The Role of IL-2Rβ-dependent Signaling and CD103 in Regulatory T Cells for Mucosal Tolerance

Xiaomei Yuan  
*University of Miami, yuanxiaomei2235@hotmail.com*

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THE ROLE OF IL-2Rβ-DEPENDENT SIGNALING AND CD103 IN REGULATORY T CELLS FOR MUCOSAL TOLERANCE

By

Xiaomei Yuan

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

August 2014
THE ROLE OF IL-2Rβ-DEPENDENT SIGNALING AND CD103 IN REGULATORY T CELLS FOR MUCOSAL TOLERANCE

Xiaomei Yuan

Approved:

Thomas R. Malek, Ph.D.  Eckhard R. Podack, M.D., Ph.D.
Professor of Microbiology and Immunology Professor of Microbiology and Immunology

Wasif N. Khan, Ph.D.  Maria T. Abreu, M.D.
Professor of Microbiology and Immunology Professor of Medicine

Krishna V. Komanduri, M.D.  M. Brian Blake, Ph.D.
Professor of Medicine Dean of the Graduate School

Todd M. Brusko, Ph.D.
Assistant Professor of Pathology, Immunology, and Laboratory Medicine
University of Florida
A network of mechanisms operates to maintain tolerance in the gut mucosa. CD103 marks many lymphoid cells, including regulatory T cells (Tregs), within the gut. CD103+ Tregs represent a subset of Tregs with potent suppressor function \textit{in vitro} and \textit{in vivo}. However, other features of this subset remain undefined. In current study, we found that CD103+ Treg subset exhibited a more activated phenotype and showed increased suppressive activity in preventing inflammation in the small intestine. We also showed that low IL-2R signaling had localized effect on CD103+ Treg subset in the peripheral lymphoid organ vs. tissue site.

With respect to the function of CD103 in intestinal tolerance, CD103 may be part of a redundant pathway as CD103+ mice do not exhibit autoimmunity. To reduce such redundancy, CD103-/- mice were crossed to mice (designated Y3) whose T cells expressed mutant IL-2Rβ chains that lower IL-2R signaling. Unlike Y3 mice that only exhibit symptoms of a mild autoimmune attack, all Y3/CD103-/- mice rapidly developed severe colitis. The large intestine of these mice contained an increase in CD4+ T helper (Th)1 and Th17 effector cells and a reduced ratio of Tregs. Importantly, colitis was effectively prevented by the transfer of wild type (WT) Tregs into Y3/CD103-/- mice. Impaired intestinal tolerance was not attributed to an obvious lack of CD103-dependent
gene regulation or intestinal homing/retention by Tregs nor a lack of functional activities typically associated with CD103+ dendritic cells (DCs), such as peripheral induced Treg (pTreg) development or imprinting CCR9 and α4β7 homing molecules on Treg and T effector cells. Transcriptome analysis of Tregs was consistent with altered homeostasis due to impaired IL-2Rβ-dependent signaling with minimal dysregulation added by the absence of CD103. Rather the absence of CD103 functioned to further dysregulate Treg homeostasis through a mechanism primarily associated with improper localization of the cells within the gut microenvironment that may prevent receiving optimal survival signals. Thus, IL-2Rβ-dependent signaling and CD103 normally cooperate through distinctive processes to promote Treg homeostasis and immune tolerance.
DEDICATION

I would like to dedicate this thesis work to my parents, Xueqin and Yongchang Yuan for their great love and support during the challenges of my graduate studies and life. Thank you and I love you!
ACKNOWLEDGEMENT

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I also want to thank Jay Enten, Shannon Opiela and Patricia Guevara in the Flow lab, and Marcia Boulina, Allison Bayer, and Oliver Umland in the Diabetes Research Institute for their expertise and enormous help.

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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<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>
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<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CCL</td>
<td>CC chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC chemokine receptor</td>
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<tr>
<td>CNS</td>
<td>Conserved noncoding sequence</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytolytic T lymphocyte-associated antigen-4</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine receptor</td>
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<tr>
<td>DAPI</td>
<td>4’,6’-diamidino-2-phenylindole</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
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<tr>
<td>DSS</td>
<td>Dextran sulfate sodium</td>
</tr>
<tr>
<td>DT</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EG</td>
<td>Enrichment group</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FCS</td>
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</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
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<td>γC</td>
<td>Common gamma chain</td>
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<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GVHD</td>
<td>Graft-versus-host disease</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
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<td>hr</td>
<td>Hour</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>ICOS</td>
<td>Inducible costimulator</td>
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<tr>
<td>IEL</td>
<td>Intra-epithelial lymphocyte</td>
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<td>IEC</td>
<td>Intestinal epithelial cell</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>iTreg</td>
<td><em>in vitro</em> induced Treg</td>
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</table>
i.v. Intravenous
KlrG1 Killer cell lectin like receptor G1
LI-LP Lamina propria of the large intestine
mAb Monoclonal antibody
MACS Magnetic-activated cell sorting
MAIT Mucosal-associated invariant T cell
miR MicroRNA
mg Milligram
MHC Major histocompatibility complex
min Minute
ml Milliliter
MLN Mesenteric lymph node
mRNA Messenger Ribonucleic acid
MyD88 Myeloid differentiating factor 88
n Number in study or group
ng Nanogram
ND Not determined
NK Natural killer
NOD Non-obese diabetic
Nrp-1 Neuropilin-1
ns Not significant
OCT Optimal cutting temperature
OVA Ovalbumin

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>P</td>
<td>Probability</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>pLN</td>
<td>Peripheral lymph node</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PPs</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>pTreg</td>
<td>Peripheral-induced Treg</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination-activating gene</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SFB</td>
<td>Segmented filamentous bacterium</td>
</tr>
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<td>SI-LP</td>
<td>Lamina propria of the small intestine</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tfh</td>
<td>T follicular helper</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Tr1</td>
<td>T regulatory type 1 cell</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSDR</td>
<td>Treg-specific demethylated region</td>
</tr>
<tr>
<td>tTreg</td>
<td>Thymic-derived Treg</td>
</tr>
<tr>
<td>V</td>
<td>Variable region</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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CHAPTER 1

INTRODUCTION

1.1 Tolerance and Tregs

Self-tolerance, a state in which immune system does not attack its own body, can be divided into two broad categories: central tolerance where most of the self-reactive cells are removed in the bone marrow (BM) or thymus due to clonal deletion and peripheral tolerance where self-reactive cells are suppressed by a variety of mechanisms such as antigen segregation, anergy, cytokine deviation, or suppression by regulatory cells in the periphery (1-4). It is well accepted that CD4⁺ Foxp3⁺ regulatory T cells (Tregs) are a dedicated lymphocyte population that mediates peripheral tolerance.

Tregs were first identified as a subpopulation of CD4⁺ T cells which are CD25⁺, capable of suppressing autoimmunity in athymic nu/nu mice induced by the transfer of CD4⁺ CD25⁻ cells (5). Since then, these cells have been extensively studied and it is known that Tregs not only suppress autoreactive T cells, but also help regulate immune responses to infectious agents and tumors. At the molecular level, transcription factor Foxp3, which is encoded by X-chromosome, has been recognized as a faithful marker for Tregs (6, 7). The essential role for Foxp3 in Treg programming has been shown in animal models and in humans that express mutations in the Foxp3 gene (8-13). In these cases, Tregs are not produced and lethal autoimmunity ensues. Furthermore, Foxp3 must be induced for Treg development and its expression is actively maintained in mature Treg cells for their suppressive function (12-16).
Natural occurring Tregs develop within the thymus (tTregs) after expression of Foxp3 at a relatively late stage of thymopoiesis that is primarily confined to “single positive” (SP) CD4\(^+\) T cells. tTregs represent a minor population of thymocytes, roughly 4% of the SP CD4\(^+\) cells (17). T cell receptor (TCR), co-stimulatory, and IL-2 signals are required for thymic development of tTregs. After exiting the thymus, tTregs are shaped by basal environmental cues and inflammatory responses that regulate their suppressive program, migration and homeostasis (18).

Foxp3 can also be expressed by conventional T cells in the periphery to generate suppressive induced Tregs (pTregs). These cells have been implicated in maintaining tolerance in tissues sites, for example when encountering food antigens and commensal bacteria within the gut mucosa. The overall contribution of pTregs to the total pool of peripheral Tregs under basal and inflammatory conditions remains under debate. TCR repertoire analyses of peripheral Tregs in lymphoid tissues have been estimated to be from 5-20% of all Tregs (19, 20). However, the extent these cells might dominate the Treg pool within tissues at the site of immune responses remains unknown.

1.2 Homeostasis and maintenance of Tregs in the periphery

Treg homeostasis is a process of maintaining stable and constant generation, proliferation and survival of functional Tregs in the periphery. Peripheral Treg homeostasis is a prerequisite to maintain normal Treg suppressor function and thus reinforces tolerance. The homeostasis of Tregs might be analogues to CD4\(^+\) and CD8\(^+\) conventional T cells, i.e. requires self-antigen MHC, co-stimulatory signals and cytokines (Fig. 1.1). Numerous
Factors are thought to influence Treg homeostasis and they vary remarkably at lymphoid tissue vs. local tissue sites, in steady state vs. inflammatory responses.

**Fig. 1.1 Treg homeostasis in lymphoid and non-lymphoid tissues.**
TCR, co-stimulatory, and IL-2 signals are required for thymic development of Tregs. After exiting the thymus, peripheral Tregs also depend on self-peptide/MHC interaction with TCR, co-stimulatory signals and cytokines such as IL-2, TGF-β for their survival, proliferation and maintenance. After entering the tissue sites, Tregs may acquire additional local environmental cues for their homeostasis.
Low-affinity TCR and co-stimulatory requirements

Tregs are more activated than T conventional cells, which raises the possibility that the homeostasis of Tregs might be different from naïve T conventional cells and would be independent of self-peptide MHC interaction. However, the proliferative rate of Tregs was remarkably diminished after adoptive transfer of WT Tregs into MHCII-deficient lymphopenic mice (21), suggesting that auto-antigens are required for the maintenance of Tregs in the periphery. In line with this, depletion of DCs was correlated with the decrease of Tregs and this was MHCII-dependent (22). More strikingly, the increase of Treg numbers by increasing DC numbers effectively prevented autoimmunity in mice. These findings clearly point to the critical role of DCs in Treg homeostasis. TCR repertoire analysis of Tregs revealed that the specificities of Treg TCRs from different anatomical locations largely differ (19). The latter finding provides further evidence implying that local antigens shape the TCR repertoire and therefore play an important role for the homeostasis of local Tregs.

Anti-CTLA4 or anti-B7 treatment to block CD28/B7 co-stimulation in Non-obese diabetic (NOD) mice resulted in a marked reduction of peripheral Tregs (23, 24). The decrease of peripheral Tregs could also be observed from adult thymectomized mice after anti-B7 treatment (23), which excludes the possibility that this reduction was secondary to the failed thymic generation of Tregs due to co-stimulation blockade. Moreover, adoptive transfer of Tregs to recipient mice followed by anti-B7 treatment resulted in a similar effect on Tregs (23), suggesting that CD28/B7 is mandatory for peripheral Treg survival and self-renewal. It turns out that CD28/B7 co-stimulation regulation of Treg homeostasis is achieved probably by increasing IL-2 availability (23). Additionally,
another co-stimulatory molecule, ICOS has been shown to influence Treg expansion and survival (25). Collectively, these findings support a notion that self-antigens and co-stimulatory signals are indispensable for peripheral Treg homeostasis.

Role of γC cytokine in Treg homeostasis

Cytokines which are produced in a secreted form are the most robust factors promoting T cell homeostasis through cytokine receptors either constitutively expressed or induced by inflammatory stimuli. IL-2 is the most studied cytokine which profoundly affects Treg homeostasis. Several pieces of evidence support this notion. IL-2-and IL-2R-deficient mice contain a dramatic reduction in the Treg frequency and number in the periphery, which are characterized as CD25lo Foxp3lo (26, 27). However, the caveat of these studies is there is no way to clearly distinguish that the decrease of Tregs is due to failed thymic output or poor homeostasis in the periphery. Consistently, anti-IL-2 treatment was shown to decrease Tregs in the periphery (28). In contrast, administration of low dose IL-2 or agonist IL-2-anti-IL-2 complex resulted in a rapid expansion of peripheral Tregs (29-31), suggesting that IL-2 represents a critical cytokine for Treg expansion and survival in the periphery.

Adoptive transfer of WT Tregs to neonatal IL-2Rβ−/− mice sufficiently restored tolerance and rescued mice from developing fatal disease (32). Analysis of these “cured” adult IL-2Rβ−/− mice revealed that the peripheral Treg pool constituted the large majority of WT Tregs with very few host-derived Foxp3lo Tregs. These observations clearly demonstrated that IL-2Rβ−/− Tregs are unable to compete with WT Tregs which are responsive to IL-2 and donor WT Tregs are capable of maintaining the life-long
homeostasis without continuous thymic output. These results support a view that IL-2R signaling is essential for Treg homeostasis. In line with this view, adoptive transfer of CD4+ T cells from IL-2−/− mice prevented the autoimmunity in mice with spontaneous experimental autoimmune encephalomyelitis (EAE) (33). Donor-derived IL-2−/− Tregs readily engrafted and functioned in an IL-2 sufficient environment to suppress the otherwise EAE in recipient mice.

Transgenic expression of IL-2Rβ under lck promoter on IL-2Rβ−/− background resulted in normal Treg development and homeostasis (27, 34). This finding apparently contradicts with the notion that IL-2 plays a non-redundant role in Treg homeostasis in the periphery, because in this case, Tregs express low, if detectable IL-2Rβ after exiting the thymus. Two possibilities perhaps account for this observation. The normal frequency and number of Tregs found in these transgenic mice could be due to continuous thymic output of Tregs to seed the periphery. Alternatively, Tregs maintain their homeostasis by low or minimal IL-2R signaling or other survival signals. Indeed, peripheral Tregs were well-maintained after adult thymectomy and these transgenic mice lacked autoimmunity associated with IL-2Rβ deficiency (27). Furthermore, the proliferation and turnover of peripheral Tregs after adult thymectomy did not change. Thus, normal Treg homeostasis in these mice is unlikely due to continuous output of thymic Tregs. In addition, elegant mixed thymus and BM chimeras revealed that WT precursor-derived Tregs had an advantage over Tregs with impaired IL-2R signaling and reconstituted almost the whole Treg pool in the periphery (27). Taken together, these findings indicate IL-2 is dominant in controlling Treg homeostasis and weak or transient IL-2R signaling is sufficient for Treg homeostasis in a non-competitive setting. The latter notion is further supported by
the evidence derived from IL-2Rβ−/− mice with transgenic expression of mutant IL-2Rβ chain, where low IL-2R signaling is ready to support normal Treg homeostasis in the periphery and sustain tolerance (35).

The comparison of gene expression profile of Tregs from IL-2−/− mice treated with IL-2 or control PBS revealed that IL-2 mainly regulates the genes involved in proliferation, survival and metabolism fitness in Tregs (26). The mechanism for how IL-2 regulates Treg homeostasis is partially attributed to microRNA155 (miR155) (36). In mixed BM chimeras of miR155−/− and miR155+/+, peripheral Tregs remained at a 1:1 ratio before BM was fully reconstituted whereas miR155+/+ BM derived Tregs largely populated the peripheral Treg pool after full BM reconstitution. This observation suggests that in lymphoreplete environment, miR155 is essential for Tregs to compete for limited survival cytokines, such as IL-2. Indeed, in Tregs, miR155 is required to suppress the expression of SOCS1 which is an inhibitory factor for IL-2-induced STAT5 phosphorylation.

IL-2 is thought to provide survival signals to the Tregs in the periphery. Recently, one group demonstrated that IL-2-dependent survival might occur through regulating the anti-apoptotic factor Mcl-1 (37). Depletion of 50% Tregs by administration of diphtheria toxin (DT) in female mice, which genetically bear Foxp3/DTR on one X chromosome and congenic marker on the other, resulted in a rapid expansion followed by a contraction of Treg numbers to a normal range. This pattern of expansion-contraction is IL-2-dependent. The interesting observation led the authors to investigate the intrinsic pathways of apoptosis and survival of Tregs. Indeed, proapoptotic factors, Bim, Bax and Bak, are responsible for Treg apoptosis whereas anti-apoptotic factors, Bcl2 and Bclx, are
dispensable for Treg survival. However, the IL-2-dependent anti-apoptotic Mcl-1 is non-redundant and essential for Treg survival and deficiency of this factor in Tregs leads to early autoimmunity in mice (37).

Recent findings from Campbell’s group further showed that IL-2 is indispensable for the homeostasis of naïve-like Tregs and the co-stimulatory signals, such as DC and ICOS are required for the tissue effector-like Tregs (38). Collectively, these studies elaborated the role of IL-2 in maintaining Treg homeostasis and the mechanisms underlying how this cytokine regulates this process.

Other cytokines and factors influencing Treg homeostasis

TGF-β has been known to support the development of pTregs, but it is still controversial for the development of tTregs. Nevertheless, it is essential for the peripheral Treg homeostasis (39, 40). Other cytokines, such as TNF, play a role. Both in vitro and in vivo experiments have showed that TNF promotes Treg expansion (41-43). Moreover, TNF receptor superfamily member 25 (TNFRSF 25), upon agonist activation, led to rapid expansion of peripheral Tregs (44).

Tregs not only exist in lymphoid tissues, but also reside in the non-immune tissues (18, 45). Thus, it is not surprising that local environmental cues are the additional essential players to maintain the local homeostasis of Tregs. Such as Vitamin A and D metabolites, these factors have been shown to influence intestine and skin tissue-specific Tregs and thus regulate local Treg homeostasis (46-50). During the course of inflammatory responses, Tregs are further affected by the inflammatory cytokine milieu.
1.3 Treg stability and plasticity

Another important issue about Tregs in the context of autoimmunity is whether Tregs represent a stable cell lineage or they have the capacity to be reprogrammed into T effector cells. This is becoming an important consideration in the application of reagents to boost Treg expansion to suppress unwanted immune responses. There is an emerging picture that some Tregs lose Foxp3 expression and de-differentiate into other T effector cell lineage.

*Tregs lose Foxp3 expression in several settings*

Numerous reports have been shown that Tregs are unstable in various settings. For example, *in vitro* derived Tregs (iTregs) easily lost Foxp3 in the presence of IL-6 and they differentiated towards Th17 cells upon adding additional cytokines, such as TGF-β, IL-1 and IL-21 (51). Even tTregs showed capacity to differentiate to Th17 cells in the presence of IL-6 *in vitro* (52). *In vivo*, it has been shown that Tregs lost Foxp3 expression and acted as T follicular helper (Tfh) cells to promote germinal center (GC) formation and IgA production in the gut after adoptive transfer into lymphopenic mice (53). In agreement with these, in other lymphopenic settings, Tregs lost Foxp3 and produced inflammatory cytokines (54-56). In these cases, Foxp3 expression in Tregs is unstable and Tregs de-differentiate into T effector cells probably due to lack of IL-2 (54, 55). The idea that Tregs are unstable is also supported by the studies showing Tregs were reprogrammed to Th or T effector cells under inflammatory cytokine milieu (57-61). By developing sophisticated reporter mice to trace the stability of Foxp3⁺ Tregs, Bluestone’s group showed that the large majority (>80%) of Tregs were stable whereas nearly 20% of
cells were at one time in the Treg lineage (ex-Tregs) and unstable Tregs increased under inflammatory conditions (59). These ex-Tregs expressed an activated/memory phenotype with T effector functions. Given the observation that Tregs lose suppressor function and gain effector cell function under certain conditions, a model has been proposed, i.e. the CD4+ T cell effector program is a default program and Foxp3 acts as a repressor to inhibit the effector program (62). This model is supported by the finding of SATB1, a genome organizer and transcription factor, repressed by Foxp3 in Tregs (62). Enforced expression of SATB1 in Tregs resulted in loss of suppressor function in Tregs and acquisition of Th effector phenotype and transcription program. Collectively, these findings are consistent with the notion that Tregs exhibit plasticity and they have the potential to differentiate into Th cell types under some inflammatory settings.

Other studies, however, argue against the above notion but rather claim that Tregs are a committed cell lineage. The latter idea is supported by evidence from the adoptive transfer of Treg subsets based on CD25 expression into lymphopenic mice where only a small population among the CD25- subset was unstable and lost Foxp3 (55). Taking the advantage of $Foxp3^{GFP\text{-}Cre^{ROSA26^{RFP}}}$ reporter mice, it was further demonstrated that there is only a small proportion of unstable Tregs which are characterized as Foxp3+ RFP- cells originated from newly generated Tregs (63). These unstable Tregs are enriched among those CD25- RFP- cells which contain fully methylated Treg-specific demethylated region (TSDR) and confer defective suppressor activity. However, the partially demethylated RFP+ Foxp3- (ex-Tregs) are capable of re-acquiring Foxp3 expression and becoming fully demethylated upon TCR stimulation. Consistent with these findings, tracking Treg cell fate by administration of tamoxifen in $Foxp3^{GFP\text{-}Cre^{ERT2}}$ knockin
ROSA26<sup>YFP</sup> mice, ex-Tregs were found to be rare (<5%) and were not more prevalent even under lymphopenic, autoimmune or inflammatory conditions (64). Therefore, a “heterogeneity model” has been proposed where Tregs represent a mostly committed cell lineage with a small population being unstable Tregs (65, 66).

It is still controversial and elusive whether Tregs are largely reprogrammed to Th cell types and to what extent this reprogramming promotes inflammatory immune responses. To reconcile these different observations from various settings, I agree with the view that Tregs are a committed cell lineage under the steady state. However, Tregs have the potential to differentiate into Th cells and produce inflammatory cytokines largely determined by the local environmental cues.

**Molecular determinants for stable expression of Foxp3 in Tregs**

Despite the controversies on Treg plasticity, demethylation of CpG at conserved noncoding sequence (CNS)2 within Foxp3 locus has been linked to the heritable expression of Foxp3 in Tregs (67, 68). tTregs and pTregs show similar pattern of demethylation at CNS2 whereas iTregs lack this pattern of demethylation (67-69). In particular, though TGF-β signaling can induce Foxp3 expression in CD4<sup>+</sup> T conventional cells, it is unable to induce demethylation at CNS2, which accounts for the unstable Foxp3 expression in iTregs (67, 69, 70).

At the transcriptional level, Runx/Cbf-β binding to CNS2 of Foxp3 is required to maintain stable expression of Foxp3 and functional activity of mature peripheral Tregs (71, 72). The binding of Runx/Cbf-β to the Foxp3 promoter and CNS2 leads to an active chromatin state rather than directly inducing gene expression. Deletion of CNS2 does not
abolish thymic development of Tregs in neonatal mice, but eventually leads to diminished numbers of Foxp3\textsuperscript+ Tregs in the periphery (73). Foxp3 appears to stabilize its own expression by binding to CNS2 through a Runx/Cbf-β complex (73). Ets-1, another transcription factor that acts as an enhancer, was identified to bind to the demethylated CNS2 of Foxp3 (74). Recent studies further revealed that many transcription factors bind directly or indirectly to CNS2 in a form of a large complex (73, 75). Collectively, these findings indicate that transcriptional regulation mediated by factors associated with CNS2 is required to maintain Foxp3 expression in developing Tregs.

From a degrading view, stable expression of Foxp3 is also controlled by the ubiquitination (76). More recently, E3 ubiquitin ligase Stub1 has been shown to interact with Foxp3 to promote its degradation whereas deubiquitinase USP7 was found to decrease Foxp3 degradation and sustain its expression (77, 78).

1.4 Inter-relationship between Th cells and Tregs

\textit{In vitro}, upon TCR activation, Naïve CD4\textsuperscript+ T cells are able to differentiate towards Th1, Th2, Th17, and iTregs in the presence of specific cytokines with or without blocking antibodies to other cell lineages (79). However, \textit{in vivo}, many cytokines are present at the same time in the context of diverse immune responses and this complexity raises the questions about how and to what extent these environmental cues integrate and determine a cell fate.
**Molecular mechanisms for Treg cell fate decision**

Examining the epigenetic modification pattern of signature cytokine genes with H3K4me3, which is associated with gene activation, and H3K27me3, which is associated with gene inactivation, has revealed that the H3K4me3 pattern is correlated with specific cell lineage cytokine transcription while H3K27me3 is not exclusively true. These results suggest that although epigenetic modification of the cytokine signature genes is active for that cell lineage, the other lineage cytokine genes are not repressed and have the potential to get activated (80). Indeed, this is reflected by the potential of CD4+ T cells to differentiate into Th1, Th2, Th17, or iTregs lineages.

In a cell *per se*, how does the signaling pathway vary in a Treg vs. a T conventional cell to determine a cell fate? In this regard, the PI3K/Akt pathway is differentially activated. TCR, co-stimulation, and IL-2 all readily activate the PI3K pathway in conventional T cells, but this pathway is attenuated in tTregs (81, 82). For example, constitutively expressing an active form of Akt resulted in a decreased induction of iTregs (83). Conversely, blockade of the PI3K pathway promoted Treg cell maturation (84). Thus, relatively low PI3K/Akt activation is an important determinant for development to Tregs. mTOR, downstream of PI3/Akt, has been found to play an important role to determine a cell fate. CD4+ T cells lacking mTOR failed to respond to Th1 (IL-12, IFN-r), Th2 (IL-4) and Th17 (IL-6) cytokines and phosphate downstream STAT4, STAT6 and STAT3, respectively, and therefore failed to differentiate into Th1, Th2 and Th17 cells (85). Conversely, deficiency of mTOR in T cells lowered their threshold to differentiate into Tregs even in the absence of TGF-β. Moreover, it seems that downregulation of both mTORC1 and TORC2 are involved in Foxp3 induction, as
depletion of mTORC1 failed to recapitulate the effect of mTOR deficiency in facilitating iTreg induction and deficiency of an essential subunit of mTORC2 only impaired Th1 and Th2 differentiation (85, 86).

Many factors exploit this PI3/Akt/mTOR pathway to affect lineage choices between Th and Treg. For example, S1p(1), one of the receptors that recognize S1P which is a natural lysophospholipid in the plasma, recently has been found involved in determining differentiation of T cells into Th1 cells or Tregs (87). Over expression of S1P(1) in CD4\(^+\) T cells abrogated their ability to differentiate into iTregs, even in the presence of TGF-\(\beta\). Instead, these CD4\(^+\) T cells favored differentiation towards IFN-\(\gamma\) producing Th1 cells. Mechanistically, this is attributed to S1P(1) activation of Akt/mTOR, which in turn antagonizes TGF-\(\beta\)-induced activation of Smad3. Diminished activation of PI3K pathway appears to be important to maintain active levels of transcription factors Foxo1 and Foxo3, which favor the Foxp3 transcription (88-90). When the PI3K pathway is active, Foxo1 and Foxo3 are phosphorylated, which represent inactive forms that do not support Foxp3 transcription but increase Th1 and Th17 development. More recently, another transcription factor BACH2 has been identified as a regulator for CD4\(^+\) T cell differentiation (91). In Tregs, BACH2 is essential to maintain Treg suppressor function. However, in T conventional cells, it has been shown to curtail effector function.

**Inter-relationship between induced Treg and Th17 lineages**

There is a clear inter-relationship between the development of Th17 and induced Treg cells (pTregs and iTregs), due to the shared cytokine TGF-\(\beta\) for their development. TGF-\(\beta\), acting along with IL-6 or other inflammatory mediators, supports Th17 cell
development (92-94). However, TGF-β, in the presence of IL-2 favors iTregs and opposes Th17 development (95-98). At the molecular level, the cell fate choice between Th17 and Treg cells is in part determined by the levels of several transcriptional regulators. For example, Foxp3 has been shown to interfere with the activity of RORγt and RORα, key transcriptional regulators for Th17 development (51, 99, 100). However, RORγt and RORα do not affect Foxp3 activity, rather STAT3 downregulates Foxp3 (51). In fact, the ratio of STAT5:STAT3 determines whether a cell adopts a Treg or a Th17 cell fate, the former which is supported by IL-2 and the latter by IL-6 and IL-21(101, 102).

Despite the important role of cytokines in determining the cell fate, metabolic products and immune mediators are also involved in regulating this interrelationship. It is well-studied that retinoic acid (RA) promotes iTregs/pTregs induction and antagonizes Th17 differentiation (46, 47, 103). Mechanistically, RA might lower the threshold of co-stimulation in T cells to facilitate Treg development (48). In addition, RA antagonizes IL-6-induced Th17 induction thus enhances TGF-β signaling to induce Tregs (104). Memory cells synthesizing IL-4, IL-21, and IFN-γ are more resistant to iTreg conversion. RA acts to relieve this inhibition, which facilitates iTreg production (105). At the molecular level, it has been revealed that the binding of RA and its receptor RAR and retinoid X receptor (RXR) at enhancer I and near promoter region of Foxp3 gene increases histone acetylation at the enhancer I region which contains Smad3 binding sites and thus facilitate pSmad3 binding to enhancer I region (106).

Another regulator which has been identified involved in T cell differentiation is aryl hydrocarbon receptor (AHR). Interestingly, binding of different ligands to AHR
mediates entirely different effects on T cell differentiation (107). Additionally, expression of HIF-1α, a transcription factor induced in hypoxia, in T cells, leads to differentiation of Th17 cells and reduction of Tregs and this is attributed to its multiple roles in activating RORγt, regulating Th17 signature cytokine gene transcription and facilitating Foxp3 degradation (108-110). Taken together, these findings suggest environmental factors play a critical role in regulating a cell fate decision and immune responses, providing promising therapeutic targets to manipulate the immune responses.

1.5 Treg heterogeneity

Foxp3 is ubiquitously expressed in Tregs all over the body and it represents a faithful marker of Tregs. However, Tregs are not identical, rather they synergize local environmental cues to acquire diverse phenotypes and functions. For instance, the gene expression profile of tTregs, pTregs and iTregs remarkably differs, even the expression profile of pTregs generated in different conditions varies (111). Heterogeneity of Tregs mirrors their differences in origin, activation, function and anatomical locations. It can be reflected by expression of various arrays of markers at different developmental stages in the periphery, at different anatomical locations and during the course of various inflammatory responses.

Developmental heterogeneity

Tregs are derived either from the thymus or periphery and they are designated to have different properties due to their different origins. Despite their common requirement for
TCR, co-stimulatory signaling and IL-2, the development of pTregs requires an extra indispensable signal from TGF-β and are more dependent on environment cues (112-115).

Mucosa is a preferential site for pTreg induction. This is attributed to the abundance of TGF-β and RA produced by local epithelial cells, CD103+ DCs and macrophages (46-48, 103, 116, 117). It has been shown that the binding of pSmad3, which is activated by TGF-β, to CNS1 of Foxp3 is required for pTreg development (73). Accordingly, CNS1-deficiency in mice are devoid of pTregs and consequently results in Th2-type inflammation in the gut and lung (115), suggesting an indispensable role of pTregs in maintaining the mucosal tolerance. Another unique factor contributing to pTreg development is commensal bacteria, food antigens in the gut and inhaled environment allergens in the airways of the lung (117-120). These foreign antigens educate pTregs to express unique TCR specificities to mucosa (121). The direct piece of evidence comes from analysis of the TCR repertoire of colonic Tregs. The TCR repertoire largely differs from those in other lymphoid tissues and some of the TCRs are reactive to colonic content. In addition, retroviral expression of these unique TCRs in thymocytes failed to generate Tregs, suggesting that these TCRs are selected by foreign antigens rather than self-antigens. In another study, some tTregs, however, showed reactivity to microflora-derived antigens (122). Unique TCR specificities to gut antigens appear essential to maintain the gut tolerance. This notion is supported by the observation that both tTregs and pTregs are required to rescue Foxp3-deficient newborn mice from severe autoimmunity and prevent chronic inflammation and immune activation effectively (123). This finding dissects the roles of tTregs vs. pTregs in preventing autoimmunity. Although the global gene expression profile is similar between tTregs and pTregs, apparently, in
this case, the TCR repertoire matters. Indeed, some of groups identified specific commensal bacteria, the clostridium genus and species of *B. fragilis*, which facilitate pTreg induction (118-120). Taken together, these findings support the notion that pTregs are essential to sustain the mucosal tolerance.

Noteworthy, another contribution of the pTregs is to maintain maternal-fetal tolerance (124). In Foxp3 CNS1-deficient mice where the development of pTregs is aborted, the presence of tTregs failed to suppress semi-alloantigen autoimmunity at the interphase of maternal-fetus and consequently increased the resorption of the fetus. Collectively, these results revealed the indispensable role of pTregs in maintaining tolerance in a broad spectrum.

Although it is commonly accepted that the conversion of activated T cells in the periphery contributes to peripheral tolerance, it is still elusive to what extent the pTregs constitute to the peripheral Treg pool. It has been estimated that the proportion of pTregs is around 5-20% of total Tregs by TCR repertoire analysis (19, 20, 123). However, these results remarkably vary based on the settings. Therefore, looking for unique markers to distinguish tTregs and pTregs becomes urgent and of great interest. A transcription factor of the IKaros family, Helios, has been reported as a marker for tTregs (125). However, later studies revealed that the expression of Helios is more associated with cell activation and induced expression of Helios could be found in pTregs in a different TCR transgenic model (126, 127). More recently, Nrp-1 has been suggested as a promising marker to distinguish tTregs from pTregs and iTregs (128, 129). Nevertheless, pTregs have been found to upregulate Nrp-1 expression during inflammation (129). Thus, so far, there is still no definitive marker to distinguish tTregs, pTregs and iTregs.
A unique feature of tTregs is the demethylated status of CNS2 in Foxp3 gene which is also related to its lineage stability (130). iTregs are unstable and readily lose Foxp3 upon TGF-β retrieval, which is consistent with their methylated status of CNS2 (51, 67, 68). However, pTregs display a heterogeneous pattern of demethylation of CNS2 which probably reflect their development in different conditions (63, 68, 69, 123).

**Phenotypic heterogeneity of Tregs**

Newly generated thymic Tregs are phenotypically homologous and are characterized as CD62L⁺ CCR7⁺ CXCR4low Foxp3⁺ (131). After exiting the thymus, Tregs migrate to secondary lymphoid organs and later non-lymphoid organs upon encountering local antigens. Therefore, a two-step switch model of chemokine receptor expression profile has been proposed to depict the journey of Treg migration from the thymus to peripheral lymphoid and non-lymphoid tissues. The first switch occurs in the thymus and most importantly, Tregs acquire CCR7 expression for homing to the secondary lymphoid tissues. The second switch occurs in the secondary lymphoid tissues where Tregs are shaped by local antigens to express an array of chemokine receptors and integrins, such as α4β7 and CCR9 in the mesenteric lymph node (MLN), whereas expression of CD62L and CCR7 are downregulated. Generally, Tregs express increased levels of trafficking molecules, particularly those CD62L⁻ Tregs in the secondary lymphoid tissues compared to conventional T cells, which likely accounts for their advantage to migrate to non-lymphoid tissue more efficiently to maintain local immune homeostasis.

Numerous studies have been shown that expression of these combinations of homing molecules and trafficking to the non-lymphoid tissues are essential for local
immune homeostasis. Various sets of chemokine receptors and integrins are expressed on Tregs for their trafficking to tissues such as the lung, liver, BM, skin, pancreases and intestine at the steady state and additional homing molecules are required during the course of immune responses, such as those against tumor, infection and allo-grafts (18). For instance, expression of CCR4, P- and E-selectin ligand on Tregs in peripheral lymph node (pLN) is essential for their trafficking to the skin and lung airway (132, 133).

T cell homing to the gut mucosa is more complicated because this process involves lymphocytes entry into lamina propria (LP) and migration to the intra-epithelial lymphocyte (IEL) compartment. Moreover, redundancy of homing molecule expression adds another layer of complexity. It is commonly accepted that the expression of α4β7 and CCR9 is required for T cell trafficking to the small intestine (134, 135). However, in mice deficient in CCR9 or its ligand CCL25, CD4+ T cells still exhibited normal capability for gut entry, especially in distal of intestine where the source of CCL25 is less abundant, implying that there is a CCR9-independent pathway (136). Perhaps, other chemokine receptors, such as CXCR3 and CXCR4, supplement CCR9/CCL25 axis for T cell homing to the gut (137-139). Tregs lacking integrin β7 were largely impaired in their homing to the colon, suggesting that integrin β7 functions to facilitate Treg trafficking to the colon (140). However, integrin β7-deficient Tregs were still capable of suppressing inflammation in a T cell transfer model of colitis, suggesting that either Tregs suppress immune responses in the draining MLN or limited number of Tregs in the colon is sufficient to control intestinal inflammation induced by effector T cells. Other study showed that the chemokine receptor CCR4 might be critical for Treg trafficking and function in a T cell-mediated model of colitis, because the absence of CCR4 delayed
Treg homing to the MLN to suppress immune responses (141). More recently, GPR15 has been identified as an essential molecule expressed on Tregs for their homing to the large intestine (142). Tregs that lack of GPR15 showed remarkably reduced capacity of homing to the colon and thus failed to prevent intestinal inflammation. Unlike small intestine tropic homing molecules which are regulated largely by RA, GPR15 is typically TGF-β dependent and regulated by colonic microbiota, suggesting that local environmental cues are involved in shaping Tregs with unique properties, partly by affecting their homing.

During the course of immune responses, certain chemokine receptors are required for Tregs to suppress local inflammation and abnormal expression of these homing molecules will result in the failure of suppression. For example, CXCR3 has been recognized as a Th1 chemokine receptor (143). During Th1 responses, IFN-γ causes the induction of local chemokines and upregulates CXCR3 both on T cells and Tregs (144). The expression of CXCR3 endows Tregs with the ability to trafficking to the site of inflammation and is essential to limit local immune responses and prevent tissue damage. CCR8 is a chemokine receptor for T cell trafficking to the skin and is upregulated during Th2 immune responses (18, 145). Interestingly, recent findings showed that the expression of CCR8 on Tregs is indispensable to prevent GVHD (146). Further studies revealed that CCR8 expression promoted Treg survival by facilitating the interaction between Tregs and DCs at tissue sites. Chemokine receptor CCR6 has been shown to be essential for Th17 cell recruitment in rheumatoid arthritis and EAE models and its expression on Tregs is also required for Treg trafficking to the sites of inflammation during Th17 immune responses (147-150). Collectively, these findings indicate that
Tregs are educated in the second lymphoid tissues by local antigens and equipped with an array of chemokine receptors and other molecules for trafficking to the non-lymphoid tissues to suppress immune responses.

Functional heterogeneity of Tregs

In vitro studies of Treg function have revealed that the mechanisms utilized by Tregs to suppress immune responses can be divided into two categories (151, 152). First, Tregs suppress responder T cells by IL-2 deprivation, secreting IL-10, IL-35, TGF-β, and expressing granzyme and Galectin-1. Second, the suppressive function also can be achieved by suppressing DC maturation, through the expression of CTLA4, CD39, CD37, Nrp-1 and Fgl-2. In vivo, Treg suppressor function even extends beyond adaptive immunity. It has been reported that Tregs can suppress innate immune cells, such as mast cells, macrophages, natural killer (NK) cells (151, 153).

The presence of multiple mechanisms raises the question concerning what is the main suppressive mechanism used by Tregs in vivo and to what extent these mechanisms cooperate to suppress certain immune responses. The knockout of several mediators only in Tregs provides answers to this question. For example, deficiency of CTLA4 in Tregs markedly affected Treg suppressor function, though thymic generation and homeostasis of Tregs in the periphery were normal (154). Mice bearing CTLA4 deficiency in Tregs developed severe autoimmunity manifested by lymphadenopathy and multi-organ cell infiltration. These observations suggest that the lack of CTLA4 in Tregs is sufficient to override tolerance and result in systemic autoimmunity in mice. Thus, CTLA4 has been proposed to be the core suppressive mechanism used by Tregs to regulate tolerance (155).
In contrast, mice containing Tregs with IL-10 deficiency developed inflammatory bowel disease (IBD) at 3-6 months of age rather than systemic autoimmunity, suggesting that IL-10 production by Tregs is the crucial mechanism of Treg suppressive program to maintain intestinal tolerance (156). Additionally, lack of IL-10 in Tregs also resulted in lung and skin hypersensitivity. These results indicate that other Treg suppressive mechanisms are also active but not sufficient to fully control unwanted immune responses in local tissue sites that encounter environmental antigens. Collectively, all these findings suggest that Tregs suppress abnormal immune responses and maintain tolerance at multiple levels.

In order to suppress various immune responses, Tregs might integrate the signals from the local environment and adapt to function properly. Recent identification of a population of Tregs in adipose tissue supports this view. Adipose Tregs express not only Treg signature genes, but also a set of genes associated with unique trafficking, anti-inflammatory properties, and lipid metabolism (157). Depletion of Tregs in fat tissue largely correlated with increased inflammation and insulin resistance. Reciprocally, expanding Tregs in this tissue increased the anti-inflammatory cytokine IL-10 and decreased glucose and insulin levels in the blood. The unique property of Tregs in adipose tissue has been proposed to be attributed to the PPAR-γ, a key regulator of adipocyte differentiation (158). It has been shown that PPAR-γ not only determines the phenotype of fat Tregs, but also regulates local Treg homeostasis. These findings provide a new and alternative mechanism underlying how chronic inflammation contributes to obesity. Apparently, in this case, Tregs in adipose tissue integrate the local master
regulator PPAR-γ to endow a new phenotype and function to suppress chronic inflammation and regulate metabolism.

Table 1.1 Distinct Treg subsets in suppressing Th responses.

<table>
<thead>
<tr>
<th>Subset</th>
<th>Cytokine</th>
<th>Transcription factor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Th1</td>
<td>IFN-γ, IL-12</td>
<td>T-bet</td>
<td>Production of IFN-γ; expression of CXCR3 on γδ cells</td>
</tr>
<tr>
<td>Ts1</td>
<td>IFN-γ, IL-27</td>
<td>T-bet</td>
<td>Differentiation of CXCR3+ Tregs; high expression of GITR, CTLA4, CD103, IL-10, TGF-β on Tregs</td>
</tr>
<tr>
<td>Th2</td>
<td>IL-4, IL-2</td>
<td>Gata3, IRF4</td>
<td>Production of IL-4, IL-5, IL-13, IL-10 cytokines; Ig production</td>
</tr>
<tr>
<td>Ts2</td>
<td>?</td>
<td>IRF4</td>
<td>Upregulate ICOS, Fgl2, IL-10, granzyme B, Maf, CCR8, IL-1R1 on Tregs</td>
</tr>
<tr>
<td>Th17</td>
<td>IL-6, TGF-β</td>
<td>EORγt</td>
<td>Production of IL-17A, IL-17F, IL-21, IL-22 and GM-CSF</td>
</tr>
<tr>
<td>Ts17</td>
<td>IL-10</td>
<td>STAT3</td>
<td>Upregulate IL-10, Ebi3, granzyme B, Prf1 and CCR6 expression on Tregs</td>
</tr>
<tr>
<td>Th</td>
<td>IL-6, IL-21</td>
<td>Bcl6</td>
<td>Expression of CXCR5 on T cells; germinal center formation; affinity maturation</td>
</tr>
<tr>
<td>Ts</td>
<td>?</td>
<td>Bcl6</td>
<td>Expression of CXCR5, SAP on Tregs</td>
</tr>
</tbody>
</table>

To combat immune responses, Tregs respond to local stimuli and differentiate into subtypes of Tregs equipped with distinctive suppressive programs. In recent years, intensive studies have been focused on how Tregs suppress different types of immune responses, in particular, Th1, Th2 and Th17 immune responses (Table 1.1). Encountering pathogens activates the innate immune system and consequently inflammation initiates the adaptive immune responses, Th1, Th2, and Th17 etc. Cytokines play an essential role in determining the development of Th cell lineage and the corresponding immune responses. It is commonly accepted that STAT activation by cytokines drives gene expression of transcription factors of Th lineage: IFN-γ activates STAT1 and IL-12.
activates STAT4 to drive T-bet expression for Th1 cells, IL-4 activates STAT6 to drive Gata3 and IRF4 expression in Th2 cells, and IL-6 and TGF-β activates STAT3 to drive RORγt expression for Th17 cells (79). The expression of these signature transcription factors is responsible for the differentiation and function of Th cells to combat infections. While such responses represent competent protective immunity to foreign antigens, it has the risk to cause overwhelming chronic inflammation and autoimmunity, if these immune responses are not well-regulated. Therefore, the question has been raised as how Tregs adapt to control multiple types of immune responses induced by various pathogens.

Tregs utilize the same transcriptional regulators to generate suppressive responses to counteract the respective Th responses, designated as Ts1, Ts2 and Ts17 in Table 1.1. It has been shown that during a Th1 response, Tregs highly express the Th1 signature transcription factor T-bet and expression of T-bet by Tregs is required for their migration, proliferation and function (144). Tregs with deficiency of T-bet were unable to express the homing molecule CXCR3, exhibited impaired capacity to persist during the infection and consequently failed to suppress Th1 immune response in mice, suggesting that Tregs utilize T-bet to fulfill several key properties of Th1 cells in order to suppress Th1 response. However, Tregs only partially adopt a Th1 phenotype and they do not express the Th1 effector cytokine IFN-γ. Along with this, further studies revealed that the cytokines promoting T-bet+ Tregs are only partially overlapped with the pro-inflammatory cytokines that promote Th1 cells (159). Analogues to Th1 cells, Tregs that differentiate into T-bet+ Treg are IFN-γ-STAT1 dependent. However, unlike Th1 cells, which require further IL-12-STAT4 signaling to drive Th1 responses, Tregs are unresponsive to IL-12. Such IFN-γ-STAT1, but not IL-12-STAT4, activation in Tregs
might be important for Tregs to express T-bet to acquire some Th1 properties and maintain suppressor function while not acquiring effector characteristic of Th1. In addition, IL-27, another member of IL-12 family, via STAT1 activation has been implicated in promoting the differentiation of CXCR3+ T-bet+ Tregs (160). Whereas IFN-γ is indispensable for the differentiation of T-bet+ Tregs in peripheral lymphoid organs, IL-27 signaling is required for the induction of T-bet+ Tregs at the site of Th1 responses. The latter finding indicates that the pathway by which Tregs differentiate into T-bet+ Tregs diverges from that for T cells to differentiate into Th1 cells.

Similarly, IRFR4, a transcription factor essential for Th2 differentiation, is indispensable for Tregs to suppress Th2 responses (145). Mice with targeted deletion of IRFR4 in Tregs developed severe Th2 autoimmunity manifested by high amount of IL-4, IL-5 and serum immunoglobulin and multi-organ plasma cell infiltration. Although gene expression associated with Treg suppressive activity such as CTLA4, TGF-β and GITR was unchanged, other molecules, such as Fgl2, IL-10, granzyme B, and especially Th2-related genes, ICOS, Maf and CCR8, were decreased, suggesting that lacking IRF4 in Tregs only affects part of the Treg gene expression program which is associated with Th2 responses. At the molecular level, both Foxp3 and IRF4 have been found to bind at the ICOS promoter. Thus, it is likely that IRF4 is required in Tregs to co-operate with Foxp3 to regulate Th2 type genes, such as ICOS, to endow Tregs with additional suppressive activity to control Th2 responses. However, another Th2 master regulator, Gata3, plays a different role in Tregs. When Gata3 was deficient in Tregs, mice developed autoimmunity manifested by enhanced production of Th1, Th2, and Th17 cytokines and multi-organ lymphocyte infiltration rather than skewed Th2 autoimmunity (161).
Moreover, the lack of Gata3 resulted in a more profound effect in Tregs which was reflected by compromised competitive fitness during homeostasis and impaired suppressor function. This broad effect resulted from Gata3 deletion also includes the reduced expression of Foxp3 and decreased expression of Treg signature genes. Further analysis of the Foxp3 gene revealed that Gata3 normally binds to CNS2 region and Foxp3 expression requires both Gata3 and Foxp3 itself. In agreement with these findings, other studies showed that Foxp3 binds many protein partners and form a large complex in Treg cells (75). Foxp3 and some of the partners, such as Gata3, form a feedback loop and regulate reciprocally to enforce Treg properties and function. Therefore, IRF4 and Gata3 have distinct functions in Tregs, i.e. Gata3 has a broader function in controlling Treg maintenance, homeostasis and function whereas IRF4 in Tregs is dominant in regulating its suppressor function specialized to target Th2 responses.

Resembling Treg suppression of Th1 and Th2 responses, Tregs regulate Th17 immune responses in a STAT3-dependent manner (150). In T conventional cells, STAT3 activation by IL-6 and TGF-β signaling is indispensable to induce the transcription factor RORγt for their differentiation to Th17 cells (94, 162). Analogous to T conventional cells, STAT3 is required for Tregs to control Th17 responses. Mice containing Tregs with STAT3 depletion spontaneously developed IBD characterized by pronounced production of Th17 cytokines (150). The defect resulted from STAT3 deficiency in Tregs are exclusively linked to the impaired function of Tregs to suppress Th17 responses, while suppression of Th1 and Th2 immune responses are not affected. In line with these results, gene expression profiling further revealed that STAT3 deletion in Tregs downregulated the expression of a set of genes such as IL-10, granzyme B, Ebi3, Prf1 and CCR6, which
are putatively important to suppress colitis or to direct homing to the mucosa while upregulated the expression of other genes such as IL-6, TGF-β and Vip, which are related to Th17 differentiation. Nevertheless, unlike in T conventional cells where STAT3 activation is induced by proinflammatory cytokines, such as IL-6 and IL-23, STAT3 activation in Tregs is anti-inflammatory cytokine IL-10 dependent (94, 163). Collectively, all these findings support a model that Tregs adopt Th1, Th2, and Th17 transcriptional regulators to acquire distinct properties in order to suppress the corresponding immune responses.

Besides distinct types of Th cells, a subset of T cells which are characterized as Bcl6+CXCR5+CD4+ cells, namely Tfh, is specialized in regulating the GC reaction (164-168). Given the notion that Tregs suppress various types of immune responses by co-opting the corresponding Th transcriptional regulators, it raises the question whether suppression of Tfh cells by Tregs also fits this scenario. Gene expression profiling revealed that Tregs expressed signature genes, as well as a set of genes unique to Tfh, such as Bcl6, CXCR5, CXCL13 and Pdcd1 after immunization (169). Bcl6 is critical for expression of CXCR5 in Tregs, a chemokine receptor for trafficking to B cell follicle areas (169-171). Tregs lacking either CXCR5 or Bcl6 showed impaired suppressor function, which led to enhanced germinal center responses characterized by pronounced antibody production, affinity maturation and accumulation of plasma cells.

In recent years, some other Th cells have been identified, such as Th22 and Th9 which are involved in the immune responses in psoriatic lesion and allergic inflammation, respectively (172-174). Th22, which is a Th17 related cell type, produces IL-22 and requires transcription factor AHR (175). Th9, related to Th2, produces IL-9 and low
amount of IL-4, is dependent on the transcription factor PU.1 and IRF4 (176, 177).
Likewise, Tregs might express corresponding transcription regulators to suppress these
Th22, Th9 immune responses (178).

*Naïve, effector/memory Treg subsets*

Tregs at development stages may resemble T conventional cells, i.e. they undergo naïve,
effector/memory phenotypic changes. In comparison with T conventional cells, Tregs
exhibit an activated phenotype characterized by higher expression of activated markers,
such as CD25, CD69, CD38, CTLA4, 4-1BB, OX40 and lower expression of CD62L,
CD45RB, probably due to its stronger TCR strength and IL-2R signaling (179, 180). This
property potentially confers Tregs an advantage to outcompete autoreactive T cells for
accessing ligand, favoring immune suppression over autoimmunity.

Subdividing the Treg population based on activation marker, such as CD103
revealed that CD103+ Tregs exhibited a more activated phenotype and suppressed better
than their negative counterparts per cell basis (180, 181). In line with this finding,
CD103+ Tregs showed robust suppressor activity in the T cell transfer model of colitis
(182). Furthermore, this subset of Tregs not only expressed a profile of activation
markers which assembled the effector/memory cells, but also displayed homing property
to non-lymphoid tissue at the steady state and to inflamed tissue during acute skin
infection (183). Therefore, only CD103+ Tregs, but not CD103- Tregs, suppressed
immune response in an acute arthritis model. These findings supported a notion that
CD103 is a useful marker to distinguish effector/memory Tregs from naïve Tregs. In
agreement with these findings, gene expression profiling also revealed that CD103+ Tregs favor tissue migration and enhanced suppressive activity (111).

Klrg1 has been used as a marker to identify terminally differentiated Treg subset with potent suppressor function (184-186). Klrg1+ Tregs display effector/memory phenotype, similar to CD103+ Tregs. Consistent with these characteristics, this subset is preferentially found in the tissue sites, such as the lamina propria of the small intestine (SI-LP) (184). Klrg1+ Tregs appear at a later developmental stage and are terminally differentiated as they lose the capacity to differentiate to the Klrg1− counterparts.

At the transcriptional level, IRF4 and Blimp-1 are the key transcription factors involved in promoting effector Tregs (187). Blimp-1+ Tregs are characterized by high expression of CD103, CD44, ICOS, GITR and lower expression of CD62L. The expression of Blimp-1 is upregulated in Tregs stimulated with IL-2 and other inflammatory cytokines, indicating that Blimp-1 functions to regulate an effector Treg subpopulation. Although Blimp-1 is not required for the development of effector Tregs, this transcription factor is indispensable for homeostasis of effector Treg in mucosal tissue sites and production of IL-10. In contrast, activated Tregs are substantially reduced in mice with IRF4 deficiency, indicating that IRF4 is the key transcription factor which controls the development of effector Tregs. Gene expression profiling demonstrated that a set of genes is regulated by both IRF4 and Blimp-1, but IRF4 is required for Blimp-1 expression in Tregs. Taken together, these findings indicate that IRF4 functions upstream of Blimp-1 in the development of effector Tregs and these two regulators cooperate to regulate the activity of effector Tregs.
The observation that Treg suppressed repeated antigen-induced skin inflammation more efficiently, indicating the presence of memory Tregs (188). In this model, transgenic OVA-specific CD4⁺ T conventional cells and Tregs could be followed over time in response to transient expression of OVA in the skin. The initial self-antigen expression in the skin led to the inflammation in the skin followed by activation, proliferation and migration of Tregs to the tissues site to control the autoimmunity. After antigen removal, these Tregs were still maintained at the tissue site with an activated phenotype and they suppressed the inflammation quickly and efficiently upon re-exposure to antigens. The importance of memory Tregs is also evidenced from the maternal-fetus tolerance (189). Tregs contribute to maintaining maternal-fetus tolerance during pregnancy. Importantly, after delivery, these tissue-specific Tregs still persist and rapidly proliferate during a second pregnancy.

Tregs in the periphery undergo naïve, effector and memory developmental switches, analogous to conventional T cells, which raises the question concerning what drives this process. γC cytokines are essential players that are involved in Treg differentiation. Resembling T conventional cells, IL-2 is required for the development of effector memory Tregs (190). Consistent with this notion, in IL-2Rβ-deficient mice with transgenic expression of mutant IL-2Rβ chains, the development of Klrg1⁺ Tregs which represent terminally differentiated effector Tregs are largely hampered and the decrease of Klrg1⁺ Tregs is directly proportional to the level of impaired IL-2R signaling (184). Characterizing memory Tregs in the skin after the inflammation revealed that they expressed high levels of CD127, CD44, CD27 and CCR6 and lower level of CD62L, suggesting that IL-7 might play a role for the maintenance of this population (190).
Deprivation of IL-2, either by neutralizing antibody or depleting IL-2 producing T cells, did not affect the persistence of memory Tregs, suggesting IL-2 is not the main cytokine for the maintenance of memory Tregs. However, blocking IL-7 signaling remarkably resulted in a decrease of memory Tregs in the skin. Thus, these findings suggest that Tregs at different developmental stages depend on distinct γC cytokines, i.e. IL-2 for the generation of effector/memory Tregs and IL-7 for the maintenance of memory Tregs.

1.6 Intestinal tolerance and IBD

Intestine is a unique tissue site where body encounters tremendous levels of food antigens and commensal bacteria. Nevertheless, the immune regulatory network in the gut mucosa readily maintains tolerance to these antigens at homeostatic conditions while being poised to elicit protective immune responses to pathogenic organisms. The gut epithelium, host immune system and gut microflora are commonly thought to represent the main players that cooperate for mucosal tolerance (Table 1.2). Aberrant regulation of this network has the potential to breakdown intestinal homeostasis and to cause IBD.

*Intestinal epithelium and barrier function*

The intestinal epithelial cells (IECs) do not simply function as a physical barrier to prevent invasion of pathogenic microorganisms in the gut lumen. Rather, IECs comprise some types of cells with unique functions (191-193). Typically, the intestinal stem cells at the crypt base are responsible for the renewal of IEC. Goblet cells are specialized mucin-secreting cells and the mucins form the mucus layer, not only preventing bacterial penetration but also maintaining the high concentration of antimicrobial products, such as
defensins and secretory IgA, at the apical surface of the IECs. In addition, Paneth cells produce antimicrobial peptides in an inducible manner and these antimicrobial peptides secreted by Paneth cells are crucial to prevent localization and penetration of luminal bacteria (194).

**Table 1.2 Components mediating tolerance vs. immunity in the gut mucosa.**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Tolerance</th>
<th>Immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IEC</strong></td>
<td>Goblet cells secret mucin; Paneth cells produce antimicrobial peptides; TLR signaling in IECs protects the barrier integrity.</td>
<td>Dysregulation of autophagy and ER stress of IECs.</td>
</tr>
<tr>
<td><strong>APCs</strong></td>
<td>CD103+DCs produce TGF-β and RA, and support the pTreg development (lose tolerogenic properties during colitis); CX3CR1+ microphages prevent commensal bacteria translocation, limit Th17 responses, and drive IL-10 producing Treg expansion in the LP.</td>
<td>Sustained PRR signaling in APCs induce the production of proinflammatory cytokines; E-cadherin+ DCs are colitogenic, which accelerate the development of colitis.</td>
</tr>
<tr>
<td><strong>CD4+ T cells</strong></td>
<td>Tregs producing IL-10, TGβ-β and IL-35.</td>
<td>IL-23/Th17 axis, IFN-γ producing Th1 and Th2 effector cells.</td>
</tr>
<tr>
<td><strong>Other immune cells</strong></td>
<td>CD8α T cells and TCR γδ T cells in IEL compartment contribute to intestinal homeostasis; ILCs, first line of defense, maintain the barrier function; IL-10 producing Tr-1 cells in the small intestine.</td>
<td>Dysregulation of ILCs</td>
</tr>
<tr>
<td><strong>Microflora</strong></td>
<td>Polysaccharide A of <em>B. frasill</em> promote pTreg induction; Clostridia promote pTreg development and colonic Treg accumulation.</td>
<td>SFB promotes Th17 cell development.</td>
</tr>
</tbody>
</table>
Recently, dysregulation of autophagy and Endoplasmic reticulum (ER) stress of IECs has been linked to IBD, largely due to the abnormalities in the Paneth cells and goblet cells (195, 196). For example, deficiency of XBPI in IECs, a transcription factor involved in ER stress pathway, led to loss of Paneth cell and marked reduction of goblet cells (197). Consequently, such reduction of specialized IEC poorly limited bacteria invasion. Therefore, mice with this deficiency showed enhanced inflammation in experimental colitis. Collectively, these studies demonstrated that the dysfunction of IECs, especially those specialized IECs render the risk to develop intestinal inflammation.

To sense luminal microorganisms, IECs express pattern recognition receptors (PRRs) and signals through these receptors are essential to maintain the barrier function (198, 199). For example, toll-like receptors (TLRs) have been detected to be constitutively expressed on mouse IECs at a low level. However, the expression of these TLRs is enhanced during inflammation. One function of TLR signaling on IECs is to promote cell proliferation and injury recovery (200-202). In the steady state, IECs from TLR4−/− or TLR downstream adaptor protein Myd88−/− mice showed comparable level of proliferation. However, during DSS-induced experimental colitis, mice with these deficiencies were more susceptible to colitis because IECs in these mice showed decreased proliferation and increased apoptosis. TLR4 signaling on IECs induces the production of COX2, PGE2 and amphiregulin which are essential for the IEC proliferation (203, 204). Moreover, TLR signaling drives the reposition of the stromal cells to the intestinal stem cell niche, facilitating the regeneration of the IECs (202, 205). Another mechanism by which TLR signaling protects barrier integrity is to improve tight junctions between adjacent IECs, especially during injury (206-208). For example,
administration of TLR2 ligand during DSS-induced colitis ameliorated inflammation by decreasing IEC apoptosis and promoting tight junctions (206). TLR signaling is also protective by inducing the secretion of antimicrobial peptides in IECs to prevent bacteria translocation (209-212). Deficiency of Myd88 abrogated Paneth cells’ capacity of producing antimicrobial lectin REG3γ (194, 213). Reciprocally, transgenic expression of Myd88 in Paneth cells restored their ability to produce antimicrobial peptides.

**Immune cells and IBD**

Multiple specialized lymphocyte populations are located in the intestinal tract where they initiate appropriate immune responses to pathogens or maintain tolerance to microflora. These lymphocytes either reside within the gut-associated lymphoid tissue (GALT), including MLNs, Peyer’s patches (PPs), and isolated lymphoid follicles (ILFs) or distribute diffusely in the IEL compartment and LP.

IEL compartment is composed of a large population of CD8αα T cells and very few CD4+ T cells. Unlike CD8+ T conventional cells found elsewhere, which bear αβ TCRs, the majority of CD8αα T cells in the IEL express γδ TCRs (214). Several reports have been shown that CD8αα T cells and TCR γδ Tcells are implicated in intestinal epithelium repair and immune regulation (215). In the LP, CD4+ T cells comprise the major T cell compartment, with fewer CD8+ T cells. Complementary to these major T cell types, a small population of NKT cells and mucosal-associated invariant T cells (MAITs), which utilize invariant TCR α chain, are also present in LP throughout the intestine (216). Recently, innate lymphoid cells (ILCs), as a newly defined population, have been shown to play a role in intestinal homeostasis (217, 218). ILCs share some
characteristics with CD4$^+$ T conventional cells while their development is independent of somatic recombination. So far, three groups of ILCs have been described based on the distinct transcription factors, T-bet, Gata3 and RORyt, respectively (218). The development of ILCs and the composition of commensal bacteria are regulated reciprocally, partially accounting for their activity in regulating inflammation and epithelium homeostasis. Collectively, these different specialized cell types constitute a complicated network to regulate the intestinal homeostasis.

DCs, the major cell population of antigen-presenting cells (APCs), act to connect innate immune responses with adaptive immune system. DCs and other APCs, such as macrophages, express TLRs (191). Despite the role of TLR signaling in IECs that promotes repair and prevents colonization of gut flora, TLR signaling by DCs and macrophages is attributable to the intestinal pathology. Sustained TLR signaling leads to intestinal inflammation and has been associated with colorectal cancer (191, 198). For example, the BM chimera of RAG$^{-/-}$ mice reconstituted with RAG$^{-/-}$ Myd88$^{-/-}$ BM failed to develop intestinal inflammation in $H. hepaticus$ induced colitis, suggesting that Myd88, the main adaptor of TLR signaling, in hematopoietic cells, is indispensable for the development of inflammation (219). In line with this finding, IL-10$^{-/-}$ mice spontaneously develop intestinal inflammation (220). In contrast, mice with double deficiency of IL-10 and Myd88 appear healthy and lack severe colitis (191, 221). These studies indicate that TLR signaling has different effects on intestinal immune homeostasis: while TLR signaling on IECs is protective in maintaining barrier function, sustained TLR signaling in APCs induces production of proinflammatory cytokines which is pathogenic.
CD103 and CX3CR1 have been used as markers to define the subsets of DC population with distinct origin and function (222, 223). In general, CD11C_{hi} CD103^{+} DCs are tissue resident DCs which are derived from common DC progenitors whereas CD11C_{hi} CX3CR1^{+} DCs are derived from Gr1_{hi} monocytes. CD103^{+} DCs are dispersed in the LP and they are true APCs with ability to migrate to the draining MLN, to initiate adaptive immune responses (224). Extensive studies of this subset of DCs have revealed that CD103^{+} DCs possess specialized functions, such as cross-presentation of antigen to CD8^{+} T cells, Ig class switch, imprinting T cells to express gut homing molecules and mediating tolerance by induction of pTregs (47, 225, 226). These unique features of CD103^{+} DCs in part depend on their production of TGF-\beta and RA. Given that this DC population produces TGF-\beta and promotes the development of pTregs, CD103^{+} DCs have been defined as a tolerogenic DC subset. Nevertheless, these tolerogenic properties are not hard-wired, as observed during colitis, CD103^{+} DCs showed decreased expression of genes related to tolerance but increased expression of genes related to inflammation, and consequently they lost the ability to support development of iTregs but rather favored the accumulation of Th1 effector cells (227). Moreover, recent study of CD103^{+} DCs revealed that CD11b^{+} CD103^{+} DCs are indispensable for the generation of Th17 cells in an IRF4-dependent manner, suggesting that CD103^{+} DCs also express inflammatory properties (228).

CX3CR1^{+} DCs are more heterogeneous and they are located adjacent to the epithelium (222). These DCs do not have the ability to migrate to the MLN or to prime T cells. Rather, they extend their dendrites to the lumen to sample and transfer the antigens to CD103^{+} DCs. Nevertheless, in a setting of dysbiosis, CX3CR1_{hi} cells were capable of
transporting non-invasive bacteria antigen and migrating to the MLN, which argues against the non-migratory and poor immunostimulatory properties of CX3CR1\textsuperscript{hi} cells (229). Due to their heterogeneity, CXCR1\textsuperscript{+} DCs showed various functions. For instance, E-cadherin\textsuperscript{+} DCs which originate from monocytes have been described as a pro-inflammatory DC subset (230). During colitis, these cells accumulated, expressed a pro-inflammatory gene profile and produced colitogenic cytokines. In a T cell-induced model of colitis, co-transfer of T effector cells with E-cadherin\textsuperscript{+} DCs, markedly exacerbated colitis. It is worth noting that CX3CR1 is also expressed on LP macrophages. Studies of LP CX3CR1\textsuperscript{+} macrophage have revealed several distinctive features. For example, CX3CR1\textsuperscript{+} macrophages were shown to prevent commensal bacteria translocation and limit the Th17 responses (231). Consistent with this finding, in another study, it has been shown that CX3CR1\textsuperscript{+} macrophages were required for the IL-10 producing Treg expansion in the LP (232). Taken together, these studies have shown a complexity of DC populations, as well as other antigen presenting cells in the intestine and further studies are needed to clarify the functions of various subsets of DCs.

Adoptive transfer of CD4\textsuperscript{+} CD45RB\textsuperscript{hi} T cells into immune deficient mice induces IBD in the host, suggesting that CD4\textsuperscript{+} T effector cells play a crucial role in the development of IBD. Indeed, CD4\textsuperscript{+} T cells act as the driver of IBD and the accumulation of CD4\textsuperscript{+} T cells has been thought as a hallmark of IBD (233). Distinct types of immune responses have been shown to contribute to the development of IBD.

The presence of IFN-\textgreek{g}\textsuperscript{+} producing CD4\textsuperscript{+} T cells has been described in the steady state and inflamed intestine of murine models, suggesting Th1 effector cells are important players of IBD (234, 235). Several other lines of evidence further support this notion.
Adoptive transfer of T cells with deficiency of key Th1 transcription regulators, such as T-bet or STAT4, failed to induce colitis in immunodeficient mice (236, 237). Moreover, early treatment with anti-IFN-\(\gamma\) prevented the development of IBD in several colitis models and blocking Th1 cytokine, IL-12, significantly prevented the IBD (238-241). This is further supported by the evidence derived from patients with Crohn’s disease. Accumulation of IFN-\(\gamma^{+}\) CD4\(^{+}\), T-bet\(^{+}\) CD4\(^{+}\) and enhanced expression of IL-12R\(\beta2\) has been found in the LP of these patients (235, 237, 242). Nevertheless, some studies revealed that early but not late administration of anti-IFN-\(\gamma\) prevented the ongoing IBD in mice models and administration of monoclonal anti-IFN-\(\gamma\) to patients with Crohn’s disease were not curative (238, 243), suggesting that Th1 effectors cells may be the initial driver of the IBD and are not the only effectors in the pathogenesis of IBD.

Several studies showed that Th2 effector cells mediated colitis in mice (234). In line with this, increased production of IL-5 and IL-13 has been observed in patients with ulcerative colitis and further study revealed that IL-13 is produced by non-typical NKT cells (235, 244). More recently, lacking pTregs due to deficiency of CNS1 of Foxp3 leads to Th2-type colitis in mice (115). The latter finding provides another line of evidence that IBD can be mediated by different types of immune responses.

Recent studies favor a view that Th17 cells, as a new emerging type of immune responses, play a dominant role in the development of IBD. Th17 immune responses have been shown to play an important role in preventing fungi infection and impaired Th17 responses result in chronic mucocutaneous inflammation in patients (245-247). In the small intestine, an increased proportion of Th17 cells could be observed in steady state and in autoimmune settings without distinct symptoms (248). However,
accumulation of IL-17 producing CD4+ T cells is a feature of IBD. Direct supporting evidence from the T cell transfer model of colitis revealed that T effector cells produced elevated amount of IL-17 and T cells with deficiency of IL-23, which is important for Th17 pathogenicity, markedly impaired their capacity to induce colitis (249, 250).

IL-1β, IL-6 and TGF-β are essential to drive Th17 cell differentiation (94, 162, 251-253). However, it is contradictory concerning the indispensable role of each cytokine in the development of Th17 cells in different experimental models. For example, several studies showed that IL-1β is dispensable for the induction of Th17 cells in the small intestine whereas IL-6 is required for the development of Th17 cells (162, 251). In contrast, by using RORγt-gfp reporter mice, it showed that the development of CD4+ RORγt-gfp+ Th17 cells were greatly impaired in IL-1R−/− mice but not in IL-6−/− mice (253). Moreover, it is still controversial for the requirement of TGF-β. The presence of intestinal IL-17+ CD4+ T cells in transgenic mice bearing dominant negative form of TGF-β subunit receptor II in CD4+ T cells argues against an indispensable role of TGF-β in Th17 cell differentiation (254). Additionally, IL-6 and IL-23, combined with IL-1β, readily induce Th17 cells in the absence of TGF-β. These discrepancies might suggest that the developmental, functional and anatomical heterogeneity of Th17 cells is determined by the combination of available cytokines. In line with this, TGF-β3 was shown to induce Th17 cells with enhanced colitogenicity and a distinct transcription signature (255). To reconcile these findings, recent studies might favor a view that a localized regulation of Th17 cell development differs in the periphery vs. mucosal tissues (252).
Although IL-23 is not required for the initial induction of Th17 cells, IL-23R signaling in T cells is critical for expansion and accumulation of IL-17^+ IFN-γ^+ T cells and is responsible for the pathogenesis of IBD (250, 254, 256, 257). The effective prevention of colitis by administration of anti-IL-12p40 was further assessed and revealed that such an effect is achieved by blocking not only IL-12 signaling, but also IL-23 signaling, since IL-12p40 is one of the subunit of IL-23 (258). IL-23/Th17 axis was shown to induce the enhanced proliferation of hematopoietic stem and progenitor cells in lymphoid organs, such as the BM and spleen, and increased production of granulocyte-monocyte progenitors in the intestine, which consequently led to the accumulation and infiltration of inflammatory monocytes and neutrophils (259). In addition, this cytokine is colitogenic partly due to its capacity of inhibiting iTreg induction and IL-10 production by T regulatory type 1 (Tr1) cells (250, 257). In humans, the polymorphism of IL-23R gene has been linked to IBD (260). Overall, these findings point to the important role of IL-23 in the pathogenicity of Th17 cells in the intestinal inflammation.

Foxp3^+ Tregs play a non-redundant role in maintaining tolerance in gut mucosa. Numerous studies have shown that Tregs are capable of preventing T cell induced colitis or curing established colitis (261-263). The intestinal Treg pool comprises a mixture of tTregs and pTregs and the latter are indispensable to sustain the tolerance to commensal bacteria and other foreign antigens derived from the gut lumen (121, 123).

In the gut mucosa, Tregs mediate suppressor function through multiple pathways. Among these, production of IL-10 is most critical. Mice with IL-10 deficiency in Tregs spontaneously developed IBD, which recapitulates the phenotype of mice with global deficiency of IL-10 (156). IL-10 production by Tregs not only acts as a suppressive
mediator on T effector cells, but also affects their own suppressor function (163, 264). IL-10 signaling in Tregs is required to induce STAT3 activation to suppress Th17 immune responses. TGF-β, acting as another important suppressive mediator, is important for Treg-mediated suppression in the intestine. Tregs unable to produce TGF-β failed to suppressed colitis in a T cell transfer model of colitis (265).

The knockout of several other suppressive mediators in Tregs revealed other regulatory mechanisms of Treg-mediated suppression in gut mucosa. For example, Tregs genetically manipulated not to express ebi3 or IL-12a (IL-12p35) failed to prevent T cell-induced colitis, suggesting that IL-35 activity by Tregs is important to suppress intestinal inflammation (266). Similarly, Tregs with OX40 deficiency were unable to suppress the IBD in a T cell transfer model of colitis, implying that OX40 expressed by Treg is required to mediate suppressor function (267).

The dialogue between T cell subsets and microbiota

Numerous studies are emerging to demonstrate that the gut microflora and host immune system are affected reciprocally. On one hand, the immune responses in the intestine exert the impact on the composition of gut microflora. For example, lacking pTregs in the gut resulted in an altered distribution of gut microbiota (115). On the other hand, commensal bacteria have a profound effect on the development of T cells. The latter notion is first supported by earlier studies showing that germ-free mice lack intestinal Th17 cells (268, 269). Later studies specifically demonstrated that colonization of segmented filamentous bacterium (SFB) promoted Th17 development (270, 271). While SFB favors the induction of Th17 cells, several other species of microflora facilitate the
induction of pTregs. For example, polysaccharide A of *B. fragilis* promoted pTreg induction via TLR2 signaling on T cells (272). Other studies reported that a clostridia cocktail promoted the induction and accumulation of colonic Tregs in germ-free mice through microbiota-derived short-chain fatty acid, butyrate (119, 120, 273, 274). More recently, one group showed fungi species also had an effect on immune system, signaling through innate immune receptor Dectin-1 (275). Collectively, these studies clearly demonstrated that factors involved in regulating intestinal homeostasis are interacts with each other and cooperate to form a sophisticated network to maintain the intestinal homeostasis.

### 1.7 Open questions and research purpose

Owing to redundant mechanisms that contribute to mucosal tolerance, a defect in a single regulatory component, even one crucial to maintain tolerance, such as IL-10, does not necessarily immediately tip the balance from tolerance to inflammation and IBD. The rapid development of IBD in the absence of IL-10 depends on an agent that triggers strong inflammatory responses, such as infection by the pathogenic bacteria *H. hepaticus* (239). These observations strongly suggest that regulatory mechanisms co-operate to form a redundant network that functions to maintain mucosal tolerance. However, these complexities make establishing the function of one component within regulatory circuits more difficult.

αE-integrin (CD103) is highly expressed by IELs, a subset of DCs, and Tregs in the LP in the gut mucosa. CD103, paired with β7 integrin, interacts with E-cadherin
which is abundantly expressed by gut epithelial cells and some DCs (230, 276). The expression of E-cadherin by epithelial cells and CD103 by lymphocytes are expected to promote intercellular interactions that have been hypothesized to mediate long-term retention of lymphocytes in the IEL compartment (276). Several lines of evidence support such a role for CD103 on T effector cells. For example, graft-versus-host disease (GVHD) induced by CD103−/− donor cells was characterized by fewer CD8+ T cells in the gut and less destruction of gut epithelium (277). In responses to infection, CD8+ T resident-memory cells in the IEL were reduced, if they did not express CD103 (278, 279). In addition, blockade of CD103 in the context of Foxp3- or IL-2-deficiency resulted in less severe autoimmunity (280, 281). Thus, deficiency of CD103 on T cells attenuates immune responses and has been associated with impaired T effector cell retention.

CD103−/− mice exhibit a decrease in IELs and to a lesser extent LP lymphocytes in some strains (282), which is consistent with a defect in T cell retention within the gut. CD103−/− mice, however, do not exhibit pathological abnormalities, including those related to IBD, suggesting that if Tregs require CD103 for retention, this deficit may be balanced by impaired retention by T effector cells or another redundant pathway. Overall, evidence is limited and contradictory concerning a requirement for CD103 expression by Tregs to effectively mediate tolerance. For example, expression of CD103 was reported to be required for Treg retention in the skin for control of inflammation during *L. major* infection (283). In contrast, CD103−/− Tregs readily suppressed an intestinal immune response in a T cell transfer model of colitis (263), suggesting that CD103 expression by Tregs is not required for mucosal tolerance. Indeed, some evidence suggests that CD103 expression by DCs may be more relevant for tolerance in this model (263). These
discrepancies with respect to a role for CD103 in Treg retention are likely due to redundant and context dependent mechanisms in the gut mucosa. Thus, it still remains uncertain the extent by which CD103 facilitates Treg mediated suppression in intestinal homeostasis and tolerance.

CD103 expression on Tregs marks a subset of Tregs with increased capacity to suppress T cell proliferation in vitro and colitis induced by CD4+ T conventional cells in lymphopenic recipients (180-182). However, it is unknown whether CD103 expression contributes to the robust suppressive function of CD103+ Tregs or CD103 just acts as a marker. Moreover, other features of this CD103+ Treg subset remain undefined. Thus, important objectives of this dissertation are to further characterize the CD103+ Treg subset and to determine whether CD103 plays a functional role in Tregs to maintain tolerance.

Proper IL-2R signaling represents another activity essential for tolerance in the gut mucosa (284). Polymorphisms in IL-2, IL-2Ra, and IL-2Rβ are genetic risks for several autoimmune diseases, including IBD (285-287). Our lab has developed a mouse model that permits the evaluation of the outcome of impaired IL-2R signaling on Treg function and the risk for autoimmune disease (35). IL-2RβY3 are transgenic mice on the IL-2Rβ−/− genetic background (referred to as Y3 in this dissertation) where all T cells express a transgenic IL-2Rβ chain whose cytoplasmic tail contains three tyrosine to phenylalanine mutations that impairs IL-2-dependent PI3K and STAT5 activation. A low level of STAT5 activation occurs upon IL-2 binding to this IL-2Rβ mutant molecule and this amount of signaling readily supports outwardly normal thymic Treg development and peripheral homeostasis. Nevertheless, some IL-2-dependent functions remained
impaired in these Tregs. Y3 mice do not develop severe autoimmunity associated with parental IL-2Rβ−/− mice and are long-lived, but upon aging (>16 weeks) some exhibit immune activation and mild to moderate lymphocytic infiltrates in several tissues, principally the lung and salivary gland, with less frequent involvement of the intestine. Nevertheless, the intestinal inflammation in the context of low IL-2R signaling has not been characterized and it is uncertain to what extent such impaired IL-2R signaling contribute to intestinal inflammation.

In this dissertation, I will first compare the CD103+ Treg subset with CD103− Treg subset with respect to their anatomical locations, activation stages, function, and role of IL-2R signaling in regulating the development of Treg subsets in Chapter 2.

In order to reveal the role of CD103 in maintaining mucosal tolerance, CD103−/− mice were crossed to Y3 mice to assess whether more severe autoimmune disease may develop. We predicted that generally lowering Treg activity through impaired IL-2R signaling may help to reveal the potential contribution of CD103 to mucosal tolerance. In Chapter 3, I will summarize the main findings from the study as following: 1. The IL-2R-related risks for intestinal inflammatory responses that may contribute to IBD and the role of CD103 deficiency in cooperating with low IL-2R signaling that resulted in severe colitis in mice. 2. The development of colitis in Y3/CD103−/− mice was at the level of Tregs. 3. The CD103-dependent component for IBD was not due to impaired Treg homing/retention to the gut or impaired Treg activity, but rather to altered Treg homeostasis.
CHAPTER 2
CHARACTERIZATION OF CD103⁺ TREG SUBSET

2.1 CD103⁺ Tregs displays activated phenotype

CD103 is expressed on most Tregs in the gut mucosa but is on fewer Tregs in secondary lymphoid tissues (Fig. 2.1). Many fewer conventional CD4⁺ T cells express CD103, with the highest proportion found in the IEL compartment of the small intestine (Fig. 2.1).

CD103⁺ Tregs from the spleen and SI-LP express an activated phenotype (i.e. increased CD44, CD69, ICOS, Klrg1, Blimp-1) and higher levels of some suppressive molecules (i.e. CD39 and CTLA4) (Fig. 2.2A, B). These trends were more noticeable for the spleen as it contains may fewer CD103⁺ Tregs. Collectively, these data demonstrate that CD103⁺ Tregs are predominant in gut mucosal and CD103 marks an activated Treg subset which expresses higher levels of suppressor molecules.

Fig. 2.1 CD103 expression by CD4⁺ T cells in various tissues. The proportion of CD103⁻ Tregs and CD103⁺ CD4⁺ T conventional cells (T convs) in various tissues were assessed by flow cytometry (n = 3-9 mice).
**Fig. 2.2 Comparison of CD103^- vs. CD103^+ Treg subsets.** (A, B) CD103^- and CD103^+ Tregs from spleen (A) and SI-LP (B) of WT mice were analyzed for the expression of marks as indicated and the percent of expression or relative mean fluorescence intensity (MFI) was determined by flow cytometry. The expression of Blimp-1 in Tregs was measured according to the GFP expression from Blimp-1/GFP/Foxp3/RFP reporter mice. The relative MFIs of CD103^- and CD103^+ Tregs were normalized to CD103^- Tregs (n =3-5 mice).
2.2 CD103+ Tregs mark a Treg subset with increased suppression

CD103+ Tregs showed increased capacity to suppress T cell proliferation in vitro and colitis induced by CD4+ conventional T cells in lymphopenic recipients (180-182). To investigate whether this also holds true in in vivo lymphoreplete hosts, Treg subsets were transferred to day 2 IL-2Rβ−/− neonates. IL-2Rβ−/− mice rapidly develop lymphoproliferative autoimmunity due to lack of functional Tregs and display overt symptoms between 4-16 weeks of age (288, 289). We have previously shown that adoptive transfer of limited number of purified Tregs (1-2 x 10^5) in neonatal IL-2Rβ−/− mice effectively prevented the lethal autoimmunity (290). In the current study, total Tregs, fractionated naïve Tregs (CD103− CD62L^hi CD69^lo Klrg1^−^) and fractionated CD103+ Tregs (CD103^+ CD62L^lo^) were transferred into day 2 IL-2Rβ−/− neonates. Consistent with our previous studies, untreated IL-2Rβ−/− mice showed signs of disease rapidly. Thus, untreated mice were analyzed between 6-8 weeks of age. In contrast, mice treated with Tregs all survived and did not develop obvious signs of disease by the time they were analyzed (16-21 weeks). We noted that although spleen cellularity was comparable between all groups (Fig. 2.3A), some IL-2Rβ−/− mice exhibited anemia or splenomegaly (data not shown). Mice received Tregs showed 3-fold decrease of pLN cellularity (Fig. 2.3A) and a normal proportion of engrafted Tregs in the spleen and pLN (Fig. 2.3B). Moreover, treated mice exhibited less CD4+ T conventional cell activation in the spleen based on the expression of CD44, CD62L and CD69 activation markers (Fig. 2.3C). However, so far we did not observe any differences from mice received various Treg subsets, suggesting theses Treg subsets equivalently suppressed the systemic autoimmunity associated with IL-2Rβ chain deficiency.
We were interested to know whether these subsets vary in suppressing tissue-specific autoimmunity. IL-2Rβ\(^{−−}\) mice rapidly progress autoimmunity with death about 8-16 weeks, usually proceeding intestinal inflammation. Adoptive transfer of Tregs consistently prevented disease and these mice lived longer. Therefore, we were able to compare suppression by Treg subsets in the gut mucosa. 16-21 weeks post transfer, some of IL-2Rβ\(^{−−}\) mice treated with Tregs exhibited increased MLN cellularity, mostly from those receiving CD103\(^{−}\) Treg subset (**Fig. 2.4A**).
Fig. 2.4 CD103⁺ Treg subset exhibits increased suppression in the small intestine.

(A) MLN cellularity and number of total CD4⁺ T cells in the SI-LP and LI-LP from untreated (6-8 weeks old) and adoptive transferred mice (16-21 weeks old). (B) The frequency of CD4⁺ Foxp3⁺ Tregs in indicated tissues were analyzed for untreated and treated mice. (C) Representative (top) and averaged (bottom) cytokine expression by CD4⁺ Foxp3⁻ T conventional cells after culture with PMA and ionomycin for 4 hr in indicated tissues from untreated and adoptively transferred mice. Data are pooled from 2 independent experiments (n = 3-7 mice/group).
The increase of CD4+ T cells is a critical characteristic of intestinal inflammation (233). As expected, mice treated with CD103+ Treg subset showed fewer accumulation of CD4+ T cell in the SI-LP, although CD4+ T cell numbers were comparable in the lamina propria of large intestine (LI-LP) (Fig. 2.4A). Nevertheless, the proportions of Tregs were comparable in MLN, SI-LP and LI-LP from mice receiving various Treg subsets (Fig. 2.4B), suggesting that the varied suppression in the small intestine by Treg subsets is not due to varied Treg engraftment in the gut mucosa. In the small intestine, mice treated with CD103+ Treg subset also showed fewer IL-2, IL-17, IFN-γ and TNF-α producing CD4+ T effector cells (Fig. 2.4C). Collectively, these results demonstrate that Treg subsets equally suppressed the systemic immune response and particularly, CD103+ Treg subset better suppressed the inflammation in the small intestine when compared to naïve CD103- Tregs in IL-2Rβ-/- mice, implying a tissue specific suppression feature of CD103+ Treg subset.

2.3 CD103+ Treg subset exhibits distinct homeostatic characteristics

Tregs display distinct homeostasis in peripheral lymphoid organs vs. tissue sites (38, 45). The proliferation and apoptosis of ex vivo CD103- and CD103+ Treg subsets were assessed. CD103+ Treg subset was more proliferative in the spleen, measured by Ki67 expression (Fig. 2.5). Nevertheless, in the SI-LP, CD103- and CD103+ Treg subsets showed comparable level of proliferation. Moreover, both the splenic and SI-LP CD103+ Treg subsets expressed lower level of anti-apoptotic molecule Bcl-2 (Fig. 2.5). These
results are consistent with the notion that effector Tregs are highly proliferative and proapoptotic (38).

2.4 Role of IL-2R signaling in CD103$^+$ Treg homeostasis

By taking the advantage of Y3 mouse model, we were able to study the effects of low IL-2R signaling in T cells (35). It has been shown that IL-2 is sufficient to amplify CD103$^+$ T conventional cells (281). With respect to Tregs, the gene expression profile of Tregs with impaired IL-2R signaling has revealed that CD103 is IL-2-dependent (35). A decrease of CD103$^+$ Tregs was noted in the spleen and pLN from Y3 mice (Fig. 2.6A and data not shown). However, an increase of, rather than a decrease of, CD103$^+$ Tregs was observed in the SI-LP from Y3 mice (Fig. 2.6A). Further evaluation of Treg proliferation and survival activity by measuring Ki67 and Bcl-2 expression showed that CD103$^+$ Tregs in the spleen from B6 and Y3 mice expressed similar level of Ki67 and CD103$^+$ Tregs from Y3 mice expressed lower level of Bcl-2 (Fig. 2.6B). The latter finding is consistent
with the notion that IL-2 provides survival signals for Treg cell growth (26). Strikingly in the SI-LP, CD103⁺ Tregs from Y3 mice expressed more Ki67 and comparable level of Bcl-2, compared to WT CD103⁺ Tregs (Fig 2.6B), indicating that the hyper-proliferation resulting from impaired IL-2R signaling account for the accumulation of CD103⁺ Tregs in the SI-LP of Y3 mice.

![Image](image-url)

**Fig. 2.6 Proliferation and turnover of Treg subsets with impaired IL-2R signaling.** (A, B) The proportion of CD103⁺ Tregs (A), and Ki67 and Bcl2 expression by CD103⁺ Tregs (B) in the spleen and SI-LP from B6 and Y3 mice (n = 15-20 mice/group). (C) BrdU incorporation was determined for CD103⁺ Tregs in the spleen and SI-LP from B6 and Y3 at different time points after feeding mice with BrdU in drinking water for continuous 5 days and then normal water (day 0) (n = 3 mice /group/time point).

To examine the turnover of CD103⁺ Tregs, BrdU incorporation studies were performed where B6 and Y3 mice received BrdU in the drinking water for 5 days and BrdU uptake and loss were determined upon withdrawal. Consistent with Treg proliferation assessed by Ki67 expression, splenic CD103⁺ Tregs displayed similar
turnover between B6 and Y3 mice (Fig. 2.6C). Noticeably, in the SI-LP, CD103+ Tregs from Y3 mice exhibited greater turnover than WT CD103+ Tregs (Fig. 2.6C). The turnover between CD103- and CD103+ Tregs was similar when examined in spleen and SI-LP from B6 or Y3 mice (data not shown). Collectively, these results demonstrate that IL-2R signaling exerts different effects on Tregs in the peripheral lymphoid organ vs. tissue site. Particularly in the SI-LP, the low IL-2R signaling resulted in heightened proliferation of Tregs in an attempt to compensate for the rapid cell death.

Studies of the CD103+ Treg subset revealed several unique features with respect to their activation status, potent suppressive activity in the small intestine and IL-2-dependent regulation. However, these results further raise the following questions, i.e. Is CD103 functional active for Treg-mediated suppression? What is the role of CD103 in mucosal tolerance? What is the key signal, except IL-2R signaling, that controls CD103+ Treg development in the gut mucosa. In chapter 3, I will present the work that addresses these questions.
3.1 Y3/CD103\(^{\sim}\) mice develop colitis

The high prevalence of CD103 on mucosal Tregs raises the possibility that CD103 might be required to facilitate Treg-mediated suppression. We reasoned that if CD103 deficiency was expressed in autoimmune prone mice such as Y3 mice, the normal function of CD103 to maintain tolerance might be revealed. Therefore, CD103\(^{\sim}\) mice were bred to Y3 mice and their health was followed for 6 months. Strikingly, all Y3/CD103\(^{\sim}\) mice developed severe colitis characterized by diarrhea, rectal prolapse and wasting disease, usually between 12-16 weeks of age (Fig. 3.1A). These mice exhibited enlarged MLN with increased cellularity (Fig. 3.1B) and thickening of the wall of the large intestine (data not shown). Histological evaluation revealed extensive lymphoid infiltration and tissue destruction in the large intestine consistent with chronic active colitis (Fig. 3.1C). Some mice also showed mild inflammation of the pancreas, lung, liver and salivary gland and liver (data not shown), similar to that reported for Y3 mice (35). Coincident with colitis, splenic cellularity was slightly, but significantly, increased for Y3/CD103\(^{\sim}\) mice that was primarily due to an increase in Ly-6G\(^{+}\) granulocytes (Fig. 3.1D).

The LI-LP from Y3/CD103\(^{\sim}\) mice showed a large increase in the proportion and number of CD4\(^{+}\) T cells (Fig. 3.2A). A decreased frequency (2-fold) of Tregs was found in the LI-LP from Y3 and Y3/CD103\(^{\sim}\) mice, but only Y3/CD103\(^{\sim}\) mice showed a
substantial increase in the number of Foxp3$^+$ Tregs (Fig. 3.2B). In contrast, conventional CD4$^+$ T cells and Tregs each increased in the SI-LP from Y3 and Y3/CD103$^{-/-}$ (Fig. 3.2A, B), but the proportion of Tregs to conventional CD4$^+$ T cells was similar to WT and CD103$^{-/-}$ mice (Fig. 3.2B).

Fig. 3.1 Y3/CD103$^{-/-}$ mice develop severe colitis. (A) Incidence of disease. Mice from each genotype were monitored weekly for signs of colitis, and were considered diseased when they exhibit at least one of the following severe diarrhea, rectal-prolapse and/or wasting. (B) MLN cellularity. (C) Representative formalin-fixed and H&E stained colon sections from age-matched 12-24 week-old mice (n ≥ 4 mice/group) (original magnification 12.5 X). (D) Splenic cellularity (left) and the frequency of Ly6G$^+$ granulocytes (right) in the spleen were assessed by flow cytometry. (B, D) The number of mice/group is indicated within each bar or by individual symbols in scatter graphs.

Increased percentages of CD44$^{hi}$ CD62L$^{lo}$ activated CD4$^+$ Foxp3$^-$ T cells were detected in the spleen and MLN, but not the SI-LP, of Y3 and Y3/CD103$^{-/-}$ mice, but only...
in the LI-LP of Y3/CD103⁻/⁻ mice (Fig. 3.3). The LI-LP of Y3/CD103⁻/⁻ mice also showed increased number of IL-2, IL-17, IFNγ and TNF-α producing CD4⁺ T effector cells (Fig. 3.4).

Fig. 3.2 Accumulation of CD4⁺ T cells in the LI-LP of Y3/CD103⁻/⁻ mice. (A, B) The proportion and number of total CD4⁺ (A) and CD4⁺ Foxp3⁺ Tregs (B) in the LI-LP and SI-LP were determined by flow cytometry. The number of mice/group is indicated within each bar or by individual symbols in scatter graphs.

In marked contrast, the SI-LP of Y3 and Y3/CD103⁻/⁻ mice showed comparable increases of IL-17-producing CD4 T effector cells (Fig. 3.4). These findings demonstrate that a CD103-dependent induction of colitis in the context of altered IL-2Rβ signaling is due to a substantial CD4⁺ T cell inflammatory response, with a lower ratio of Treg:T conventional cells, in the LI-LP. However, with the exception of increased CD4⁺ T conventional and Treg cells in the LI-LP of Y3/CD103⁻/⁻ mice, leading to a low
proportion of Tregs, the other significant immunological changes noted were detected in Y3 and Y3/CD103−/− mice, indicating that these were primarily dependent upon the Y3 IL-2Rβ mutation.

3.2 Altered Treg homeostasis in the gut mucosa of Y3/CD103−/− mice

CD4+ conventional and Treg cells were evaluated for their proliferative activity in vivo by Ki67 expression. The increased number of Y3/CD103−/− Tregs and activated CD4+ T conventional cells (Figs. 3.2 and Fig. 3.3) in the LI-LP was associated with increased proliferation of both cell populations (Fig. 3.5). This effect is due to the absence of CD103 as Tregs in the LI-LP of Y3 mice did not show increase in Ki67+ cells. With respect to the SI-LP, conventional CD4+ T cells and Tregs from Y3 and Y3/CD103−/−
mice each showed increased proliferative Ki67+ cells, indicating that this effect is due to the Y3 IL-2Rβ mutation (Fig. 3.5), in an agreement with previous BrdU uptake and loss studies which showed that the increased proliferation, in the SI-LP of Y3 mice is due to increased turnover of these cells (184) (Fig. 2.6C).

The preceding experiments examined CD4+ T conventional and Treg proliferation in the LI-LP of Y3/CD103−/− mice when colitis was evident. The increased proliferation of Tregs, therefore, might reflect a response to the proliferating CD4+ inflammatory T cells. To address this point, the CD4+ T cell compartment within the LI-LP of younger colitis-free Y3/CD103−/− mice were examined and compared to control WT mice. At this time extensive lympho-proliferation had not yet developed as assessed by relatively low cellularity of the MLN which is comparable to that from Y3 mice (Fig. 3.6A). An increase in CD4+ T cells and a lower percentage of Tregs were also detected in the LI-LP.
of younger Y3/CD103−/− mice (Fig. 3.6B). Such effects observed from younger Y3/CD103−/− mice could be consistently found in adult Y3 and Y3/CD103−/− mice, suggesting a low IL-2R signaling associated dysregulation on CD4+ T conventional cells and Tregs. However, Y3/CD103−/− Tregs, but not CD4+ T conventional cells showed increased proliferation (Fig. 3.6C), indicating altered proliferation of Tregs precedes changes in proliferation of inflammatory CD4+ T cells and a CD103-deficiency related dysregulation on Tregs earlier before the overt disease.

3.3 WT Tregs prevent the development of colitis in Y3/CD103−/− mice

The development of colitis in Y3/CD103−/− mice and the associated immune abnormalities raise the possibility that Tregs were not able to fully maintain tolerance in the intestine. To test this possibility, the capacity of WT Tregs to suppress this form of colitis was evaluated. Y3/CD103−/− mice (12-15 days old) received FACS-purified WT Tregs from
Foxp3/RFP reporter mice. At 4 weeks post transfer, 40% to 70% of total Tregs in the peripheral blood of these recipients were of donor origin (data not shown). Cohorts of sex-matched treated and untreated Y3/CD103<sup>−/−</sup> mice were monitored for weight change, diarrhea and rectal prolapse. Disease severity as measured by weight loss was more pronounced in untreated female Y3/CD103<sup>−/−</sup> mice (Fig. 3.7A). Adoptively transferred WT Tregs prevented this weight loss and other symptoms of colitis (Fig. 3.7A, B). Accordingly, histological examination of the large intestine showed normal colonic histology (Fig. 3.7C) and significantly reduced total cellularity for the MLN (Fig. 3.7D).

Fig. 3.6 Increased Treg proliferation in younger Y3/CD103<sup>−/−</sup> mice. (A-C) 6-10 week old age-matched mice from each genotype were analyzed for MLN cellularity (A), the proportion of total CD4<sup>+</sup> and CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs in the LI-LP (B) and Ki67 expression by Tregs and CD4<sup>+</sup> Foxp3<sup>+</sup> T conventional cells (T convs) from the LI-LP (D). The number of mice/group is indicated within each bar or by individual symbols in scatter graphs.
Further analysis of the LI-LP of Treg treated mice showed a lower proportion and number of CD4⁺ T cells (Fig. 3.8A) with decreased proliferation of CD4⁺ T conventional cells (Fig. 3.8B) and an increased frequency of Tregs, which was largely donor derived (75 ± 8.9%) (Fig. 3.8C). In addition, the number of IL-2⁺, IL-17⁺, IFN-γ⁺ and TNF-α⁺ CD4⁺ T effector cells in the LI-LP was substantially decreased (Fig. 3.9). These results...
demonstrate that adoptive transfer of WT Tregs efficiently prevented colitis and the intestinal inflammation in the LI-LP of Y3/CD103\(^{-/-}\) mice. Thus, a major factor that accounts for this type of colitis is defects associated with Tregs.

**Fig. 3.8 Decrease of CD4\(^{+}\) T cells in Treg-treated Y3/CD103\(^{-/-}\) mice.** (A, B) The proportion (left) and number (right) of total CD4\(^{+}\) T cells (A) and Ki67 expression by CD4\(^{+}\) Foxp3\(^{-}\) T conventional cells (B) in the LI-LP and SI-LP from untreated and adoptively transferred mice. (A, B) The number of mice/group is indicated within each bar or by individual symbols in scatter graphs. (C) The frequency and origin of Tregs in the LI-LP and SI-LP from untreated and adoptively transferred mice (n = 5 mice for each group).

It is worth noting that the increase in proportion, cellularity (Fig. 3.8A), and proliferation of CD4\(^{+}\) T conventional cells (Fig. 3.8B) and proportion of Tregs (Fig. 3.8C) in the SI-LP were not affected by the donor WT Tregs. In addition, increased cytokine production by effector CD4\(^{+}\) T cell in SI-LP (Fig. 3.9) was also unaffected by the WT...
donor Tregs. This finding suggests that inflammation associated with the SI-LP of Y3/CD103−/− mice is cell intrinsic and independent of altered function of Tregs.

3.4 Normal trafficking/retention of CD103−/− Tregs in the gut mucosa

The effective suppression of inflammation within the colon by CD103+ Tregs might be related to higher expression of suppressive and/or gut homing molecules (111). CD103 might directly contribute to these distinct properties or alternatively may simply mark cells with these activities. CD103 has been reported to promote Treg retention in the skin (283), raising the possibility that CD103 directly regulates Treg trafficking and/or retention within the gut. To test this possibility, the fate of a 1:1 mixture of input Thy1.1+ WT and Thy1.2+ CD103−/− RFP+ Tregs were followed after transfer into lympho-replete Y3 mice (Fig. 3.10A). Past studies have shown that adoptively transferred WT Tregs
engraft in Y3 recipients (184), which easily permits following the fate of the donor Tregs, in this case based on expression of RFP and Thy1.1. Four week post transfer, Tregs of WT and CD103−/− origin were detected in a largely similar proportion in the SI-LP where most WT Tregs expressed CD103 (Fig. 3.10B). The spleen, MLN and LI-LP also contained similar proportions of Tregs derived from WT and CD103−/− donor cells (Fig. 3.10C). On average, donor WT Tregs in the spleen, MLN, SI-LP and LI-LP were approximately 40%, 75%, 75% and 50% CD103+, respectively. These results indicate that CD103 expression is not required for homing or retention within the gut mucosa. Thus, the normal Treg homeostasis in the SI-LP and LI-LP and suppression of colitis in Y3/CD103−/− mice that received Tregs is not due to CD103-dependent tissue homing and retention in the gut.

**Fig. 3.10 CD103 molecule is not required for Treg retention in the gut mucosa.** FACS-purified CD4+ RFP+ Thy1.1+ Tregs from Foxp3/RFP mice were mixed with CD4+ RFP+ Thy1.2+ Tregs from CD103−/− Foxp3/RFP mice at 1:1 ratio (4 x 10^5 total) (input donor Tregs) and injected i.v. into Y3 mice. (A) Representative FACS plots showing the purity and ratio of mixed input Tregs of WT Thy1.1+ and CD103−/− Thy1.2+ origin. Four weeks post-transfer, representative (B) and averaged (C) distribution of Tregs of WT and CD103−/− origin for the indicated tissues. Data are pooled from 2 independent experiments (n = 7 mice/group).
3.5 CD103 is required for proper localization in the gut mucosa

Although CD103\(^{-/-}\) Tregs were readily found in the gut, their localization within the intestinal microenvironment might be altered. To directly examine T cell localization within the intestine, confocal imaging was performed after immunofluorescent labeling of frozen intestine sections. The localization of CD4\(^{+}\) T conventional and Treg cells with respect to E-cadherin\(^{+}\) epithelial cells was determined for the small intestine, where CD103 is normally most prevalent (Fig. 2.1), by staining for CD4, Foxp3, E-cadherin and DAPI, the latter to generally reveal nucleated cells. In agreement with FACS analysis, CD4\(^{+}\) T cells were more numerous in the small intestine of Y3 and Y3/CD103\(^{-/-}\) mice (Fig. 3.11A), which exhibited increased CD4\(^{+}\) T cell cellularity (Fig. 3.2A). However, altered T cell localization was noted in the gut mucosa. CD4\(^{+}\) T cells were diffusely localized in the villi of Y3 small intestine (Fig. 3.11A). More Y3 CD4\(^{+}\) T cells were closely associated to epithelial cells as assessed by co-localization of CD4\(^{+}\) and E-cadherin\(^{+}\) cells. In contrast, CD4\(^{+}\) T cells were more centered in the villi of the Y3/CD103\(^{-/-}\) small intestine and fewer cells were associated with E-cadherin\(^{+}\) cells (Fig. 3.11A, B). Although many fewer Foxp3\(^{+}\) Tregs were detected, these trends likely holds true for Tregs because they co-localize with the CD4\(^{+}\) T cells.

This distinct localization of CD4\(^{+}\) T cells in the small intestine of Y3 vs. Y3/CD103\(^{-/-}\) mice indicates that CD103 is required for close interaction with E-cadherin\(^{+}\) epithelium. Consistent with this notion, DAPI stained nucleated cells in the villi were closer to the epithelial cells in small intestine from WT and Y3 mice whereas these cells were more clustered within the center of the villi in the small intestine from CD103\(^{-/-}\) and Y3/CD103\(^{-/-}\) mice (Fig. 3.11A). Thus, not only CD4\(^{+}\) T cells, but also other cell types in
the intestine require CD103 to closely associate to the E-cadherin\textsuperscript{+} epithelium.

Collectively, these findings revealed that CD103 is not required for Treg homing or retention in the gut mucosa, but rather is necessary for proper lymphoid cell localization toward the gut epithelium.

**Fig. 3.11 CD103 is important for proper T cell localization in the small intestine.** (A) Representative confocal images show E-cadherin\textsuperscript{+} epithelial cells, CD4\textsuperscript{+} T cells, Foxp3\textsuperscript{+} Tregs and DAPI stained nucleated cells in the small intestine from indicated mice. Foxp3\textsuperscript{+} Tregs are marked with yellow arrow head and co-localized CD4\textsuperscript{+} and E-cadherin\textsuperscript{+} cells are indicated with yellow arrow. (B) Co-localization of CD4\textsuperscript{+} T and E-cadherin\textsuperscript{+} cells was determined by two approaches. Left, total CD4\textsuperscript{+} T cells and CD4\textsuperscript{+} T cells that co-localized or closely associated with E-cadherin were counted. The percent of cells that were co-localized or closely associated with E-cadherin\textsuperscript{+} cells was determined after counting 674 total CD4\textsuperscript{+} T cells from 26 fields for Y3 and 576 total CD4\textsuperscript{+} T cells from 27 fields for Y3/CD103\textsuperscript{-/-} mice (PPs were excluded). Right, co-localization was measured by relative mean pixel intensity (Relative Mean) and show graphically. Each symbol represents one relative mean pixel intensity from one field (n = 6 sections per tissue obtained from 3 individual mice for each group).
3.6 Similar gene programs in Y3 and Y3/CD103⁻/- Tregs

The inability of Y3/CD103⁻/- Tregs to maintain tolerance within the gut mucosa might reflect intrinsic defects in these cells when compared to Y3 Tregs. Differences in gene expression between WT and Y3 Tregs provide information concerning properties that predispose Y3 mice to autoimmunity. CD103 might directly or indirectly influence Y3 Treg function by interaction with E-cadherin on DCs and/or epithelial cells or by permitting other molecular interactions due to proximity to these cells. In these situations gene expression is expected to vary between Y3 and Y3/CD103⁻/- Tregs. Initially to address these points, FACS analysis was performed for SI-LP and LI-LP Tregs from B6, Y3, and Y3/CD103⁻/- mice to examine several important activation, functional, and homing molecules (Fig. 3.12A). In comparison to WT Tregs, many of the molecules examined (Foxp3, CD73, CXCR3, CCR6, and CCR5) were similarly expressed by Y3 and Y3/CD103⁻/- Tregs in both the SI-LP and LI-LP. As expected, CD25 expression, which is upregulated by IL-2R signaling, was lower for Y3 and Y3/CD103⁻/- Tregs in the SI-LP and LI-LP. However, CD39, Nrp-1 and CTLA4, molecules linked to Treg function, and CCR4 were lower whereas Helios was higher in both Y3 and Y3/CD103⁻/- Tregs, indicating that their expression was altered due to impaired IL-2Rβ signaling. In each case, however, these differences in expression by Tregs showed localized effects, i.e. Nrp-1, and CCR4 was lower in SI-LP whereas CTLA4 was lower and Helios was higher only in the LI-LP. As these changes were not unique to Y3/CD103⁻/- Tregs, they may contributes to mild/moderate inflammation associated with the SI-LP of Y3 and Y3/CD103⁻/- mice and are likely permissive for colitis associated with Y3/CD103⁻/- mice.
Fig. 3.12 Similar gene programs in Y3 and Y3/CD103<sup>-/-</sup> Tregs. (A) Expression of the indicated markers in Tregs from the SI-LP and LI-LP were assessed by flow cytometry (n ≥ 3 mice/group). ND (not determined). (B) Euclidean cluster analysis of all differentially expressed genes (≥ 1.5-fold) for the indicated groups (n = 3 mice/group). (C) Euclidean clustering of sample relatedness and genes within the major enrichment groups (EG). EG 1-5 are defined in the text.

We have previously reported that Klrg1<sup>+</sup> Tregs are IL-2-dependent terminally differentiated cells that have undergone more than 8 cell divisions (184). As expected
very few Y3 and Y3/CD103+/− Klrg1+ Tregs were found in the SI-LP (Fig. 3.12A).

However, when compared to the SI-LP, WT mice showed a lower frequency of Klrg1+ Tregs in the LI-LP and these were further reduced only for Y3/CD103+/− mice. This result indicates that the LI-LP contains fewer terminally differentiated Tregs and that the development of some of the Klrg1+ Tregs may depend on CD103 rather than IL-2Rβ, suggesting a contribution of CD103 to Treg homeostasis.

Tregs were purified from the total LP (both SI-LP and LI-LP) from WT, Y3, and Y3/CD103+/− mice and the RNA was used for genome-wide expression studies to globally address the contribution of impaired IL-2Rβ signaling and the lack of CD103 function for intestinal inflammation and loss of tolerance within the colon. 204 unique annotated mRNAs were differentially expressed by ≥1.5-fold between these 3 groups (Fig. 3.12B). Very few mRNAs were uniquely up (4) or down (8) regulated in relationship to Y3 and Y3/CD103+/− gut Tregs (Fig. 3.12B, regions 1 & 2). In contrast, 175 mRNAs (88%) showed largely similar expression between Y3 and Y3/CD103+/− Tregs but were up- (139 mRNAs) or down- (36 mRNAs) regulated in comparison to WT Tregs. The former group included 28 mRNAs that showed a somewhat closer expression levels between WT and Y3/CD103+/− Tregs, but were not statistically different from Y3 Tregs due to their variability in expression (Fig. 3.12B, regions 3), perhaps due to colitis. These results indicate that the large majority of differentially expressed genes in this comparison are mainly related to impaired IL-2R signaling by the Y3 mutation rather than the absence of CD103.

Gene enrichment analysis was performed for the differential expressed mRNAs and 4 major enrichment groups (EGs), consisting of 75 mRNAs, were identified.
Euclidian clustering of these mRNAs represents a microcosm of the entire list of differentially express mRNAs (Fig. 3.12C). The gene enrichment groups are: EG1, Cytokine-cytokine receptor interaction (p=2.2 x 10^-8) and JAK-STAT signaling pathway (p=3.2 x 10^-6); EG2, regulation of lymphocyte activation (p=9.7 x 10^-9) and proliferation (p=0.020); EG3, regulation of cell death (p=2.4 x 10^-4) and the Caspase pathway in apoptosis (p=0.005), and EG4, transcription (p=0.006). EG5 consists of 8 other genes related to immune function, e.g. Itgae (CD103). Inspection of these mRNA is consistent with cells more prone to cell death due to decreases in receptors that deliver growth and survival signals (Il2ra, Il7r) and increases in pro-apoptotic molecules (Fas, Apaf1, Birc4, Casp4, Lmnb1, Gzmb). Increases in mRNA were also detected for molecules that promote T-helper activity (Il12rb2, Il12rb1, Ifng, Il21r, Tbx21 (T-bet), Stat1, and Stat3) and aspects of innate immune responses, in particular, the IL-1R pathway (Il1r1, Il1r2, Il18r1, Il18rap). All these genes were similarly expressed in Y3 and Y3/CD103^-/- Tregs suggesting that these effects are primarily related to impaired IL-2Rβ signaling. Expression of Foxp3 and most mRNAs linked to Treg function were similarly expressed by all three samples tested (data not shown). One exception was Nrp1, which was reduced, and a trend for lower IL-10, but this was judged not be statically significant. Overall, these findings are consistent with the known role of IL-2 to regulate cell growth and death and suggest that IL-2Rβ signaling may also normally function in the gut microenvironment to maintain Treg identity by inhibiting responsiveness to inflammatory mediators and expression of molecules that favor T helper activity. These alterations in Tregs, however, on their own, did not lead to colitis.
3.7 CD103⁺ DCs maintain tolerogenic functions

CD103 is not only expressed on Tregs, also it is expressed on other cells, such as DCs. CD103⁺ DCs in MLN and SI-LP mark a subpopulation of DCs that are tolerogenic due in part to their production of TGF-β and RA (291). These cytokines are required for pTreg development and expression of molecules, especially CCR9 and α4β7, on T lymphocytes required for homing to the gut mucosa (46-48, 292). To determine whether CCR9 imprinting function was active in Y3/CD103⁻/⁻ mice, T lymphocytes from the MLN were examined for CCR9 expression. CD4⁺ T conventional, Treg, and CD8⁺ T cells from the Y3/CD103⁻/⁻ mice contained normal proportions of CCR9⁺ cells when compared to WT, Y3, and CD103⁻/⁻ mice (Fig. 3.13A). The latter finding also is consistent with a lack of requirement for expression of CD103 by DCs for CCR9 (225).

To more directly assess homing and CCR9 and α4β7 imprinting in the absences of CD103 expression by DCs, we followed the fate and phenotype of OVA-specific class-II restricted TCR transgenic OT-II CD4⁺ T cells after transfer to Y3 and Y3/CD103⁻/⁻ mice. The OT-II T cells were stimulated by antigen through OVA in the drinking water to induced antigen-specific proliferation and migration to the gut mucosa. Ten days post-transfer, OT-II cells were enumerated in the MLN and SI-LP by their co-expression of the Va2 and Vβ5 subgroups. Comparable proportions (1.5-3.0%) of OT-II cells were found in the MLN and SI-LP in both recipients (Fig. 3.13B). Analysis of the phenotype of donor OTII cells in the MLN revealed that they expressed similar levels of CCR9 and α4β7 homing molecules (Fig. 3.13B). Collectively, these data are consistent with a CD103-independent role for DC-dependent gut homing and CCR9 and α4β7 of T lymphocytes.
To determine whether CD103 is required for induction of pTregs, purified CD4+ Foxp3− T cells were transferred into Y3 and Y3/CD103−/− mice. In this setting pTreg development in response to host auto or environmental antigens. At 2 weeks post transfer, pTreg development was assessed for the spleen, MLN, SI-LP and LI-LP (Fig. 3.14) and comparable proportions of pTregs were detected in both recipients. These data indicate that expression of CD103 by host cells, including DCs, is not required for pTreg development. Collectively, these data indicate that several important aspects of mucosal DCs functioned normally in the absence of CD103.

**Fig. 3.13 Deficiency of CD103 does not affect homing molecules expression.** (A) MLN T cells were analyzed for the expression of CCR9 by flow cytometry. Representative FACS plots (left) or averaged values (right) for CCR9 expression by CD4+ T conventional (T convs), Tregs and CD8+ T cells. The number of mice/group is indicated within each bar. (B) CD4+ OTII cells (1 x 10^6) were i.v. transferred into the indicated recipients and one day later they were fed with OVA (20 mg/ml) in the drinking water for 9 days. Day 10 post-transfer, the percent of OTII cells (based on the expression of TCR Vα2 Vβ5) among total CD4+ T cells in indicated tissues were determined (left). Representative (middle) and averaged (n = 3 mice/group) (right) expression of α4β7 and CCR9 on donor OTII cells in MLN was assessed. Similar results were obtained in a second experiment (not shown) for recipients fed with OVA (10 mg/ml) for 14 days.
3.8 Survival of CD103+ Tregs depends on another CD103+ cell

The transfer of WT Tregs into Y3/CD103−/− mice prevented colitis (Fig. 3.7-3.9). When we evaluated the phenotype of the donor Tregs from “cured” Y3/CD103−/− recipients 7 to 8 month post-transfer, we unexpectedly found that WT donor Tregs in the LI-LP (Fig. 3.15A), spleen, MLN and SI-LP (data not shown) did not express CD103 and contained a higher fraction of highly activated Klrg1+ Tregs than recipient-derived Tregs. To examine whether these differences were due to the absence of CD103 by recipients cells, WT Tregs were adoptively transferred into adult Y3 and Y3/CD103−/− recipients. At 4 weeks post transfer, donor WT Tregs were readily detected in Y3 and Y3/CD103−/− mice (Fig. 3.15B). The engrafted WT Tregs highly expressed Klrg1 and CD103 in Y3 recipients (Fig. 3.15B). In contrast, very few donor Tregs in Y3/CD103−/− recipients expressed CD103 and higher Klrg1+ Tregs were detected in the SI-LP and LI-LP (Fig. 3.15B). The
very few CD103^+ Tregs in Y3/CD103^-/^- mice raised the possibility that the developing CD103^+ Tregs either differentiated to CD103^- Tregs, or de-differentiated to CD4^+ T conventional cells, or died.

To further investigate these phenomena, CD103^+ and CD103^- Treg subsets were purified from the spleen and MLN of WT mice and were transferred into Y3 and Y3/CD103^-/^- recipients (Fig. 3.16A). Donor Tregs were detected in the spleen, MLN, SI-LP, and LI-LP of the Y3 recipients and expressed CD103 irrespective their original CD103 expression (Fig. 3.16A). In marked contrast, WT CD103^- Tregs developed in
Y3/CD103− and adopted a CD103− phenotype whereas WT CD103+ Tregs did not survive and were not detected in Y3/CD103− recipients (Fig. 3.16A), which suggest that the CD103 deficiency environment fails to support the development of CD103+ Tregs and favors the selection of CD103− Tregs.

**Fig. 3.16 Survival of CD103+ Tregs depends on another CD103+ cell.** FACS-purified CD4+ RFP+ Thy1.1+ T cells from Foxp3/RFP mice (5-6 x 10^6) were i.p. transferred into Y3 and Y3/CD103− recipient mice. Shown are representative FACS plots for Klrg1 and CD103 expression by donor Tregs from the spleen of the indicated recipients and the average data for engraftment by WT CD103+ and WT CD103− donor Tregs from the indicated tissue from Y3/CD103− recipient mice. Data are pooled from 3 independent experiments (n = 5 mice/group). (D) MACS-enriched CD4+ T cells from Foxp3/RFP mice (5-6 x 10^6) were injected i.p. into 10-15 day old Y3/CD103− mice. 6-8 weeks post first transfer, donor Tregs from the spleen and MLN were FACS-sorted from Y3/CD103− recipient mice according to RFP expression and injected (1.5 x 10^5) i.v. into adult Y3 recipient mice. Donor cells in spleen were analyzed for the expression of CD103 and Klrg1 2 weeks after the second transfer. Representative FACS plots show expression of CD103 and Klrg1 by donor derived Tregs (based on RFP expression) after the first transfer into Y3/CD103− mice (left) and then after the second transfer into Y3 mice. Data are from 1 experiment and are representative of 3 Y3 recipients.
WT Tregs engrafted in Y3/CD103^-/- recipients and lacked CD103 expression. If these Tregs were isolated and transferred into a secondary Y3 recipient, many of the donor Tregs now expressed CD103 (Fig. 3.16B). Thus, the absence of CD103 by these Tregs in the primary Y3/CD103^-/- recipient is not the result of an intrinsic defect in expression of CD103. Collectively, these results indicate that CD103 expression by a host cell is required for the survival of CD103+ Tregs and for the development of CD103+ Tregs from CD103- Tregs. The detection of a substantial fraction of Klrk1+ Tregs after transfer of WT CD103- Tregs into a host that lacks expression of CD103 suggests that there is not a block in Treg activation that prevents the development of terminally differentiated Klrk1+ Tregs. However, these results clearly demonstrate a CD103-dependent shaping of Treg pool, particularly in the gut mucosa, where normally the majority of Tregs are CD103+.

3.9 CD103+ DCs may be dispensable for the survival of CD103+ Tregs

Besides cytokine such as IL-2, Treg maintenance in the periphery also depends on DCs, and co-stimulatory signals (22, 23, 25). This notion suggests that CD103+ DCs most likely are involved in influencing Treg homeostasis in the gut mucosa. In addition, CD103+ DCs are capable of producing active form of TGF-β, the cytokine has been shown to upregulate CD103 expression (277-279). Thus, to reveal the role of CD103+ DCs in regulating CD103+ Treg development, we generated mixed BM chimeras that transient lacked substantial number of CD103+ DCs through diphtheria toxin-mediated deletion (Fig. 3.17A). Lethally irradiated CD103^-/- mice received T cell-depleted BM from CD103^-/- origin and CD11C^DTR origin mixed at 1:1 ratio (referred as CD103^-/-...
As well, control group received T cell-depleted BM from WT origin and CD11C<sup>DTR</sup> origin mixed at 1:1 ratio (referred as WT:CD11C<sup>DTR</sup> chimeras). This experimental design allows us to follow the fate of Tregs from CD11C<sup>DTR</sup> origin when only CD103<sup>-/-</sup> or WT DCs are present after rapid depletion of DCs from CD11C<sup>DTR</sup> origin by DT administration.

![Diagram of experimental setup](image)

**Fig. 3.17 Generation of mixed BM chimeras of CD103<sup>-/-</sup>:CD11C<sup>DTR</sup>.** (A) Experimental strategy of mixed BM chimeras and DT treatment. (B) Reconstitution of indicated lymphocyte compartments gated on total lymphocytes (except for CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs gated on CD4<sup>+</sup> T cells) (left) and proportion of CD45.1<sup>+</sup> CD11C<sup>DTR</sup> derived (right) were determined for the peripheral blood after 6-8 weeks of BM reconstitution. (C) Representative (left) and averaged CD103 expression (right) on indicated T cells were analyzed for peripheral blood. Data are pooled from 2 independent experiments (n = 8-9 mice/group).
After 6-8 weeks of BM reconstitution, T cells, DCs, as well as B cells were readily developed as examined from peripheral blood (Fig. 3.17B), with over 50% of each cell compartment was derived from CD45.1+ CD11CDTR BM (Fig. 3.17B). We also assessed the CD103 expression on T cells from peripheral blood. Tregs, as well as CD4+ T conventional and CD8+ T cells from CD11CDTR origin, readily expressed CD103 in these chimeras (Fig. 3.17C), implying that hematopoietic-derived CD103+ cells, rather than non-hematopoietic cells, are likely the candidates required for the development of CD103+ Tregs.

In young Y3/CD103−/− mice (day 10-15 old), adoptive transferred WT Tregs undergo longer period of time of proliferation (4-10 weeks) to lose CD103 (data not shown), suggesting that in a relative lymphopenic condition, frequent auto-antigen-driven TCR stimulation likely to some extent, counteract the effect resulting from the lack of CD103. More importantly, the turnover of WT CD103+ Tregs (Fig. 2.6C) has showed that it takes \( \geq 2 \) weeks for these Tregs to undergo a lot of proliferation and apoptosis to replace 90% and 60% of original Treg pool in the spleen and SI-LP, respectively. Thus, we did not expect that Tregs from CD11CDTR origin would immediately exhibit CD103− phenotype after DC depletion by administration of DT. Chimeric mice were injected with DT every other day for 3 weeks, which substantially depleted the CD11C+ DCs in the SI-LP and LI-LP, but with poor depletion in the spleen and MLN (Fig. 3.18 and data not shown). The DT treatment still caused a large decrease of CD103+ DCs in the SI-LP (Fig. 3.18). However, the proportion of CD103+ Tregs, as well as CD103+ T conventional cells was comparable between two groups of treated chimeras (Fig. 3.19). Overall, these data indicate that in a condition where DCs were mostly derived from CD103−/− origin,
CD103+ Tregs were still enormously present in the tissues we examined. These results suggest that the development of CD103+ Tregs may be not stringently dependent on CD103+ DCs.

![Image of DC depletion after DT treatment.](image)

**Fig. 3.18 DC depletion after DT treatment.** Representative (top) and averaged (bottom) proportion of CD45.1+ and CD45.2+ derived DCs and proportion of CD103+ DCs in the spleen and SI-LP after 3 weeks treatment of DT. Data are pooled from 2 independent experiments (n = 8-9 mice/group).

Unexpectedly, T cells, particularly the Tregs in CD103-/-:CD11C-DTR chimeras, were largely skewed to CD11C-DTR origin, compared to the T cell compartment in WT:CD11C-DTR chimeras (Fig. 3.20). The underrepresentation of Tregs from CD103-/- origin was observed not only in the gut mucosa, but also in the thymus and peripheral lymphoid organs. Moreover, the ratio of CD45.1+ Tregs to total Tregs in CD103-/-:CD11C-DTR chimeras remained unchanged when compared between the thymus, spleen,
MLN, SI-LP and LI-LP, suggesting that lacking CD103 does not further dampen Treg homing/retention in the intestinal mucosa and further support the notion that CD103 is not required for Treg retention in gut mucosa (Fig. 3.10). However, increased ratio of CD45.1\(^+\) CD8\(^+\) T to total CD8\(^+\) T cells was noted in the SI-LP and LI-LP compared to the thymus and peripheral lymphoid organs, indicating that CD103 is involved in CD8\(^+\) T cell retention in the gut mucosa. More importantly, these results also suggest that CD103 might function at the early stage of thymic T cell and Treg development.

**Fig. 3.19 CD103 expression on T cells after targeted DC depletion.** Representative (top) and averaged (bottom) data of CD103 expression on T cells were analyzed for the spleen and SI-LP after 3 weeks of DT treatment on indicated chimeras. Data are pooled from 2 independent experiments (n = 8-9 mice/group).
Fig. 3.20 CD103<sup>−/−</sup> T cells are underrepresented in CD103<sup>−/−</sup>:CD11C<sup>DTR</sup> chimeras. The proportions of CD45.1<sup>+</sup>CD11C<sup>DTR</sup> BM derived lymphocyte compartments were assessed for various tissues as indicated after DT treatment. Data are pooled from 2 independent experiments (n = 8-9 mice/group).
CHAPTER 4
DISCUSSION

4.1 Increased suppression of CD103\(^+\) Treg subset in the transfer model

Nearly a decade ago, several studies demonstrated that CD103\(^+\) Tregs suppress tissue-specific autoimmunity better than CD103\(^-\) Tregs (181-183). This notion is further supported by the gene expression profile of CD103\(^+\) Tregs derived from several settings (111).

It should be noted that, after adoptive transfer into IL-2R\(\beta\)\(^{-/-}\) mice or into lymphopenic recipients, donor CD103\(^-\) Tregs readily adopt an activated phenotype to express CD103 while transferred CD103\(^+\) counterparts are not terminally differentiated and they are still capable of developing into CD103\(^-\) cells. Therefore, such phenotype is not fixed and donor Tregs will eventually contain a large fraction of CD103\(^+\) progeny irrespective their initial CD103 expression. Thus, the adoptive transfer model may not clearly reveal the functional differences between CD103\(^+\) and CD103\(^-\) Treg subsets. Nevertheless, it takes at least several days for the naïve Tregs (CD103\(^-\) Klrg1\(^-\) CD62L\(^{hi}\)) to differentiate into CD103\(^+\) Tregs in the periphery (184). This time window is very important for donor Tregs to prevent the early autoimmunity in neonatal IL-2R\(\beta\)\(^{-/-}\) mice. Thus, a mild difference between Treg subset-mediated suppression in the small intestine was still observed, although the donor Tregs displayed the same level of CD103 expression (data not shown) when we analyzed these mice (16-21 weeks). Such increased tissue specific suppression mediated by CD103\(^+\) Tregs represents their initial highly expressed tissue homing and Treg suppressive molecules, rather than CD103 molecule associated activity.
4.2 Impaired IL-2R signaling have localized effect on Treg homeostasis

The gene expression profile of Tregs with low IL-2R singling identified that CD103 is an IL-2-dependent gene (35). Consistent with this finding, the proportion of CD103⁺ Tregs in the spleen and peripheral lymphoid tissues are decreased. However, this does not hold true in the intestine, rather, the normal fraction or even higher proportion of CD103⁺ Tregs were detected in SI-LP and LI-LP in Y3 mice. The former could be due to decreased IL-2-dependent survival whereas the latter could be explain by the altered the Treg proliferation in an attempt to balance the increased cell death resulted from impaired IL-2R signaling. These observations revealed a localized regulation on Tregs by IL-2R signaling. The increased turnover of Tregs with impaired IL-2R signaling in the SI-LP, rather than in the spleen, suggest that the suboptimal IL-2R signaling barely maintain the Treg homeostasis in the tissue site.

Recent study from Campbell’s group proposed that homeostasis of tissue resident Tregs are less IL-2 dependent based on the low STAT5 activation (38). Our findings argue against this notion. First, the most apparent evidence from our study that indicates deregulated Treg homeostasis in the tissue site resulting from impaired IL-2R signaling is the decrease of Klrg1⁺ effector Tregs in the SI-LP. This decrease is proportional to the levels of impaired IL-2R signaling, indicating the maintenance of this Treg subset at tissue site is stringently dependent on IL-2 (184). Second, the altered proliferation rate and turnover of Tregs in the SI-LP, but not in the spleen, was observed from Y3 mice, which further pointing to the essential role of IL-2/IL-2R signaling in Treg homeostasis in tissue sites. Furthermore, gene expression profile of LP Tregs from mice bearing Y3 IL-2Rβ mutation revealed that many genes differentially expressed were clustered in
genes regulating lymphocytes activation, proliferation, cell death and apoptosis, indicating that IL-2R signaling is critical to regulate mucosal Treg gene expression program. Lastly, such dysregulation by suboptimal IL-2R signaling in tissue site renders mice prone to autoimmunity (discuss below). Collectively, all these findings clearly demonstrated a non-redundant role of IL-2R signaling in regulating Treg homeostasis in the gut mucosa.

4.3 IL-2R signaling and CD103 cooperate to regulate Treg homeostasis

Tregs and DCs marked by expression of CD103 have been associated with maintaining tolerance in the gut mucosa (47, 182), but the direct functional contribution of CD103 in tolerance remains poorly understood. In chapter 3, our study shows that there is a dynamic and important role for CD103 to functionally participate on Tregs and a non-Treg cell for tolerance in the large intestine, primarily through a mechanism that regulates Treg homeostasis (Table 4.1). This activity of CD103 only became apparent in the context of Tregs that expressed impaired IL-2R signaling. The Y3 IL-2Rβ mutation in Tregs on its own does not result in colitis. These effects on Tregs can be viewed as a risk for colitis, perhaps in an analogous manner by which SNPs in IL-2 or IL-2R subunits are risk for other autoimmune disease, including Crohn’s disease (285-287). CD103 has not been implicated as a genetic risk for colitis. Nevertheless, the Y3/CD103-/- mice represent a new model of colitis and illustrates how an additional genetic lesion, which on its own is not associated with disease, cooperates in a highly penetrant manner with other risk factors to cause autoimmunity.
Table 4.1 Dysregulation of intestinal homeostasis in Y3 and Y3/CD103−/− mice

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<th>Defect</th>
<th>Small intestine</th>
<th>Large intestine</th>
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<tr>
<td>Y3</td>
<td>Impaired IL-2Rβ signaling</td>
<td>Normal Treg/Tconv ratio, Rapid Treg turnover, Klr1+ Tregs↓, Ki67+ Tregs↑, CD4+ T cells ↑, Th17↑</td>
<td>Treg/Tconv ratio↓, Normal Klr1+ Tregs, Normal Ki67+ Tregs, CD4+ T cells slightly↑</td>
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<td></td>
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<td>Altered intestinal Treg gene expression profile</td>
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<td>Y3/CD103−/−</td>
<td>CD103 deficiency</td>
<td>Altered CD4+ T cell localization</td>
<td>Klr1+ Tregs↓, Ki67+ Tregs↑, CD4+↑, Inability to support the survival of CD103+ Tregs</td>
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<tr>
<td>Others</td>
<td></td>
<td>Tr1 cells (tolerogenic)</td>
<td>Microflora (trigging factors)</td>
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The importance for CD103 in mucosal tolerance at the level of Tregs is apparent based on the finding that WT Tregs readily suppressed immune activation and colitis associated with Y3/CD103−/− mice. If inflammation and autoreactivity in Y3/CD103−/− mice was cell intrinsic or otherwise independent of a defect in Tregs, the high effective reversal of disease was not expected to occur. In contrast, when one considers the inflammatory response of the SI-LP in Y3/CD103−/− mice, which is closely paralleled in Y3 mice, donor WT Tregs did not reverse the increase in total CD4+ T cell numbers and IL-17 producing cells. These aspects of altered immunity likely represent intrinsic effects at the level of T conventional cells due to impaired IL-2R signaling. This conclusion is in line with the known role of IL-2 to repress Th17 development (101, 102), which also did not function normally by T cells that expressed the Y3 IL-2Rβ mutation.

Genome-wide mRNA expression profiling of Tregs from the intestine provides several important clues concerning how impaired IL-2R signaling and the absence of
CD103 contribute to the development of colitis. First, very few mRNAs were found differentially expressed between Y3 and Y3/CD103−/− mice and these were not included in the IL-2-dependent gene enrichment groups including those related to T cells activation and proliferation and regulation of cell death. This finding suggests that CD103 does not directly signal to promote the homeostasis of CD103+ Tregs. Second, mRNAs differed between WT and Y3 Tregs and were similarly over-expressed in Y3 and Y3/CD103−/− Tregs, including mRNAs important for mediating cell death, e.g. Casp4, Apaf1, and Birc2. This finding indicates that the one important effect of the Y3 mutation is to render Tregs to be more prone to cell death.

The current study indicates that homeostasis of Y3 and Y3/CD103−/− Tregs from the SI-LP is altered (184). Although Treg numbers and proportion in the SI-LP are relatively normal, these Tregs showed increased level of proliferation, so the increased proliferative rate must be offset by increased cell death to maintain a normal homeostatic level of cells. The SI-LP of Y3 and Y3/CD103−/− contain a near 10-fold reduction in Klrg1+ Tregs, consistent with cells that have died before the 8-10 divisions required for becoming terminally differentiated short-lived Klrg1+ Tregs. Some aspects of altered Treg homeostasis related to the Y3 mutation was also noted for cells in the LI-LP, especially a lower ratio of Tregs to CD4+ T cells. This IL-2R signaling defect on its own is not sufficient to cause severe pathology or disease. However, two unique features related to the lack of CD103 expression by Y3/CD103−/− Tregs in the LI-LP is heightened proliferative activity coupled with lower levels of Klrg1+ Tregs. This represents a scenario of CD103-dependent aberrant Treg homeostasis accompanied by increased proliferation and cell death over that associated with Y3 Tregs. This altered homeostasis
likely results in a failure in Tregs to effectively suppress autoreactive T cells and represents at least one aspect by which the lack of CD103 leads to colitis.

4.4 The breakdown of intestinal tolerance in Y3/CD103⁻/⁻ mice

Y3 mice showed a clear tendency for immune inflammation in the SI and LI, yet only Y3/CD103⁻/⁻ mice developed severe pathology, and only in the LI. Why was the SI spared from a more severe autoimmune attack? First, even though Foxp3⁺ Tregs in the SI-LP have impaired gene expression due to the Y3 IL-2Rβ mutation and altered localization due to the absence of CD103, Tr1 cells, which are abundant in the SI, and other regulatory mechanisms may help to counteract autoreactive T cells (293-295). In contrast, tolerance in the colon is more dependent on Tregs because these cells represent the major cell type that produces IL-10. Second, the small intestine contains much less microflora compared to large intestine (296). The substantially larger bacterial load of the microbiota in the large intestine represents a potential trigger to drive colitis. Thus, tolerance in the large intestine depends more stringently on relatively normal Treg activity. Lastly, a unique mechanism operates within the small intestine to control Th17 cells where SI-LP IL-17⁺ cells are eliminated via lumen leading to a less colitogenic phenotype (248).

4.5 A niche for CD103⁺ Treg survival and intestinal Treg homeostasis

CD103 has been implicated in other functions that promote immune tolerance, including homing/retention of Tregs within tissue sites, CD103⁺ DCs-mediated imprinting of CCR9 and α4β7 on T cells, and promoting the development of pTregs (47, 226, 283). However,
these activities were generally comparable in Y3 and Y3/CD103⁻/- mice. These finding indicate that CD103 largely serves as a marker on DCs, rather than a functional participant in these tolerogenic activities. Thus, colitis associated with Y3/CD103⁻/- mice is not accounted for by these activities associated with CD103, further pointing to the importance of CD103-dependent homeostasis of CD103⁺ Tregs as an important contributing factor for tolerance in the colon.

If CD103 does not directly deliver a survival signal, as suggested by gene profiling data, how does it contribute to Treg survival? The most likely possibility is that CD103 expression is required for the proper localization of Tregs in a niche with other lymphoid cells, and perhaps epithelial cells, and these latter cells provide a survival signal through secreted molecules or cell-cell interactions. One candidate cytokine is TGF-β, which is highly produced by epithelial cells and CD103⁺ DCs, and might directly mediate Tregs survival or indirectly influence Tregs survival by its ability to upregulate CD103 on lymphocytes (40, 277-279, 297).

Another interesting finding from current study is the inability of CD103⁺, but not CD103⁻, Tregs to survive and persist in Y3/CD103⁻/- mice. The lack of CD103 in these mice represents one defining feature for CD103⁺ Treg survival, because both CD103⁻ and CD103⁺ Tregs readily survive and persist in Y3 mice. These findings revealed that CD103 expression on a non-Treg cell is involved in regulating tolerance through supporting the development of CD103⁺ Tregs. The requirement for CD103 to promote Treg survival represents a new function for CD103 in the context of immune tolerance.

Past studies using the T cell transfer model of colitis showed WT Tregs failed to prevent colitis in RAG⁻/- CD103⁻/- recipients and proposed that CD103 expression on a
non-Treg cell type, likely DCs, is indispensable to mediate tolerance (263). This finding now might be interpreted as a failure in CD103$^+$ donor Tregs to persist and suppress colitis in these recipients.

The role of CD103 in supporting Tregs survival is important only for the CD103$^+$ Treg subset. When CD103$^+$ Tregs were transferred into Y3/CD103$^{-/-}$ recipient mice, very few Tregs were detected 2-4 weeks post-transfer. Thus, once a Treg acquires CD103 expression, its survival then depends upon another CD103$^+$ cell. However, there is also a separate CD103 independent pathway for Treg survival and persistence, as unfractionated or CD103$^-$ WT Tregs persist in Y3/CD103$^{-/-}$ mice.

T cell trafficking from LP to epithelium is accompanied by upregulating $\alpha_E\beta_7$ and downregulating $\alpha_4\beta_7$ (298), suggesting that the CD103 expression likely depend on the close proximity with epithelium where there is abundant TGF-$\beta$ (277, 278, 299). To gain a survival signal and express CD103, Tregs may require an microenvironment where CD103$^+$ cells act as a bridge to bring Tregs in close proximity to E-cadherin$^+$ epithelium or CD103$^+$ cells which interacting with epithelium directly provide survival signals to Tregs. These findings are consistent with above discussed niche model where proper localization of Tregs and other lymphocytes with gut epithelium is the prerequisite for Treg survival and homeostasis. In addition, the observation that WT CD103$^-$ Tregs that persist in Y3/CD103$^{-/-}$ mice reacquire the ability to express CD103 upon transfer into CD103-sufficient Y3 recipients suggests that CD103$^-$ Tregs may enter this niche and receive signals that upregulate CD103 that facilities their survival.

The nature of this non-Treg CD103$^+$ cell remains to be determined. One obvious candidate is CD103$^+$ DCs as they produce TGF-$\beta$ which provides direct source for
upregulating CD103 expression or Treg survival (22, 40, 297). In addition, Treg proliferation and activity was largely DC-dependent as intestinal Tregs were found closely associated with DCs during cure of colitis (262). However, at this point we have been unsuccessful in providing data to support this notion, including the development of mixed chimeras that transient lacked substantial number of CD103+ DCs through DT-mediated deletion. Several caveats in this experiment might account for the failure in revealing the role of CD103+ DC. First, in order to achieve long-term depletion of DCs, low dose DT treatment was performed on mice, which failed to deplete all the DCs from CD11C\textsuperscript{DTR} origin. This incomplete depletion of DCs might influence the results. Second, chimeric mice developed diarrhea and wasting disease after long-term DT treatment, which may or may not affect Treg cell fate. Although we cannot entirely exclude a role for CD103+ DCs in Tregs survival, this finding raises the possibility that CD103+ conventional T cells, which are prevalent in the gut, might be candidates to provide a survival signal for CD103+ Tregs.

It is worth noting that the absence of CD103+ Tregs in Y3/CD103\textsuperscript{-/-} mice after adoptive transfer may represent another immunological mechanism, i.e. immune responses to eliminate non-self antigens, rather than niche mechanism. Y3/CD103\textsuperscript{-/-} host immune system might initiate responses to CD103 antigen because host lymphocytes have never encountered CD103 antigen in the primary lymphoid organs during clonal deletion and, as a consequence, CD103 reactive T cells and B cells could exist in the periphery. Thus, it remains possible that, after adoptive transfer, CD103 expressing cells are recognized as foreign antigens and will be cleared by the host adaptive immune responses and only CD103\textsuperscript{-} cells could survive and develop. Nevertheless, we still
believe that this mechanism is unlikely true. Although CD103 expressed on cells can be recognized as a foreign antigen by Y3/CD103−/− host immune system, the donor cells transferred are Tregs with known role of suppression which will immediately suppress the ongoing immune responses if there are. At this point, although we cannot completely rule out this possibility, further studies are needed to determine this mechanism.

In summary, at this juncture we favor the following model, which provides a framework for future studies. The IL-2Rβ Y3 mutation lowers IL-2 and IL-15-dependent signaling on Treg and T effector cells. Lower IL-2R signaling is primarily responsible for mild subacute inflammation associated with Y3 mice in part due to altered Treg homeostasis with a tendency for increased Treg apoptosis and lower negative regulation of Th17 development. This IL-2-dependent risk, however, does not generally progress to severe autoimmune disease. Other aspects of low IL-2 or IL-15 signaling might interfere and lower T effector responses, memory programming, and memory homeostasis (300), but these effects should not increase self-reactivity, and might counteract lower Treg activity. The absence of CD103, which on its own is not pathogenic, consistently leads to severe colitis in the context of impaired IL-2R signaling imposed by the Y3 mutations. This form of IBD is a result of an inability of these Tregs to maintain tolerance in the gut mucosa. Altered activity of a gut specific niche promotes colitis through a failure of CD103 to properly localize Tregs to receive growth and/or survival signals. In the absence of CD103, the defects associated with the Y3 IL-2Rβ mutation cannot compensate the failure in producing this suppressive niche, leading to tissue-specific severe self-reactivity. The lack of autoimmunity associated with CD103−/− mice, indicates that redundant mechanisms compensate for the lack of CD103 to prevent IBD. It seems
highly likely that some of this redundancy depends on molecules that are normally expressed in an IL-2Rβ-dependent fashion.
CHAPTER 5
MATERIALS AND METHODS

Mice
C57BL/6 (B6), B6.129S2(C)-Itgaetm1Cmp/J (CD103−/−), CD11c-DTR mice were originally obtained from The Jackson Laboratory. Foxp3/RFP mice were kindly provided by R.A. Flavell (16). Blimp-1/GFP mice were kindly provided by S.L. Nutt (301) and further crossed to Foxp3/RFP mice. IL-2Rβ−/− and Y3 mice were generated in our lab as previously described (35). Y3 mice were crossed to CD103−/− mice to generate Y3/CD103−/− mice. To perform adoptive transfer experiments and microarray assays, CD103−/−, Y3 and Y3/CD103−/− mice were each crossed to Foxp3/RFP reporter mice. OTII transgenic mice were kindly provided by Dr. E.R. Podack. All mice were on C57BL/6 background and maintained in the animal facility at the University of Miami. Animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at the University of Miami.

Cell preparation
LP cells were prepared as previously described with minor modifications (184). Briefly, small intestine was cut 0.5 cm below the stomach and 1 cm above the cecum. Large intestine was cut from the cecum to anus. After removing the fat and connective tissue, the intestine was flushed with Ca²⁺-Mg²⁺ free HBSS containing 5% FBS and PPs were dissected. The intestine was cut longitudinally and sliced into 0.2-0.5 cm pieces, followed by incubating in 20 ml Ca²⁺-Mg²⁺ free HBSS containing 10% FBS, 15mM HEPES, 5mM
EDTA at 37°C on 200 rpm shaker for 20 min to remove IELs. The Intestine fragments were further digested in 20 ml RPMI 1640 containing 5% FBS, collagenase VIII (0.24mg/ml) (Sigma Aldrich) and trypsin inhibitor (0.24mg/ml) (Sigma Aldrich) at 37°C on 200 rpm shaker for 60 min. Samples were passed through 70 µm cell strainer, LP cells were collected, and they were then purified on a 44%/67% Percoll (GE Healthcare) gradient by centrifugation (800g, 20 min at room temperature).

Antibodies and flow cytometry

The following reagents were used for FACS staining or confocal microscopy: CD4 (Gk1.5), CD8 (53-6.7), CD44 (pgp-1), Ly6G (1A8), CD45.1 (A20), were prepared in our laboratory; CD4 (Gk1.5), CD62L (MEL-14), CD69 (H1.2F3), CD103 (2E7), CCR5 (HM-CCR5), CCR4 (2G12), CCR6 (29-2L17), CD19 (6D5), CD45.2 (104), CTLA4 (UC10-4B9), Bcl2 (BCL/10C4), Helios (22F6), IL-17 (TC11-18H10.1), IFN-γ (XMG1.2), PE Streptavidin were obtained from BioLegend; Klrg1 (2F1), CD127 (A7R34), ICOS (15F9), CD39 (24DMS1), CD73 (eBioTY/11.8), Thy1.1 (HIS51), CCR9 (eBioCW-1.2), α4β7 (DATK32), CXCR3 (CXCR3-173), Nrps (3DS304M), MHCI (IA/IE) (M5/114.15.2), Foxp3 (FJK-16s), IL-2 (JES6-5H4), TNF-α (MP6-XT22), Streptavidin PE-Cy7, Streptavidin PerCP-eFluor 710 were obtained from eBioscience; CD25 (PC61), Thy1.2 (53-2.1), TCR Va2 (B20.1), TCR Vβ5 (MR9-4), CD11C (HL3), Ki67 (B56), E-Cadherin (36/E-Cadherin) were obtained from BD Biosciences; Alexa Fluor 568 Streptavidin was obtained from Invitrogen. Intracellular staining of Foxp3, Ki67, Bcl2, CTLA4, and Helios was performed using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer’s instruction. FACS data were acquired on LSR
II and FACSFortesa (BD) and analyzed with BD-FACSDiva software. In general, 200-300 x 10³ total events were collected for spleen and MLN samples and roughly 500 x 10³ total events were collected for LP samples. Cell sorting was performed on FACS Aria II.

**BrdU incorporation assay**

Mice were fed with BrdU (0.8mg/ml) in the drinking water for continuous 5 days. Then, BrdU containing water was replaced by normal water and mice were analyzed at different time points. To assess the incorporation of BrdU, FITC BrdU Flow kit (BD Biosciences) was used according to manufacturer’s instruction. In brief, cells were first stained with surface mAbs for 15 min on ice. After washing, cells were fixed with Cytofix/Cytoperm Buffer for 30 min, incubated with Cytoperm Plus Buffer for 10 min and fixed with Cytoperm Buffer for another 5 min. Cells were then treated with DNase (300µg/ml) for 1h at 37°C. After washing, cells were stained with anti-BrdU and other intracellular Abs for 20 min at room temperature. Cells were analyzed by flow cytometry.

**Ex vivo cytokine analysis**

To measure the intracellular cytokine production, unfractionated cells (2-2.5 x 10⁶) were cultured in 24-well plates in 1 ml RPMI 1640 complete medium in the presence of brefeldin A (GolgiPlug; BD Biosciences), PMA and Ionomycin (Sigma Aldrich) at 37°C incubator for 4h. Cells were harvested and subjected to surface staining with antibodies for 15 min. Cells were then fixed, permeabilized with Foxp3/Transcription Factor Staining Buffer Set and stained with mAbs to IL-2, TNF-α, IL-17, IFN-γ and Foxp3 according to manufacturer’s instruction. Cells were analyzed by flow cytometry.
Adoptive transfer

In general, lymphocytes from spleen, peripheral lymph node and MLN were collected and CD4+ cells were enriched using anti-CD4 magnetic beads (Miltenyi Biotec). Cells were stained for desired surface antibodies and subjected to cell sorting. Total, CD103+, CD103+ CD4+ WT Tregs, CD103-/- Tregs or CD4+ T conventional cells were purified based on the Foxp3/RFP reporter. The purity of sorted cells was always ≥ 98%. In particular, the purity of sorted CD4+ T conventional cells to assess pTreg development in vivo was > 99.9%. In some experiments, total CD4+ T cells were MACS-enriched and transferred. Recipient mice received purified cells by i.v. or i.p. injection. Details of each transfer are provided in the Figure legends.

Histopathology

Tissues/organs were removed and fixed with 10% formalin solution. Fixed tissue blocks were paraffin-embedded, sectioned and stained with hematoxylin and eosin (H&E) for pathological analysis.

Microarray analysis

Tregs from Foxp3/RFP reporter mice were purified by FACS sorting from the SI-LP and LI-LP. Total RNA was prepared and processed to probe Affymetrix Mouse Gene ST2.0 arrays as previously described (184). In brief, 10-20 ng of RNA was used in a single round of linear RNA probe amplification using Ovation Pico WTA System V2 and biotin-labeled using Encore Biotin Module (NuGEN, San Carlos, CA, USA). The resulting data were normalized by the RMA method using software at GeneSifter (Seattle,
WA) and expressed as log₂ signal intensity. Group comparisons of the transformed data of 3 independent biological replicates were performed using ANOVA, applying the Benjamini Hochberg correction for false positives. Data analysis was limited to annotated mRNAs after setting a lower signal intensity limit of 4 units (one group must pass). Genome-wide expression data in the article have been submitted to the Gene Expression Omnibus data base (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE56438.

Confocal microscopy and image analysis

The intestine was cut, flushed with PBS and then snap frozen in OCT. Frozen tissues were cut into 6μm thickness and fixed in ice-cold acetone for 10 min. Prior to staining, sections were blocked with 20% BSA containing 0.3% Triton-X 100 for 30 min. Sections were stained with Biotin-Foxp3 at 4°C overnight, washed with PBS and then stained with Alexa Fluor 568 Streptavidin, Alexa Fluor 647 anti-CD4, and FITC anti-E-Cadherin. Stained sections were mounted with Fluoroshield with DAPI (Sigma Aldrich). Images were acquired on TCS-SP5 II Leica microscope, using 40 X objective lens, NA = 1.25 and processed with ImageJ software.

Prior to analysis, images were individually background subtracted. Background was defined as mean pixel intensity of the area that carried no tissue. Localization analysis was performed as follows: regions of interest (ROIs) were selected using the CD4 channel as guidance. Individual CD4⁺ T cells were enclosed into separate ROIs, leaving an additional ½ cell diameter around the cell. ROIs were then transferred onto the E-cadherin channel images. Mean pixel intensity was calculated for ROIs in the E-
cadherin channel, and the value was normalized against mean pixel intensity of E-cadherin in the E-cadherin channel for the current image. These data were evaluated by the Relative Mean pixel intensity = (M_{ROIs in E-cadherin channel} - M_{background})/(M_{E-cadherin in E-cadherin channel} - M_{background}), as one approach to define the extent of co-localization.

**Bone marrow chimeras and Diphtheria toxin treatment**

Recipient mice received a single dose of total body irradiation (900 rad). The following day, mice were given a mixture of T cell-depleted bone marrow cells (10 x 10^6 cells total) by i.v. injection. Mice were fed with gentamicin (1mg/dl) in drinking water for 4 weeks after receiving BM. Then, mice were maintained with normal water. After 6-8 weeks of BM reconstitution, mice were treated with Diphtheria toxin (16ng/g) (Sigma Aldrich) by i.p. injection every other day for 3 weeks.

**Statistical Analysis**

Data were shown as the mean ± SD in most of the figures. Statistical analysis was performed using one-way ANOVA with Turkey’s multiple comparison test when comparing groups (>2) and unpaired Student’s t test when comparing 2 groups. Some data (Incidence of disease Fig. 3.1A and Disease free Fig. 3.7B) were analyzed using Log-rank test. Statistical significance were considered as * (P<0.05), **(P<0.01), *** (P<0.001) and ****(P<0.0001).
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