANOVA applying the Benjamini Hochberg correction for false positives. Genes expressed ≥2.0-fold up or down (p<0.05) between groups were considered differentially expressed. GEA for differentially expressed genes were performed using GeneSifter.
Allergic Asthma Induction

Mice were sensitized by i.p. injection of 66 µg ovalbumin (crystallized chicken egg albumin, grade V; Sigma-Aldrich, St. Louis, MO) adsorbed to 6.6 µg alum (aluminum potassium sulfate; Sigma-Aldrich) in 200 µl PBS on day 0, with an i.p. boost on day 5. On day 12, mice were aerosol challenged with 0.5% w/v ovalbumin (Sigma-Aldrich) in PBS for 1 hour using a BANG nebulizer (CH Technologies, Westwood, NJ) into a Jaeger-NYU Nose-Only Directed-Flow Inhalation Exposure System (CH Technologies). Mice were sacrificed either on day 12 without aerosol or on day 15 after aerosol. Bronchoalveolar lavages obtained as well as lung lobes perfused and processed for single cell suspension made from lung homogenate for flow cytometry analysis as described previously [135]. Spleen and draining bronchial LN were also procured for subsequent flow cytometry analysis.

Treg suppression and survival assays in vitro

Suppression was assessed by the capacity of purified Treg subsets to inhibit proliferation of responder conventional CD4+ T cells as previously described [74]. Briefly, purified WT Treg subsets were co-cultured with conventional CD4+ T cells (5×10^4) at a ratio of 1:1 or 1:2 in a 96 well round bottom plate with anti-CD3 (0.25 µg/ml) and APC (5×10^4) from mitomycin C (50 µg/ml) treated splenocytes. The proliferation by responding cells was eventually assessed by the incorporation of ^3H-thymidine which was added into the culture for the last 4 hours of a 72-hour culture.

To assess the survival of Treg subsets, magnetic bead enriched splenic CD4+ T cells from WT mice were cultured in a 24-well plate (1-2 x 10^6/well) with RPMI1640
containing 5% FCS for 3 days. At the indicated time, the viability of Treg subsets identified with distinct surface markers was assessed by using the Live/Dead fixable stain kit (Life Technologies, Grand Island, NY). Briefly, cells were harvested from the culture and incubated with the reconstituted fluorescent reactive dye from the kit for 30 minutes at room temperature. After washing by Hank’s buffer, cells were stained with subset surface markers and then fixed for intracellular Foxp3 staining. The data were analyzed by FACS and the number of recovered Treg cells was calculated.

**Adoptive transfer**

The indicated purified Treg population was adoptively transferred by i.v. injection through the tail vein into B6, Y3, CD45.1 congenic B6, or TCRα−/− recipients, as indicated in each Figure. In most cases TCRα−/− recipients were co-injected with Treg-depleted conventional CD4+ T cells using Foxp3/GFP reporter mice to achieve ~1:10 ratio of Treg:T conventional cells. To study developmental progression, Treg cells were labeled with CFSE using the Vibrant CFDA SE Cell Tracer Kit (Sigma-Aldrich) prior to transfer. The Treg cells (5×10^5/ml) were incubated with 5 µM CFSE in RPMI1640 containing 5% FCS for 15 minutes at 37°C according to the manufacturer’s instruction.

**Anti-IL-2/IL-2 complex treatment**

Mouse IL-2 and the JES6-1A12 mAb against mouse IL-2 were purchased from eBioscience. Anti-IL-2/IL-2 complexes were prepared as previously described [120]. In brief, IL-2 and JES6-1A12 were incubated at a molar ratio of 2 to 1 in PBS at room temperature for 30 minutes such that each mouse received 1 µg of IL-2 and 5 µg of JES6-
1A12 in 200 µl PBS by i.p. injection for 3 consecutive days. Control mice received 200 µl PBS for 3 consecutive days. Mice were analyzed 2 days after the last injection of the complex.

**BrdU incorporation assay**

Mice received 0.8mg/ml BrdU contained drinking water for 5 days. The incorporation of BrdU was detected by following the manufacturer’s instructions (BD pharmpingen) and analyzed by FACS. Briefly, splenic cells were stained with surface markers for 15 minutes on ice. After washing, cells were fixed with Cytofix/Cytoperm Buffer for another 15 to 30 minutes and then incubated with Cytoperm Plus Buffer for 10 minutes on ice. Cells were washed again and re-fixed with Cytofix/Cytoperm Buffer for 5 minutes. Following DNase (300µg/ml) treatment, cells were then stained with fluorescent anti-BrdU and other intracellular antibodies for 20 minutes at room temperature.

**Statistical analysis**

Data were analyzed using Prism 5.0 software. Multi- and two-group statistical analyses were performed using in a one-way ANOVA with Tukey’s multiple comparison test and t-test. P<0.05 was considered significant. Significant difference are designated as * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. BrdU turnover data were subjected to linear regression analysis and assessed for significantly different slopes.
Works Cited


