DEVELOPMENT AND FUNCTION OF THYMOCYTE-SELECTED CD4 T CELLS

by

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Abstract

CD4 T cells play important roles in adaptive immune responses. Conventional understanding of CD4 T cell development is that the MHC class II molecules on cortical thymic epithelial cell are necessary for selection, as shown in mouse models. However, increasing evidence suggests that CD4 T cell selection mediated by hematopoietic cells such as thymocytes also occurs in humans as well as in mouse models. Thymocyte-selected CD4 T cells (T-CD4 T cells) are shown to be different from epithelial cell-selected CD4 T cells (E-CD4) in many aspects including developmental requirements and functional characteristics.

In this study, the strength of TCR signaling necessary for T-CD4 T cell development and the role of T-CD4 T cells during bacteria infection was investigated. In contrast to E-CD4 T cells, T-CD4 T cells were selected more efficiently when TCR signaling was weakened. In addition, T-CD4 T cell development relied on the presence of the promyelocytic leukemia zinc finger protein, a transcription factor essential for invariant NKT cell generation. The distinct developmental process mediated by thymocytes resulted in T-CD4 T cells possessing a suppressive function. Instead of promoting host immunity as E-CD4 T cells do, T-CD4 T cells suppressed anti-*Listerial* responses, evidenced by the reduced frequency and cytotoxicity of *Listeria*-specific CD8 T cells during both the primary and the memory immune response. Studies in this dissertation revealed the novel suppressive function of T-CD4 T cells, which is most likely caused by the differential signaling delivered during thymocyte-thymocyte interactions in the thymus.

Chapter I

Introduction

1.1 Mammalian immune system

1.1.1 Innate immunity

Living organisms are continuously exposed to a great complexity of nonpathogenic and pathogenic microorganisms. Mammalians have evolved an immune system composed of innate and adaptive compartments as a defense against these invaders (1, 2). The innate immune system is mobilized within hours after infection and is responsible for destroying the vast majority of microorganisms (3). A couple of days later, the adaptive immunity system is recruited to fight against prolonged infection and establishes enhanced protection in case of repeated exposures to the same pathogen.

Both the inflammatory environment and cell recruitment in innate immunity are initiated by cytokines and other molecules produced by infected and activated cells (4-7). Inflammation causes locally increased vascular diameter and permeability, facilitating cell attachment and extravasation (8, 9). Professional phagocytes, including neutrophils, macrophages and monocytes that can differentiate into macrophages, are important for the clearance of intracellular pathogens (10, 11). The engulfed pathogens are destroyed intracellularly by a number of oxygen-containing compounds generated through oxygen-dependent pathways (12, 13) or by pre-formed substances released from granules and

lysosomes once infused with phagosomes (14, 15). Another important innate cell population, the nature killer (NK) cells, constantly survey the periphery and receive regulatory signals through activating and inhibitory receptors to control their activity (16). They are activated when the infected cells down-regulate NK cell inhibitory receptors such as MHC class I and Ly49, which results in the derepression of NK cell activating signals through receptors such as NKG2D. NK cell activation leads to the production of large amounts of IFN-γ as well as perforin/granzyme-mediated cytolysis (17). Extracellular pathogens can be destroyed by mast cells and polymorphonuclear leukocytes (PMNs) such as neutrophils, eosinophils and basophils (18, 19). These cells can be activated by physical destruction or IgE ligation, and by the process of degranulation, release preformed inflammatory mediators stored in secretory granules (20). The secreted mediators include biogenic amines such as histamine and proteoglycans, toxic oxygen-derived and nitrogen-derived products, as well as a spectrum of neutral proteases, lysozymes, and acid hydrolases. Most of these components function to destroy the microorganisms directly, and some are also toxic to host tissues.

There are other humoral elements with crucial roles in the innate immune response system, foremost of which is the complement cascade (21). A major function of the complement cascade is to form a membrane-attack complex on target cells, which perforates cell membranes to cause cell death. In addition, complement cascade fragments C3a, C4a, and C5a cause mast cells to release inflammatory mediators such as histamine to increase vascular permeability, and C5a also acts as a powerful neutrophil chemoattractant (22). Cytokines play diverse roles in the process of pathogen clearance

as well. For example, type I interferons stimulate the synthesis of antiviral proteins to limit viral replication (23-25), the nuclear myxovirus-resistance proteins sequester viral ribonucleoproteins in specific subcellular compartments, the 2',5'-oligoadenylate synthetase as well as RNase L breaks down RNAs, and the RNA-dependent protein kinases inhibit translational processes (26). IL-1 and IL-6 induce the production of acute phase proteins from the liver (27) to enhance the resistance to infection and to promote the repair of damaged tissue (28, 29). TNF-α and IFN-γ synergistically enhance the antimicrobial action of the macrophages, particularly by inducing the production of nitric oxide and superoxide ions (30).

Although innate immunity is prompt and essential for the initial control of infection, it is pathogen-nonspecific. It identifies and distinguishes self from foreign pathogens by recognizing pathogen-associated molecular patterns (PAMPs) that are shared by groups of related microbes but are rarely associated with pathogen infected host cells (31, 32). In addition, innate immunity does not improve with repeated exposure to a given infection, and therefore lacks the potential for immunogenic memory.

1.1.2 Adaptive immunity

As the innate immune response goes on, adaptive immunity is developed in secondary lymphoid organs. Cells from the infected site carry pathogens to draining lymph nodes through the high endothelial venules (HEV) and afferent lymphatic vessels (33, 34), and to the spleen through the blood vessels in the red pulp.

T cells are activated in T cell zones by recognizing pathogen-derived peptides presented by MHC Class I or II complexes on antigen presenting cells (APC) (35, 36). T cell receptors (TCRs) are clonally specific (37, 38) in that they differ from one another in gene sequences and tertiary structures to distinguish slightly different epitopes. As a result, despite the presence of millions of T cells, only a few recognize and respond to a specific pathogen. With the assistance of co-stimulatory factors, T cells recognizing antigen undergo dramatic clonal expansion and acquire effector functions in a couple of days. Activated CD8 T cells up-regulate chemokine receptors such as CCR5 causing them to migrate to the infected site (39), where they produce large amounts of IFN-γ to promote cellular immunity as well as directly kill infected cells by inducing Fas-mediated apoptosis and perforin/granzyme-mediated cytolysis (40). Additionally, antigen activated CD4 T cells down-regulate CCR7 and migrate toward the edge of the T cell zone to interact with B cells that are migrating from the opposite direction (40). Meanwhile, CD4 T cells further differentiate into T helper cells with different migration potentials: T helper 1 (Th1) cells produce IFN-γ and up-regulate CCR5 and CXCR3 to migrate in response to chemokines such as IP-10, MIP-1α and MIP-β that are induced by pro-inflammatory cytokines (41-43). T helper 2 (Th2) cells produce IL-4, IL-5 and IL-13 cytokines, preferentially express CCR3 and CCR4 and migrate in response to chemokine gradients of eotaxin and MCPs induced by type 2 cytokines (44, 45). IL-17-producing CD4 T cells express CCR6 and therefore preferentially migrate to tissues that express its ligand CCL20 (46). Follicular helper cells (Th_{FH}), produce IL-21, up-regulate CXCR5 and migrate to the B cell zone in lymphoid tissues to help with B cell functional differentiation (47, 48).

B cells reside in the B cell zone at steady state. Like TCRs, B cell receptors (BCRs) are generated with clonal specificity by gene rearrangement during B cell development. But unlike T cells that require antigens to be presented by MHC molecules for recognition to occur, B cells recognize and engulf free antigens. Antigen binding to BCRs induces the down-regulation of CXCR5 and the up-regulation of CCR7 to instruct B cells to migrate to the edge of the B cell follicles (49), where they receive help from the T cells migrating from the T cell zone. The interaction of B and T cells is antigen-sepcific, in that B cells process the antigen recognized by the BCR and present the resulting peptides to T cells in concert with MHC class II molecules. The interaction with activated CD4 T cells results in B cell activation through the CD40-CD40L signaling pathway (50). Then with several rounds of proliferation, B cells differentiate into antibody-secreting plasma cells and undergo antibody class switching that is regulated by various cytokines. In the mouse, IFN-γ preferentially induces class switching to IgG3 and IgG2a, IL-4 to IgG1 and IgE, and IL-5 to IgA (50, 51).

To fight against invasion effectively, the host must mount an appropriate immune response, which depends on the nature of the infection (52, 53): cellular immunity mainly deals with intracellular pathogens including various bacteria and viruses; antibody-mediated humoral immunity is effective to cope with extracellular pathogens such as parasites. The immune response to antigens varies depending upon the differentiation direction taken by CD4 T cells. Strong TCR stimulation with pro-inflammatory cytokines such as IL-12 promote the differentiation of Th1 cells to produce IFN-γ, a cytokine

critical for the promotion of CD8 T cell function and the full activation of macrophages (54) against intracellular pathogens (55). Low antigen levels and early IL-4 production by innate immune cells drive Th2 differentiation. Th2 cytokines facilitate B cells to produce antibodies and promote the killing of bacteria and parasites by eosinophils, basophils and mast cells. More recently, IL-17-producing CD4 T cells have been identified that are distinct from Th1 and Th2 cells and have been named Th17 cells. Their differentiation requires regulatory cytokine TGF-β and pro-inflammatory IL-6 (56, 57). The roles of Th17 cells have been largely associated with various inflammatory autoimmune diseases (58), although it is also suggested that the massive inflammation induced by Th17 cells is necessary for the clearance of specific types of pathogens that are not adequately dealt with by Th1 or Th2 immunity (59). It should be noted that both cellular and humoral immunities are involved in most immune responses although on a case by case basis, one type of response is often dominant and more responsible for pathogen clearance than the other.

1.1.3 Immune memory

When the immune response to pathogen is appropriate and successful, the immune response abates after pathogen clearance. Most of the active antigen-specific immune cells are susceptible to apoptosis and rapidly die with the absence of antigenic stimuli and survival cytokines (60). This contraction process is very important for T and B cell homeostasis. Some of these cells, however, survive this phase and persist in the periphery as memory immune cells, which are responsible for a faster and more potent recall response upon a second and later encounter with the same pathogen (61-65).

Humoral memory is provided by long-lived plasma cells and is marked by a persistent antigen-specific antibody level in the periphery (66-68). Long-lived memory B cells reside in specific niches in the bone marrow or secondary lymphoid organs, where they are maintained by TNF, IL-5 and IL-6 cytokines, as well as by certain co-stimulatory molecules such as the CD44 ligand hyaluronic acid (69-71). Memory T cells reside in the periphery and are maintained by IL-7 and IL-15 (72-75) as well as through MHC-dependent homeostatic proliferation (76).

Memory T cells can be categorized into two major types: the central memory T cells (T_{CM}) and effector memory T cells (T_{TM}). These two memory cell types differ in their location as well as function (77). T_{CM} express high levels of CD62L and CCR7, the two receptors required for the extravasation of cells through HEV and for their migration to T cell areas of secondary lymphoid organs (78, 79). Although naïve T cells also express CD62L and CCR7, T_{CM} are equipped with higher sensitivity to TCR stimulation and their activation is less dependent on co-stimulation. The activated T_{CM} quickly become effector cells, down-regulating CCR7 and CD62L and then migrating to the infected site where they produce large amounts of effector cytokines (80). $T_{EM,}$ in comparison, display low levels of CCR7 and CD62L but express chemokine receptors and adhesion molecules that are responsible for the homing to inflamed tissues. Therefore, T_{EM} either circulate in the periphery or populate the site of former infection, and are ready to exert rapid effector function within hours upon re-infection (80).

The quality of T cell memory is determined by multiple factors during primary responses. First, TCR signaling quality is critical for T cell expansion and survival, which in turn affects the frequency of memory precursors (81-84). In addition, co-stimulatory signals through B7 and TNF family receptors also contribute to increasing the size of the memory T cell populations (85-89). Second, inflammatory cytokines such as IL-12 and IFN-γ play key roles in determining whether the generated effector T cells are with or without T cell memory potential (72, 90-92). For a given dose of antigen, a low inflammatory environment promotes memory T cell development, while increased inflammatory signals decrease the ratio of memory precursors to terminally differentiated effector T cells (93, 94). Third, studies with various mouse models have shown the indispensable role of CD4 T cells for the immune memory of cytotoxic T lymphocytes (CTLs) (55, 95-97). During the primary response, T helper cells assist in dendritic cell (DC) maturation through the CD40L-CD40 signaling pathway (98) and enhance the chemotactic response of naïve CD8 T cells to mature DCs (99). It has also been reported that CD4 T cells are needed for the long-term maintenance of memory CTLs (100). In addition, the early production of IL-2 by memory CD4 T cells upon re-infection facilitates the effective expansion and robust recall capacity of the memory CTLs (92).

1.2 αβ T cell development

1.2.1 The generation of αβ T cells in the thymus

T cells are generated from bone marrow (BM)-derived lymphoid progenitors. They enter the thymus at the corticomedullary junction and migrate outward to the cortex while undergoing a series of genetic programming. The early development of CD4/CD8 double negative (DN) cells can be tracked by the expression of CD25 and CD44 and thus

divided into four stages: DN1 ($CD25^{lo}CD44^{hi}$), DN2 ($CD25^{hi}CD44^{hi}$), DN3 ($CD25^{hi}CD44^{lo}$) and DN4 ($CD25^{lo}CD44^{lo}$) (101). At the DN3 stage, thymocytes undergo TCRβ chain rearrangement. Each TCR gene contains multiple variable (V), diversity (D), and joining (J) region segments. The recombination of different V, D, and J segments with deletions and random insertions of nucleotides at the junction regions give rise to millions of different gene sequences (102). TCRβ rearrangement is allelic exclusive, and the first in-frame β chain rearrangement stops further somatic recombination (103). The generated TCRβ chain pairs with a surrogate of the TCRα chain to assemble the pre-TCR complex to be expressed on the cell surface.

The pre-TCR signaling leads to cell expansion and CD4/CD8 up-regulation (104). At the CD4/CD8 double positive (DP) stage, thymocytes rearrange the TCRα chain to express TCRαβ antigen receptors and to undergo positive selection (105). During positive selection, DP thymocytes are tested for their responsiveness to self-MHC molecules. Only those thymocytes that recognize the TCR ligands appropriately survive and continue with the developmental process (105). If the current αβ pair does not interact appropriately with the MHC complexes, the α chain rearrangement can be continued for new α chain products (106, 107). However, still about 90% of the DP thymocytes are eliminated at this stage because of inadequate TCR-MHC interaction (105).

Coincident with the positive selection, two major T cell lineages are generated: CD4 T cells that recognize peptide-MHC class II complexes and CD8 T cells that recognize peptide-MHC class I complexes (108). These positively selected single

positive (SP) thymocytes migrate to the medulla (109) for further maturation and undergo screening for self-reactivity, a process called negative selection (110, 111). Medullary thymic epithelial cells express and process various tissue-specific antigens to present them to thymocytes through MHC complexes (112). TCRs that recognize these self peptide-MHC complexes with high affinity elicit strong TCR signals that lead to cell apoptosis. By negative selection, most of the self-reactive thymocytes are eliminated from the immune system and self-tolerance in the periphery is established. Thus, positive and negative selection together generates a repertoire of T cells bearing TCRs with self-MHC restriction and the ability to distinguish self from foreign antigens.

1.2.2 TCR signaling during development

TCR signaling quality, which is a function of the strength of the TCR-MHC interaction, is the major determinant of the fate of thymocytes. On the cell surface, TCR is expressed as a complex of a TCR αβ pair, a CD3 γε and a CD3 δε heterodimer as well as a CD3ζζ homodimer (113). The cytosolic tails of TCR chains are short and lack signaling domains. Instead, CD3 dimers contain the immunoreceptor tyrosine-based activation motifs (ITAMs) and function to initiate the intracellular signals after TCR activation (114, 115). As shown in Figure 1.1, the aggregation of TCR complexes results in transphosphorylation and activation of the Src family of protein tyrosine kinases (PTKs) Lck and Fyn that associate with the CD4 or CD8 co-receptor (116-119). The Src family PTKs phosphorylate ITAMs (120) to provide binding sites for other PTKs including ZAP70. Upon binding to the ITAMs, ZAP70 becomes activated (121, 122), and in turn phosphorylates the docking sites of adaptor proteins LAT and SLP-76 to recruit

specific SH2 domain-containing proteins (122, 123). LAT serves as a docking scaffold for Grb2 family adapter proteins and kinases such as PI3K and PLC-γ1 (124). SLP-76

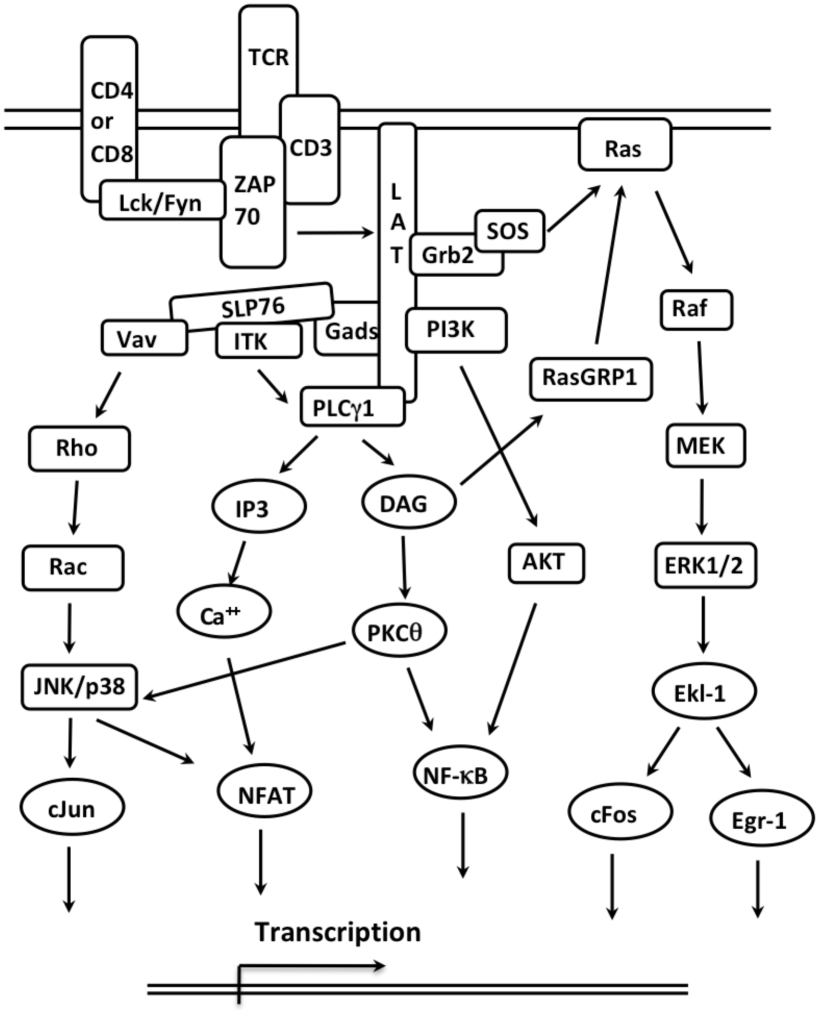

Figure 1.1 TCR signaling. Detailed description can be found in the text.

binds to the LAT complex through Gads and provides binding sites for proteins such as VAV, NCK, and ITK through its phosphorylated N-terminal tyrosine residues (125-128). SLP-76 association with Gads also contributes together to PLC-γ1 phosphorylation (131). VAV and NCK are thought to integrate the activation of different pathways responsible for gene transcription and cytoskeletal rearrangement (129, 130), and ITK has been

suggested to be important for the optimal activation of PLC-γ1 (132).

Once activated, PLC-γ1 cleaves phosphatidylinositol 4,5-bisphosphate into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binding to receptors on the endoplasmic reticulum initiates the release of intracellular calcium store. Increased intracellular calcium concentration activates phosphatase calcineurin, which dephosphorylates the cytoplasmic component of the NFAT transcription factors (133) and results in their translocation into the nucleus, where they form heterodimers with members of the AP-1 family to regulate gene transcription (134, 135). DAG responds to the increased intracellular calcium by activating PKCθ and the downstream NF-κB pathways (136). DAG also activates the guanine nucleotide exchange factor (GEF) RasGRP1 and the downstream Ras-MEK-MAPK/ERK1/2 pathway (137). This signaling pathway is also regulated by another GEF, the son of sevenless (SOS) protein, which can be recruited by the adaptor protein Grb2. Ras-MEK-MAPK/ERK1/2 pathway leads to the activation of the transcription factors such as Egr-1 and Elk-1 (148). Various transcription factors form a network to regulate the expression and activity of the genes involved in thymocyte development.

1.2.3 TCR signaling pathways responsible for positive and negative selection

Several signaling pathways downstream of proximal TCR signaling are important for thymocyte positive selection (138, 139). One of these is the calcium–calcineurin–NFAT signaling pathway (140). Mice with an inactivated regulatory subunit of calcineurin are clearly deficient in positive selection, as demonstrated by diminished $TCR\beta^{hi}CD69^{hi}$ DP populations (141). NFAT4 deficient mice exhibit impaired positive

selection by increased apoptosis of DP thymocytes and reduced SP thymocyte number. Conversely, constitutive activation of calcineurin results in increased CD4 SP cell generation in a TCR transgenic mouse model (142).

Ras-MEK-MAPK/ERK1/2 is another well-studied signaling pathway with a role in thymocyte positive selection. RasGRP1 deficient mice display profoundly impaired positive selection as seen by the very few SP thymocytes generated (143). Consistent with this observation, the disruption of this pathway with a dominant negative Ras (144-146) or an inactivated ERK1 (147) selectively inhibits positive selection of thymocytes without altering negative selection. Egr-1seems to be the downstream target that is uniquely important for positive selection (148), and this is supported by the observation that enforced expression of Egr-1 partially rescued the positive selection of thymocytes in a HY TCR transgenic model (149).

TCR signaling is very sensitive; a small increase in the affinity of TCR ligation can cause dramatic changes in the signaling kinetics of the downstream pathways. MAPKs have been suggested to account for the distinction between positive and negative selection on the basis of the TCR signaling intensity (150). At low stimulation levels, Ras is activated mainly by RasGRP1 at the Golgi aparati, which leads to the moderate activity of the Ras-ERK cascade and favors cell survival (151-153). Strong TCR ligation, however, results in enhanced phosphorylation of LAT and the recruitment of Grb2-SOS in addition to RasGRP1 from the cytosol to the plasma membrane (154). This results in strong and transient activation of the Ras-ERK cascade at the plasma membrane and the

activation of two other MAPKs, JNK and p38 (107, 155), which are not activated by RasGRP1 alone because of a higher activation threshold than that of ERK1/2 (156). These two MAPKs are thought to be specifically involved in negative selection, and further, the selective disruption of their activity impairs negative but not positive selection (156-158). Thymocyte apoptosis during negative selection seems to be Fas-independent and instead is mediated by Bcl-2 family members and Nur77 (159). Bim deficient mice develop autoimmune disease due to undeleted auto-reactive thymocytes (160) and exhibit impaired superantigen-mediated negative selection (161). Similarly, the negative selection is impaired when the activity of Nur77 is inhibited (162).

1.3 Thymocyte-selected CD4 (T-CD4) T cells

1.3.1 The generation of T-CD4 T cells

Conventionally, CD4 T cell generation is believed to be TEC-dependent. However, there has been increasing evidence suggesting that human CD4 T cells can be generated through an alternative pathway. For example, patients with bare lymphocyte syndrome (BLS) lack CD4 T cells due to defective MHC class II expression, and currently bone marrow transplantation (BMT) is the only curative treatment. The BMT treatment results in the restoration of functional CD4 T cells in many patients (163-166), indicating that in humans CD4 T cell generation is not absolutely dependent on TECs and can be mediated by bone marrow-derived cell types. Further studies support thymocytes as a mediator of CD4 T cell selection using the OP9-DL1 stromal cell line that supports T cell development (167). Mouse T cell precursors develop into CD8 but not CD4 T cells in the OP9-DL1 system because the stromal cells and mouse thymocytes express MHC class I but not class II. By contrast, human thymocytes that can express a substantial

amount of MHC class II molecules on their surface (168, 169) are generated when co-cultured with OP9-DL1 cells (167). Physiologically speaking, it is hard to assess thymocyte-selected CD4 T cells in humans because there are not cell surface markers available to distinguish them from TEC-selected CD4 T cells, although a recent study reported that the transcription factor promyelocytic leukaemia zinc finger (PLZF) protein is expressed in thymocyte-selected but not TEC-selected human CD4 T cells (170).

Considering the evidence of thymocyte-generated CD4 T cells in humans and the limited knowledge of this population, it became necessary to establish a similar CD4 T cell developmental system using mouse models. In 2005, we and others independently reported the generation of mouse CD4 T cells independent of the TEC-mediated pathway (171, 172). Both groups induced MHC class II expression on mouse thymocytes using a human CIITA transgene ($CIITA^{Tg}$). CIITA is a master transactivator of MHC class II expression. It is conserved between humans and mice, and the transgenic expression of CIITA results in efficient MHC class II expression on mouse thymocytes (171, 172). The transfer of $CIITA^{Tg}$ BM to MHC class II-deficient hosts results in CD4 T cell restoration, which cannot be accomplished by wild type (WT) BM transfer to the same kind of host, confirming that thymocyte-mediated selection is independent of TEC-mediated selection in CD4 T cell generation. Thymocyte-selected CD4 T cells are named T-CD4 T cells, and conventional CD4 T cells selected by TEC are named E-CD4 T cells.

In addition to selecting cell types, the developmental process of T- and E-CD4 T cells also differs in the requirement of co-stimulatory signals. One example is the type I

transmembrane proteins of the signaling lymphocyte activation molecule (SLAM) family. These are expressed restrictively in hematopoietic cells (173) and signal through the adaptor SLAM-associated protein (SAP) (174). Whereas SLAM pathways are dispensable for E-CD4 T cell generation, they are critical for T-CD4 T cell development. Leaving E-CD4 T cell generation intact, SLAM or Ly108 deficiency decreases and SAP deficiency almost abolishes T-CD4 T cell generation. (175).

1.3.2 T-CD4 T cell functional characteristics

E-CD4 T cells are conventional adaptive immune cells. Upon stimulation, they undergo several days of clonal expansion, during which the cells are programmed and differentiate into different T helper cells according to the type of the stimulation. Therefore, it takes several days for them to be ready for functional performance. By contrast, T-CD4 T cells exert effector function within hours after TCR stimulation both *in vivo* and *in vitro* (176). This functional property is reminiscent of cells of the innate immune response, and therefore is referred to as "innate-like". Moreover, the Th1 and Th2 cytokine production of E-CD4 T cells is mutually exclusive, but certain T-CD4 T cells produce both Th1 and Th2 cytokines (176) under un-polarized conditions, and more remarkably, under Th1-skewing conditions as well (177). Further studies have shown that T-CD4 T cells contain pre-formed mRNA of the Th2 cytokine IL-4 at steady state that is STAT6-independent (176).

The physiological role of T-CD4 T cells has been investigated in several mouse models of immune diseases. First, T-CD4 T cells repress experimental autoimmune encephalomyelitis (EAE), indicated by lower disease scores and reduced IFN-γ

production of $MOG_{35–55}$-specific CD4 T cells in $CIITA^{Tg}$ mice compared to WT littermates (178). Second, T-CD4 T cells were protective in antigen-induced airway inflammation (176). Different from polyclonal stimulation by anti-CD3 antibody, T-CD4 T cells did not enhance Th2 cytokine production in sensitized and aerosolically challenged $CIITA^{Tg}$ mice. Instead, these mice demonstrated decreased allergen-specific IgE levels in the serum, limited IL-13 and IL-17 transcripts in the lungs as well as reduced cell numbers in lung infiltrate compared to wild type mice. Third, in contrast to the reduced disease progression in the two models above, $CIITA^{Tg}$ mice exhibited aggravated disease development in a oxazolone-induced colitis model (179), although the mechanism of T-CD4 T cell functional disparities in these mouse models is not yet clear. In contrast to these adaptive immune responses, innate responses do not seem to be significantly affected by T-CD4 T cells, as $CIITA^{Tg}$ and wild type mice showed similar innate inflammation in dextran sodium sulfate-induced acute colitis (177).

1.3.3 Other unconventional mouse T cell populations

Besides T-CD4 T cells, several other T cell populations have been identified with unconventional developmental characteristics and immediate effector functionality. One of the most studied populations is the invariant natural killer T (iNKT) cells. They undergo TCR αβ rearrangement as conventional T cells and are either $CD4^+$ or CD4/CD8 double negative. As an innate-like T cell population, they produce IFN-γ and IL-4 upon TCR stimulation and have been shown to regulate immune-related diseases such as asthma and cancer (182-184). They are called iNKT cells because they express certain NK cell markers including NK1.1, 2B4 (CD244) and CD94/NKG2 (180, 181), and because their TCR repertoire is much more limited than that of conventional T cells, and

preferentially express Vα14-Jα18/Vβ8.2 and Vβ7 in mice, and Vα24-Jα18/Vβ11 in humans (185, 186). Unlike conventional CD4 T cells, the TCRs of iNKT cells do not recognize peptide-MHC complexes expressed on TEC, but instead recognize glycolipids presented by the MHC class Ib molecule CD1d expressed on thymocytes (187). Moreover, iNKT cells express the transcription factor PLZF, which further distinguishes them from E-CD4 T cells (185, 186). Interestingly, not only do iNKT cells share the same selecting cell type with T-CD4 T cells, they also require the SLAM-SAP signaling pathways for their development (188).

Unconventional CD8 T cells are another widely identified and investigated innate-like T cell population (189-195). Their antigen-independent cytokine secretion and NKG2D-dependent cytotoxicity are observed early after bacterial and viral infections (196-198). The generation of these innate-like CD8 T cells has heterogeneous causes. They can be selected by either TEC or hematopoietic cells, and they recognize MHC class Ib molecules such as H2-M3 and Qa-1 (199). H2-M3-restricted CD8 T cells recognize formyl-methionine-modified peptides typically found in bacterial and mitochondrial encoded proteins that are uncommonly found in the mammalian cytosol (200). Qa-1 molecules associate with peptides or lipids to positively select CD8αα T cells that are also known as intestinal intraepithelial lymphocytes (201, 202). The TCR specificity of innate-like CD8 T cells does not seem to be as restrictive as that of conventional T cells. For example, a H2-M3-restricted TCR can recognize multiple unrelated peptides as long as they are N-terminal formylated (203). Moreover, the signaling requirement for innate-like CD8 T cell development is also different from that for conventional T cell development. First, reduced TCR signaling that compromises

conventional CD8 T cell development enriches innate-like CD8 T cell populations. Second, co-stimulatory pathways mediated by SLAM and CD28 that do not affect conventional CD8 T cell generation are critical for innate-like CD8 T cell generation and their functional properties (199).

1.4 *Listeria monocytogenes* infection

1.4.1 Infection course and the host innate immune response

Listeria monocytogenes are gram-positive, rod-shaped facultative intracellular bacterium (204-206), which are ubiquitous in the environment and are recognized as an important food borne pathogen. Although it rarely causes serious symptoms in immunocompetent populations, *L. monocytogenes* causes septicaemia and meningitis in immunocompromised individuals as well as chorioamnionitis and septic abortion in pregnant women (207).

L. monocytogenes infection and the immune responses triggered by the infection have been intensively characterized (207-213). The natural route of *L. monocytogenes* infection is through the gastrointestinal tract. The bacteria can traverse the epithelial cell layer into the bloodstream and spread systemically, which makes the spleen and liver the major infection sites. Macrophages and neutrophils are recruited within hours after infection, followed by monocytes that can differentiate into macrophages by TLR and/or cytokine stimulation (214, 215). These cells function to internalize and destroy the bacteria through reactive oxygen intermediate-dependent mechanisms (216, 217), which is critical for the initial control of the scale of the infection. The depletion of these cells remarkably enhances host susceptibility to *L. monocytogenes* infection (218-220). In

addition, NK cells and macrophages produce the cytokines IFN-γ and TNFs that are important for *L. monocytogenes* clearance (221-223). IFN-γ promotes macrophage activation (224) and MHC up-regulation (167, 225); TNFs recruit neutrophils and functionally synergize with IFN-γ. Mice lacking these cytokines rapidly succumb to infection (212, 226, 227).

1.4.2 Adaptive immunity to *Listeria monocytogenes*

After being phagocytosed, *L. monocytogenes* can escape from the phagosome by secreting listeriolysin O (LLO) to permealize the phagosomal membrane. Once inside the cytosol, *L. monocytogenes* are processed through the MHC class I antigen-presenting pathway and thereby cause robust CD8 T cell responses (228, 229). Although adaptive immunity does not develop into full momentum until a couple of days into the infection, its programming starts early after infection: in the spleen and the draining lymph nodes, T cells are primed in the T cell zone by APCs such as CD11c-expressing DCs (230) within 24 hours, and the later cell expansion is largely dependent on the antigen presentation within the first day (231). In addition, T cell survival at the contraction phase is inversely correlated with the pro-inflammatory environment early on (232, 233). In comparison, the innate immune responses after the first 24 hours have only a small effect on the kinetics and magnitude of CD8 T-cell response (233).

L. monocytogenes-specific CD4 and CD8 T cells expand synchronically (234), with their frequencies in the spleen peaking approximately eight days after a primary infection and three days after a secondary infection. Studies using adoptive transfer models have shown that CD4 T cells promote the immune responses by IFN-γ production

(212, 235). IFN-γ in this case is likely to activate macrophages to become fully bactericidal, as *in vitro* studies have showed that treating macrophages with IFN-γ prevents bacteria escaping from the phagosome (236). CD8 T cells mediate antigen-specific lysis of infected cells through two major molecular pathways (237, 238). The granule exocytosis pathway requires the coordinated activity of perforin and granzymes released from the granules of activated CD8 T cells to activate the caspase cascade of the target cells to trigger apoptosis (239). Alternatively, activated CD8 T cells express Fas (CD95) ligand, which activates the Fas pathway in target cells to induce apoptosis (237, 238). CD8 T cells with perforin deficiency display intact priming and expansion but compromised cytolytic activity and protective capacity against *L. monocytogenes*, especially in controlling cell-cell contact-dependent bacterial spread (240, 241). Remarkably, mice deficient in both perforin and Fas have dramatically reduced capacity for *L. monocytogenes* clearance compared with mice defective in only one of these, suggesting that the two killing pathways play complementary roles in host defense (242).

L. monocytogenes infection is a useful model to study the dependency of CD4 T cell help in the establishment of CTL memory immunity. CD4 T cell deficient mice can recover from the primary infection efficiently, but they display defective bacterial clearance during a secondary *L. monocytogenes* infection (231, 243, 244) with dramatically compromised antigen-specific CTL proliferation (177). It seems that the CD4 T cell assistance is important during CD8 T cell priming, because their absence at this stage impairs CTL memory immunity but their depletion at a later stage does not affect the efficiency of CTL memory response (231, 243, 244). The role of CD4 T cells

has also been revealed in the homeostasis of memory CD8 T cells after the resolution of the infection. The number of *Listeria*-specific CTLs in CD4 T cell deficient hosts declines much faster over time than that in CD4 T cell sufficient hosts (100). The molecular mechanism of CD4 T cell function in the development of anti-*Listerial* CTL memory immunity remains elusive, except that pathways such as the CD40-CD40L co-stimulatory pathway (245) have been shown to be dispensable for the process.

1.5 Goals of the thesis

The goals of this thesis are: first, to investigate the TCR signaling requirement for T-CD4 T cell development, and second, to study the function of T-CD4 T cells in a bacterial infection.

To investigate TCR signaling requirement for T-CD4 T cell development, the effect of TCR signaling modulation on T-CD4 T cell development will be compared with that of E-CD4 T cells in different gene knockout models.

To study the function of T-CD4 T cells in anti-bacterial immunity, antigen-specific primary and memory responses will be assessed using a mouse model of *L. monocytogenes* infection.

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Chapter II

Development of innate CD4 T cells expressing promyelocytic leukemia zinc finger protein is regulated by the strength of TCR signaling

2.1 Abstract

Studies have shown that CD4 T cells can be selected by MHC class II-expressing thymocytes in addition to TEC in experimental mouse models. This observation offers an explanation for CD4 T cell generation by MHC class II-expressing hematopoietic cells in humans. Here, we report that TCR signaling regulates the frequency of thymocyte-selected CD4 (T-CD4) T cells. T-CD4 T cell development was enhanced when TCR signaling strength was weakened due to ITK deficiency, whereas the same deficiency compromised E-CD4 T cell selection by TEC. The frequency of T-CD4 T cells was decreased when Ras activity was augmented by eliminating the Ras deactivating enzyme RASA1. Furthermore, T-CD4, but not E-CD4 T cells, expressed the transcription factor promyelocytic leukaemia zinc finger (PLZF) protein that is known to be essential for iNKT cell development. Furthermore, the CD4 T cells that developed in ITK deficient mice, in which TCR signaling was decreased, also expressed PLZF. These findings suggest that the TCR signaling quality required for T-CD4 development is different from that required for E-CD4 T cell development and is associated with PLZF expression.

2.2 Introduction

CD4 T cells are developed by the interaction of T cell receptors (TCRs) with their ligand major histocompatibility complex (MHC) class II molecules, and they play a key role during an adaptive immune response (1-4). The fate of T cells during their development is largely determined by the magnitude of TCR signaling, and it is known that a narrow window of TCR signaling strength is required for the survival of thymocytes (5-9). Upon TCR ligation, a series of protein tyrosine kinases are recruited to the cytoplasmic domains of the TCR complex (10-12), where they are activated by phosphorylation and in turn activate components of distinct downstream signaling pathways. This cascade of signaling events results in the activation of transcription factors for new gene products that are required for thymocyte development (13). Particularly, signaling pathways mediated by changes in intracellular calcium flux, the NF-κB pathway, and the Ras- MAPK/ERK1/2 cascade are known to regulate positive selection of thymocytes (13).

On the level of proximal TCR signaling, the IL-2 inducible T cell kinase (ITK) plays a vital role for thymocyte positive selection (5, 14, 15). Activated by Lck, ITK activates phospholipase C-γ1, leading to calcium mobilization and MAPK/ERK activation (16). $ITK^{-/-}$ mice display impaired conventional CD4 and CD8 T cell positive selection without altering their lineage decisions (17). Interestingly, $ITK^{-/-}$ CD8 T cells exhibit innate cell characteristics that differ from conventional CD8 T cells (18, 19). However, the development of T-CD4 T cells in ITK-deficient mice has not been characterized.

The Ras-MAPK/ERK1/2 cascade is known to be important for thymocyte positive selection (13) in that positive but not negative selection of thymocytes expressing the H-Y male-specific transgenic TCR is severely compromised by expression of a dominant negative form of Ras expressed under the control of the Lck proximal promoter (13, 20, 21). In addition, gene disruption of ERK1, one of the Ras downstream kinases, also partially blocked positive selection (22). The activity of the small GTPase Ras is modulated by positive and negative regulators (13). Ras guanine nucleotide exchange factors (GEFs) promote Ras activity by catalyzing the exchange of GDP for GTP (23, 24). One of the GEFs, RasGRP1, has been shown to be required to mediate TCR signaling to the Ras-ERK1/2 cascade during positive selection (24). RasGRP1 deficiency completely abrogates ERK activation and thus T cell development (23, 25). On the other hand, Ras GTPase-activating proteins (RasGAPs) facilitate GTP hydrolysis and thereby function to inactivate Ras (25-27). RASA1 (p120-RasGAP), one of the RasGAPs, is critical for normal Ras activity (28) in various cell functions, including cell survival, proliferation, and movement (28, 29). However, the role of RASA1 in T cell development has not been characterized (27).

In the mouse thymus, cortical TEC are recognized to be the major cell type mediating positive selection of conventional CD4 and CD8 T cells (30-32). However, observations in humans argue against TEC being the only cell type capable of supporting positive selection. Patients who are not able to produce their own CD4 T cells due to defects in MHC class II expression can restore CD4 T cells upon bone marrow transplantation, which strongly suggests that hematopoietic cells can provide TCR ligands for CD4 T cell selection (33-35). Moreover, human thymocytes are likely to be the TCR ligand providers, considering that

they express MHC class II molecules (36) and that they are the major cell population in the thymus. In order to test whether thymocytes indeed can mediate CD4 T cell selection using mouse models, we constructed a transgenic mouse line to express MHC class II molecules on mouse thymocytes by introducing the MHC class II transactivator (CIITA) transgene after a mouse CD4 promoter. Using this CIITA^{Tg} mouse model, we have established that MHC class II molecules expressed exclusively on thymocytes can support the development of unconventional CD4 T cells (37). To distinguish between CD4 T cells that are dependent on either TECs or thymocytes for their selection, we will refer to these cells as E-CD4 and T-CD4 T cells, respectively.

T-CD4 T cells possess unique properties that are not found in E-CD4 T cells. Unlike E-CD4 T cells, they produce both Th1 and Th2 cytokines immediately after TCR stimulation by anti-CD3 antibody *in vitro* and *in vivo* (38). Moreover, T-CD4 T cells but not E-CD4 T cells can express IL-4 under Th1-skewing conditions. In addition, T-CD4 T cells produce IL-4 in a Stat6-independent manner (38), which is due to preformed IL-4 mRNA in T-CD4 T cells (38). The functional differences between T- and E-CD4 T cells have been demonstrated further by the observation of a protective role of T-CD4 T cells in an airway inflammation model (37) as well as in an EAE model (39). In addition, T- but not E-CD4 T cells require the SLAM-SAP signaling pathway for their selection. Interestingly, many of these characteristics of T-CD4 T cells are also observed in invariant NKT (iNKT) cells (40). iNKT cells are also selected by thymocytes (41, 42) and depend on the SLAM-SAP signaling pathway for their development (43). More recently, the promyelocytic leukemia zinc finger

(PLZF) protein has been recognized as a lineage marker of iNKT cells that is critical for their development (44, 45).

The differences and similarities among T-CD4, E-CD4, and iNKT cells prompted us to investigate the role of the TCR signaling strength in the development and functions of these cells. Here we report that the strength of TCR signaling regulates the efficiency of T-CD4 T cell generation. Weakened TCR signaling achieved by eliminating ITK augmented the development of T-CD4 T cells while reducing the generation of E-CD4 T cells. By contrast, elevated Ras activity in mice lacking RASA1 resulted in diminished T-CD4 but enhanced E-CD4 T cell generation. We also observed that PLZF was not only expressed in T-CD4 T cells but also in $ITK^{-/-}$ CD4 T cells selected by bone marrow-derived cells in a non-$CIITA^{Tg}$ system. Therefore, thymocytes bearing TCRs that received a certain range of signaling qualities undergo selection mediated by hematopoietic cells and develop into innate-like CD4 T cells that express PLZF.

2.3 Materials and Methods

Mice

Mice carrying the human type III CIITA transgene (Tg) were described previously (46). Tg mice were bred to carry both the CD45.1 and CD45.2 congenic markers. Non-Tg littermates from heterozygous Tg breeding were used as wild type (WT) controls. All mice were housed in the animal facility at the University of Michigan Medical School under specific pathogen-free (SPF) conditions and used at 6-12 weeks of age. All animal experiments were performed under protocols approved by the institutions.

Bone marrow chimeric mice

For bone marrow (BM) transfer experiments, the recipient mice were lethally irradiated with 960 rads 24 hr before receiving BM transfers. Total BM cells were harvested from the femurs and tibias of donor mice (2–3 months of age) and depleted of mature T cells, B cells, and MHC class II-positive lymphocytes by using a cocktail of antibodies containing anti-CD4 (RL172), anti-CD8 (TIB105, TIB210), anti-CD19 (1D3), and anti-MHC class II (M5/114) followed by complement-mediated lysis. BM cells from two different types of donor mice were mixed at the indicated ratio. Each recipient mouse received 4–6 × 10^6 cells in 200 μl of 1× PBS via tail vein injection. All BM chimeras were reconstituted for 4-6 weeks before analysis of T cell development and function.

Flow cytometry

All antibodies used for flow cytometry were purchased from BD PharMingen (San Diego, California). Cells were pre-incubated with the anti-FcγR mAb 2.4G2 to block

nonspecific antibody binding before they were stained with the following FITC-, PE-, PerCP5.5-, CyChrome-, APC-, or biotin-conjugated antibodies: TCRβ (H57), CD4 (L3T4), CD8 (53-6.7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD44 (IM7), TCRb (H57-597), NK1.1 (NK1.1), a-GalCer analog-loaded CD1d-trtramer (from NIH tetramer core facility), anti-IL-4 (11B11), and anti-IFN-γ (XMG1.2). Events were acquired on a FACSCanto (Becton Dickinson) flow cytometer, and the data were analyzed with FlowJo software.

CD4 T cell preparation and differentiation

CD4 cells were purified from single-cell suspensions of splenocytes from chimeric mice with anti-mouse CD4 microbeads (Miltenyi Biotec, Auburn, California). CD4 T cells (1×10^6/ml) were stimulated with 5 μg/ml plate-bound anti-CD3 (145-2C11), 1 μg/ml soluble anti-CD28 (37.51), and 50 U of IL-2 (Source: Roche, Indianapolis, Indiana) for 5 days. For Th1 differentiation, 3.5 ng/ml of IL-12 and 10 μg/ml of anti-IL-4 (11B11) were added.

Cytokine intracellular staining

Differentiated Th1 CD4 T cells were stimulated with 50 ng/ml phorbol myristyl acetate and 1.5 μM ionomycin (Source: Calbiochem, San Diego, California) for 5 hr. Monensin (Source: Sigma, St. Louis, Missouri) at 3 μM was added during the last 3 hr of stimulation. Activated Th1 cells were stained with anti-CD45.1, anti-CD45.2 antibodies. Cells were then fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.2% saponin (Source: Sigma), followed by staining with anti-IL4 (11B11) and anti-IFN-γ (XMG1.2) mAbs for flow cytometry.

Real time PCR:

A Lightcycler and SYBR Green Master Mix reagents (Source: Roche) were used for quantitative real-time PCR of PLZF. Results were normalized by comparison to PCR amplification of the gene encoding HPRT (hypoxanthine guanine phosphoribosyl transferase). Primers were as follows: PLZF forward, 5'-TCTGACAAAGATGGGGATGATCC-3', and reverse, 5'-CAGTATTCCGTGCAGATGGTACAC-3'; HPRT forward, 5'-GGATACAGGCCAGACTTTGTTG-3', and reverse, 5'-GAGGGTAGGCTGGCCTATGGCT-3'.

Statistical analysis

P values were calculated between groups by a two-tailed student's t test (Prism 5; Graph-Pad Software, San Diego, CA). Differences were considered significant at $p < 0.05$ by the Student's t test (unpaired).

2.4 Results

2.4.1 Enhanced T-CD4 T cell development in the absence of ITK

Previously, we have shown that, different from E-CD4 T cells, T-CD4 T cell generation requires signaling through SLAM receptors (47), which led us to investigate whether or not T-CD4 T cell development requires the same TCR signaling quality as E-CD4 T cells.

ITK has been reported to be important for E-CD4 T cell selection (5, 16). To test whether it is similarly required for the generation of T-CD4 T cells, we employed a strategy of mixed BM reconstitution. We generated T-CD4 T cells by co-transferring ITK$^{-/-}$ BM cells with CIITA^{Tg} BM cells into lethally irradiated Aβ$^{-/-}$ hosts (ITK$^{-/-}$ + CIITA^{Tg}→Aβ$^{-/-}$: T-BMT). In these BM chimeras, TEC do not express MHC class II molecules. Instead, hematopoietic cells including thymocytes originating from CIITA^{Tg} BM cells express MHC class II. Therefore, all CD4 T cells including those originating from ITK$^{-/-}$ BM in the chimeras must have undergone selection mediated by CIITA^{Tg} BM driven cells, most likely thymocytes. WT cells can be efficiently selected by CIITA^{Tg} thymocytes in the mixed BM chimeras (52). To generate ITK$^{-/-}$ cells that are E-CD4 T cells, we transferred BM cells from ITK$^{-/-}$ and WT mice to WT hosts (ITK$^{-/-}$+WT → WT: E-BMT). In these mice, MHC class II expressing TEC in the host support ITK$^{-/-}$ E-CD4 T cell development. In all cases, we used CD45 congenic markers to distinguish cells from the two donors and the recipient.

When ITK$^{-/-}$ cells were selected by TEC (ITK$^{-/-}$+WT → WT), the CD4 single positive (CD4 SP) compartment was reduced in comparison to the WT partner cells (Fig 2.1 A, left

group). By contrast, the percentage of ITK$^{-/-}$ T-CD4 T cells was greater than that of the CIITA^{Tg} BM driven cells in T-BMTs (Fig 2.1 A, right group), suggesting that the absence of ITK is beneficial to the generation of T-CD4 T cells. To get a quantitative evaluation, the CD4 T cell selection efficiency was calculated as the ratio of the percentage of CD4 SP to that of CD4/CD8 double positive (DP) thymocytes. We then used the ratio of ITK$^{-/-}$ CD4 T cells to that of the co-transferred partner cells in the same host to get the selection efficiency. A ratio of 1 would indicate the equivalent selection efficiency of CD4 T cell generation from the two donor BM cells. If ITK$^{-/-}$ CD4 T cells are selected more efficiently than the co-transferred partner cells, the ratio would be greater than 1. By contrast, a ratio less than 1 indicates poorer selection of ITK$^{-/-}$ cells. ITK$^{-/-}$ E-CD4 T cell selection was reduced (Fig 2.1 B), which is consistent with the published observations that the loss of ITK substantially impairs but does not totally abolishes T cell development (5, 16). Surprisingly, T-CD4 T cell selection was significantly enhanced in the absence of ITK (Fig 2.1 B), suggesting T-CD4 T cell generation is favored by weak TCR signals.

In the periphery, both T- and E-BMT hosts had reduced numbers of CD4 and CD8 T cells from ITK$^{-/-}$ BM compared to their partner cells (Fig 2.1 C), consistent with prior reports that ITK deficiency resulted in reduced CD4 T cells in the periphery (14, 16). However, when ITK$^{-/-}$ and ITK$^{+/+}$ cells were compared within the T- and E-BMT group, T-CD4 cells were reduced by two fold in ITK$^{-/-}$ cells compared to ITK$^{+/+}$ cells while this reduction was five fold for E-CD4 T cells. In addition, the numbers of T-CD4 T cells in the periphery were also higher than the numbers of E-CD4 T cells when ITK was absent (Fig 2.1 D, compare first and third panels). Therefore, the status of ITK affected E and T-CD4 T cell selection and

cellularity. Our previous work showing that mice containing T-CD4 T cells often have a decreased CD8 T cell population in the periphery (37) was confirmed here (Fig 2.1 D).

T-CD4 T cells in the periphery exhibit an effector/memory-like phenotype (37). These characteristics were maintained in ITK$^{-/-}$ T-CD4 T cells as shown by high expression of CD44 and low expression of CD62L, which suggests that ITK contributes to the selection process but not to the phenotype of the resulting cells (Fig 2.1 E).

2.4.2 Requirement of RASA1 for the development of T-CD4 T and iNKT cells

Enhanced T-CD4 T cell development in the absence of ITK suggests that weakened TCR signals may favor the generation of T-CD4 T cells. If this were the case, increased signaling strength would result in reduced T-CD4 T cells. To test this, we utilized a mouse model of T cell-conditional RASA1 deficiency, in which the increased Ras activity leads to enhanced E-CD4 T cell positive selection (unpublished data by Philip Lapinski and Philip King). Using the same mixed BM chimera approach as above, we constructed T- and E-BMT mice with RASA1$^{-/-}$ BM cells. RASA1$^{-/-}$ BM cells yielded greater CD4 SP than WT cells in the E-BMT group (RASA1$^{-/-}$ + WT → WT) and the overall selection efficiency was also increased in the E-BMT group (Fig 2.2 A, left group and Fig 2.2 B). In the T-BMT group (RASA1$^{-/-}$ + CIITA^{Tg} → Aβ$^{-/-}$), however, the selection efficiency of RASA1$^{-/-}$ CD4 T cells was much lower than that of CIITA^{Tg} CD4 T cells, suggesting that RASA1 deficiency compromised thymocyte-mediated CD4 T cell selection (Fig 2.2 A, right group and Fig 2.2 B).

In the periphery, CD4 and CD8 T cells did not show significant differences in cellularity regardless of RASA1 and the selection pathway (Fig 2.2 C and D). Further, similar to ITK$^{-/-}$ T-CD4 T cells, RASA1$^{-/-}$ T-CD4 T cells were CD44^{hi}CD62L^{lo} and therefore maintained typical T-CD4 phenotype (Fig 2.2 E).

T-CD4 T cells share many similarities with iNKT cells, as we have previously reported (37, 47). Because RASA1 deficiency affected T-CD4 T cells and because its role in iNKT cells is unknown, we also examined the role of RASA1 in iNKT cell development. To do this, we constructed RASA1$^{-/-}$+WT→WT chimeras. iNKT cells identified by CD1d tetramers were greatly diminished in RASA1$^{-/-}$ originated T cell populations compared to WT populations (Fig 2.2 F). The data thus suggest that RASA1 plays an important role in the generation of both T-CD4 T and iNKT cells.

2.4.3 Critical role of RasGRP1 for both T-CD4 T and iNKT cell generation

Having observed that enhancing Ras activity by RASA1 deficiency increased E-CD4 T cells but reduced T-CD4 T cells, we investigated whether repressing Ras activity would enhance T-CD4 selection. For this purpose, we used mice deficient in RasGRP1, an activator of Ras. In the absence of RasGRP1, E-CD4 T cell development is greatly reduced due to the lack of Ras activation and, as a result, positive selection of E-CD4 T cells is severely diminished (13).

RasGRP1$^{-/-}$ E- and T-CD4 T cells were generated by transferring RasGRP1$^{-/-}$ BM cells with WT BM to WT hosts and with CIITA^{Tg} BM to Aβ$^{-/-}$ hosts, respectively. As

expected, CD4 SP thymocytes in E-BMT hosts were severely diminished (Fig 2.3 A, left group). Similarly, we observed the disappearance of T-CD4 T cells lacking RasGRP1 (Fig 2.3 A, right group). The severe reduction of T-CD4 T cells in the thymus resulted in few CD4 T cells in the periphery (Fig 2.3 B). Both groups of chimeras also showed reduced CD8 T cells in the thymuses and the spleens. Together, RasGRP1 plays an indispensable role in both T- and E-CD4 T cell as well as CD8 T cell development. In addition, iNKT cells were barely detectable in the thymus and the spleen (Fig 2.3 C).

2.4.4 Effect of ITK and RASA1 on the effector function of T-CD4 T cells

So far, we have shown that the modulation of TCR signaling affected T-CD4 T cell development. We next asked whether CD4 T cells developed under different signaling environments would possess the same effector functions or not. T-CD4 T cells are functionally distinct from E-CD4 T cells in many aspects (37, 38). Most notably, T-CD4 T cells produce IL-4 in addition to IFN-γ under conditions that favor Th1 differentiation (38). Therefore, to ascertain the role ITK and RASA1 in cytokine production, CD4 T cells from the spleens of the ITK$^{-/-}$ or RASA1$^{-/-}$ BM chimeras were enriched and differentiated into Th1 cells followed by intracellular staining of IFN-γ and IL-4. The number of RasGRP1$^{-/-}$ CD4 T cells was too low to do the functional assessment.

When ITK$^{-/-}$ and WT CD4 T cells were selected by epithelial cells, both ITK$^{-/-}$ and WT CD4 T cells produced IFN-γ but not IL-4, which is the typical profile of Th1 cells (Fig 2.4 A, left group). However, we observed that the IFN-γ expressing cell population was substantially lower when ITK was absent, suggesting a role for ITK in IFN-γ expression (Fig

2.4 B). In contrast to E-CD4 T cells, ITK$^{-/-}$ and WT T-CD4 T cells expressed IL-4 in addition to IFN-γ even though they were Th1 differentiated cells (Fig 2.4 A, right group and B). Therefore, ITK seems to be less critical for the effector function of T-CD4 T cells.

We next examined IFN-γ and IL-4 expression in Th1 differentiated cells with or without RASA1. As shown in Figure 2.4 C, RASA1$^{-/-}$ and WT E-CD4 T cells showed a comparable IFN-γ-expressing population, and the proportions of IL-4-expressing cells were insignificant. Therefore it seems that RASA1 is not involved in Th1 differentiation of E-CD4 T cells. However, when RASA1$^{-/-}$ cells developed into T-CD4 T cells, they expressed IL-4 together with IFN-γ just as RASA1$^{+/+}$ T-CD4 T cells did (Fig 2.4 C and D). We also observed that RASA1$^{-/-}$ cells tend to have more IL-4^{+} cells at the expense of IFN-γ cells, although the difference was not statistically significant. Nevertheless, the key feature of T-CD4 T cells to produce both Th1 and Th2 cytokines after Th1 differentiation was not altered by RASA1 deficiency.

2.4.5 Selection of ITK$^{-/-}$ CD4 T cells by hematopoietic cells

Data presented above were generated by using a murine model in which thymocytes express MHC class II. Based on the fact that ITK$^{-/-}$ + WT→WT chimeras were not able to generate ITK$^{-/-}$ CD4 T cells efficiently as ITK$^{-/-}$ + CIITA^{Tg}→Aβ$^{-/-}$ chimeras, one might conclude that thymocytes but not other hematopoietic cells are capable of mediating thymocyte selection. To address whether thymocyte-expressed TCR ligands are indeed required for T-CD4 T cell selection, we performed BM transfer experiments using ITK$^{-/-}$ BM cells into WT or mice lacking both MHC class I and II. In these hosts, T cell development

has to be mediated by hematopoietic cells from the donor cell origin, and importantly the donor thymocytes do not express MHC class II. Figure 2.5 A showed that ITK$^{-/-}$ cells developed poorly in WT mice compared to that of WT→WT. However, when MHC molecules were absent from host TEC, ITK$^{-/-}$ BM cells did better than WT BM cells in developing into CD4 as well as into CD8 SP cells. Therefore, the data suggested that MHC class II expressing hematopoietic cells can support CD4 T cell development and that the frequency of the selected cells increases when ITK is absent.

2.4.6 PLZF expression in hematopoietic cell-selected CD4 T cells

Recently, PLZF has been recognized as an essential transcription factor for iNKT cell development (44, 45). PLZF is expressed in both human and mouse iNKT cells but not in E-CD4 T cells. However, a recent report showed that human T-CD4 T cells also express PLZF (48). In agreement with this study, we observed a distinct PLZF^{+} CD4 T cell population in CIITA^{Tg} mice that are CD1d-tetramer negative and thus not iNKT cells (Fig 2.5 B). Consistent with our previous study, iNKT cells were reduced in CIITA^{Tg} mice (49). In CIITA^{Tg} mice, CD4 T cells can be developed by TEC as well as by thymocytes because both cell types express MHC class II. Therefore, it is likely that PLZF expressing and non-expressing CD4 T cells are selected by thymocytes and TEC, respectively. If so, we hypothesized that forcing all CD4 T cells to undergo selection exclusively by thymocytes could enrich PLZF-expressing cells in CD4 T cell compartment. To test this, we constructed chimeras to generate T- and E-CD4 T cells and examined CD4 SP cells in the thymus. Indeed, PLZF^{+} CD4 T cells were greatly induced in CD4 SP population from T-BMT mice (Fig 2.5 C) although PLZF was not expressed in all CD4 SP cells in T-BMT mice. As

expected, neither E-CD4 T cells nor CD8 T cells expressed appreciable levels of PLZF. Additionally, in line with T-CD4 T cells, CD4 T cells from $ITK^{-/-}$ mice but not those from WT mice expressed a high level of PLZF mRNA (Fig 2.5 D). We also found that the majority of $PLZF^{+}$ CD4 SP cells lost MHC class II (Fig 2.5 E). The CIITA transgene and thus the MHC class II expression was down-regulated when $CIITA^{Tg}$ CD4 T cells were activated by CD3 stimulation (data not shown). Therefore, the $PLZF^{+}$ CD4 SP cells have undergone more TCR stimulation than the $PLZF^{-}$ cells.

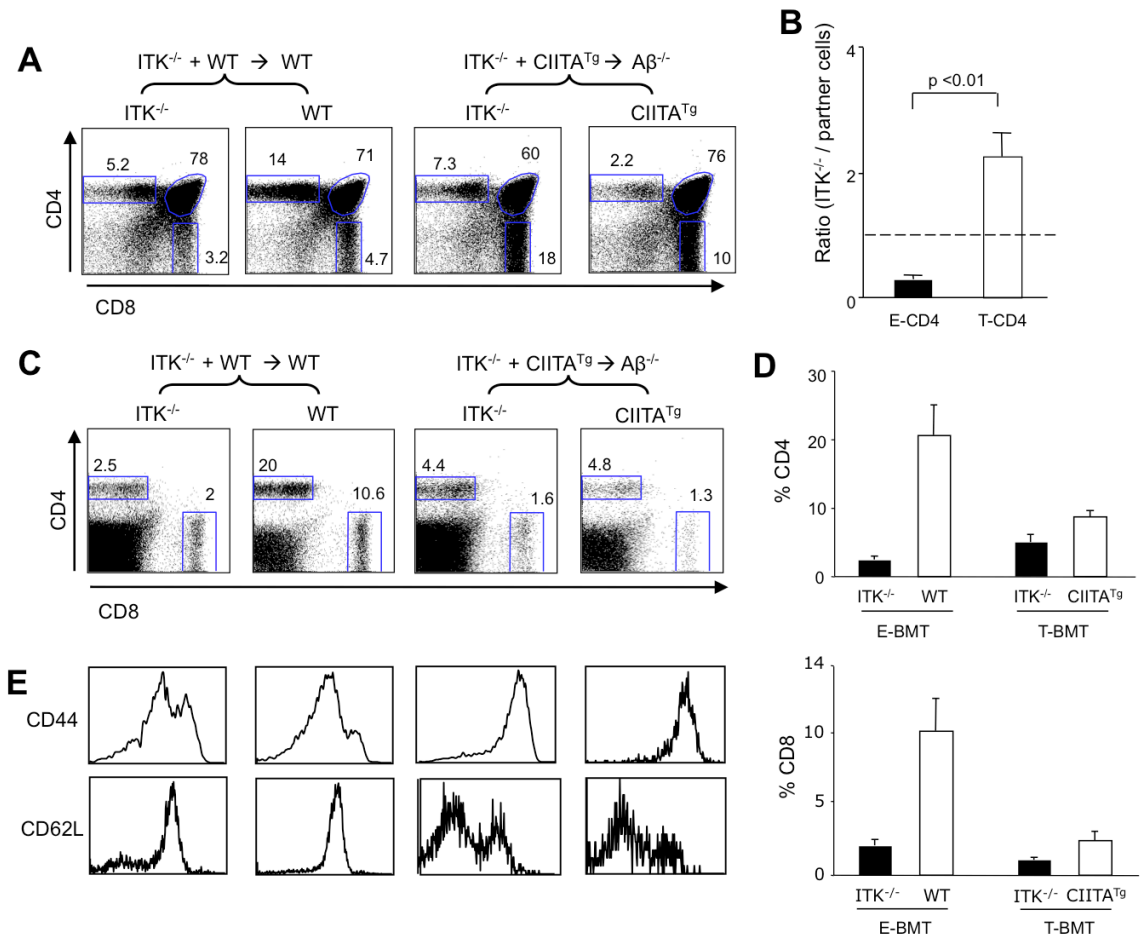

Figure 2.1 ITK deficiency enhances T-CD4 T cell generation.
(A) ITK$^{-/-}$ BM (CD45.2) and wild type (WT) BM (CD45.1 and CD45.2) were mixed and co-transferred into B6.SJL hosts (CD45.1) (ITK$^{-/-}$ + WT → WT) at the ratio of 3:1 to generate E-CD4 T cells. To produce T-CD4 T cells, ITK$^{-/-}$ BM (CD45.2) and CIITA^{Tg} BM (CD45.1 and CD45.2) were introduced to Aβ$^{-/-}$ hosts (CD45.1) (ITK$^{-/-}$ + CIITA^{Tg} → Aβ$^{-/-}$) at the ratio of 3:1. Mice were sacrificed 5-6 weeks after the BM transplantation. Total thymocytes were used to analyze T cell subsets. The numbers in the dot plots indicate the percentages of gated cells derived from each BM type using the CD45 allele. Data are representative of four mice in each group. (B) CD4 T cell selection efficiency was expressed as a ratio using the formula (% CD4 SP / % DP of ITK$^{-/-}$) / (% CD4 SP / % DP of the partner) as described in the text. The dotted line indicates the ratio of 1. (C and D) Splenic cells from the same chimeras in (A) were used to assess peripheral CD4 and CD8 T cell compartments. Representative staining data are shown in (C) and the bar graphs in (D) are mean±SE from 4 mice in each group. (E) CD44 and CD62L expression of splenic CD4 T cells in (C) was analyzed. ITK$^{-/-}$ BM was from Dr. Pam Schwartzberg's lab.

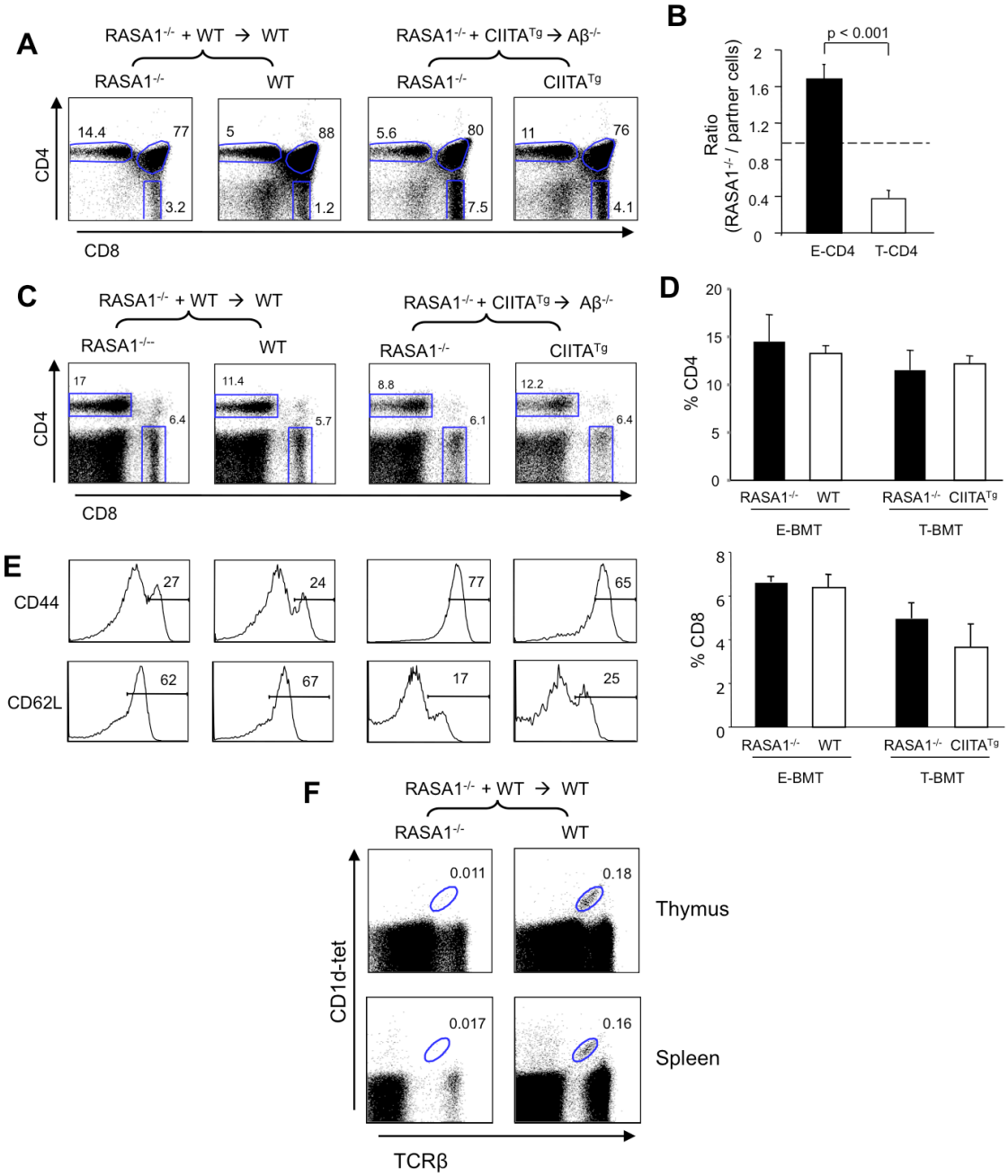
A
RASA1-/- + WT → WT
RASA1-/- + CIITATg → Aβ-/-
RASA1-/-
WT
RASA1-/-
CIITATg
CD4
CD8
14.4
77
3.2
5
88
1.2
5.6
80
7.5
11
76
4.1
B
p < 0.001
Ratio
(RASA1-/- / partner cells)
2
1.6
1.2
0.8
0.4
0
E-CD4
T-CD4
C
RASA1-/- + WT → WT
RASA1-/- + CIITATg → Aβ-/-
RASA1-/--
WT
RASA1-/-
CIITATg
CD4
CD8
17
6.4
11.4
5.7
8.8
6.1
12.2
6.4
D
% CD4
20
15
10
5
0
RASA1-/-
WT
RASA1-/-
CIITATg
E-BMT
T-BMT
% CD8
8
6
4
2
0
RASA1-/-
WT
RASA1-/-
CIITATg
E-BMT
T-BMT
E
CD44
27
24
77
65
CD62L
62
67
17
25
F
RASA1-/- + WT → WT
RASA1-/-
WT
CD1d-tet
TCRβ
0.011
0.18
Thymus
0.017
0.16
Spleen

Figure 2.2 T- and E-CD4 T cell development in the absence of RASA1
(A) BM chimeras were constructed as indicated using the ratio of 1:1 of BM cells. Mice were sacrificed 5-6 weeks after transplantation and total thymocytes were analyzed. The numbers in the dot plots indicate the percentages of gated cells derived from each BM types (N=6). (B) CD4 selection efficiency of $RASA^{-/-}$ cells relative to the co-transferred partner cells were calculated as in Figure 2.1. The dotted line indicates the ratio=1. (C and D) Splenic cells from the same chimeras in (A) were used to assess peripheral CD4 and CD8 T cells. Representative staining data are shown in (C) and the bar graphs in (D) are mean±SD (N=6). (E) CD44 and CD62L expression of splenic CD4 T cells in (C). (F) iNKT cell development was severely reduced in the absence of RASA1. Numbers indicate the percentages of iNKT cells among total thymocytes or splenocytes. $RASA1^{-/-}$ BM was from Dr. Philip King's lab.

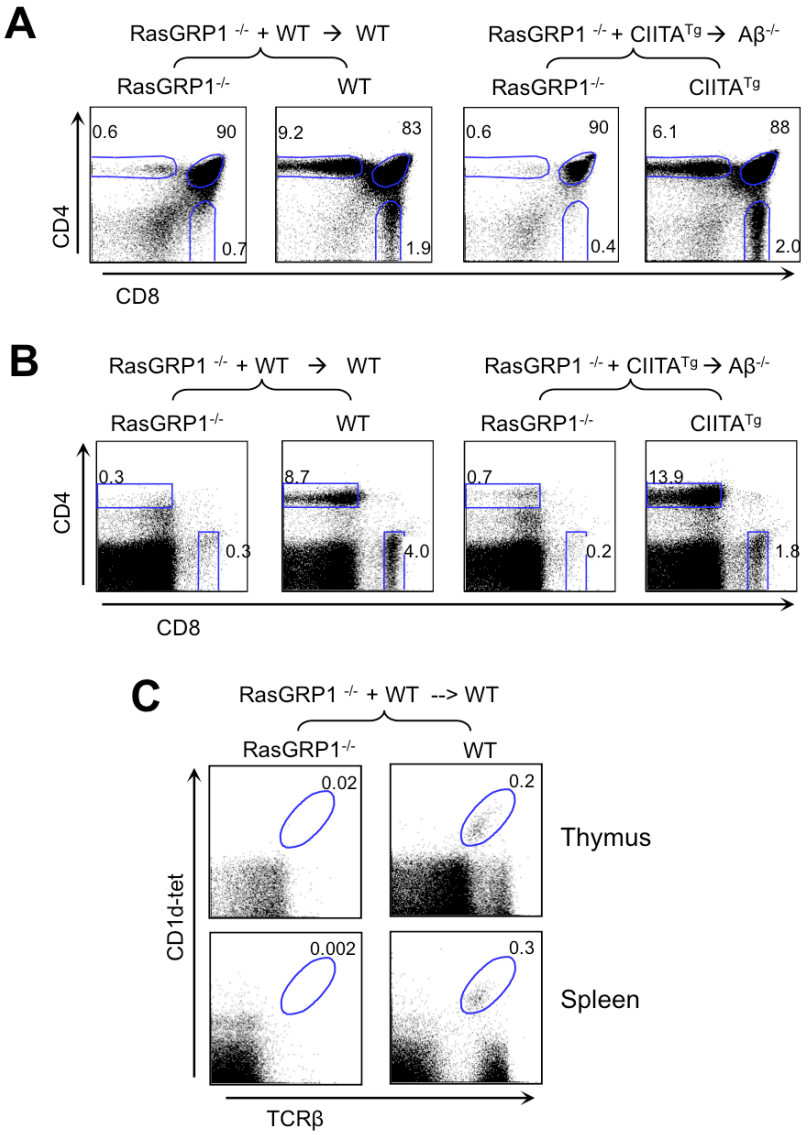

Figure 2.3 RasGRP1 is essential for both T- and E-CD4 T cell generation
(A) To generate E-CD4 T cells, RasGRP1$^{-/-}$ BM (CD45.2) and WT BM (CD45.1 and CD45.2) were mixed and co-transferred into B6.SJL hosts (CD45.1) (RasGRP1$^{-/-}$ + WT → WT) at the ratio of 3:1. RasGRP1$^{-/-}$ bone marrow (CD45.2) and CIITA^{Tg} BM (CD45.1 and CD45.2) were mixed and co-transferred into Aβ$^{-/-}$ hosts (CD45.1) (RasGRP1$^{-/-}$ + CIITA^{Tg} → Aβ$^{-/-}$) at the ratio of 3:1 to generate T-CD4 T cells. Mice were sacrificed 5-6 weeks after transplantation. Total thymocytes (A) and splenocytes (B) from indicated mice were analyzed to assess CD4 and CD8 T cell compartments. The numbers in the dot plots indicate the percentages of gated cells derived from each BM types. (C) iNKT cell development was severely reduced in the absence of RasGRP1. Numbers indicate the percentages of iNKT cells among total thymocytes or splenocytes. Data are representative of five mice. RasGRP1$^{-/-}$ BM was from Hung-Sia Teh's lab. The experiments were done by Lingqiao Zhu.

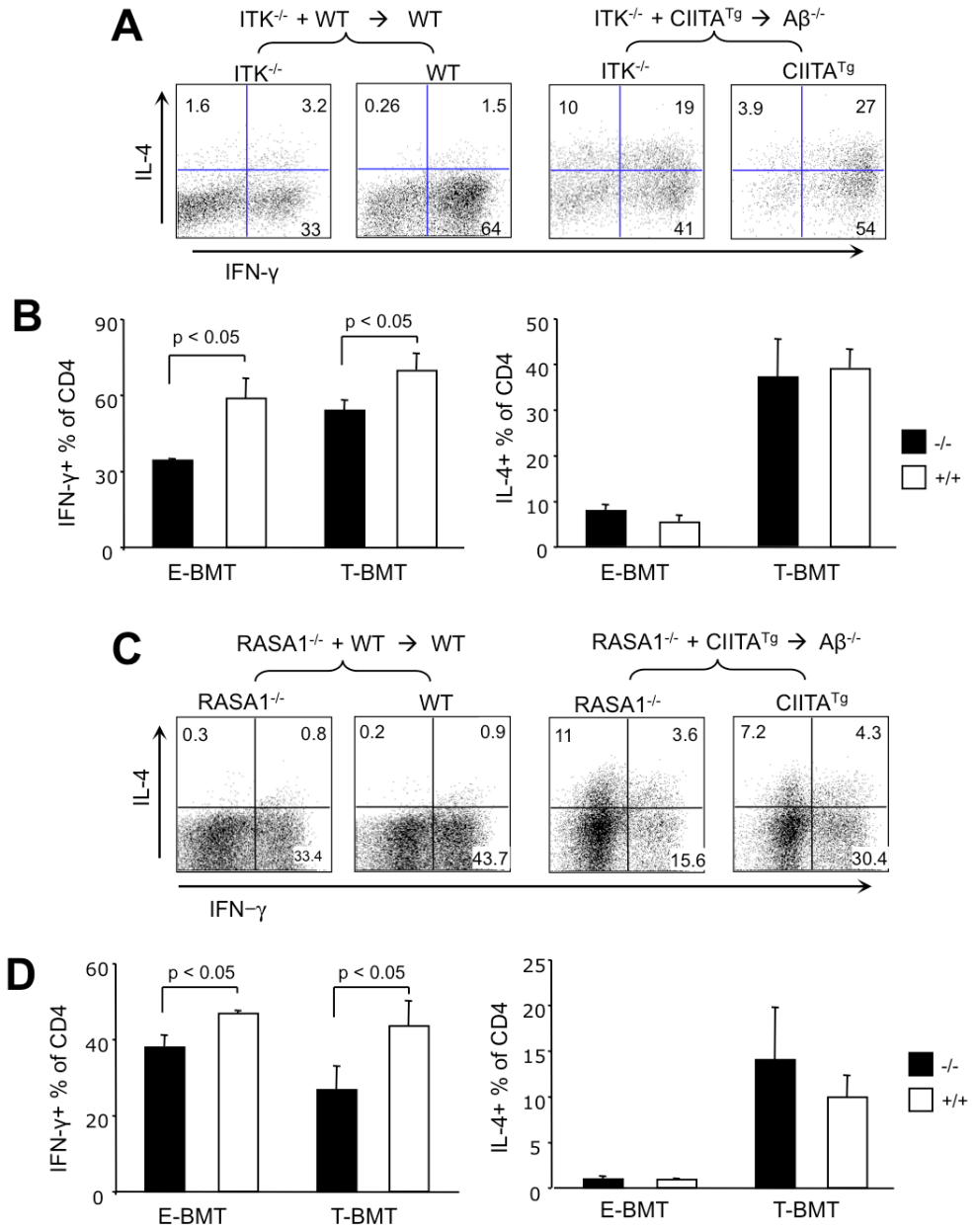

Figure 2.4 Role of ITK and RASA1 in the cytokine expression
Splenic CD4 T cells from indicated mice were enriched and cultured under the Th1 differentiating conditions as described in the Materials and Methods followed by intracellular staining of IFN-γ and IL-4. (A, C) Representative staining of ITK$^{-/-}$ (A) or RASA1$^{-/-}$ (C) BM chimeras. The numbers in the dot plots indicate the percentages of gated cells derived from each BM type. (B, D) Summary of the percentage of IFNγ- or IL-4-producing cells in the experiments of (A) and (C), respectively. Shown are mean±SD of 3 and 4 mice of ITK and RASA1, respectively.

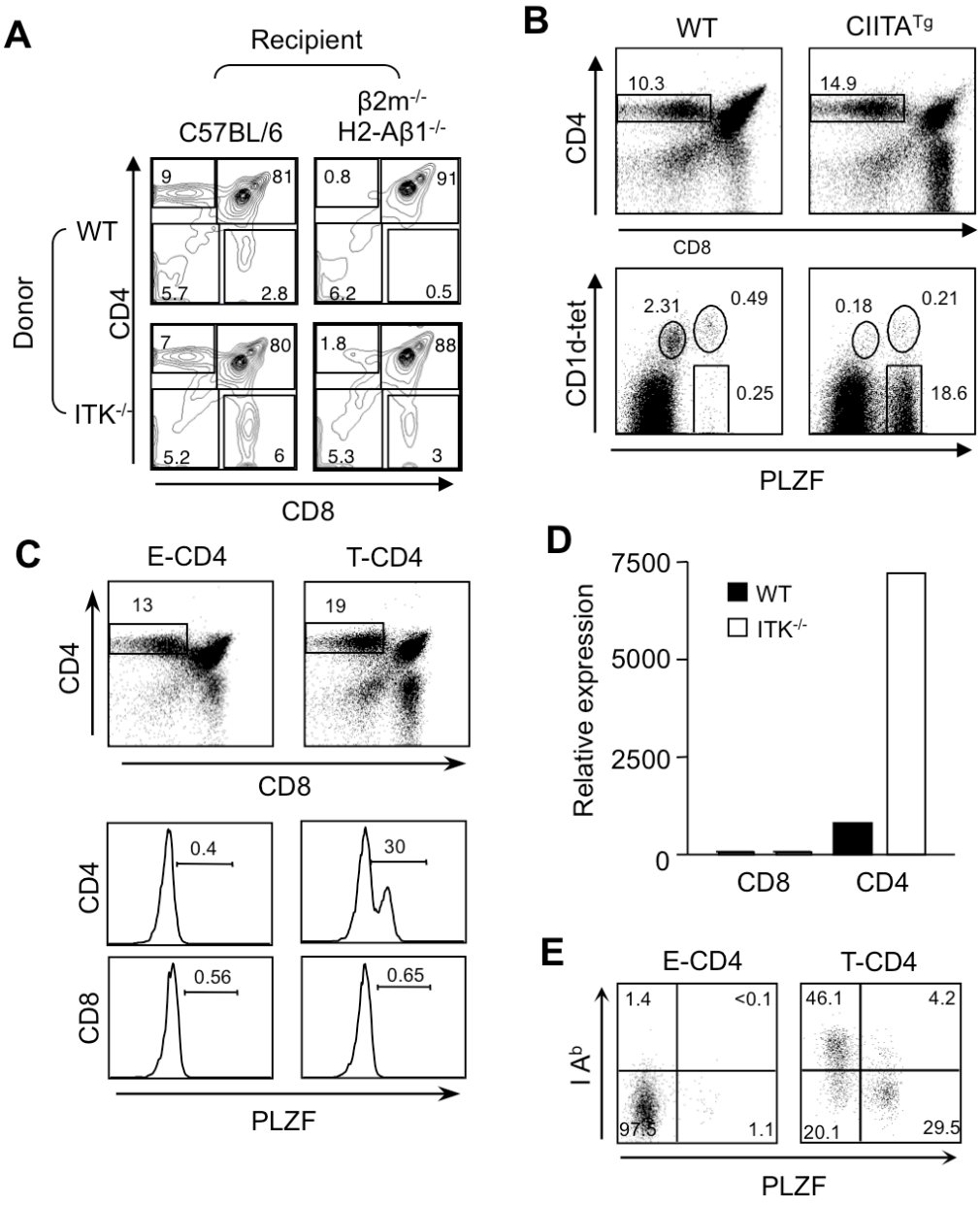

Figure 2.5 Expression and role of PLZF in hematopoietic cell-selected CD4 T cells. (A) WT or ITK$^{-/-}$ BM cells were transferred to B6 or mice lacking both MHC class I and II. Representative CD4 and CD8 profiles of total thymocytes were shown. The numbers in the dot plots indicate the percentages of donor cells. (B) Representative CD4 and CD8 staining of total thymocytes from WT and CIITA^{Tg} mice (top panels) and PLZF staining of CD4 SP gated population shown above (bottom panels). (C) T- and E-CD4 T cells were generated using WT→WT and Tg→Aβ$^{-/-}$ mice, respectively. Total thymocytes were stained to assess PLZF expression. Shown are CD1d-tetramer negative CD4 and CD8 SP cells. (D) CD4 and CD8 T cells from WT and ITK$^{-/-}$ mice were sorted and used to prepare RNA and the level of PLZF mRNA was measured using quantitative RT-PCR. (E) The mice used in (B) were examined to assess PLZF and IA^{b} expression in CD4 SP thymocytes. (A) and (D) were done by Dr. Pam Schwartzberg's lab.

2.5 Discussion

In the current study we showed that the same TCR signaling modulation affected T- and E-CD4 development oppositely. Decreased TCR signaling due to ITK deficiency reduced E-CD4 T cell selection efficiency as expected but enhanced that of T-CD4 T cells. On the contrary, increased Ras activity in the RASA1$^{-/-}$ model resulted in more E-CD4 but less T-CD4 T cell generation (Table 2.1). Based on these observations, we propose that T-CD4 T cell selection prefer weak TCR signals compared to E-CD4 T cell selection. Since thymocyte survival requires a certain amount of intracellular signaling, it is likely that additional signaling pathways are involved to compensate the differences in TCR signaling properties. One good candidate is the SLAM-SAP pathway, which is essential for T-CD4 but not required for E-CD4 T cell generation.

Table 2.1 Summary of TCR signaling modulations on T- and E-CD4 selection

	TCR signaling	E-CD4 selection	T-CD4 selection
ITK$^{-/-}$	↓	↓	↑
RASA1$^{-/-}$	↑	↑	↓
RasGRP1$^{-/-}$	↓↓	N/A	N/A

Contrary to RASA1 deficiency that compromised both iNKT and T-CD4 T cell development (Fig 2.2), ITK deficiency that enhanced T-CD4 T cell generation (Fig 2.1) resulted in decreased iNKT cell generation (50). One potential reason for the different effect of ITK deficiency on iNKT and T-CD4 development lies in the diversity of the TCR repertoire. iNKT cells are known to have a limited TCR repertoire and thus the range of TCR signaling strength that allows for their survival would also be very narrow. In contrast, T-CD4 T cells with a diverse TCR repertoire respond to a wider signaling window and thus survive better. Alternatively, ITK may play a crucial role for the maturation of iNKT cells, and yet may not be required for T-CD4 T cell development.

We have demonstrated similar requirements with T-bet and IL-15Rα, both of which are important for iNKT cell but not for T-CD4 T cell development. Therefore, the developmental processes of T-CD4 T cells and iNKT cells are distinct from each other despite certain similarities.

Both ITK$^{-/-}$ and RASA1$^{-/-}$ T-CD4 T cells maintained their potential to express IL-4 under Th1 differentiation conditions. We suspect that although the selection efficiency was affected in the absence of ITK or RASA1 due to the change in signaling quality overall, CD4 T cells in the periphery must have received an adequate amount of signaling during development in the thymus and thus have survived. Therefore, it is not surprising that these cells possess the typical T-CD4 T cell properties. In addition, RASA1$^{-/-}$ T-CD4 T cells seem to have more IL-4^{+} cells and fewer IFN-γ expressing cells compared to their WT partner T-CD4 T cells, indicating that increased Ras activity enhances the potential of T-CD4 T cells to express IL-4. This is consistent with a previous report that reduced Ras activity results in decreased IL-4 production (51).

PLZF was expressed not only in T-CD4 T cells but also in ITK$^{-/-}$ CD4 T cells. It has been reported that ITK deficiency impairs E-CD4 T cell generation but enriches an innate-like CD4 T cell population (47). It is likely that the PLZF-expressing sub-population of CD4 T cells in ITK$^{-/-}$ mice were indeed these innate-like cells. If this were the case, PLZF expression during CD4 T cell development correlates well with their signaling quality in determining T cell fate. Moreover, PLZF^{+} CD4 T cells are present in humans (48), consistent with the idea that they represent a similar innate-like CD4 T cell

population. Together, these observations suggest the presence of a CD4 T cell population that has gone through hematopoietic cell-mediated selection in both humans and WT mice. Supporting this notion, Tanaka *et al.* have reported that a fraction of CD4 T cells in WT mice possess the memory/effector phenotype and constitutively express IL-4 (52). However, the frequency of these cells seems to be very low in WT mice presumably due to dominant TEC-mediated selection.

We observed that not all T-CD4 T cells express PLZF in the thymus. A recent study also reported the same pattern of PLZF expression in thymocyte-selected CD4 T cells in humans (53). Moreover, $PLZF^+$ cells express a higher level of IL-4 than CD4 T cells that do not express PLZF (48). A similar observation was made in γδ T cells and it is proposed that $PLZF^+$ and $PLZF^-$ γδ T cells represent two distinct lineages (53). It is plausible, therefore, that T-CD4 T cells are comprised of two sub-populations with different characteristics associated with PLZF expression. Perhaps, the TCR repertoires are different between the two populations. It is well known that there are two types of NKT cells that are CD1d restricted. One population, named iNKT cells, shows the limited TCR usage, and the other has a more diverse repertoire. It is not clear what regulates the generation of these two populations. However, as developmental requirements of the two are not identical, it is likely that the difference in signaling during development could account for the different characteristics of the two. The other possibility is that, upon positive selection, T-CD4 T cells receive an additional signal to up-regulate PLZF expression. This suggests a two-step process of T-CD4 T cell generation in the thymus. The signal given at the first step is primarily via the TCR-MHC

interaction and results in thymocyte survival, while a subsequent and unidentified second signal induces PLZF expression.

Together, the current study suggests that thymocytes with TCRs that deliver weak signals are directed to get selected by hematopoietic cells, express PLZF, and become effector cells immediately after selection. It is likely that human CD4 T cells are generated by similar mechanisms, and further studies on human T-CD4 T cells are warranted to gain a better understanding of the development and function of these innate CD4 T cells in humans.

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Chapter III

Thymocyte-selected CD4 T cells suppress CD8 T cell-mediated immunity against *Listeria monocytogenes*

3.1 Abstract

Conventional CD4 T cells developed by thymic epithelial cells (E-CD4 T cells) play critical roles in adaptive immunity by regulating immune responses and the development of immune memory. Previously, we discovered a new CD4 T cell population that is selected by MHC class II expressing thymocytes and named T-CD4 T cells. In addition to different developmental requirements, T-CD4 T cells exhibit distinct effector functions by producing cytokines immediately after stimulation. Here, we report the suppressive role of T-CD4 T cells on CD8 T cell responses during bacteria infections. Using a mouse model of *Listeria monocytogenes* infection, we observed that T-CD4 T cells control immune responses during the adaptive, but not the innate phase. In the presence of T-CD4 T cells, *Listeria*-specific CD8 T cells decreased in frequency as well as in killing efficiency. Furthermore, T-CD4 T cells suppressed the development of CD8 T cell-mediated immune memory, leading to high bacterial burden upon re-infection. In addition, this suppression function was dependent on cell-cell contact. Thus, our study revealed a previously unknown suppressor function for T-CD4 T cells in anti-bacterial immunity.

3.2 Introduction

Conventionally, CD4 T cells modulate adaptive immunity by differentiating into helper cell subsets and producing cytokines according to the environmental signals. For example, Th1 cells produce the pro-inflammatory Th1 cytokine IFN-γ, and facilitate CD8 T cell-mediated cellular immunity against intracellular pathogens, whereas Th2 cells produce cytokines IL-4, IL-5 and IL-13, which are critical for B cell differentiation and antibody-mediated humoral immunity. Moreover, CD4 T cells are required for the development of CD8 T cell and B cell immune memory in various infection models (1-6).

It is well established that conventional murine CD4 T cells are selected on thymic epithelial cells (TEC) that express TCR ligands, the peptide-MHC class II complexes (7). However, studies have indicated a non-conventional way to generate CD4 T cells in humans, which is independent of TEC-expressed MHC class II (8-10). The alternative cell types supporting CD4 T cell development seem to be hematopoietic cells, particularly thymocytes (11-13). Using mouse models, we have demonstrated that indeed MHC class II-expressing thymocytes successfully mediate CD4 T cell selection independent of TEC-expressed MHC class II (14). CD4 T cells selected by MHC class II on thymocytes are called thymocyte-selected CD4 (T-CD4) T cells, as distinguished from conventional TEC-selected CD4 (E-CD4) T cells. Unlike E-CD4 T cells, T-CD4 T cells secrete IL-4 even after having undergone Th1 differentiation (15). Moreover, T-CD4 T cells rapidly produce Th1 and Th2 cytokines upon TCR stimulation both *in vitro* and *in vivo* (14, 15). These characteristics suggest that T-CD4 T cells resemble invariant natural

killer T (iNKT) cells in their innate-like functional characteristics (16-20), although these two populations differ in TCR ligands and NK cell marker expression. In addition, mice possessing T-CD4 T cells are protected from allergen-induced airway inflammation (21) and experimental autoimmune encephalomyelitis (EAE) (22), which are clear demonstrations of T-CD4 T cell functional characteristics distinct from those of E-CD4 T cells. However, the role of T-CD4 T cells in immune responses against bacterial infections has not been addressed.

Listeria monocytogenes is a facultative intracellular pathogen. The pathogenesis and the immune responses triggered by the infection have been widely studied in mouse models (23). Upon infection, *L. monocytogenes* are taken up primarily by phagocytes and killed within phagosomes. However, they can escape from phagosomes into the cytosol through listeriolysin O (LLO)-dependent mechanisms (24), and thence are processed and presented through the MHC class I pathway, inducing robust cellular immunity (25-28). *L. monocytogenes*-specific CD4 and CD8 T cells are major mediators of primary as well as memory immune responses. They exhibit similar responding kinetics with regards to activation, expansion and contraction (27), and produce large amounts of the Th1 cytokine IFN-γ, which is critical for the anti-microbial activity of macrophages (29-31) and the up-regulation of MHC expression on antigen presenting cells (APCs) (32-34). In addition, CD8 T cells (also called cytotoxic T lymphocytes, CTLs) can directly kill infected cells, which is an important mechanism of bacterial clearance (35-38).

Immunological memory protects host organisms by clearing recurrent infections with enhanced rapidity and effectiveness. Studies have reported the essential role of E-CD4 T cells in the establishment and development of memory immunity against *L. monocytogenes* infection (1, 2, 39, 40). When E-CD4 T cells are present, mice are able to mount a primary immune response resulting in the generation of *L. monocytogenes*-specific effector CD8 T cells and the elimination of bacteria. Meanwhile, memory CD8 T cells are generated, which effectively eliminate the bacteria upon re-infections. In the absence of E-CD4 T cells, CD8 T cells suffer from a defective memory immune response upon re-infection, although during a primary *L. monocytogenes* infection, the mice clear bacteria with a similar efficiency to when E-CD4 T cells are present (1, 2, 39-41). This suggests that E-CD4 T cells play a critical role in establishing immunological memory to *L. monocytogenes* infection. Here we report that, in contrast to E-CD4 T cells, T-CD4 T cells resulted in suppressed CTL responses against *L. monocytogenes* during both primary and secondary infection, as evidenced by reduced frequency of *L. monocytogenes*-specific CD8 T cells and high bacterial burden. Moreover, this suppressive function of T-CD4 T cells is cell-contact dependent and is distinct from that of Foxp3-expressing regulatory T cells. Thus, our work has identified a novel regulatory role of T-CD4 T cells in the immune response to infections by bacterial pathogens.

3.3 Materials and Methods

Mice

CIITA^{Tg} (Tg) and WT littermates were bred and kept under specific pathogen–free conditions in the animal facility at the University of Michigan Medical School. C57BL/6 mice at 7-8 wk of age were purchased from Jackson or NCI. CD45.1^{+} B6 mice (B6.SJL-*Ptprc*^{a}/BoyAiTac) and CD45.1^{+} Aβ$^{-/-}$ mice (B6.SJL-*Ptprc*^{a}/BoyAiTac *H2-Ab1*^{tm1Gru}) (7-8 wk of age) were purchased from Taconic. All mice used were 6-12 wk of age. All experimental procedures and protocols were approved by the University Committee on Use and Care of Animals.

Bacteria strain

The recombinant strain of *L. monocytogenes* expressing a secreted form of ovalbumin (rLM-OVA) was previously described (42). rLM-OVA was grown in brain heart infusion broth (Difco) to mid-exponential phase prior to injection *i.v.* into mice. 5×10^{5} rLM-OVA are equivalent to 0.5 LD_{50} for infection (43).

T- and E-CD4 T cell generation

T- and E-CD4 T cells were generated by transferring BM cells from CIITA^{Tg} (Tg) or WT to lethally irradiated Aβ$^{-/-}$ (MHC class II-deficient) or WT hosts, respectively. Eight weeks after the transfer, the hosts were sacrificed and CD4 T cells were enriched from total splenocytes using MACS anti-mouse CD4 microbeads (Miltenyi Biotec).

Adoptive transfer model

The recipients were sub-lethally irradiated (500 rad). Three days later, each of them received ~3x10^7 CD4 T cell-depleted splenocytes from WT mice mixed with 10^7 E-CD4 or T-CD4 T cells obtained from BM chimeric mice using anti-mouse CD4 microbeads (Miltenyi Biotec). The mice were rested overnight and then infected with rLM-OVA as indicated in each experiment via the *i.v.* route.

Quantification of bacterial load

Livers were removed and put into 14-ml tubes containing 10 ml PBS with 0.2% NP40. The tissues were homogenized by using a homogenizer (The Lab Depot, Inc.) at maximum speed for 30 seconds. Tissue homogenates were subjected to 10-fold serial dilutions and then plated onto Luria broth agar plates. The number of colonies formed were counted after 24 hour of incubation at 37°C.

Stimulation of rLM-OVA-specific T cell population ex vivo and cytokine intracellular staining

Splenocytes from infected and naïve mice were stimulated with SIINFEKL peptide (1ug/ml, Biomatik Corporation) to detect rLM-OVA-specific CD8 T cells or with LLO (listeriolysin O) 190-201 to detect rLM-OVA-specific CD4 T cells. Splenocytes were incubated with 1ug/ml peptide for five hours, and monensin was added before the last three hours to stop the secretion of the synthesized cytokines. After five hours, cells were washed and stained with anti-CD4 and anti-CD8 antibody. Next, cells were fixed in

2% paraformaldehyde for 30 min at room temperature, permeabilized with 0.2% saponin (Sigma), and stained with anti-IFN-γ (XMG1.2) for flow cytometry.

Flow cytometry

Antibodies specific for CD4 (GK1.5), CD8 (53-6.7), CD45.1 (A20), CD45.2 (104), TCRβ (H57-597), NK1.1 (PK136), Ly-6G (Gr-1), CD11c (HL3), CD11b (M1/70), F4/80 (6F2), I-A^b (AF6-120.1), H-$2K^b$ (AF6-88.5) were from PharMingen, BD Bioscience. The antibody against Foxp3 (FJK-16s; eBioscience) was used according to the staining protocol provided by the company. Samples were analyzed using a FACS Canto flow cytometer (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

In vivo CTL assay

Splenocytes from naive mice, depleted of red blood cells, were split into two portions. One was labeled with a high concentration of CFSE (5.0 nM, 2 x 10^7 cells/ml) and pulsed with OVA 257-263 peptides as the target population. The other was labeled with a low concentration of CFSE (0.5 nM, 2 x 10^7 cells/ml) without peptides as a control. Cells were washed and then the two populations were mixed at 1:1 ratio (4-5 x 10^6 cells each). Cells were injected into rLM-OVA infected or PBS-treated mice. Mice were sacrificed at indicated time points, and single-cell suspensions of spleens were analyzed by flow cytometry. The killing efficiency was calculated as follows: 100 - ([(% peptide pulsed in infected/% unpulsed in infected)/(% peptide pulsed in uninfected/% unpulsed in uninfected)] x 100).

CFSE proliferation assay

Cells were purified and incubated with CFSE (5 nM) at 37°C for 10 minutes, washed three times, and then cultured for 3-4 days under indicated conditions. Cell proliferation was measured by CFSE dilution using flow cytometry.

Real-time RT-PCR

Total RNA of the splenocytes from infected and control mice were extracted using TRIzol (Invitrogen). The PCR reactions were performed and analyzed using the iCycleriQ™ (BioRad). Conditions: 95°C for 5min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec. Primers: GAPDH Forward (F): ctccactcacggcaaattca, Reverse (R): cgctcctggaagatggtgat. IL-1 F: caaccaacaagtgatattctccatg, R: gatccacactctccagctgca. IL-10 F: ggttgccaagccttatcgga, R: acctgctccactgccttgct. IFN-γ F: tcaagtggcatagatgtggaagaa, R: tggctctgcaggattttcatg. TNF-α F: ccccaaagggatgagaagtt, R: cacttggtggtttgctacga

Statistical analysis

The statistical analysis was done using Prism software. A two-tailed *t*-test was used for statistical analysis. p values of ≤ 0.05 were considered significant, and p values > 0.05 were not indicated and were considered statistically insignificant.

3.4 Result

3.4.1 CTL immune responses were decreased in mice with T-CD4 T cells

To ascertain the role of T-CD4 T cells during bacterial infection, we employed a well-established murine model of *L. monocytogenes* infection, and compared the immune responses between two groups of mice: wild type (WT) and CIITA^{Tg} (Tg) mice. Tg mice express MHC class II on thymocytes in addition to TEC, and thus thymocytes can be selected by both cell types to become T- or E-CD4 T cells. As a consequence, Tg mice have both T- and E-CD4 T cells. By contrast, WT mice have only E-CD4 T cells because they express MHC class II only on TEC in the thymus. Therefore, the differences in the immune responses between WT and Tg mice is likely caused by the presence of T-CD4 T cells in addition to E-CD4 T cells in Tg mice.

To facilitate the detection of the dominant *Listeria*-specific cell populations, we chose a recombinant strain of *L. monocytogenes* expressing a secreted form of chicken ovalbumin (rLM-OVA). The primary immune response was analyzed on day 7 after *i.v.* infection. The host bacterial loads in the spleen and the liver were under the limit of detection at the time of analysis, and the numbers of splenic CD4 and CD8 T cells were comparable between Tg and WT groups (Fig 3.1 A). To measure the pathogen-specific T cell response, CD4 and CD8 splenocytes were stimulated separately *ex vivo* with rLM-OVA-specific peptides for five hours. T cells recognizing the peptide responded by producing IFN-γ, which was detected by intracellular staining. The two groups of mice exhibited different antigen-specific T cell responses; Tg mice decreased in rLM-OVA-

specific CD8 T cells (Fig 3.1 B) but increased in the percentage and the number of rLM-OVA-specific CD4 T cells in comparison with WT mice (Fig 3.1 C).

Another important indicator of the efficiency of the response is the potency of CTL cytotoxicity (35-38). Therefore, an *in vivo* killing assay was employed to measure *L. monocytogenes*-specific killing efficiency (3, 44). In brief, total splenocytes were either labeled with a high concentration of CFSE and pulsed with OVA peptides as a target population, or labeled with a low concentration of CFSE without peptides as a control. These two populations were mixed 1:1 and injected into infected Tg and WT mice and into uninfected naïve mice as controls. While the ratio of the two populations injected would stay steady in naïve mice, the $CFSE^{hi}$ cells would decrease in infected mice as they were recognized and killed by rLM-OVA-specific CTLs. Three hours after the injection, mice were sacrificed, and the composition of the injected cells in the spleens were analyzed by flow cytometry. As expected, the ratio of $CFSE^{hi}$ to $CFSE^{lo}$ populations stayed around 1:1 in naïve mice but decreased in infected mice (Fig 3.1 D). However, the difference in the killing efficiency between the two infected groups was not significant.

3.4.2 Memory immune responses against *L. monocytogenes* were impaired in the presence of T-CD4 T cells

It has been demonstrated that E-CD4 T cells play a critical role in the establishment of optimum CTL immune memory against *L. monocytogenes* (1, 2, 39, 40). To study the role of T-CD4 T cells in the course of CTL memory generation, Tg and WT mice were infected with a low dose of rLM-OVA, rested for one month, and re-infected with a high dose of rLM-OVA. Three days after the second infection, the numbers of

total CD4 and CD8 splenocytes were comparable between Tg and WT mice (Fig 3.2 A). However, Tg mice had decreased rLM-OVA-specific CD8 T cells (Fig 3.2 B), although rLM-OVA-specific CD4 T cells were comparable between the two groups (Fig 3.2 C). To test the effector function of CTLs *in vivo*, we measured the capability of mice to clear bacteria by counting the number of live bacteria harvested from the liver. We observed higher bacterial burdens in Tg than in WT mice (Fig 3.2 D), further suggesting the immune capacities of Tg mice were less potent than that of WT mice.

3.4.3 T-CD4 T cells were responsible for the reduced anti-*Listerial* response

The reduced efficiency of anti-*Listerial* responses in Tg mice led us to hypothesize that T-CD4 T cells function to diminish anti-*Listerial* immunity. However, it was unclear whether the difference was due to T-CD4 T cell function directly or secondary to some other unknown differences between Tg and WT mice. In fact, we previously demonstrated that iNKT cells are decreased in Tg mice (45). Therefore, to eliminate the possibility that other immune cells influence the outcome of infection, we established an adoptive transfer mouse model (Fig 3.3 A). T-BMT (Tg →A$\beta^{-/-}$) and E-BMT (WT →WT) mice were constructed to obtain T- and E-CD4 T cells, respectively. These chimeric mice were sacrificed eight weeks after BM transplantation to prepare CD4 T cells from splenocytes. Total T- or E-CD4 splenocytes were mixed with CD4 T cell-depleted splenocytes from naïve WT mice and adoptively transferred to C57BL/6 mice that were sub-lethally irradiated three days prior to cell transfer. The transferred cells can be distinguished from recipient cells by different CD45 allotype markers. The sub-lethal irradiation of hosts eliminates a large portion of host lymphocytes facilitating plantation of the incoming cells and also abolishes the immune responsiveness of the

remaining host cells (46-48). Thus, the T cell immune responses are primarily attributable to the transferred cells. Flow cytometry analysis of E-CD4 and T-CD4 T cells prior to transfer showed a similar cell composition between the two (Fig 3.3 B).

Recipient mice were infected with rLM-OVA one day after cell transfer and sacrificed 7 days after infection (Fig 3.3 A). The total number of transferred CD8 T cells remained unperturbed after seven days (Fig 3.3 C), but the proportion of rLM-OVA-specific CD8 T cells was significantly lower in T-CD4 hosts than in E-CD4 hosts (Fig 3.3 D). In contrast, although the total T-CD4 T cell numbers were reduced (Fig 3.3 C), IFN-γ-expressing CD4 T cells were present at comparable levels between the two groups (Fig 3.3 E). Therefore, rLM-OVA-specific T-CD4 T cell numbers were much less than that of E-CD4 T cells. Next, we investigated whether the cytotoxic function of CD8 T cells also was affected by T-CD4 T-cells. Consistent with the reduction of IFN-γ expressing CD8 T cells, the *in vivo* CTL assay performed as in Fig 3.1 D showed significantly decreased CTL cytotoxicity of T-CD4 hosts (Fig 3.3 F). Thus, we concluded that T-CD4 T cells are responsible for suppressive effects on CTL effector function.

3.4.4 T-CD4 T cells suppressed anti-*Listerial* memory immunity

The data from adoptive transfer experiments clearly showed the role of CD4 T cells on CTLs during primary immune responses. It was anticipated that a poor primary CTL response would lead to a deficient memory response. To test this, we prepared cells and conditioned the recipient mice as described in Fig 3.3, and then followed this with rLM-OVA infection (Fig 3.4 A). We also included an additional group of host mice

receiving donor cells that did not contain CD4 T cells. Three groups of mice receiving different populations of cells (Fig 3.4 B) were infected, rested for one month, and then infected again with a high dose of rLM-OVA. Three days after the secondary infection, we found that the number of total splenocytes was lower in mice receiving T-CD4 T cells (Fig 3.4 C). Furthermore, the percentages as well as cell numbers of both CD8 and CD4 T cells specific for rLM-OVA were greatly decreased in T-CD4 hosts (Fig 3.4, D and E), indicating a greater deficiency in memory responses than in primary responses. When we measured *in vivo* responses, T-CD4 hosts showed the high median values of the bacterial burdens, which were comparable to those of mice that did not receive CD4 T cells (Fig 3.4 F).

3.4.5 T-CD4 T cells did not affect the innate response but suppressed CTL proliferation

Next, we sought possible mechanisms for the inhibitory function of T-CD4 T cells on CTL responses. We have shown that T-CD4 T cells can produce IL-4 upon stimulation *in vivo*, and also under Th1 differentiating conditions *in vitro*. Therefore, it is possible that T-CD4 T cells produce IL-4 during *L. monocytogens* infection, which might have a negative effect on CTL function not to express IFN-γ. However, IL-4 producing CD4 T cells were not detected on day seven (Fig 3.5 A). Considering the prompt responsiveness of T-CD4 T cells upon anti-CD3 stimulation, we speculated that T-CD4 T cells might respond early and alter the course of innate immune responses, which in turn might account for the suppressed adaptive immune responses (49, 50). To test this hypothesis, we first measured cytokine levels in the spleen from Tg and WT mice on day three of the primary infection. Contrary to our prediction, the mRNA expressions of IFN-

γ, TNF-α and IL-1β were comparable between the two groups (Fig 3.5 B). In addition, when we examined innate cell populations expressing Gr-1 and F4/80, both Tg and WT groups showed increased $Gr1^+$ $F4/80^+$ populations at a comparable level after infection (Fig 3.5 C).

Next, we investigated the effect of T-CD4 T cells on CTL proliferation and function. CD4 T cell-depleted splenocytes containing mainly CD8 T cells and B cells were labeled with CFSE and then stimulated with anti-CD3 antibody in the presence of E- or T-CD4 T cells obtained from the same kind of BMT mice in Fig 3.3. Three days later, CTL proliferation was measured by CFSE dilution and cytokine production was measured by intracellular staining. CTLs co-cultured with T-CD4 T cells underwent less proliferation than those cultured with E-CD4 T cells (Fig 3.5 D, left panel). However, their IFN-γ production was not significantly altered upon re-stimulation by PMA and ionomycin (Fig 3.5 D, right group).

To find out whether the suppressive effect of T-CD4 T cells on CTL proliferation is mediated by soluble factors or requires cell-to-cell contact, E- or T-CD4 T cells were stimulated for two days and the supernatants of the cell culture were collected. CFSE-labeled WT CTLs were stimulated with the anti-CD3 antibody and cultured with E- or T-CD4 T cell supernatant for 3-4 days. The CFSE dilution of CTLs was not significantly different between the two samples, and thus the supernatants from the T-CD4 T cell cultures did not alter CTL proliferation (Fig 3.5 E), indicating that soluble factors secreted by T-CD4 T cells were not involved in the suppressive function. The cell contact

dependent inhibition prompted us to assess whether T-CD4 T cells could be regulatory T cells (Treg). To test this, we examined Foxp3-expressing Treg populations in the E-CD4 or T-CD4 cells. The percentage of Foxp3^{+} cells was not significantly different between the two (Fig 3.5 F). We have shown previously that the suppressive functionality of Treg in T-CD4 T cells was not different from that seen in E-CD4 T cells (15). Therefore, the suppression observed in our study was not likely due to Treg cells.

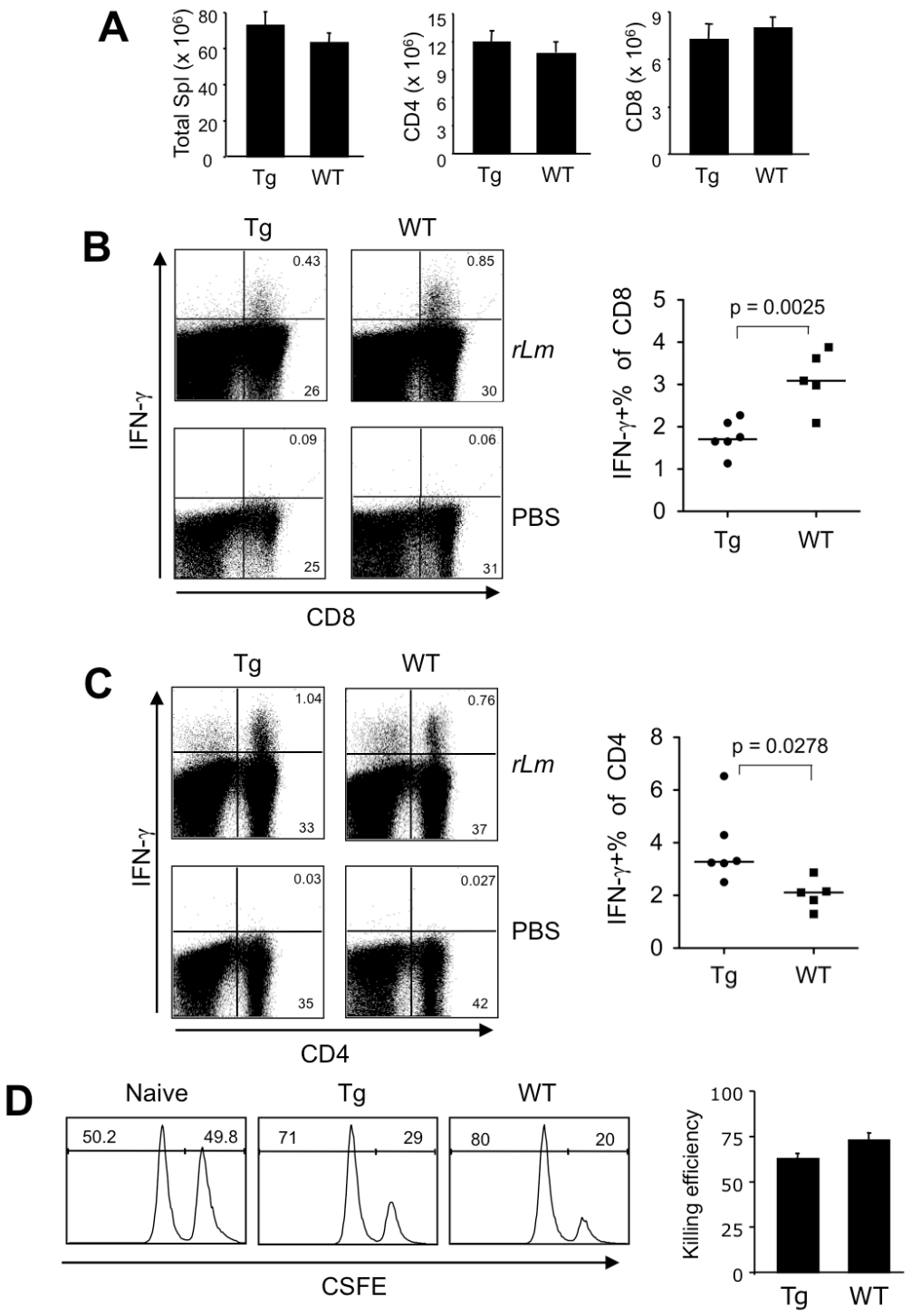
A
Total Spl (x 10^6)
80
60
40
20
0
Tg
WT
CD4 (x 10^6)
15
12
9
6
3
0
Tg
WT
CD8 (x 10^6)
9
6
3
0
Tg
WT
B
Tg
WT
0.43
26
0.85
30
rLm
0.09
25
0.06
31
PBS
IFN-γ
CD8
IFN-γ+% of CD8
p = 0.0025
5
4
3
2
1
0
Tg
WT
C
Tg
WT
1.04
33
0.76
37
rLm
0.03
35
0.027
42
PBS
IFN-γ
CD4
IFN-γ+% of CD4
p = 0.0278
8
6
4
2
0
Tg
WT
D
Naive
Tg
WT
50.2
49.8
71
29
80
20
CSFE
Killing efficiency
100
75
50
25
0
Tg
WT

Figure 3.1 Reduced anti-*Listerial* responses in Tg mice during primary infection
Tg and WT littermates were infected intravenously with rLM-OVA (5 x 10^4) or PBS. Mice were euthanized and analyzed 7 days after infection. (A) Numbers of the total splenocytes and of the indicated cell populations are shown. (B, C) Frequencies of IFN-γ-producing rLM-OVA-specific CD8 (B) and CD4 (C) T cells. Total splenocytes were stimulated in the presence of rLM-OVA peptides for 5 hours followed by intracellular cytokine staining. The values in representative FACS profiles are percentages of the total splenocyte population; the graphs on right show the percentage of CD8 (B) and CD4 (C) T cells that produced IFN-γ. The bars indicate the median value. (D) *In vivo* killing assay. A mixture of cells comprised of the OVA peptide loaded target cells ($CFSE^{hi}$) and the control cells ($CFSE^{lo}$) were injected into recipient mice. Mice were euthanized 3 hours after cell transfer. The killing efficiency was calculated as following: 100 - ([(% peptide pulsed in infected mice/% unpulsed in infected mice)/(% peptide pulsed in uninfected mice/% unpulsed in uninfected mice)] x 100). The bar graph shows mean±SE of 4 mice.

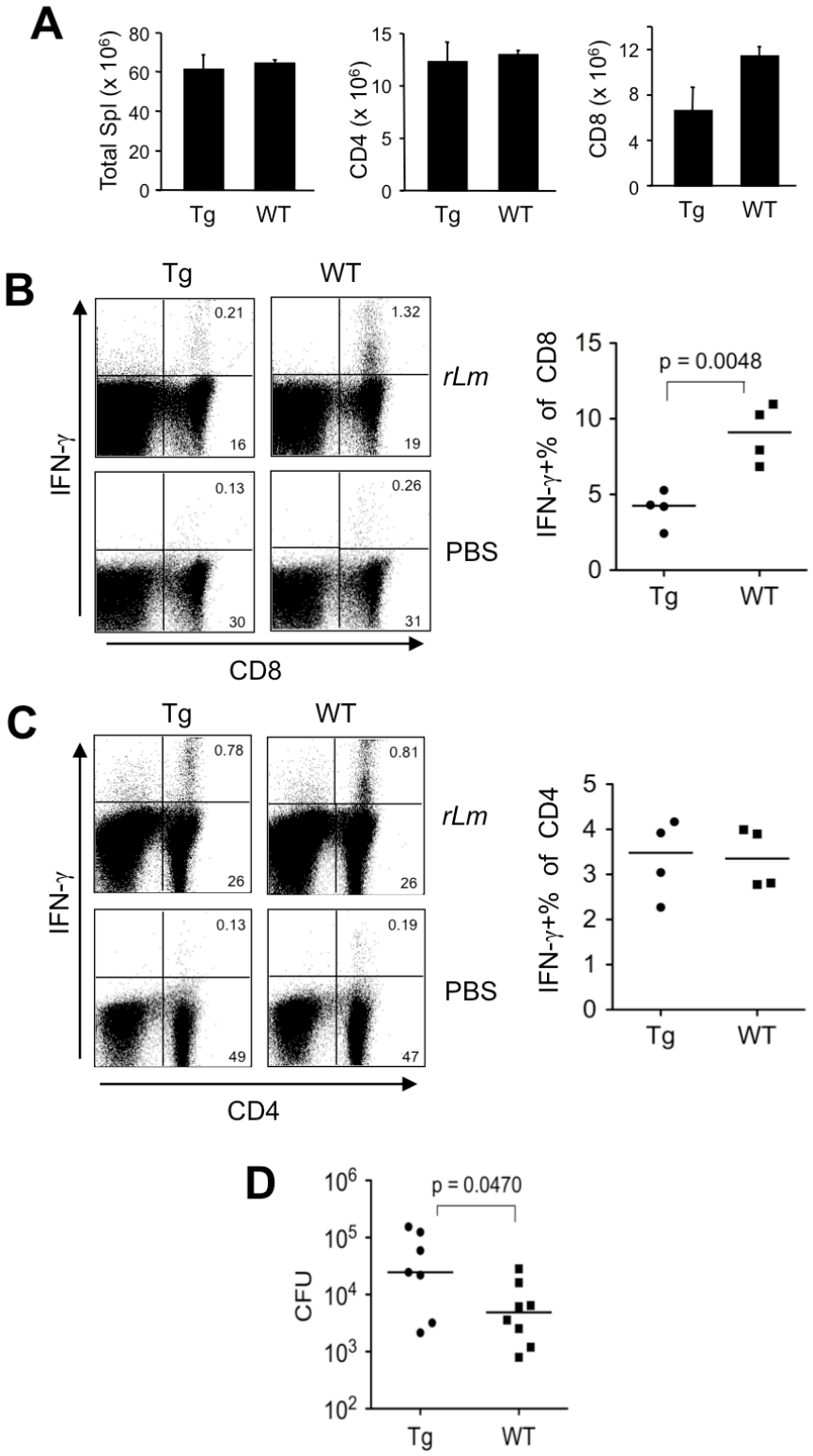
A
Total Spl (x 10^6)
80
60
40
20
0
Tg
WT
CD4 (x 10^6)
15
10
5
0
Tg
WT
CD8 (x 10^6)
12
8
4
0
Tg
WT
B
Tg
WT
0.21
16
1.32
19
rLm
0.13
30
0.26
31
PBS
IFN-γ
CD8
IFN-γ+% of CD8
p = 0.0048
15
10
5
0
Tg
WT
C
Tg
WT
0.78
26
0.81
26
rLm
0.13
49
0.19
47
PBS
IFN-γ
CD4
IFN-γ+% of CD4
5
4
3
2
1
0
Tg
WT
D
CFU
p = 0.0470
10^6
10^5
10^4
10^3
10^2
Tg
WT

Figure 3.2 Poor memory CTL response in Tg mice
Tg and WT littermates were infected intravenously with 4×10^3 rLM-OVA and rested for one month before a secondary infection with 5×10^5 rLM-OVA or PBS as a control. All the mice were sacrificed and analyzed 3 days after the secondary infection. (A) Numbers represent the total splenocytes and the indicated cell populations. (B, C) Frequencies of IFN-γ-producing rLM-OVA-specific CD8 (B) and CD4 (C) T cells. Experiments and data analyses were done as described in Fig 3.1 B and C. (D) Numbers of viable bacteria from liver homogenates are depicted. The bars indicate median values. The colony forming units from mice treated with PBS were below the detection limit of 100 CFU/mouse liver.

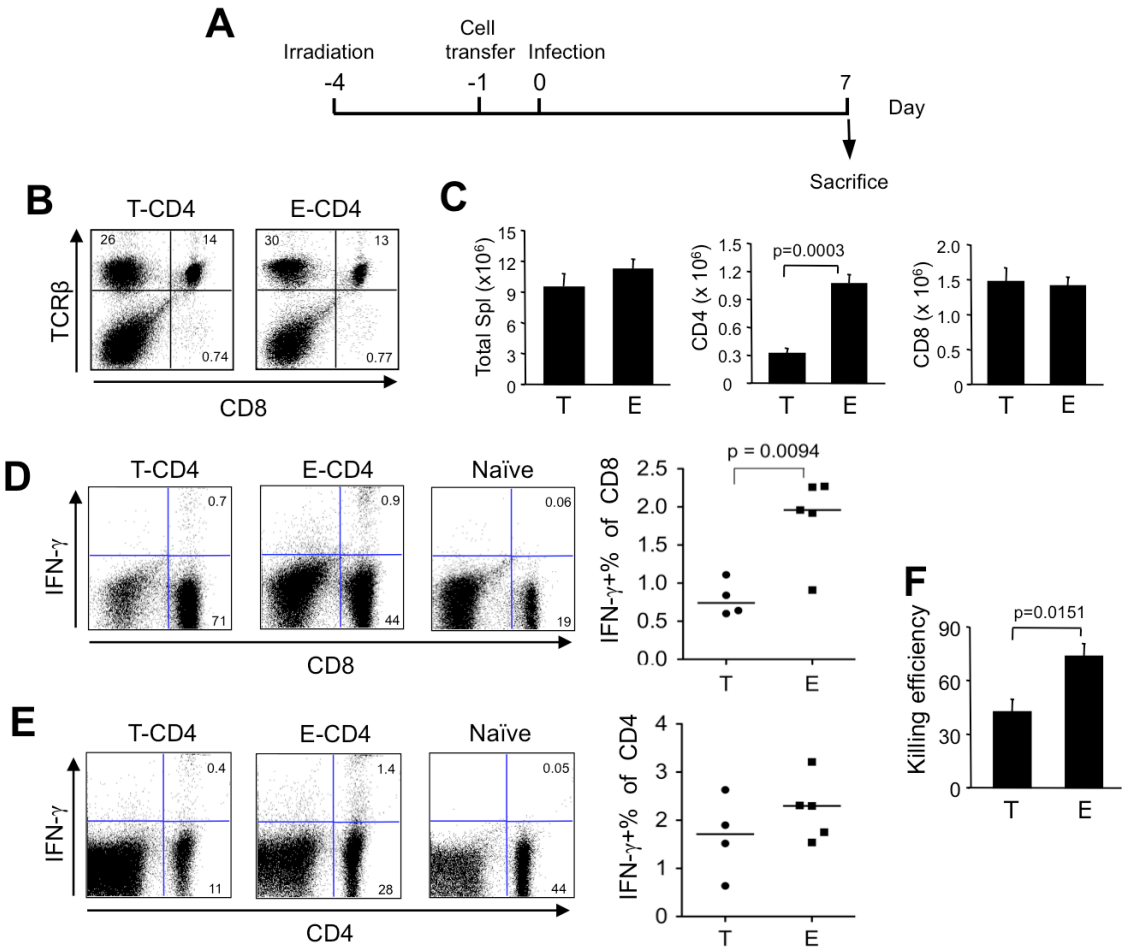

Figure 3.3 Effector CD8 T cell generation is compromised in the presence of T-CD4 cells

(A) A scheme of the experiment protocol. The infection dose was 2×10^4 rLM-OVA. (B) The composition of the CD45.1$^+$ input cell population prior to the transfer. (C) The numbers represent the total splenocytes and the indicated cells originating from the donor on day 7. p is > 0.05 if not indicated. (D and E) Frequencies of IFN-γ-producing rLM-OVA-specific CD8 (D) and CD4 (E) T cells after *in vitro* stimulation as in Fig 3.1. The FACS data shown were gated on donor populations. The values in representative FACS profiles are percentages of total donor splenocytes; the graphs on right represent percentages of total donor CD8 (D) or CD4 (E) T cells that produced IFN-γ. The bars indicate the median value. (F) *In vivo* killing assay. Experiments were performed as described in Fig 3.1 D, except that mice were sacrificed 24 hours after cell transfer.

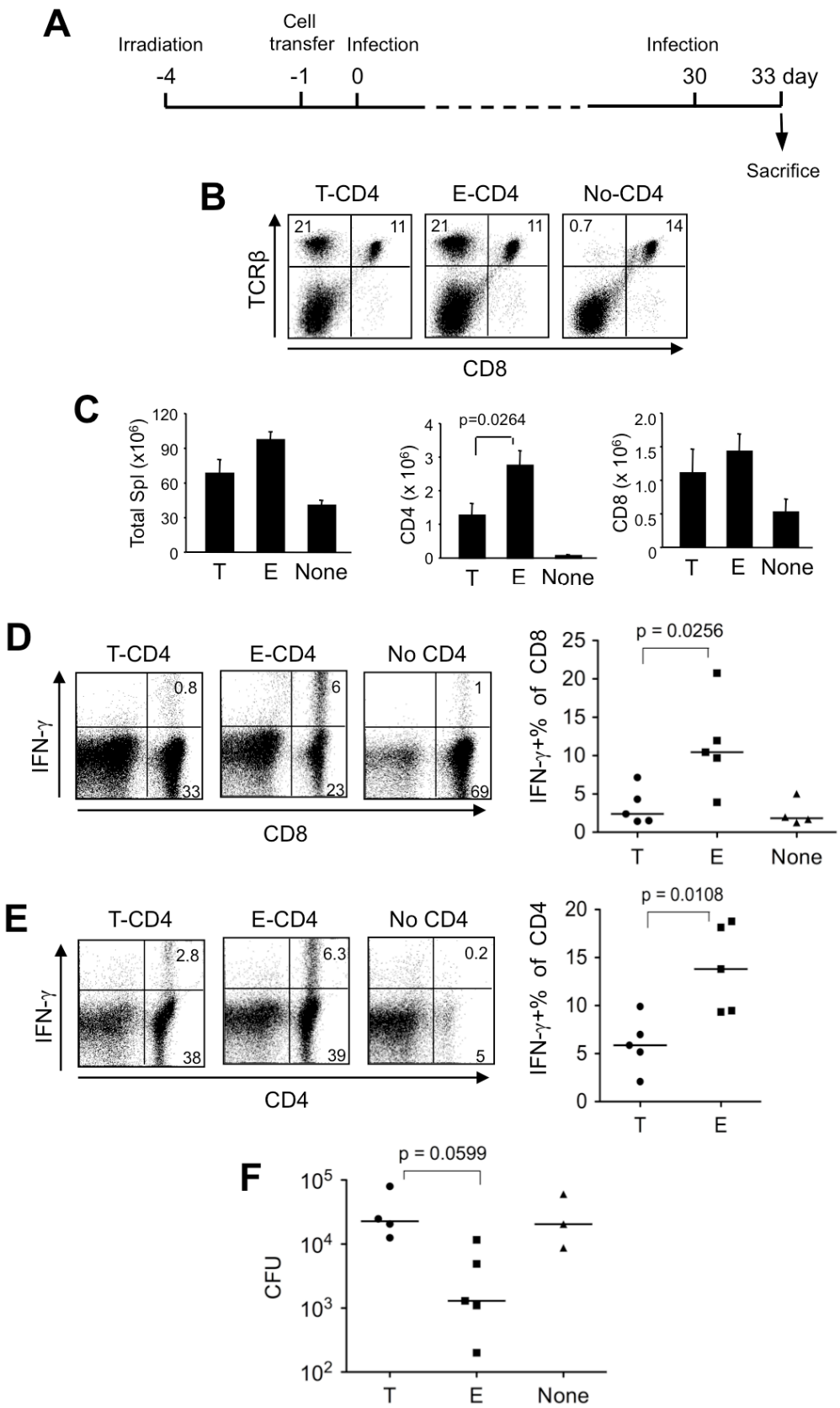
A
Irradiation
Cell transfer
Infection
Infection
-4
-1
0
30
33 day
Sacrifice
B
T-CD4
E-CD4
No-CD4
TCRβ
CD8
21
11
21
11
0.7
14
C
Total Spl (x10^6)
CD4 (x 10^6)
CD8 (x 10^6)
p=0.0264
T
E
None
D
T-CD4
E-CD4
No CD4
IFN-γ
CD8
0.8
33
6
23
1
69
IFN-γ+% of CD8
p = 0.0256
E
T-CD4
E-CD4
No CD4
IFN-γ
CD4
2.8
38
6.3
39
0.2
5
IFN-γ+% of CD4
p = 0.0108
F
CFU
p = 0.0599

Figure 3.4 T-CD4 cells inhibit development of memory CD8 effector cells
(A) A scheme of the experiment protocol. Mice were infected with 2×10^3 and 5×10^5 rLM-OVA for primary and secondary infection respectively, and then sacrificed three days after the second infection. (B) The composition of the CD45.1$^+$ input cell populations prior to the transfer. (C) The numbers represent total splenocytes and the indicated cells originating from the donor. (D and E) Frequencies of IFN-γ-producing rLM-OVA-specific CD8 (D) and CD4 (E) T cells (N=5). Experimental design and data analysis were performed as described in Figure 3 D and E. (F) Numbers represent viable bacteria isolated from liver homogenates. The bars indicate median values. The CFU counts of PBS-treated mice were below the detection limit.

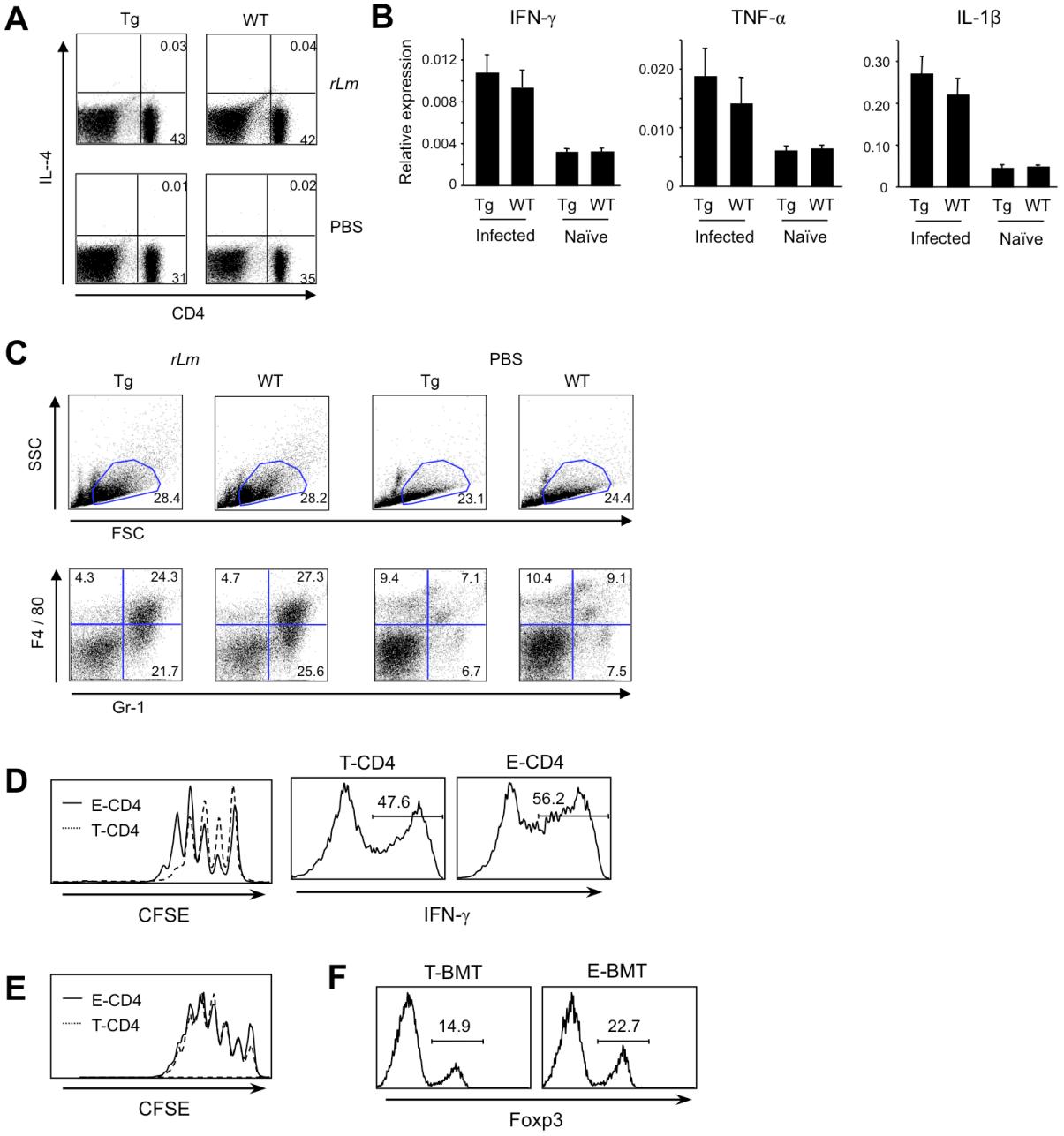
A
Tg
WT
0.03
0.04
rLm
43
42
IL--4
0.01
0.02
PBS
31
35
CD4
B
IFN-γ
TNF-α
IL-1β
Relative expression
0.012
0.008
0.004
0
0.020
0.010
0.30
0.20
0.10
Tg WT Tg WT
Infected
Naïve
C
rLm
PBS
Tg
WT
SSC
28.4
28.2
23.1
24.4
FSC
F4 / 80
4.3
24.3
21.7
4.7
27.3
25.6
9.4
7.1
6.7
10.4
9.1
7.5
Gr-1
D
E-CD4
T-CD4
CFSE
47.6
56.2
IFN-γ
E
F
T-BMT
E-BMT
14.9
22.7
Foxp3

Figure 3.5 T-CD4 T cells suppress proliferation of CD8 T cells in a cell contact-dependent manner

(A) Antigen-specific T-CD4 T cells did not produce IL-4 after re-stimulation on day 7 of infection. Experimental design and data analysis were as described in Fig 3.1 B. (B) Tg and WT mice were infected intravenously with 5×10^4 rLM-OVA. Three days later, RNA was extracted from the spleen and quantitative RT-PCR was performed to measure cytokine gene expression. Relative expression of each cytokine was normalized to GAPDH. (C) Gr-1^+ and/or F4/80^+ subsets of Tg and WT splenocytes. Hosts were *i.v.* infected with 1×10^5 rLM-OVA and sacrificed on day 3 of the primary infection. (D) CD4 depleted total splenocytes were labeled with CFSE and cultured in the presence of E- or T-CD4 T cells for three days. CFSE intensity (left) and IFN-γ expression after short-term re-stimulation (right) are shown. (E) The CFSE profiles of CD8 T cells cultured with E- or T-CD4 supernatant for 4 days. (F) Representative profiles of Foxp3 expression in splenic CD4 T cells from BM chimeric mice (T-BMT: Tg→A$\beta^{-/-}$; E-BMT: WT→WT). All experiments were done at least twice.

3.5 Discussion

The present study reported a novel suppressive role of T-CD4 T cells in adaptive immune responses against *L. monocytogenes*, evidenced by the low number of rLM-OVA-specific CD8 T cells and compromised *in vivo* CTL killing potential. This is rather surprising since previously we have shown that T-CD4 T cells produce effector cytokines immediately after TCR stimulation (15, 21). This disparity is a good example of the differences between polyclonal stimulation and antigen-specific responses.

The suppressed CTL response was evident in the memory phase, indicating T-CD4 T cells also differ from E-CD4 T cells in their impact on CTL memory development. Several reports have demonstrated a critical role of E-CD4 T cells in establishing CTL-mediated immunological memory against intracellular pathogens such as *L. monocytogenes* (1, 2, 39, 40). Without E-CD4 T cells, hosts are capable of clearing pathogens efficiently during the primary response, but succumb after a high dose re-encounter of the pathogen due to the defective function of memory CTLs. T-CD4 T cells, however, do not function analogously to E-CD4 T cells. As we demonstrated, immunological memory was compromised in the presence of T-CD4 T cells. The sub-optimal CTL response in T-CD4 T cell hosts, especially during the memory response, cannot be attributed to a lower number of total or antigen-specific T-CD4 T cells than E-CD4 T cells in the hosts. Instead, the suppressive effect appeared to be dominant and lasted throughout the entire period, because this suppression was also readily observed in Tg mice, which possess a good proportion of E-CD4 T cells in addition to T-CD4 T cells. Also intriguing is the observation that T-CD4 T cells could regulate CD8 T cell responses

by repressing expansion, rather than by altering the CD8 cells potential to produce IFN-γ. Further studies will attempt to identify the cell surface molecules mediating suppression and to investigate the molecular mechanisms by which T-CD4 T cells repress CD8 T cell expansion in a cell contact-dependent manner.

T-CD4 T cells share similarities with another innate T cell population known as iNKT cells (16, 19, 20). iNKT cells recognize glycolipid antigens presented by the nonpolymorphic MHC class I-like molecule CD1d (18). Similar to T-CD4 T cells, iNKT cells exhibit an effector cell phenotype, and respond quickly to TCR stimulation by producing effector cytokines. Moreover, iNKT cells have been reported to participate in immune regulation and in the protection against certain diseases (51-54). However, the functional characteristics of T-CD4 T cells seem to be quite different from iNKT cells in *L. monocytogenes* infection. First, the rapid iNKT cell response to infection can be readily observable by 4 days post infection (55). Consistent with this observation, cytokines such as IL-12 produced during an early stage of an immune response regulate iNKT cell activation in a non-antigen-specific way (17). Furthermore, the expression of the NK T cell marker NK1.1, which allows the recognition of "missing-self", seems also to play a role in iNKT cell regulation of *L. monocytogenes* infection, independent of TCR involvement (17, 56). By contrast, the presence of T-CD4 T cells conferred little detectable difference to the host environment regarding inflammatory cytokine levels at the early stage, indicating a lack of bystander response. Second, it has been reported that iNKT cells fail to expand after *L. monocytogenes* infection, and instead result in a prolonged loss in the spleen (57). T-CD4 T cells, on the other hand, seemed to expand

synchronously with E-CD4 T cells in an antigen-specific manner, but to a much milder extent. Finally, no suppressive role has been suggested thus far regarding iNKT cell function during *L. monocytogenes* infections as we have reported here for T-CD4 T cells.

The suppressed proliferation of CD8 T cells co-cultured with T-CD4 T cells was not likely due to Treg populations, because T-CD4 T cells used in the assay did not contain more Treg than E-CD4 T cells based on Foxp3 staining and that Treg from T-BMT hosts are functionally equivalent to those from E-BMT hosts (15). Furthermore, despite the suppressive function, T-CD4 T cells are distinct from Treg. Treg cells neither have pre-made IL-4 mRNAs nor release effector cytokines immediately after TCR stimulation. Instead, similarly to conventional CD4 T cells, Treg undergo several rounds of proliferation before functional maturation. They function to produce large amounts of suppressive cytokines including TGF-β and IL-10 instead of inflammatory cytokines such as IL-6 and IFN-γ produced by T-CD4 T cells. Moreover, T-CD4 T cells seem to have less capacity to produce IL-10 and TGF-β than E-CD4 T cells (data not shown, by Jihoon Chang and Tim Voorhees).

Previously, we have demonstrated that mice containing T-CD4 T cells are protected from airway inflammation (21). In addition, T-CD4 T cells also seem to suppress the onset of EAE (22). Based on these reports and our current study, it is likely that T-CD4 T cells act to down-regulate the host immune response in many other scenarios. A recent report showed the existence of T-CD4 T cells in humans (13). If human T-CD4 T cells bear a similar suppressive function to that shown here for murine

T-CD4 T cells, understanding the mechanisms underlying the phenomena described here can have clinical applications to infectious and autoimmune diseases in humans.

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Chapter IV

Discussion

4.1 TCR signaling characteristics during T-CD4 T cell generation

TCR gene rearrangement during T cell development gives rise to millions of thymocytes with distinct TCRs to interact with self-MHC complexes. The TCR-ligand interaction is correlated with TCR signaling intensity, which in turn determines T cell fate – weak TCR signaling fails positive selection and strong TCR signaling leads to negative selection; only those thymocytes with moderate TCR ligation and signal transduction survive the screening process. With the notion that most of the thymocytes die from lack of surviving signals during positive selection, the TCR selection window is believed to be on the right half of the distribution curve (Fig 4.1 A, dash line). This model is also supported by the observation that weakened TCR signaling compromises E-CD4 T cell development (1-4) and that mildly elevated TCR signaling, enhances E-CD4 T cell generation (5). However, this model applies to E- but not T-CD4 T cells. TCR signaling modulation affected T-CD4 T cell selection oppositely to E-CD4 T cell selection – reducing TCR signaling enhanced (Fig 2.1 B) and increasing TCR signaling impaired (Fig 2.2 B). T-CD4 T cell generation, suggesting that TCR signaling elicited by thymocyte-thymocyte (T-T) interaction is fundamentally different from that by epithelial cell-thymocyte (E-T) interaction. This is not surprising considering that thymocytes and TEC likely differ in many ways, including the intensity of MHC Class II expression, the

pool of selecting self-peptides, the expression of different kinds of adhesion molecules and the dynamics of cell interactions. All these factors possibly influence the avidity and/or the longevity of TCR ligation, and thus in turn, the signaling quality.

Based on our data, we propose that the T-T interaction results in stronger TCR signaling than does the T-E interaction. According to this hypothesis, the distribution curve of TCR signal resulting from T-T interactions is to the right of that resulting from E-T interactions (Fig 4.1 A). This model predicts that decreasing TCR signaling strength favors T-CD4 T cell generation but compromises E-CD4 T cell generation (Fig 4.1 B), which is consistent with the observation in ITK deficiency. Similarly, increasing TCR signaling strength would produce an opposite effect that is consistent with our study with the RASA1$^{-/-}$ model (Fig 4.1 C). It should be mentioned that, in order to focus on the comparison between T- and E-CD4 TCR signaling characteristics in this model, we defined other factors such as the selection windows for T- and E-CD4 T cells to be identical. This is reasonable because thymocytes are instructed by the intrinsic signaling qualities but not by the interacting cell types *per se*. And even if the two windows do not completely overlap in reality, it does not necessarily contradict our current conclusion.

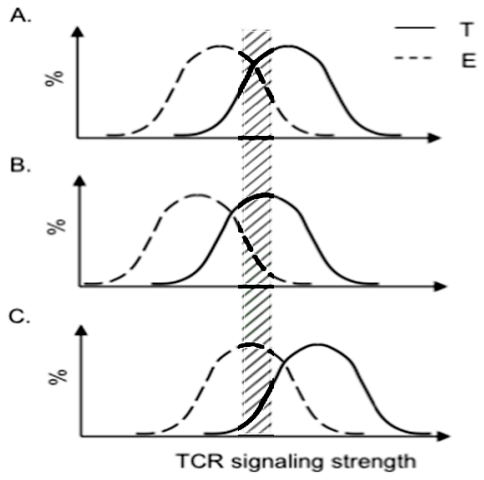

Fig 4.1 T cell selection model. Y-axes are the percentages of thymocytes. Shadows are selection windows. (A) (B) and (C) are TCR signaling distributions of T- and E-thymocytes under wild type, $ITK^{-/-}$ and $RASA1^{-/-}$conditions, respectively.

As shown in Fig 4.1, this model indicates that T- and E-CD4 T cells possess different TCR repertoires. And in fact, although FACS profiles did not show significant differences in TCR α and β chain usage, there is accumulating evidence to support this hypothesis: AND T cells, having a transgenic TCR Vα11Vβ3 pair that recognizes a pigeon cytochrome c peptide-IE^k complex in the periphery, can be efficiently selected by IA^b or IE^k expressed on TEC in the thymus (6). However, thymocytes bearing AND transgenic TCR fail to be selected by thymocyte-expressed MHC class II molecules to become T-CD4 T cells (7). Similarly, the DO11.10 and OT-II (8) CD4 TCR^{Tg} thymocytes that are efficiently selected by TEC cannot be selected by thymocytes ((7) and Fig A1.2). The reason for the differences between T- and E-selection of these transgenic TCRs is still elusive. It seems that AND or OT-II cell selection on thymocytes cannot be rescued by TCR signaling modulation, at least based on the study using the SAP, RASA1 or ITK deficient systems (Fig A1.1 and A1.2). While there is a possibility that the signal alternations in these models were not optimal for T-CD4 T cell selection, it is equally possible that these transgenic TCRs intrinsically belong to the E- but not to the T-CD4 TCR repertoire, the shaping of which can be influenced by factors such as self

peptides in addition to signaling properties. Evidence supporting this latter point of view arises from the generation of a transgenic TCR cloned from T-CD4 T cells. These TCR^{Tg} T cells express TCR Vα2Vβ8 and are named T3 CD4 T cells (Appendix 1). They were poorly generated on TEC in E-BMT hosts when compared to polyclonal partner cells (T3 + WT →WT), but were efficiently generated with a highly increased percentage of T3 CD4 SP population in T-BMT hosts (T3 + $CIITA^{Tg}$ → $A\beta^{-/-}$) (Fig A1.3). It is yet to be determined whether the T3 E-CD4 T cell generation can be rescued by modulating TCR signaling events. It is reasonable that, like AND and OT-II thymocytes in T-CD4 T cell selection, T3 E-CD4 T cells may fail to be rescued by TCR signaling modulation, which would suggest that T3 is specific for the T- but not the E-CD4 repertoire. It would not be surprising, however, if T3 E-CD4 T cell generation was rescued by signaling modulation, because T- and E-CD4 TCR repertoires may partially overlap (Fig 4.1 A). Whatever the case, the current data derived from AND, OT-II and T3 TCR^{Tg} models serve as strong indications of the differences between T- and E-CD4 TCR repertoires.

4.2 T-CD4 T cell functional characteristics

In our study with *L. monocytogenes* infection, the experiments using the adoptive transfer model clearly showed that T-CD4 T cells suppressed *Listeria*-specific CTL expansion (Fig 3.3 E) and decreased CTL killing capacity *in vivo* at a primary stage (Fig 3.3 G). Our current data suggested that the suppressive function of T-CD4 T cells was performed by a population other than Foxp3-expressing Treg cells, in that T-CD4 T cells do not contain more Treg than E-CD4 T cells (Fig 3.5 E), and that Treg from T-BMTs do not suppress cell proliferation with stronger potency than Treg from E-BMT hosts (7).

Nevertheless, more studies are needed to establish the mechanisms underlying the suppressive nature of T-CD4 T cells.

Similar to Tregs, T-CD4 T cells suppress cell proliferation through cell contact-dependent pathways *in vitro*. Treg suppression depends on accessory molecules such as CTLA-4 (CD152) and lymphocyte-activation gene 3 (LAG3) interacting with their ligands on APCs (9). It remains to be determines whether these two molecules play a role in T-CD4 T cell suppressive function. Another possible mechanism of T-CD4 T cell suppression may be granzyme-mediated cytolysis, since our microarray analysis showed that T-CD4 T cells had higher mRNA levels of multiple granzymes than E-CD4 T cells. Studies have shown that granzyme-mediated cytolysis is employed by Tregs to kill the APCs and thereby limits antigen-presentation to other T cells for activation and proliferation (10-12). We further need to determine whether T-CD4 T cells also employ this mechanism to kill the APCs or other T cells they are interacting with. Regarding cytokine regulation, unlike Tregs that produce the suppressive cytokines IL-10 and TGF-β, which are important for immune regulation in mouse models such as colitis (13) and allogeneic skin graft rejection (14), T-CD4 T cells do not produce higher amounts of IL-10 or TGF-β than E-CD4 T cells *in vitro*, and the status of IL-10 and TGF-β production by T-CD4 T cells *in vivo* is unknown.

Participation of CD8 T cells and iNKT cells in an immune response can be observed much earlier than that of conventional T cells. T-CD4 T cells also distinguish themselves from E-CD4 T cells by the immediate effector cytokine production after anti-

CD3 stimulation *in vitro* and *in vivo* (15). Therefore, it seems reasonable to expect that T-CD4 T cells will react with effector T cells shortly after infections *in vivo*. During *L.monocytogenes* infection, however, the function and influence of T-CD4 T cells were mainly in adaptive immune responses rather than at earlier stages, as shown by the measurement of cytokine expression (Fig 3.5 A) and by the innate cell profile (Fig 3.5 G). One explanation for the disparity between polyclonal and antigen-specific systems as well as for that between T-CD4 and other innate-like T cell populations may lie in the nature of their TCR ligands. Most of innate-like T cells such as iNKT and innate CD8 T cells have either a conserved TCR repertoire or less specific TCR recognition than that of conventional T cells. For example, iNKT cells recognize glycolipids presented by nonpolymorphic CD1d, and the whole iNKT population can be detected by αGaler-CD1d complexes (16). H2-M3-restricted CD8 T cells have been shown to recognize a broad spectrum of N-terminally formylated peptides by a single TCR (17). Therefore, the relatively large pools of these CD8-T responding cells facilitate their expansion-independent detection, and thus their activity can be readily assessed early on. T-CD4 T cells, on the other hand, use peptide-MHC class II complexes as TCR ligands, which are much more specific than MHC class Ib complexes and thus tend to select TCRs of higher specificity. The frequency of T-CD4 T cells specific for a certain pathogen in a host is likely comparable to that of E-CD4 T cells. It would be extremely difficult to detect these few cells even if they were producing cytokines immediately after stimulation *in vivo*. And their low cell numbers also made them unlikely to exert a dramatic influence on the whole inflammatory environment at early stages. However, if antigen-specific T-CD4 T cells indeed produce cytokines while they are interacting with the APCs, these cytokines

may affect APC function and in turn the programming of the later responses. The investigation of this aspect remains to be done.

An alternative explanation for the dual functional features of immediate cytokine production and proliferation suppression during infection is T-CD4 T cell heterogeneity. It has been noted that T-CD4 T cells are comprised of different subsets. T-CD4 T cells with an IL-4 mRNA-GFP reporter (18) express GFP in approximately 50% of the CD4SP cells in the thymus and in 30-40% of the splenocytes (H. Sofi *et al*., paper submitted). In addition, in the thymus, GFP^+ T-CD4 T cells express higher levels of Ly108 and CD44 than GFP^- T-CD4 T cells. Furthermore, the transcription factor PLZF is also expressed heterogeneously in T-CD4 T populations. And interestingly, PLZF is mostly expressed in the IL-4 mRNA-expressing T-CD4 SP thymocytes, whereas non-IL-4 mRNA-expressing cells are mostly $PLZF^-$. Therefore, it seems that T-CD4 T cells are comprised of sub-populations of IL-4^+PLZF^+, IL-4^+PLZF^- and IL-4^-PLZF^- cells. These subsets might possess different functional characteristics. For example, IL-4 mRNA-expressing cells might be the immediate IL-4 producers. It is also not known whether the suppressive T-CD4 T cells belong to certain subsets.

It is also known that T-CD4 T cells continue producing IL-4 under Th1-skewing conditions *in vitro*. But on day 7 of *L.monocytogenes* infection, no *Listeria*-specific IL-4-producing CD4 T cells were detected in T-CD4 T cell hosts. This is a good example showing that polyclonal stimulations *in vitro* do not always mirror the antigen-specific responses under physiological conditions. It is likely that there are *Listeria*-specific T-

CD4 T cells with IL-4 producing potential upon infection, but they may not expand well or may lose the IL-4 producing capacity during *L.monocytogenes* infection.

The immune deficiency caused by T-CD4 T cells was preserved in immune memory (Fig 3.4 E). However, it is not yet clear whether the deficiency at the memory stage is due to dominant suppression by memory T-CD4 T cells or is due to the defective development of CTL memory as a consequence of deficient primary adaptive immune response. To arrive at a conclusive answer, a model of secondary adoptive transfer is needed to separate the role of T-CD4 T cells in establishing CTL immune memory from their function in memory immune responses. Nevertheless, current data clearly showed that, under physiological conditions, the presence of T-CD4 T cells resulted in deficient CTL responses at both primary and memory phases.

4.3 Acquisition of functional characteristics during T-CD4 T cell development

The functional characteristics of T-CD4 T cells are shaped in the thymus, but the mechanism is still a subject for discussion and speculation.

TCR signaling events during and after positive selection might be critical for shaping T-CD4 T cell function. TCR signals for positive selection at the DP stage have been known to be important for lineage decisions. Upon positive selection, thymocytes differentiate into CD4 or CD8 T cells. CD4 T cell differentiation requires stronger TCR signals than CD8 T cell differentiation does, but purely weakening TCR signaling does not necessarily affect lineage decision as shown in an $ITK^{-/-}$ mouse model (3). Moreover, Treg development is regulated by TCR signals differently from effector E-CD4 T cells,

as reflected by the inherited differences of the TCR characteristics of the two. Treg TCRs display higher ligation affinity than effector E-CD4 TCRs based on the studies with TCR^{Tg} models, and Tregs have been proposed to recognize self-antigens in the periphery (19). In addition, iNKT cell TCR-CD1d interaction has been suggested to be of higher affinity and longer half life than E-CD4 T cell TCR-MHC class II interaction as measured using hybridoma systems (20). Thus, T-CD4 lineage decisions might also be made through T-T interaction during positive selection.

Beyond positive selection, the functional maturation of T-CD4 T cells might employ a secondary step that probably involves a unique type of TCR stimulation. This stimulation leads to slow T-CD4 T cell proliferation indicated by a $CD71^{+}$ FSC^{hi} phenotype (data not shown), the up-regulation of CD44 and the transcription of IL-4 mRNAs, instead of inducing apoptosis as in negative selection. There are at least two potential reasons for T-CD4 T cells escaping apoptosis by TCR stimulation at this stage. First, T-CD4 T cells might be better at resisting apoptosis than E-CD4 T cells because of some special programming during the T-T interaction earlier. Second, according to our hypothesis that T-T interactions produce higher TCR signaling strength for a given TCR than does the E-T interaction, thymocytes with too weak a TCR-ligand interaction for E-CD4 positive selection by TEC might survive T-CD4 positive selection by thymocytes. These T-thymocytes respond to a lesser extent than E-thymocytes to the same TCR ligation in the medulla (Fig 4.1 A). It is possible that, because of the weak TCR signals produced, some of these T-thymocytes are sub-optimally stimulated by recognizing self-

peptides instead of being depleted through negative selection. Consequently, they display activated phenotype.

In addition to TCR signaling, co-stimulatory pathways might also contribute to shaping the T-CD4 T cell functional characteristics. CD28 and SLAM-SAP pathways have been suggested to be critical for Treg (21) and iNKT development (22), respectively, and both pathways are important for the innate-like functional phenotype of a CD8 T cell population (22, 23). As for T-CD4 T cells, SLAM family receptors signal at the DP stage and are important for optimal selection. This pathway partially affects T-CD4 T cell phenotype, as demonstrated by SAP deficiency studies that showed a two-fold decrease in the IL-4-producing population under Th1-skewing conditions (24). Since SAP deficiency does not completely abolish IL-4 production by T-CD4 T cells, other pathways probably participate in the process as well. In the medulla, the CD28 pathway is a good candidate for driving the T-CD4 T cell functional phenotype, since the role of CD28 signals in Treg and innate CD8 T cell development has been established.

The differentiation of different T cell lineages is quite often marked by up-regulation of specific transcription factors. Foxp3 is a critical transcriptional factor for Treg; its deficiency abolishes Treg generation and leads to autoimmune disorders. PLZF has recently been identified as a crucial transcriptional factor for iNKT cell generation and function; PLZF deficient iNKT cells exhibit a naive phenotype, defective expansion in the thymus and compromised cytokine production after activation (25). Similarly, PLZF is also expressed in T-CD4 T cells (Fig 2.5 C), and likely plays a role in shaping T-

CD4 phenotype. However, unlike iNKT cells, only one third of T-CD4 SP thymocytes express PLZF, an indication that T-CD4 T cells contain different subsets. Moreover, over-expression of PLZF in E-CD4 T cells does not increase IL-4 production in these cells (26), suggesting that other factors in addition to PLZF are required for expression of T-CD4 T cell functional characteristics. PLZF is induced at the SP stage after positive selection, but the exact up-stream signaling events that induce or regulate its expression are poorly defined. At this point, PLZF target genes are not well identified either. Addressing these two questions would help reveal the molecular mechanisms that relate T-CD4 T cell function to their development.

4.4 The implications of this study

This study focused on T-CD4 T cells and provided insights into their generation and function in the hopes that some of our findings will have diagnostic implications and potentially influence clinical treatments in disease. Our studies in mice indeed have human implications: Human thymocytes express MHC class II molecules (27-29), and CD4 T cells generated through T-T interaction have been found in human fetus tissues (30). Moreover, these human T-CD4 T cells expressed PLZF and produced elevated IL-4 cytokine similar to mouse T-CD4 T cells. The identification of these cells opened a new avenue of T cell research along with giving rise to many immediate questions to be addressed further, such as their generation in adults and their persistence and physiological functions in the periphery. It is technically a challenge to address these questions in humans directly. Therefore, mouse models that have been and will be constructed can provide fertile testing grounds to facilitate the design and conduct of future human studies.

The results of our study of T-CD4 T cells have implications for both healthy humans and human disease. In patients receiving bone marrow transplant treatment, for example, both T- and E-CD4 T cells can exist. Moreover, in BLS patients who receive bone marrow transplants, all of the restored CD4 T cells (31, 32) are selected by BM-derived cells, and therefore resemble T-CD4 T cells. Whereas E-CD4 T cells are host MHC-restricted, T-CD4 T cells are donor MHC-restricted, which should be taken into consideration for host-graft tolerance. More importantly, the immune responses of these patients cannot be predicted solely with conventional concepts of the immune system, and the functional differences between T- and E-CD4 T cells need be considered. If human T-CD4 T cells possess the same suppressive function as mouse T-CD4 T cells do, they could be beneficial in reducing autoimmune reactions. In situations where the suppressive immune function is desired, T-CD4 T cell adoptive transfer is a feasible strategy, considering human T-CD4 T cells have been successfully generated *in vitro* (33) and *in vivo* (34). Importantly, these T-CD4 T cells display functional maturity *in vitro* and *in vivo*, shown by cytokine production and the responsiveness to vaccination and viral infection (34, 35). However, the suppressive function of T-CD4 T cells might be disastrous for immune responses against foreign pathogens. Strategies such as drug administration to boost T cell activities should be employed to cope with the potentially blunted immunity, and further identification of T-CD4 T cell specific markers would be very helpful for cell-specific depletion.

The studies presented in this thesis have provided a novel perspective on the immune system and CD4 T cell biology, which can help foster better design of the strategies needed to treat certain diseases. In addition, it also raised new questions for future research so as to better understand the mechanisms underlying pathologies that relate to the immune system.

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Appendix 1: Development of thymocytes expressing TCRs with a single specificity

As an attempt to further characterize TCR signaling characteristics in T- *vs* E-CD4 development, we modulated and evaluated the selection efficiency of a homogenous T cell population bearing the transgenic TCR AND. AND TCR transgenic (TCR^{Tg}) CD4 T cells (1) express Vα11 and Vβ3 and can be efficiently selected by MHC class II expressed on TEC (2, and Fig A1.1 A). However, they failed to develop into T-CD4 T cells (Fig A1.1 A). Moreover, this failure of thymocyte-mediated selection was not rescued when the TCR signaling quality was altered by eliminating ITK (Fig A1.1 B), RASA1 (Fig A1.1 C), or SAP (Fig A1.1 D). Similarly, OT-II (2) ($V\alpha2^{+}V\beta5^{+}$) TCR^{Tg} thymocytes were efficiently selected by TEC but were poorly selected by thymocytes (Fig A1.2 A and C), and their selection was not improved by ITK deficiency (Fig A1.2 B and C).

The results above raised the possibility that T- and E-CD4 T cells possess different TCR repertoires, and that the developmental failure of AND and OT-II T-CD4 T cells is because these two transgenic TCRs were cloned from E-CD4 T cells that are not shared by T-CD4 T cells. If so, thymocytes bearing TCRs expressed in T-CD4 T cells would be selected better by thymocytes than by TEC. To test the hypothesis, we generated a new line of TCR transgenic mice named T3 (T-CD4 TCR Transgenic). To obtain T3, we first constructed T-BMT chimeras by transferring TCR $V\beta8^{Tg}$ BM cells with $CIITA^{Tg}$ BM cells to $A\beta^{-/-}$ hosts. Eight weeks later, $Va2^{+}V\beta8^{+}$ splenic CD4 T cells originated from TCR $V\beta8^{Tg}$ BM were sorted and Va2 genes from these T-CD4 T cells were sequenced. The most frequently expressed Vα2 gene and the Vβ8 gene was cloned

to an expression vector and the standard protocol generating transgenic mice was followed to make T3 mice.

The selection preference of T3 CD4 T cells was tested using the BM chimeric strategy. CD4 SP thymocytes originated from T3 BM expressed the transgenic TCR Vα2 and Vβ8, and importantly, T3 CD4 T cell development was better by thymocytes than by TEC (Fig A1.3), which is in agreement with our hypothesis.

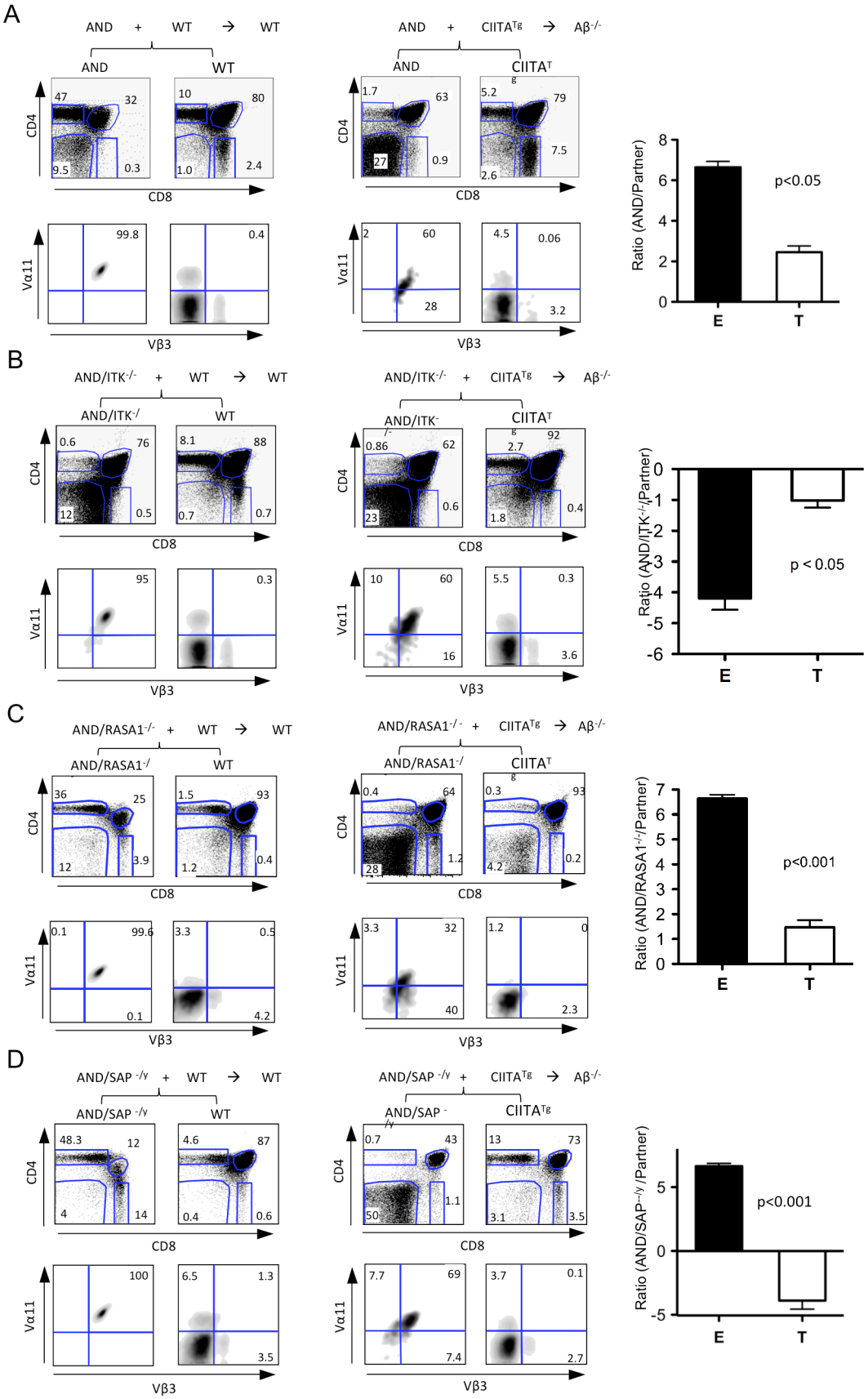
A
AND + WT → WT
AND
WT
CD4
CD8
47
32
9.5
0.3
10
80
1.0
2.4
Vα11
Vβ3
99.8
0.4
AND + CIITA^Tg → Aβ^-/-
CIITA^Tg
1.7
63
27
0.9
5.2
79
7.5
2.6
2
60
28
4.5
0.06
3.2
Ratio (AND/Partner)
p<0.05
E
T
B
AND/ITK^-/- + WT → WT
AND/ITK^-/-
0.6
76
12
0.5
8.1
88
0.7
0.7
95
0.3
AND/ITK^-/- + CIITA^Tg → Aβ^-/-
0.86
62
23
0.6
2.7
92
1.8
0.4
10
60
16
5.5
0.3
3.6
Ratio (AND/ITK^-/-/Partner)
p < 0.05
C
AND/RASA1^-/- + WT → WT
AND/RASA1^-/-
36
25
12
3.9
1.5
93
1.2
0.4
0.1
99.6
0.1
3.3
0.5
4.2
AND/RASA1^-/- + CIITA^Tg → Aβ^-/-
0.4
64
28
1.2
0.3
93
4.2
0.2
3.3
32
40
1.2
0
2.3
Ratio (AND/RASA1^-/-/Partner)
p<0.001
D
AND/SAP^-/y + WT → WT
AND/SAP^-/y
48.3
12
4
14
4.6
87
0.4
0.6
100
6.5
1.3
3.5
AND/SAP^-/y + CIITA^Tg → Aβ^-/-
0.7
43
50
1.1
13
73
3.1
3.5
7.7
69
7.4
3.7
0.1
2.7
Ratio (AND/SAP^-/y/Partner)
p<0.001

Figure A1.1 Poor selection of AND CD4 T cells by thymocytes
BM cells ($CD45.2^{+}$) from AND (A), AND/$ITK^{-/-}$ (B), AND/$RASA1^{-/-}$ (C), and AND/$SAP^{-/y}$ mice (D) were co-transferred with WT BM ($CD45.1^{+}CD45.2^{+}$) into B6.SJL hosts ($CD45.1^{+}$) to generate E-CD4 T cells (left), and with $CIITA^{Tg}$ BM ($CD45.1^{+}CD45.2^{+}$) into $A\beta^{-/-}$ hosts ($CD45.1^{+}$) to generate T-CD4 T cells (right). The transfer ratio of AND TCR-expressing cells to partner cells was 10:1 in all groups. Mice were sacrificed 5-6 weeks after the BM transplantation. Total thymocytes were used to analyze thymocyte subsets (upper panels of the FACS profile in each Figure). The numbers in the dot plots indicate the percentages of gated cells derived from each BM type using the CD45 allele. The lower panels are TCR expression of CD4 SP thymcoytes of the indicated donor cells. The numbers are percentages of the cells within each CD4 SP population. The bar graphs shown are selection efficiencies expressed as mean±SE (right) were calculated as in Fig 2.1. The y axis's are in log 2 scale, and the numbers are the logarithms of 2. (A: N=4, 3; B: N=4, 4; C: N=6, 6; D: N=4, 5.)

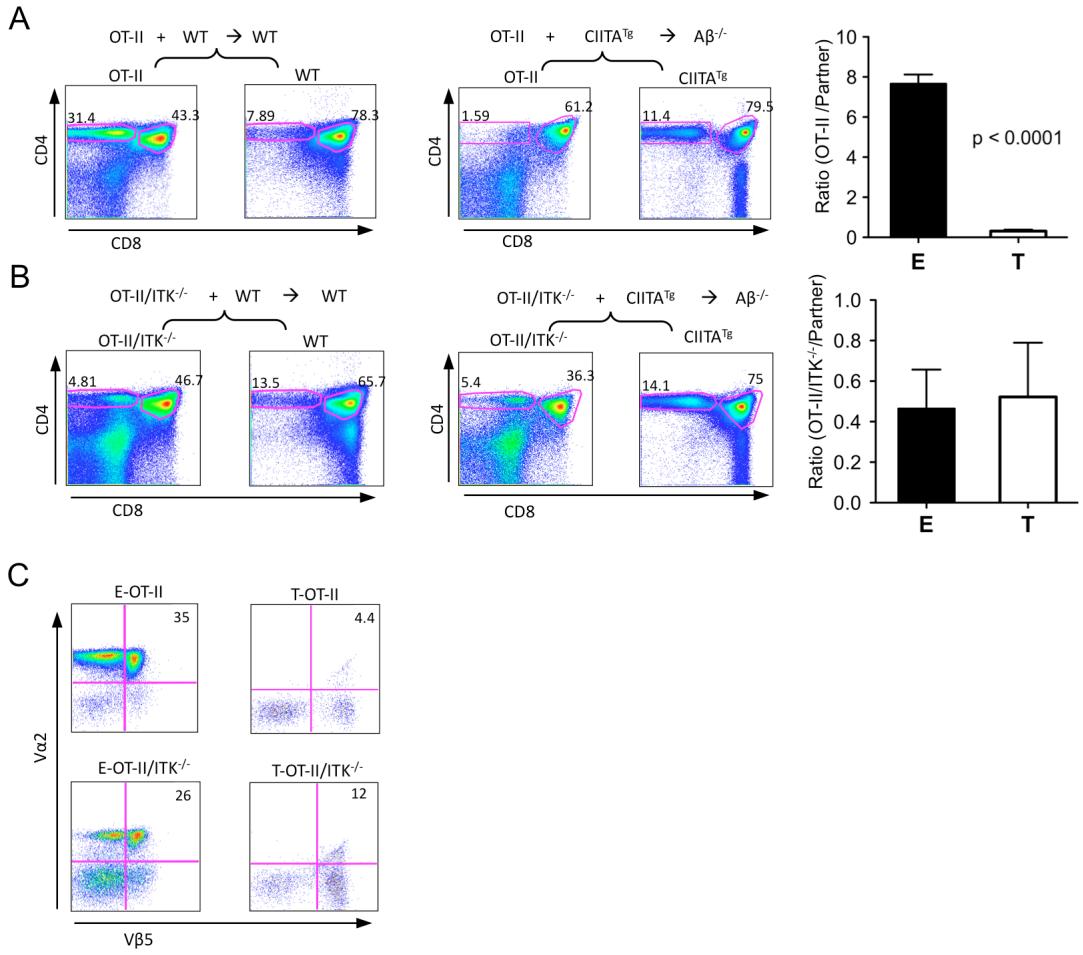

Figure A1.2 Inefficient development of OT-II TCR^{Tg} CD4 T cells by thymocytes. OT-II (A) and OT-II/ITK$^{-/-}$ (B) BM (CD45.2^{+}) were co-transferred with WT BM (CD45.1^{+}CD45.2^{+}) into B6.SJL hosts (CD45.1^{+}) to generate E-CD4 T cells (left), and were co-transferred with CIITA^{Tg} BM (CD45.1^{+}CD45.2^{+}) into Aβ$^{-/-}$ hosts (CD45.1^{+}) to generate T-CD4 T cells (right). The transfer ratio of OT-II TCR-expressing cells to partner cells was 10:1 in all groups. Mice were sacrificed 5 weeks after the BM transplantation, and the thymocytes were analyzed as in Fig A1. The selection efficiencies shown in bar graphs were expressed as mean±SE (right) as in Fig 2.1. The y axis's are in log 2 scale, and the numbers are the logarithms of 2. (A: N=2, 3; B: N=4, 2.) (C) TCR expression of gated on CD4 SP thymocytes of the indicated donor cells.

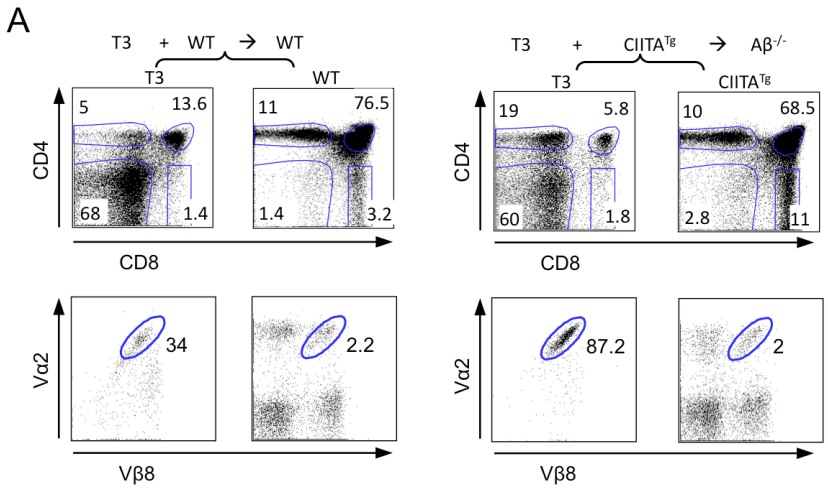

Figure A1.3 Better selection of T3 CD4 T cells by thymocytes than TEC
T3 BM ($CD45.2^+$) were co-transferred with WT BM ($CD45.1^+CD45.2^+$) into B6.SJL hosts ($CD45.1^+$) to generate E-CD4 T cells (left), and were co-transferred with $CIITA^{Tg}$ BM ($CD45.1^+CD45.2^+$) into $A\beta^{-/-}$ hosts ($CD45.1^+$) to generate T-CD4 T cells (right). The transfer ratio of T3 cells to partner cells was 3:1 in both groups. Mice were sacrificed 4 weeks after the BM transplantation. TCR expression of CD4 SP was shown in the bottom panels. The data are representative of multiple experiments.

References

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2. Robertson, J.M., P.E. Jensen, and B.D. Evavold. 2000. DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J Immunol* 164:4706-4712.