FUNCTION OF IL-2-INDUCIBLE T CELL KINASE (ITK) IN INNATE T CELLS AND MAST CELLS

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FUNCTION OF IL-2-INDUCIBLE T CELL KINASE (ITK) IN INNATE T CELLS AND MAST CELLS

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IL-2-inducible T cell kinase (ITK) is expressed in T lymphocytes and mast cells (MC), and functions as a critical signaling mediator downstream of numerous cell surface receptors. ITK regulates both adaptive and innate immunity, via regulation of cell differentiation and activation.

Lack of ITK results in spontaneous memory acquisition in αβ T cells termed "innate memory phenotype (IMP) T cells". The development and function of IMP T cells, and the role of ITK in their development remains unknown. This dissertation describes an alternative bone marrow transplantation system, which generates murine models with predominant naïve or IMP T cells, allowing comparative investigation of the functions of these cells *in vivo*; and demonstrates that: 1) IMP T cell development requires hematopoietic expression of major histocompatibility complexes; 2) IMP $CD8^+$ T cells are not the result of T cell homeostatic proliferation (HP), and they can rapidly and potently respond to primary antigenic stimulation; and 3) IMP $CD4^+$ T cells can suppress autoimmune graft-versus-host disease. In addition, this dissertation also investigates the T cell-intrinsic role of ITK in the development of both IMP and HP $CD8^+$ T cells: 1) ITK tunes IL-4/TcR signaling synergy to regulate IMP $CD8^+$ T cell differentiation; and 2) ITK suppresses $CD8^+$ T cell HP and anti-tumor immunity.

Loss of ITK in mice also results in hyper-production of immunoglobulin E

(IgE), through which MC regulate the development of allergy. Differential functions of ITK and the homologous Bruton's tyrosine kinase (BTK) in MC response to allergen/IgE-mediated stimulation have been illustrated. This dissertation further shows a redundant function for ITK/BTK in MC response to the bacterial endotoxin lipopolysaccharide (LPS), in that lack of ITK and BTK leads to hyper-production of MC-derived TNF-α, which exacerbates LPS-induced septic hypothermia.

This work provides insights into the function of ITK in innate T cells, and the value of targeting ITK to enhance antigen-specific primary response by IMP $CD8^+$ T cells, regulatory function of IMP $CD4^+$ T cells, and expansion of anti-tumor HP $CD8^+$ T cells, for therapeutic purposes. However, the MC response to LPS also raises concern on the potential use of ITK/BTK cross-reactive kinase inhibitors.

BIOGRAPHICAL SKETCH

Weishan Huang graduated from Tsinghua University in Beijing, China, with honors from Beijing City and Tsinghua University, with dual degrees: 1) BS. in Biology, and 2) BE. in Economics, 2008. She was active in student leadership and student research programs. She initiated and co-founded Tsinghua student practice base, "Bird Island in Qinghai Lake", during 2006 to 2008. This base was one of the first successful endeavors in Tsinghua and Beijing City, and is now the base for student summer ecology research, natural reserve representative for public education, and popular subject of nature literatures by students. Weishan's thesis in Biology about immunological and bioinformatics research on cytotoxic T lymphocyte epitope of influenza A nucleoprotein, was awarded outstanding academic thesis.

Weishan then was enrolled in Ph.D. program, Cell & Developmental Biology, in the Pennsylvania State University, directed by Dr. Hong Ma and Dr. Zhi-chun Lai, mentored by Dr. Avery August. In 2010, she moved with Dr. Avery August to Cornell University, and converted her research to her MS. thesis on the interaction of ITK and Gα13. She was awarded summer course grant from Cold Spring Harbor Laboratory in the summer of 2009, and received training there on Computational Cell Biology.

From 2010, Weishan was involved in research in Dr. August's lab at Cornell to investigate the role of Tec kinases in innate immunity as a Ph.D. candidate in Pharmacology. She actively participated in Cornell, national, and international research events. Her work has been recognized and presented at multiple national and international conferences. Her work added to our knowledge the complex function of Tec kinases in antigenic response, autoimmune disease and inflammation. She was honored by the American Association of Immunologists Young Investigator Award, The Hsien Wu and Daisy Yen Wu Scholarship /The Liu Memorial Award and Caroline Coffey Travel Fund, for her performance and potential in academic research.

Dedicated to my father, Yanqiu Huang,

who survived highly differentiated squamous cell carcinoma

with two major surgeries during 2011 - 2013.

ACKNOWLEDGMENTS

I thank my parents, sister and brothers for their understanding and support of my choice of this academic journey. Their support was never reduced by my absence from almost all major family events during 2008 to 2013.

Being on this journey, I have to thank Dr. Hong Ma, who was the chair of recruiting committee for Cell & Developmental Biology program in the Pennsylvania State University, and accepted my application post deadline of the recruitment season in 2008. The entry in CDB program in PSU, allowed me the opportunity to rotate in Dr. Avery August's laboratory, which further led to my participation in work presented in this dissertation.

My greatest appreciation in my Ph.D study goes to Dr. Avery August, who provided me advice on research and academic career development with extraordinary supports, which had helped the construction of my view of life value. In the August's lab, sciences have been set as priority and truth has been leading the progress of the group. In this lab, I have experienced straightforward communications, fairness and transparency, which facilitated my research with confidence in direction, efficiency in work progress, and precision in data analysis. Together with my thanks to Dr. August's mentoring, my appreciation goes to the August's lab members, who guided me into the scientific community, shared my happiness and struggle as my peers, helped me through the journey of Ph.D. study. My experience in the August's lab in both PSU and Cornell will be my lifelong treasure.

The last of my acknowledgement goes to my special committee of graduate study, which is composed of Drs. Avery August, Maurine E. Linder, Ruth Collins, Andrew Yen, and Cynthia A. Leifer; and Dr. Margaret S. Bynoe who is outside the committee but also provided essential advice to my work and served as my referee together with Dr. August and Dr. Linder during my postdoctoral position hunting.

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CHAPTER ONE

Introduction

Tec family non-receptor tyrosine kinases: structure and expression

IL-2 inducible T-cell kinase (ITK), the main focus of this dissertation, is a member of the Tec family kinases (TFKs). TFKs, including ITK, Bruton's tyrosine kinase (BTK), TEC, BMX and TXK is the second largest non-receptor protein-tyrosine kinase family (second to Src family) (Takesono et al., 2002). These kinases are classified based on the unique Tec-Homology (TH) domain, which is composed of a zinc-binding Btk-homology (BH) motif and/or proline-rich regions (PRR) (Felices et al., 2007). Among all TFKs, ITK, BTK and TEC share the highest homology in structure consisting, from protein N terminal to C terminal, pleckstrin homology (PH), TH (BH + PRR), Src-homology (SH) 3, SH2, and kinase domains (Felices et al., 2007). In BMX, PRR is absent from the TH domain; while in TXK, PH domain is replaced by a cysteine-string motif and BH motif is absent form the TH domain (Berg et al., 2005).

TFKs are predominantly expressed in the hematopoietic cells, with distinct cell-specific patterns and differential levels in various cell types. ITK is the predominant among those expressed in T lymphocytes (ITK, TEC, and TXK), and is found in both differentiating and mature T cells at ~ 100 fold higher than the highly homologous TEC (Berg et al., 2005) and 3-10 fold higher than the less homologous TXK (Hu et al., 1995), at the mRNA level. ITK is critical in T cell development and initial activation, mainly because of its fundamental role downstream of T cell receptor (TcR) (Liao and Littman, 1995). In contrast, TEC is much less important in T cell differentiation and early activation through TcR signaling, but is involved in the mature T cell response to restimulation (Tomlinson et al., 2004). T cells developed in

the absence of TXK show no defects, but when ITK is deficient, additional loss of TXK can further exacerbate the T cell defects due to the loss of ITK function, suggesting some ITK-predominant and ITK/TXK redundant function during T cell differentiation (Schaeffer et al., 1999).

BTK, the other TFK highly homologous to ITK, is ubiquitously expressed, in all myeloid cells such as mast cells and macrophages (see review (Brunner et al., 2005)), and in most lymphoid cells, most highly expressed in differentiating and resting B cells (but not in T cells and mature effector plasma B cells) (Smith et al., 1994). The interesting fact that BTK is less abundant in fully activated effector B cells suggests the major function of BTK in B cells is during development and initial activation, which is very similar to the role of ITK in T cells. Along with BTK, TEC is also expressed in B cells, and similar to the ITK/TXK redundancy in T cells, loss of TEC alone does not affect B cell differentiation, but when BTK is absent, additional loss of TEC results in more severe B cell deficiency (Ellmeier et al., 2000).

Although ITK and BTK are highly homologous, they are selectively expressed in T and B cells respectively, and both are important for the cell differentiation, maturation and initial activation. Loss of ITK or BTK expression causes immunodeficiency in T or B cells, leading to severe immune disorders (details discussed below). In some cells, such as mast cells, ITK and BTK are both expressed, but are not required for the cell development, instead, they play some redundant as well as distinct functions in cell activation (Iyer et al., 2011). The main focus of this dissertation is ITK and ITK/BTK redundant functions, thus T cells with predominant ITK expression and mast cells with ITK/BTK co-expression are under investigation.

ITK in T cell receptor (TcR) signaling

The TcR is the common T cell surface receptor utilized by conventional T cells in recognizing peptide antigens presented on Major Histocompatibility complex (MHC) I or II by antigen presenting cells (APCs), over different T cell developmental stages, from antigen driven development in the thymus, to the response of naïve T cells to specific antigen in the periphery during an immune response to generate effector and memory T cells, and to the response of the latter cells during antigen re-exposure (Anderson et al., 1996). Activation of TcR by peptide/MHC complexes triggers its downstream signaling cascade that can contribute to various outcomes for each stage of the T cell's life (Anderson et al., 1996). ITK is abundantly expressed in T cells, and plays a critical role in TcR signaling for normal T cell development (Liao and Littman, 1995).

As mentioned above, ITK is composed of multiple functional domains, including PH, TH (BH + PRR), SH3, SH2 and kinase domains, which are all involved in the ITK function downstream of TcR (see scheme of signaling pathways in **Figure 1-1** (adapted from review (Kannan et al., 2012)[a]), and details to be found in the following text). Upon TcR activation, Src family kinase Lck is activated, leading to activation of TcR co-receptor CD3 phosphorylation on its tyrosine-based activation motif, which recruits and activates ZAP-70, that further phosphorylates adaptor proteins LAT and SLP-76 (Bogin et al., 2007; Shan and Wange, 1999). Meanwhile,

[a] Arun K. Kannan*, Weishan Huang*, Fei Huang*, and Avery August. (2012) Signal Transduction via the T cell Antigen Receptor in naïve and effector/memory T cells. *The International Journal of Biochemistry & Cell Biology* 44(12):2129-34.
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PI3 kinase is activated by Lck, and catalyzes the generation of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) lipids, which interact with PH domain to recruit ITK onto plasma membrane (August et al., 1997). This PH domain-mediated translocation is critical for the function of ITK as membrane interaction is a requirement for its full activation by upstream receptors via Lck (August et al., 1997; Ching et al., 1999; Woods et al., 2001), and possibly through ITK-ITK intermolecular phosphorylation as ITKs form clusters in a PH domain-dependent manner in the vicinity of recruiting receptors (Qi et al., 2006). TH, SH3 and SH2 domains all mediate protein-protein interactions that regulate the signaling transduction proximal to ITK (August and Ragin, 2012). Zinc-binding motif in ITK TH domain is requisite for the transient interaction with Gα13 and their synergistic effect on the activation of the distal downstream serum response factor (SRF) (Huang et al., 2013b). SH2 and SH3 domains are involved in ITK interaction with upstream and downstream signaling nodes and substrates, including activated adaptor proteins LAT and SLP-76 (Bunnell et al., 2000; Ching et al., 2000). The ITK/SLP-76 interaction is critical for the efficient activation of ITK as well as in TcR signaling. The SLP-76 tyrosine 145 (Y145) residue is involved in signaling downstream of ITK, although it is not required for the interaction; and T cells expressing the Y145F mutant of SLP-76 exhibit similar developmental and functional defects to those lacking ITK (Gordon et al., 2011; Jordan et al., 2008). This ITK/SLP-76 coupling is part of multi-protein complexes that are able to regulate the actin cytoskeleton and downstream signaling activation (Andreotti et al., 2010; August and Ragin, 2012; Berg et al., 2005; Kannan et al., 2012).

Once activated, ITK performs cis auto-phosphorylation on its SH3 domain, which in turn regulates its binding to adaptor proteins and other substrates (Joseph et al., 2007a; Wilcox and Berg, 2003). The tightly regulated interaction between ITK and the adaptor proteins brings ITK in close proximity with its substrate PLC-γ, which is directly phosphorylated by ITK (Joseph et al., 2007b; Perez-Villar and Kanner, 1999). Phosphorylated PLCγ catalyzes the breakdown of phosphatidylinositol-4,5-bisphosphate (PI4,5P) to second messengers- inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). ITK also enhances this process by recruiting the enzyme PIP5K to the membrane, which is able to replenish PI4,5P at the site of action (Saito et al., 2003). IP_3 binds to the IP_3 receptor in the membrane of ER, resulting in the release of Ca^{2+} from the ER. This depletion of ER Ca^{2+} stores results in the opening of Ca^{2+} channels in the plasma membrane, triggered through the sensor STIM1 communicating with CRAC channels in the plasma membrane, including the Orai1 channels (reviewed in (Hogan et al., 2010)). ITK has critical functions in regulating the initiation and maintenance of calcium influx from the extracellular store into the T cells (Fowell et al., 1999; Liu et al., 1998; Schaeffer et al., 1999). The resulting Ca^{2+} influx leads to the activation and nuclear translocation of NFAT through calcineurin and calmodulin (Smith-Garvin et al., 2009). DAG on the other hand activates PKCθ as well as the RAS guanine nucleotide releasing protein, RASGRP. The CARMA proteins are also recruited to ADAP (which also interacts with SLP-76), and are phosphorylated by PKCθ, along with Akt/PKB, leading to activation of the NF-κB transcription factor via the PKCθ-dependent pathway (Blonska and Lin, 2011; Narayan et al., 2006; Wegener et al., 2006). RASGRP on the other hand, initiates the

MAPK pathway by activating Ras, leading to activation of Raf and ERK. Ras can also activates JNK and p38 MAPKs downstream of the TcR (Smith-Garvin et al., 2009).

The ITK/SLP-76 complex generated during antigen induced TcR signaling form unique microclusters within the cell that is separate from the TcR clusters on the cytoplasmic membrane, and includes LAT, Grb-2, ZAP-70, PLC-γ and others (Seminario and Bunnell, 2008). These microclusters are important for activation of downstream pathways critical for activating the T cell (Seminario and Bunnell, 2008). The ITK/SLP-76 core can also traffick apart from the membrane to the cytoplasmic compartment, where it activates certain small G-proteins. The activation of other small G-proteins by TcR signals occur in part by the guanine nucleotide exchange factor Vav, which can activate CDC42 and Rac, both of which are upstream of MAPKs JNK and p38. CDC42 and Rac, along with the actin regulators Arp2/3 and WASP, are also important for regulating actin polymerization downstream of the TcR (for review see (Andreotti et al., 2010)). This TcR regulation of F-actin and retrograde actin flow also sustains the phosphorylation of PLC-γ and the release of intracellular Ca^{2+} store (Babich et al., 2012).

Overall, ITK plays critical roles in regulating the signaling pathways downstream of TcR, as a signaling amplifier by initiating and sustaining the production of second messengers, as well as a unique signaling modulator for actin cytoskeleton. However, the absence of ITK does not ablate downstream signaling following TcR activation, but substantially reduces the consequential activities, leading to for example, impaired calcium influx (Lewis and Cahalan, 1995), MAPK activation (Miller and Berg, 2002; Schaeffer et al., 1999; Schaeffer et al., 2000),

NFAT regulated transcription (Crabtree and Olson, 2002), cytokine production (Gomez-Rodriguez et al., 2009; Liao and Littman, 1995; Liu et al., 1998) and actin rearrangement (Labno et al., 2003). Thus ITK deficiency does not abrogate TcR mediated T cell development and activation, but instead, alters T cell lineage differentiation in the steady state and T cell activation and function in pathogenic conditions (see details in later sections).

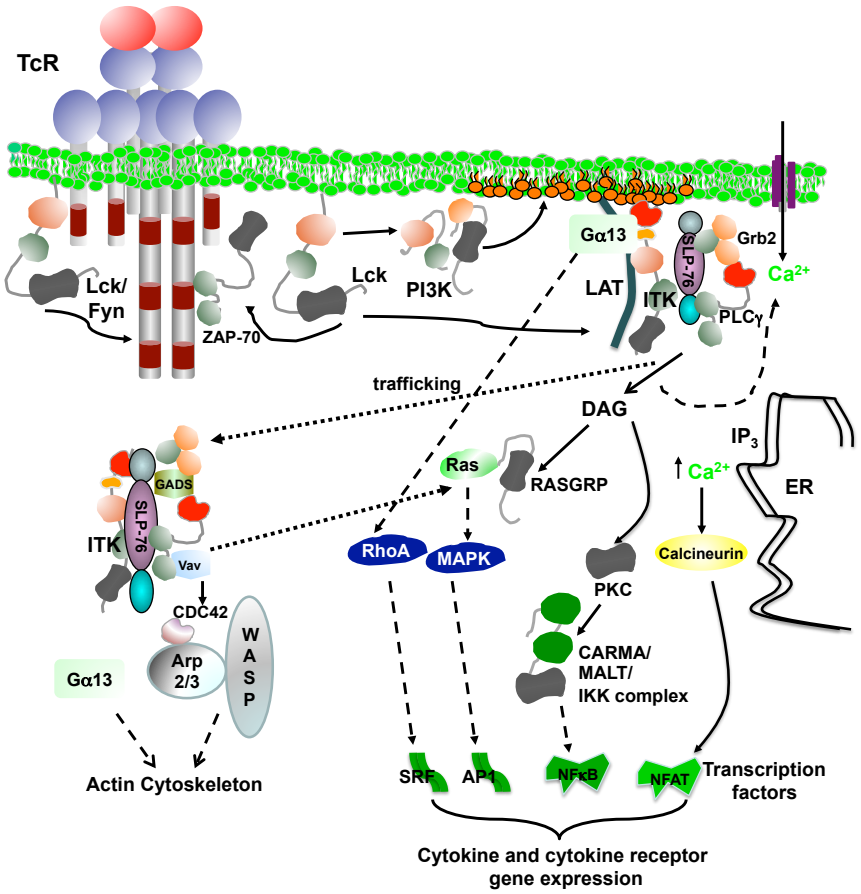

Figure 1-1. Signaling pathways mediated by ITK downstream of TcR. Dashed lines indicate indirect interactions or connections. Note the role of ITK as part of the signalosome regulating second messenger production and small G-protein activation, which contributes to transcriptional activation and rearrangement of actin cytoskeleton. Details are to be found in text.

ITK in T cell lineage development

TcR signaling governs T cell lineage fate from the very early stages of T cell differentiation. Its engagement in tonic interaction with other cells provide T cell progenitors survival and developmental signals, and because of the substantial function of ITK downstream of TcR, ITK plays critical roles in development of conventional T cells as well as the non-conventional T cells (or innate T cells, such as the newly defined innate memory T cells). This section summarizes the conventional and innate T cell differentiation models and the role of ITK in these processes (**Figure 1-2**, with details to be found in the following text).

In the most accepted model for T cell differentiation, conventional T cells develop in the thymus, which is composed of cells of hematopoietic origin that can give rise to T cells, and thymic structure related stromal cells. In the thymus, the differentiating T cells derived from hematopoietic cells are named thymocytes. TcR signaling strength and duration helps thymocytes survive, differentiate and become mature T cells. These differentiating thymocytes are defined into 4 different classes in the conventional T cell differentiation model, based on their expression of CD4 and CD8 molecules. From early to late stages they are: CD4/CD8 double negative (DN), CD4/CD8 double positive (DP), and CD4/CD8 single positive (SP, CD4 SP and CD8 SP share the same hierarchy in the lineage developmental tree) (Fowlkes et al., 1985). DN cells are the common T cell precursor for both conventional and non-conventional T cells, and may proceed toward αβ or γδ T cell fate, based on the early pre-TcR expression, in which αβ TcR is much more common than γδ TcR (Xiong and Raulet, 2007).

DN cells that have received competent signals through the αβ pre-TcR expand and progress to the next differentiating stage, the CD4/CD8 DP stage (Ciofani and Raffa, 2009). ITK can promote, although to a small extend, this DN→DP transition, as *Itk*$^{-/-}$ DN cells exhibit impaired pre-TcR signaling and these mice have smaller thyme. Furthermore, *Itk*$^{-/-}$ hematopoietic cells exhibit a disadvantage when competing with WT cells in this process (Lucas et al., 2007). The DP αβ T cell precursor diverge onto several paths, based on the type of further stimulation through αβ TcR (**Figure 1-2**).

In conventional T cell development, further progress of the DP T cell progenitors requires the interaction of the TcR and MHC expressed in the thymic stromal cells (TSC), via TcR and self-peptide/MHC complexes with help from CD4 or CD8 (Jameson et al., 1995). The DP cells, which receive moderate and adequate tonic stimulation through this interaction, will progress into CD4/CD8 SP cells, while those that receive insufficient signals undergo "death by neglect" (positive selection), those that have too high affinity with self-peptide/MHC are also eliminated through apoptosis (negative selection) (Bosselut, 2004; Singer and Bosselut, 2004). The lineage commitment towards CD4 or CD8 SP is determined by the pattern of TcR-MHC interaction (MHCI for CD8 SP, and MHCII for CD4 SP) (Singer and Bosselut, 2004). The proper DP→SP transition requires the expression of ITK, as in the absence of ITK, positive selection is defective to different degrees dependent on the strength of the selected TcR (Lucas et al., 2002), and negative selection is reduced or delayed (Liao and Littman, 1995; Lucas et al., 2002; Schaeffer et al., 2000).

The overall DN→DP→SP differentiation path in the thymus gives rise to mature but naïve conventional CD4 or CD8 SP T cells that have low expression of

CD44 ($CD44^{lo}$). These cells can migrate from the thymus to the periphery and require additional prolonged foreign antigenic stimulation to further differentiate in the periphery to become effector and/or memory T cells, forming one arm of adaptive immunity (Janeway CA, 2005).

Unlike conventional T cells described above, which are selected on thymic stroma by peptide-MHC complexes and require further activation to become effector/memory cells, non-conventional innate T cells such as invariant natural killer T (*i*NKT) cells, innate memory T cells, and γδ T cells have distinct selection pathways, express markers typically found on memory T cells, and have the ability to rapidly respond with effector function upon stimulation (Anderson et al., 1994; Behar and Porcelli, 2007; Berg, 2007; Born et al., 2006; Cho et al., 2011; Hu and August, 2008; Hu et al., 2007; Huang et al., 2013a; Oliveira et al., 2009; Urdahl et al., 2002).

Some αβ TcR expressing DP T cell precursors express TcR that contains an invariant α chain, and can recognized glycolipids (such as the protypical α-galactosyl ceramide (α-GalCer)) presented on a non-classical MHCIb molecule, CD1d, expressed by hematopoietic cells (HC) (Bendelac et al., 2007). These cells pass through a few intermediate differentiation stages and end up in a mature state with high expression of memory T cell marker CD44 and natural killer (NK) cell marker NK1.1, thus are named invariant NKT cells (*i*NKT cells) (Bendelac et al., 2007). The mature $CD44^{hi}NK1.1^{+}$ NKT cells are very capable of secreting cytokines such as IL-4 and IFN-γ when stimulated by α-GalCer through TcR activation (Godfrey et al., 2010). ITK is abundantly expressed in NKT cells, and substantially influences their maturation, survival and activation. In the absence of ITK, the final maturation stage

of NKT cells (up-regulation of NK1.1) is severely impaired, accompanied by increased cell death and impaired cytokine production (Au-Yeung and Fowell, 2007; Felices and Berg, 2008; Gadue and Stein, 2002). TXK is also expressed in NKT cells, although at a much lower level, and when over-expressed to the level of ITK in *Itk*$^{-/-}$ mice, can rescue the NK1.1 expression and cytokine production but failed to rescue NKT cell survival. Thus ITK and TXK share redundant positive functions in NKT cell maturation and activation, however, ITK plays a unique role in promoting NKT cells survival in a Fas/p53-related manner (Qi et al., 2012).

Although ITK is a substantial positive regulator for conventional T cell and the non-conventional NKT cell development, it has been found during the last decade to be, surprisingly, a negative regulator for other non-conventional innate T cell development, such as the innate memory T cells and a subset of NKT-like γδ T cells (see below).

Innate memory T cells were brought to the frontier of immunology research through the T cell phenotypic characterization in *Itk*$^{-/-}$ mice, in which, CD8 SP thymocytes were firstly found significantly increased (Liao and Littman, 1995; Lucas et al., 2002; Schaeffer et al., 2000), and later shown to express memory T cell markers CD44, CD122, and transcription factor Eomesodermin (Eomes), with rapid effector cytokine production upon stimulation (Atherly et al., 2006; Broussard et al., 2006; Dubois et al., 2006; Hu et al., 2007; Huang et al., 2013a). Although these phenotypes are typical characteristic of memory T cells derived from conventional T cell activation in the periphery, *Itk*$^{-/-}$ CD8 SP thymocytes gain them in the thymus upon completion of development independent of peripheral stimulation, thus are termed

innate memory phenotype (IMP) T cells. The development of IMP T cells share the early part of the conventional T cell differentiation path, and diverge from the DP precursors (**Figure 1-2**). However atypically, IMP $CD8^+$ T cell development is dependent on hematopoietic cell-MHCI (HC-MHCI) expression, but can be independent of the thymic MHCI and even the entire thymus, regardless the presence of ITK (Broussard et al., 2006; Huang et al., 2013a). Similar phenotypic alteration was also observed in the *Itk*$^{-/-}$ CD4 SP thymocytes, although to a lesser extent. In addition, some interesting characteristics such expression of PLZF (promyelocytic leukemia zinc finger), a transcription factor important for NKT function and IL-4 production was reported in these cells (Broussard et al., 2006; Hu and August, 2008; Raberger et al., 2008). And work described later in this dissertation found that IMP $CD4^+$ T cell development is also dependent on HC-MHC expression, in this case, HC-MHCII, expressed on dendritic cells (chapter five).

How ITK deficiency selectively enhances IMP T cell development is triggering more and more interest in the field, especially in the case of $CD8^+$ T cells, because of the highly significant IMP $CD8^+$ T cell accumulation in *Itk*$^{-/-}$ mice. Similar IMP T cell accumulative phenotypes have been observed in mice expressing SPL-76 Y145F mutant (the mutant that disrupts ITK/SPL-76 coupling, thus impairs ITK downstream signaling, as described in earlier section), mice lacking transcription factors Krüppel-like factor 2 (KLF2), inhibitor of DNA binding 3 (ID3), or CREB binding protein (CBP), and mice with over-expression of T cell factor-1 (TCF-1) or β-Catenin (Fukuyama et al., 2009; Jordan et al., 2008; Sharma et al., 2012; Verykokakis et al., 2010; Weinreich et al., 2009). A PLZF dependent IL-4 mediated pathway has

also been shown to control the generation IMP $CD8^+$ T cells in the thymus in a T cell extrinsic manner (Gordon et al., 2011; Lai et al., 2011; Weinreich et al., 2010). Ablation of IL-4 receptor from *Itk*$^{-/-}$ abrogates the excessive IMP $CD8^+$ T cell population, supporting the conclusion that IL-4 is responsible for IMP $CD8^+$ accumulation in the absence of ITK (Weinreich et al., 2010). In the presence of ITK, NKT cells, which can execute PLZF-dependent IL-4 production, is responsible for memory-like $CD8^+$ T cell development in BALB/c mice, thus was originally proposed to be the IL-4 source (Weinreich et al., 2010). However, in the absence of ITK, NKT cells are severely impaired in number as well as in IL-4 production (see above), thus it is highly possible that alternative mechanism(s) emanated by ITK deficiency is responsible for the IMP $CD8^+$ T cells development in *Itk*$^{-/-}$ mice. Some possible candidates have been proposed, such as the $CD4^+$ PLZF expressing innate T cells as described above, and a subset of NKT-like γδ T cells that are increased in *Itk*$^{-/-}$ mice (see below).

γδ T cell precursors diverge, in the very early stage, from the conventional T cell differentiation path (**Figure 1-2**). Although the cell-cell interaction requirement for γδ T cell differentiation is unknown, it was reported that a stronger TcR strength will increase the chance of γδ T cell commitment, when DN T cell precursors are faced with the decision between αβ and γδ T cell fates (Haks et al., 2005; Hayes et al., 2005), and so it was thought that ITK deficiency would reduce γδ T cell development, due to reduced TcR signaling activity in the absence of ITK. However, γδ T cells are found in dramatically increased number in the thymus and periphery in *Itk*$^{-/-}$ mice compared to WT (Felices et al., 2009; Qi et al., 2009). In *Itk*$^{-/-}$ mice, hyper-

immunoglobulin E (IgE) production (which was thought to be induced by increased IL-4 signals) can be reversed toward the WT level by removing γδ T cells (Felices et al., 2009; Qi et al., 2009), suggesting that γδ T cells participate to induce the IL-4-driven phenotype(s) in the absence of ITK. In addition, a significant subset of the *Itk*$^{-/-}$ γδ T cells appears to be PLZF^{+} IL-4 producing NKT-like cells, thus it won't be surprising if these NKT-like γδ T cells function to compensate the defective NKT cells and contribute to the IMP CD8^{+} T cell accumulation in *Itk*$^{-/-}$ mice. So together with the CD4^{+}PLZF^{+}IL-4 producing innate T cells, NKT-like γδ T cells was also proposed to be the alternative candidate for IL-4 source to induce IMP CD8^{+} T cells in *Itk*$^{-/-}$ mice. However, whether these speculations can stand remains to be investigated.

Although it is proposed that increased IL-4 production by other cells is responsible for IMP CD8^{+} T cell development in the absence of ITK, in a T cell-extrinsic manner, to date, there has been no direct evidence to show higher circulating levels of IL-4 or increased IL-4 signals influencing CD8^{+} T cell differentiation in *Itk*$^{-/-}$ mice. Hence, we cannot rule out a T cell-intrinsic ITK function in the IL-4 dependent IMP CD8^{+} T cell development. Very recently, a small subset of peripheral CD8^{+} T cells were found to carry "born memory" in mice, independently of antigenic stimulation, which is very similar to the innate CD8^{+} T cells. This newly found population have been termed "virtual memory (VM)" CD8^{+} cells, and were found with features of T cells that undergone homeostatic proliferation (Akue et al., 2012; Haluszczak et al., 2009; Lee et al., 2013). T cell maintenance/homeostasis in the periphery is mediated by the tonic interaction between TcR and self-MHC complex, and in lymphopenic conditions, in which T cell number is significantly lower than

"normal" amount, naïve T cells initiate homeostatic expansion to restore the number to the "normal" level. Naïve T cells that have undergone homeostatic expansion gain a memory-like phenotype independently of foreign antigens (Surh and Sprent, 2000). This process occurs during the neonatal lymphopenic period in both mice (Min et al., 2003) and humans (Correa-Rocha et al., 2012), and can contribute to the induction of T cell homeostatic proliferation. Thus, it is reasonable to hypothesize a role of ITK in regulating $CD8^+$ T cell number and memory acquisition through T cell homeostatic proliferation. A common and main obstacle in testing the function of ITK in the naïve $CD8^+$ response to IL-4 and homeostatic environment, particularly proliferation and memory development, is the lack of naïve $CD8^+$ T cells in *Itk*$^{-/-}$ mice, leading to the inability of using *Itk*$^{-/-}$ naïve $CD8^+$ T cells to study the effect of loss of ITK function. Work to be presented later in the dissertation established and utilized a transgenic murine model that retains naïve $CD8^+$ T cells in *Itk*$^{-/-}$ condition, thus allowing the investigation of T cell-intrinsic function of ITK in these cells.

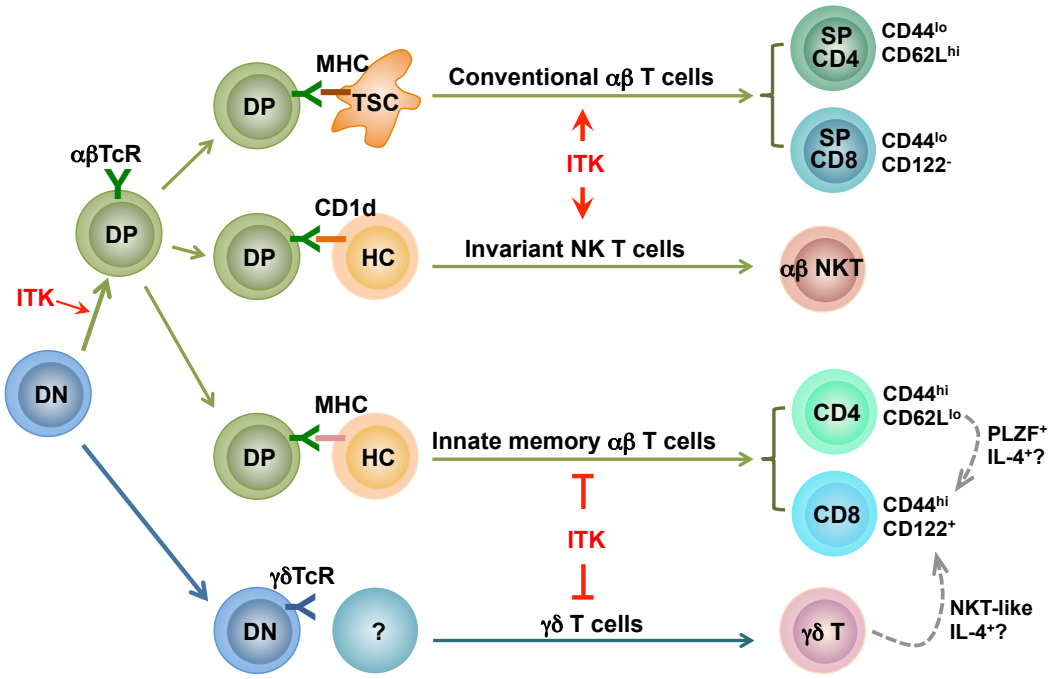

Figure 1-2. ITK function in T cell lineage development. $CD4^-CD8^-$ (DN) thymocytes differentiate into αβ or γδ T cell progenitors. ITK promotes the transition of DN cells into $CD4^+CD8^+$ (DP) αβ T cell precursors, which diverge onto different paths leading to thymic stromal cell (TSC)-MHC restricted conventional αβ T cells, hematopoietic cell (HC)-CD1d restricted αβ NKT cells, or HC-MHC restricted non-conventional innate memory (IMP) αβ T cells. ITK positively regulate conventional T cell and NKT cell development, but suppresses IMP T cell development. γδ T cell development is negatively regulated by ITK as well. In the absence of ITK, IMP $CD8^+$ T cells are significantly increased, accompanied by increased $CD4^+PLZF^+$ innate T cells and NKT-like γδ T cells that are capable of IL-4 production and are proposed as candidates for the source of IL-4 to induce IMP $CD8^+$ T cells. More details are to be found through the text.

ITK regulated T cell activation and function in disease

Given the critical role that ITK plays in TcR signaling, and thus in T cell development, ITK was found to regulate multiple types of T cell-mediated pathogenesis in both human and mouse. It was decades after the discovery of the Itk gene (Siliciano et al., 1992) when the first clinical cases with ITK deficiency in humans were reported (Huck et al., 2009). Patients, who carry homozygous missense mutation in PH, SH3, and kinase domains, or homozygous nonsense mutation causing a pre-mature stop codon in ITK kinase domain with a resultant truncated ITK expression, were found with susceptibility to severe or fatal Epstein-Barr virus (EBV) -associated lymphoproliferative disorder (Huck et al., 2009; Linka et al., 2012; Stepensky et al., 2011). These mutants were all found to have a reduced half-life of ITK protein, associated with T cell deficiency, thus EBV infection, which is positively correlated to B cell lymphoma occurrence, leading to severe lymphoproliferative disorder in these patients (Linka et al., 2012). Although defective ITK expression results in significantly higher susceptibility to EBV-associated lymphoproliferative disorder, increased ITK expression in WT mature T cells was shown in patients with Th2-mediated atopic dermatitis (Matsumoto et al., 2002). Studies of single nucleotide polymorphism (SNP) showed that variants in the ITK gene promoter region could enhance binding of nuclear factors to contribute to a more active promoter function, and this variant is observed at a significantly higher frequency in asthmatic patients (Lee et al., 2011). Thus under genetically normal conditions, increased ITK expression is also a risk factor associated with certain human diseases. Similar to ITK deficiency, BTK deficiency leads to a primary immunodeficiency, however, in B cells, named X-

linked agammaglobulinemia (XLA) in humans. XLA patients, almost exclusively males due to the X-linked heredity, are extremely susceptible to bacterial infection from early childhood, due to dramatic defect in B cell development and function that impairs serum immunoglobulin production (Tsukada et al., 1993; Vetrie et al., 1993).

In mice, the resultant consequences of the absence of ITK are not overt in non-pathogenic condition, as *Itk*$^{-/-}$ mice can live without obvious diseases until very old age (greater than one year). This is relatively mild, compared to the cases reported in humans. It is possibly due to the fact that only the cases where patients who already develop diseases and seek diagnosis were used to find out the consequence of ITK deficiency, in humans. More but not all humans with SNP associated with ITK over-expression develop asthma, a disease induced by environmental cues (Lee et al., 2011). It is possible that ITK only functions through T cell activation in pathogenic conditions to regulate disease development. *Itk*$^{-/-}$ mice have been tested with several disease-inducing experimental protocols, with main focus on the conventional T cell activation and function, including CD8^{+} T cell-mediated viral/bacterial clearance and CD4^{+} T cell-mediated inflammatory diseases.

CD8^{+} T cells play critical roles in immune responses to bacterial and viral infections. Initial antigen or pathogen recognition initiates clonal expansion of naïve CD8^{+} T cells, which develop into effector T cells, which have cytotoxicity activity on infected cells, and can clear infectious agents through secreting cytokines such as IFN-γ and TNF-α (Harty et al., 2000). In the absence of ITK, mice exhibited altered CD8^{+} T cell antiviral responses, with decreased CD8^{+} T cell cytotoxic function against *Lymphocytic Choriomeningitis Virus*, *Vaccinia Virus*, and *Vesicular Stomatitis Virus*,

with delayed viral clearance (Bachmann et al., 1999). It was originally thought that these data served as evidence to implicate a positive function of ITK in naïve $CD8^+$ T cell effector function development skewed by viral infection. However, this work was done before the discovery of the IMP $CD8^+$ T cell accumulation in *Itk*$^{-/-}$ mice. It is recently questioned that the impaired anti-viral $CD8^+$ T cell function observed in *Itk*$^{-/-}$ mice is not solely due to the lack of ITK function downstream of TcR, but also included the impact of the $CD8^+$ T cell phenotype in uninfected *Itk*$^{-/-}$ mice, which is highly distinct from that seen in WT. From the initial state of viral challenge, *Itk*$^{-/-}$ mice already has significantly higher proportion and number of memory-like cells, and whether this is the reason for the delayed anti-viral protection in *Itk*$^{-/-}$ is to date unclear.

The function of ITK in conventional $CD4^+$ T cell activation, sub-lineage differentiation and related disease development is better known, mainly because of the availability of naïve $CD4^+$ T cells in *Itk*$^{-/-}$ mice. In WT mice, peripheral naïve $CD4^+$ T cells represent up ~ 80% of total $CD4^+$ T cells, and are reduced to less than 40% in the absence of ITK, in adult mice (Hu and August, 2008), however, this still allows isolation of naïve $CD4^+$ T cells for specific investigation. Naïve $CD4^+$ T cells can be activated by antigen in combination with different cytokines to differentiate into a variety of T helper subsets, defined by their ability to secrete different cytokines. Th1 cells are those capable in secreting cytokines such as IFN-γ, and play important roles in immune response to intracellular bacterial/viral infection, and autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), multiple sclerosis (MS), diabetes, and lupus (Boehm et al., 1997; Cooper et al., 1993; Huang et al., 1993; Szabo et al., 2003). Th2 cells, by contrast, produce a representative cytokine IL-4, and

are involved in immune responses to worm infections, and are well known to promote inflammatory diseases such as allergic asthma (Busse and Lemanske, 2001; Finkelman et al., 1997; Lemanske and Busse, 2003; Maizels and Yazdanbakhsh, 2003). Whereas Th17 cells typically produce IL-17A, which is critical in recruiting neutrophils, and contributes to immune responses during infection of extracellular pathogens (including bacteria, mycobacteria and fungi), and the development of EAE and MS (Aranami and Yamamura, 2008; Curtis and Way, 2009; Oukka, 2007; Weaver et al., 2007).

The absence of ITK leads to alterations in Th1, Th2 and Th17 cell differentiation (Au-Yeung et al., 2006; Gomez-Rodriguez et al., 2009; Kannan et al., 2013b; Miller et al., 2004; Sahu et al., 2008). *Itk*$^{-/-}$ naïve CD4^{+} T cells have significantly higher accessibility in the IFN-γ gene promoter region, leading to preferential expression of IFN-γ (compared to IL-4), which further enhances other Th1 related gene expression, and suppresses Th2 cell differentiation (Kannan et al., 2013b). Mice lacking ITK fail to develop Th2-meidated allergic airway inflammation in experimental allergic asthma models, due to the impaired Th2 cell differentiation and Th2 cytokine production (Ferrara et al., 2006; Mueller and August, 2003). Some parasites such as *Leishmania major*, normally elicit Th2 response in WT mice, which is not sufficient to clear the parasitic agents, thus these parasites can cause severe sickness or death in WT mice. In contrast, in the absence of ITK, the same parasites can induce strong Th1 responses that are effective in clearing the pathogens, and help *Itk*$^{-/-}$ mice survive (Fowell et al., 1999; Sahu et al., 2008; Schaeffer et al., 2000; Schaeffer et al., 2001). However, *Toxoplasma gondii*, an intracellular parasitic protozoa that can induce strong Th1 response and IFN-γ production, resulted in more

severe pathogenesis and lethality in *Itk*$^{-/-}$ mice (Schaeffer et al., 1999), with unknown mechanism, suggested to be due to the defective memory response required to survive this infection (August and Ragin, 2012). Th17 cell differentiation and IL-17 production by CD4^{+} T cells are significantly impaired in the absence of ITK (Gomez-Rodriguez et al., 2009). Consistent to the cellular defect, *Itk*$^{-/-}$ mice are resistant to EAE, a Th17-mediated autoimmune disorder (meeting abstracts by (Douhan et al., 2008) and (Kannan et al., 2013a)).

More interestingly, in human CD4^{+} T cells, ITK enhances human immunodeficiency virus type 1 (HIV-1) infection. Productive HIV-1 infection requires CD4^{+} T cell activation, chemokine receptor expression, and actin reorganization, which are all regulated by ITK downstream of TcR signaling. And *in vitro*, loss of ITK expression, expression of ITK kinase-dead mutant, or application of ITK inhibitors can attenuate in part viral entry, replication and spread, in human CD4^{+} T cells (Readinger et al., 2008).

The pivotal function of ITK in the development of Th2- and Th17-mediated inflammatory diseases has triggered significantly interest in using ITK as an anti-inflammatory target. Although ITK is composed of multiple functional domains, its core function has been implicated to be the kinase activity, which is cooperatively regulated by other domain functions (see earlier section on ITK structure and function in signaling), thus inhibiting ITK kinase activity is currently the underlying strategy to target ITK therapeutically. A number of ITK inhibitor candidates have been found through high-throughput screen, and shown to bind to and block ITK activation with effect in reducing related T cell activation, production of pro-inflammatory cytokines

and development of inflammatory diseases (see reviews (Sahu and August, 2009; Vargas et al., 2013)).

However, the interest in ITK as a therapeutic target was mainly induced by the role of ITK in conventional T cells. Conventional T cells require ITK to develop into pathogenic effectors in Th2 and Th17 inducing conditions, thus the lack of ITK can attenuate associated diseases. But in the absence of ITK function, IMP T cells selectively develop, and this is mainly due to lack of ITK kinase activity (Hu and August, 2008; Hu et al., 2007). It is possible that treating patients with ITK inhibitors can trigger accumulation of IMP T cells, and it is so far not clear what functions IMP T cells have in disease or immune responses. IMP $CD8^+$ T cells have been shown in one study to be protective against *Listeria monocytogenes* (a bacterium) infection (Hu et al., 2007), without future details, while the function of IMP $CD4^+$ T cells awaits investigation. Work presented later in this dissertation established murine models with predominantly naïve or IMP T cells, allowing the study of the function of IMP T cells in comparison to naïve T cells *in vivo*.

More concern has been raised towards inhibitor specificity. Due to the high homology of Tec family kinases, especially among ITK, BTK and TEC, it has been a significant challenge to develop ITK inhibitors with desired potency and selectivity against other kinases (Sahu and August, 2009; Vargas et al., 2013). Cross-reactivity with BTK is one of the common problems. In allergic asthma, the disease for which scientists have the most interest in developing an ITK inhibitor, mast cells play critical roles in the pathogenesis. And because of the "quick responder" feature of mast cells, they may be one of those responding to ITK inhibition treatments. Thus it is important

to extend the study to understand the function of ITK and BTK in mast cells, to help evaluate whether ITK inhibition strategy can achieve satisfactory potency as well as specificity.

Mast cells and ITK/BTK

Mast cells are derived from hematopoietic progenitors. After the migration of their precursors into vascular system or serosal cavity, they remain as tissue residents during the differentiation and maturation phases (see review (Kalesnikoff and Galli, 2008)). Mast cells are essential effector cells in allergic responses and innate immunity (Galli et al., 2005a), and also regulate the differentiation of adaptive immunity in antigenic environments (Galli et al., 2005b).

Mast cells carry numerous granules containing preformed factors that are released by exocytosis within seconds to minutes following stimulation. The process is termed "degranulation", and results in the release of granule contents including histamine, serotonin, a variety of proteases, and a number of cytokines. Along with degranulation, activated mast cells also respond within minutes of stimulation with secretion of certain metabolic products that can enhance vascular permeability and smooth muscle contraction, and act on immune cells to trigger further immune responses. Thus, during innate immune responses, mast cells act rapidly upon stimulation, and serve as ''sentinels'' to alert the local surroundings of the potential detrimental challenges by invading agents (see reviews (Galli et al., 2005a; Galli et al., 2005b)). Mast cell immunologic specificity is mediated by Fc epsilon receptor I (FcεRI), a high affinity receptor for IgE. When FcεRI expressed on mast cell is bound with IgE, mast cells can recognize the antigens that are specifically cognate to the bound IgE, and become activated in minutes (see review (Kalesnikoff and Galli, 2008)). Antigen cross linking of IgE bound to the FcεRI on mast cells results in activation of downstream signaling events that lead to effector functions such as

degranulation and cytokine secretion. The inflammatory mediators and cytokines released from mast cells play a critical role in orchestrating the immune response against the antigen/allergen. As a result there is considerable interest in investigating critical modulators that regulate the processes of degranulation and cytokine secretion. Immediate signaling downstream of FcεRI/IgE/Antigen include activation of a variety of kinase, adaptor functions and Ca^{+} mobilization (see review (Felices et al., 2007)), among which Tec family kinases ITK and BTK were found to be involved.

Mast cells express four out of five Tec family kinases namely, ITK, BTK, TEC and TXK (Qiu and Kung, 2000). Among these the roles for ITK, BTK and more recently TEC downstream of the FcεRI have been investigated (Kawakami et al., 1994; Kawakami et al., 1995; Schmidt et al., 2009). ITK is activated in mast cells upon FcεRI-mediated signaling and *Itk*$^{-/-}$ mice have been reported to display decreased mast cell degranulation and histamine release *in vivo* (Forssell et al., 2005; Kawakami et al., 1995). However, analysis of ITK null mast cells revealed that the absence of ITK does not intrinsically affect mast cell degranulation either *in vitro* or *in vivo* (Iyer and August, 2008). Although ITK is critical for TcR-mediated induction of intracellular Ca^{2+} mobilization and Erk activation, ITK is not required for intracellular calcium signaling in response to FcεRI-mediated signaling in mast cells, and ITK-deficient mast cells secrete elevated cytokines upon activation (Iyer and August, 2008), strongly suggesting that ITK has cell type-specific functions. BTK shares very high structural homology with ITK (Felices et al., 2007), and is expressed in mast cells at similar level to ITK (meeting abstract by (Evans et al., 2009)). In mast cells, BTK is activated by the FcεRI, c-Kit and IL-3 receptors (Iwaki et al., 2005; Kawakami et al.,

1997; Setoguchi et al., 1998). Mast cell development appears normal in the absence of BTK, both *in vivo* and *in vitro*. However, more bone marrow-derived mast cells (BMMCs) are produced from BTK deficient mice in response to IL-3 (Kawakami et al., 1997). *Btk*$^{-/-}$ BMMCs also exhibit mild to moderate impairment of early and late FcεRI-mediated responses such as degranulation, histamine release, and cytokine production (Hata et al., 1998; Iwaki et al., 2005). Of note, the defects in cytokine secretion observed in *Btk*$^{-/-}$ mast cells can be partially rescued by retroviral transduction of ITK, suggesting that ITK may have redundant or compensatory functions with BTK in regulating mast cell responses (Hata et al., 1998).

Neither ITK nor BTK is required for the development of mast cells *in vivo* or *in vitro*. However, mice lacking both kinases exhibit lower histamine release in response to antigen induced systemic anaphylaxis. Furthermore, *Itk*$^{-/-}$*Btk*$^{-/-}$ mice have significantly elevated serum IgE levels, which results in increased occupancy of FcεRI on mast cells by IgE. This may explain the decreased antigen specific degranulation *in vivo*. Analysis of *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs indicates that these cells have increased growth in response to IL-3, but also have a cell-intrinsic defect in degranulation and intracellular Ca^{2+} mobilization in response to antigenic stimulation. In this response, both ITK and BTK contribute to the mast cell function, with some common and distinct features. For examples, BTK contributes positively to the secretion of IL-2, IL-3, IL-4, TNF-α and GM-CSF; while ITK contributes positively to secretion of IL-3 and GM-CSF, negatively to IL-2 and GM-CSF, and does not make significant contributions to the secretion of IL-4 and TNF-α. (Iyer and August, 2008; Iyer et al., 2011; Kawakami et al., 1994; Kawakami et al., 1995; Schmidt et al., 2009)

In addition to the well-known FcεRI/IgE mediated immunity to multi-cellular parasites and in the pathogenesis of allergy and asthma, the importance of mast cells in pathogen-associated molecular patters (PAMP)-mediated innate immune defense against bacteria and viruses is attracting more and more appreciation. Mast cells express Toll-like receptors (TLRs) and respond to TLR ligands by secreting cytokines, chemokines, and lipid mediators (Sandig and Bulfone-Paus, 2012). Gram-negative bacterial endotoxin lipopolysaccharide (LPS) is a TLR4 specific ligand (Iwasaki and Medzhitov, 2004). In mast cells, LPS was shown to bind to TLR4 (McCurdy et al., 2001) to activate MAPKs (Masuda et al., 2002) and NF-κB (Nigo et al., 2006), and induced PI3K/Akt activation that regulates NK-κB activation (Song et al., 2012). Different from the FcεRI/IgE-mediated response, LPS induced mast cell response does not execute mast cell degranulation or intracellular Ca^{2+} mobilization (Supajatura et al., 2002). BTK has been shown to bind TLR4 and be involved in NF-κB activation (Jefferies et al., 2003). In human monocytic cell line (Gray et al., 2006) and primary hematopoietic cells (Marron et al., 2012), BTK phosphorylates TLR4 downstream MyD88-adaptor like protein (MAL), leading to MAL degradation, forming a negative feedback to TLR4 signaling. However, the role of BTK in LPS-induced mast cell response is not very clear. Some reports suggest that BTK is a positive mediator in TLR4 signaling (Doyle et al., 2005; Gray et al., 2006; Jefferies et al., 2003; Mansell et al., 2006; Semaan et al., 2011), others suggest that BTK is dispensable (a negative regulator) in mast cell response to LPS, as LPS induced TNF-α and IL-6 was actually increased in 129/Sv (mouse strain) BMMCs lacking BTK, suggesting that the role of BTK in TLR4 signaling might be cell type specific or strain dependent (Zorn et al.,

2009). Whereas the role of ITK in mast cell response to LPS is not yet investigated.

Given the rapid and potent response to allergen and endotoxin, mast cells are critical promoting players in the development of allergy (Galli and Tsai, 2012) and sepsis (Ramos et al., 2010), which can lead to life-long sickness and sudden death respectively. Understanding the biological and biomedical function of ITK (and BTK) in mast cell response to related pathogenic conditions, will contribute to our knowledge of whether pharmacological inhibition of ITK is a promising strategy for disease therapy.

Overview of research goals and dissertation outline

Immunity protects hosts against environmental invasions (such as parasitic infection) and endogenous attack (such as tumorigenesis), but also can harm the hosts through auto- and over- immune responses mainly by causing inflammation. Immunity is composed of innate and adaptive immunity. Innate immunity is the first line of defense for the immune system to face incoming invasion, and initiate adaptive immunity. Tec family kinase ITK is well characterized for its function in regulating T cell acquired immunity, and has been proposed as a therapeutic target in several immune diseases. Much less is known about its role(s) in innate immune functions. This dissertation focuses on the function of ITK in innate immunity, in particular, in innate T cells and mast cells, aiming to extend our understanding of the value of targeting ITK in therapies.

Loss of ITK results in increased innate memory-like T cells, thus the first aim of this dissertation is understand the development and function of these memory-like T cells and the role of ITK in these processes. This is attempted to help us determine whether innate T cell, which are selectively developed in the absence of ITK, can influence the effect of targeting ITK. Due to the high homology ITK and BTK, it is difficult to target ITK specifically, thus this dissertation also investigated the redundant function of ITK and BTK in mast cells, which express both kinases. Work done on the compensatory function of ITK and BTK in mast cells will help us determine the possible side effects when ITK inhibitors are off-target.

Chapter one reviews the Tec family kinases with focus on ITK, on its protein structure, function in signaling, function in cell activation, and role(s) in related disease development. **Chapter two** introduces an innate memory phonotype (IMP) $CD8^+$ T cell population, which is significantly expanded in the absence of ITK, and reports the developmental and functional characteristics of such cells. This population

carries memory-like features independently of antigenic stimulation, and is distinct from cells resulted from homeostatic proliferation. This work established murine models with predominant naïve or IMP $CD8^+$ T cells, and suggests the involvement of IMP $CD8^+$ T cells in innate immune response. This work further extended the concept of innate immunity, and shed light on a novel function of IMP $CD8^+$ T cells in bridging the innate and adaptive immunity. **Chapter three** focuses on the function of ITK in regulating the development of IMP $CD8^+$ T cells. In the current model, IL-4 derived from NKT-like γδ T cells is thought to be responsible for the accumulation of IMP $CD8^+$ T cells in *Itk*$^{-/-}$ mice in a T cell-extrinsic manner, however, my work finds that γδ T cells are dispensable in this process, and that ITK functions directly downstream of IL-4 to regulate the development of innate $CD8^+$ T cells. To my knowledge, this work, for the first time, shows a T cell-autonomous function of ITK in regulating innate T cell development, which challenges the current model. **Chapter four** describes a regulatory function for ITK in population dynamics and effector memory generation of $CD8^+$ T cells undergoing lymphopenia-induced proliferation, and that ITK suppresses the anti-tumor immunity of such cells in a cell-autonomous manner. **Chapter five** establishes murine models carrying predominant naïve or innate memory $CD4^+$ T cells, and demonstrates that innate memory $CD4^+$ T cells are protective against autoimmune graft-versus-host disease post bone marrow transplantation. **Chapter six** describes the cooperative regulatory function for ITK and BTK in mast cell response to endotoxic stimulation. In the absence of ITK and BTK, mast cells exhibit hyperactive TNF-α production induced by bacterial endotoxin, and exacerbate the hypothermic septic response. This work implicates the complex function of Tec kinases in immune diseases and provides warning on using them as therapeutic targets. Due to the case specific function of ITK and BTK, drug applications should be selected based on the differential roles of these kinases.

Chapter seven summarizes the function of ITK in innate T cells and mast cells, and discusses the value and concerns of targeting ITK in inflammatory diseases.

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CHAPTER TWO

Innate memory $CD8^+$ T cells are distinct from homeostatic expanded $CD8^+$ T cells and rapidly respond to primary antigenic stimuli[b]

[b] Weishan Huang, Jianfang Hu, and Avery August. Cutting Edge: Innate Memory $CD8^+$ T Cells Are Distinct from Homeostatic Expanded $CD8^+$ T Cells and Rapidly Respond to Primary Antigenic Stimuli. *The Journal of Immunology* 190: 2490-2494.

Abstract

Innate memory phenotype (IMP) $CD8^+$ T cells are non-conventional αβ T cells exhibiting features of innate immune cells, and are significantly increased in the absence of ITK. Their developmental path and function are not clear. Here we show hematopoietic MHCI dependent generation of antigen specific IMP $CD8^+$ T cells using bone marrow chimeras. WT bone marrow gives rise to IMP $CD8^+$ T cells in $MHCI^{-/-}$ recipients, resembling those in *Itk*$^{-/-}$ mice, but distinct from those derived via homeostatic proliferation (HP), and independent of recipient thymus. By contrast, $MHCI^{-/-}$ bone marrow does not lead to IMP $CD8^+$ T cells in WT recipients. OTI IMP $CD8^+$ T cells generated via this method exhibited enhanced early response to antigen without prior primary stimulation. Our findings suggest a method to generate antigen specific "naïve" $CD8^+$ IMP T cells, demonstrate that they are not HP cells and can promptly respond in an antigen specific fashion.

Introduction

Conventional T cells arise as naïve T cells through thymic selection, and require further exposure to antigens to expand and acquire effector/memory function (Berg, 2007). While conventional T cells are selected on thymic stroma by peptide-MHC complexes, non-conventional innate T cells have distinct selection pathways, spontaneously express markers typically found on memory T cells, and have the ability to rapidly respond upon stimulation (Anderson et al., 1994; Behar and Porcelli, 2007; Berg, 2007; Born et al., 2006; Cho et al., 2011; Oliveira et al., 2009; Urdahl et al., 2002). Non-conventional $CD8^+$ T cells are distinguished by their memory-like attributes of the effector program such as secretion of IFN-γ and expression of CD44 and CD122 (IL-2Rβ) (Intlekofer et al., 2005; Pearce et al., 2003), and are thus termed "Innate Memory" or "Innate Memory Phenotype (IMP)" $CD8^+$ T cells (Berg, 2007; Broussard et al., 2006; Horai et al., 2007; Hu et al., 2007; Lai et al., 2011; Weinreich et al., 2010). IMP $CD8^+$ T cells may be selected independent of thymic MHCIa, with a dominant role played by hematopoietic MHCI (Broussard et al., 2006; Horai et al., 2007). However, it was also suggested recently that innate memory-like $CD8^+$ thymocytes are mainly selected by nonhematopoietic MHCIa (Rafei et al., 2011), strongly alluding to alternative pathways for IMP $CD8^+$ T cell development.

IMP $CD8^+$ exist in specific-pathogen-free as well as in germ-free mice (Huang et al., 2005), independent of immunization and infection, and are significantly increased in the absence of Tec kinase ITK, and the transcriptional regulators CBP, KLF2 and Id3, or mice carrying the ITK binding mutant of Slp-76 (Berg, 2007; Broussard et al., 2006; Fukuyama et al., 2009; Horai et al., 2007; Hu et al., 2007;

Jordan et al., 2008; Verykokakis et al., 2010; Weinreich et al., 2009). IL-4 has been shown to be involved in the generation of these cells in cell extrinsic manner in the absence of ITK (Weinreich et al., 2010). Although it is known that IMP $CD8^+$ are distinct from antigen-induced memory $CD8^+$ T cells, they share markers of T cells that have undergone homeostatic expansion (Haluszczak et al., 2009), and so it is not clear whether IMP $CD8^+$ T cells arise through homeostatic proliferation. Functionally, IMP $CD8^+$ T cells can facilitate the clearance of *Listeria monocytogenes* infection (Hu et al., 2007), however, it has been difficult to directly examine whether IMP $CD8^+$ T cells can respond to antigen during an immune response due to the difficulty discriminating IMP $CD8^+$ T cells from conventional memory $CD8^+$ T cells.

Materials and Methods

Mice.

All mice were on a C57BL/6 background. WT, MHCI$^{-/-}$ (*B2m*$^{-/-}$: B6.129P2-*B2m*^{tm1Unc}/J), and nude (B6.Cg-*Foxn1*^{nu}/J) mice were from the Jackson Laboratory (Bar Harbor, ME). MHCI$^{-/-}$OTI mice were derived by crossing OTI transgenic mice and MHCI$^{-/-}$ mice. All experiments were approved by the Office of Research Protection's IACUC at Pennsylvania State and Cornell Universities.

Generation of bone marrow chimeras.

Bone marrow from donor mice was injected i.v. into lethally irradiated recipients (6-9 weeks old). In some cases recipients or donors were congenic for CD45 as indicated in the figures. T cell depleted bone marrow was obtained by negative selection using biotinylated-anti-CD3ε antibody (eBioscience, San Diego, CA) and magnetic column separation (Miltenyi Biotec, Cambridge, MA). Mice were kept in specific pathogen free environment, and fed acid water containing antibiotics (1 mg/ml Gentamycin Sulfate) for 8-9 weeks post BMT prior to analysis.

Cell sorting, microarray and quantitative real-time PCR.

Cell sorting was performed on a FACSAria Cell Sorter (BD Biosciences, San Jose, CA). IMP CD8$^+$ T cells (TCRβ$^+$CD8α$^+$CD44^{hi}) were sorted from splenocytes of WT→MHCI$^{-/-}$ chimeras 8 weeks post transplantation and 8-week old *Itk*$^{-/-}$ mice. HP cells were generated by i.v. injecting naïve CD8$^+$ T cells (TCRβ$^+$CD8α$^+$CD44^{lo}) into

Rag1$^{-/-}$ recipients (0.5×10^6/mouse), followed by sorting of TCRβ$^+$CD8α$^+$CD44^{hi} cells 8 weeks post transfer. mRNA from sorted cells was extracted, amplified and used for microarray (GeneChip Mouse Genome 430 2.0 Array, Affymetrix, Santa Clara, CA) at the Cornell University Life Sciences Core Laboratories Center. Microarray data were analyzed using GeneSpring GX software (Agilent Technologies, Clara, CA). RMA-normalized probe values were used to generate correlation coefficient matrix, further converted to gene expression values with Quantile normalization, followed by analysis of gene differential expression. Data have been deposited into the NCBI GEO repository (accession # GSE41482). Quantitative real-time PCR was carried out using Taqman probe sets (Applied Biosystems, Foster City, CA).

In vitro stimulation.

Splenocytes were stimulated with PMA/Ionomycin (Sigma-Aldrich, St. Louis, MO), 100 μg/ml Ovalbumin (Sigma) or 1 μg/ml $OVA_{257-264}$ (SIINFEKL, Peptides International, Louisville, KY) as indicated. 10 μg/ml Brefeldin A (Sigma) was added on day 1, 3 and 6 for the last 6 - 7 hours, followed by staining and flow cytometric analysis.

Antibodies.

All antibodies used are listed in "fluorochrome-target" format as follows: eFluor 450-CD122, PE-MHCI, Alexa Fluor 700-CD45.2, PerCP-Cy5.5-TNF-α, PE-Cy7-IFN-γ (eBioscience); V500-CD44, FITC-CD45.1, FITC-TCRVβ5, PE-TCRVβ5, PE-TCRVα2, PE-CD44, PE-Cy5-CD44, Allophycocyanin-Cy7-TCRβ (BD Biosciences);

PE-Texas Red-CD8α (Invitrogen, Carlsbad, CA).

Data analysis.

Two-tailed Student's *t* test was performed using GraphPad Prism v5.00 (GraphPad, San Diego, CA), with $p < 0.05$ considered statistically significant.

Results

Development of IMP CD8$^+$ T cells via hematopoietic MHCI selection independent of the thymus

IMP CD8$^+$ T cells have been reported to be able to develop in the thymus independent of MHCI molecules on thymic stroma (Broussard et al., 2006). We took advantage of this to generate mice carrying predominantly IMP or naïve CD8$^+$ T cells through BMTs utilizing WT and MHCI$^{-/-}$ (*B2m*$^{-/-}$) mice. The spleens of WT→MHCI$^{-/-}$ mice had predominantly CD44^{hi}CD122$^+$ IMP CD8$^+$ T cells that rapidly produced IFN-γ upon P/I stimulation, as has been observed (Berg, 2007; Broussard et al., 2006; Horai et al., 2007; Hu et al., 2007; Lai et al., 2011; Weinreich et al., 2010). By contrast, WT recipients of MHCI$^{-/-}$→WT contained predominantly naïve CD8$^+$ T cells (**Figure 2-1** A). As expected, hematopoietic MHCI expression is necessary and sufficient for development of IMP CD8$^+$ T cells.

Further examination revealed that the thymus is dispensable for IMP CD8$^+$ T cell development, since transplant of WT bone marrow into athymic Nude mice resulted in the development of similar cells to that seen in the WT→MHCI$^{-/-}$ transplants (**Figure 2-1** B&C). Of note, while endogenous CD8$^+$ T cells in nude mice are CD44^{hi}, they do not express CD122 and fail to rapidly produce IFN-γ in response to P/I (**Figure 2-1** B).

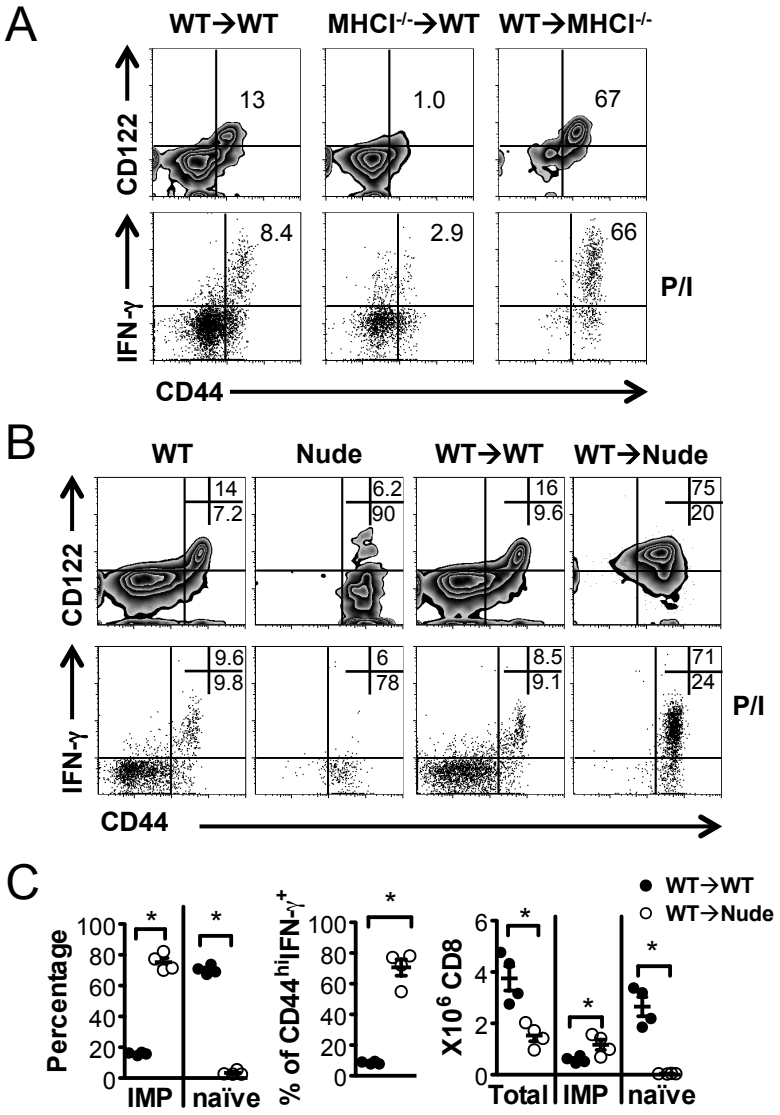


Figure 2-1. Development of IMP CD8$^+$ T cells via hematopoietic MHCI selection independent of the thymus. (**A**) Representative flow cytometric analysis of IMP CD8$^+$ T cells (upper panel, CD44^{hi}CD122$^+$) and P/I induced IFN-γ production (lower panel). Donor TCRβ$^+$CD8α$^+$ cells from WT→WT (MHCI$^+$CD45.2$^+$), MHCI$^{-/-}$→WT (MHCI$^-$CD45.2$^+$) and WT→MHCI$^{-/-}$ (MHCI$^+$CD45.2$^-$) chimeras are shown. (**B&C**) The thymus is not required for development of IMP CD8$^+$ T cells. (**B**) Bone marrow chimeras were generated as indicated and donor TCRβ$^+$CD8α$^+$ cells analyzed by flow cytometry for CD44 and CD122, and IFN-γ producing capacity induced by P/I, compared to those from WT and Nude mice. (**C**) Percentages of IMP, naïve and P/I induced CD44^{hi} IFN-γ producing CD8$^+$ T cells, along with numbers of total, IMP and naïve CD8$^+$ T cells, in "WT→WT" and "WT→Nude" chimeras. n = 4 in each group. *$p < 0.05$ by Student's *t* test.

IMP CD8$^+$ T cells are distinct from homeostatic expanded CD8$^+$ T cells

When transferred into lymphopenic environments, naïve CD8$^+$ T cells undergo significant proliferation in an attempt to restore "normal" levels of T cells. Along with this proliferation, naïve CD44^{lo}CD8$^+$ T cells acquire a phenotype resembling memory-like CD8$^+$ T cells, a process termed "homeostatic expansion" (Cho et al., 2000; Sprent et al., 2008). Naïve CD8$^+$ T cells also proliferate and differentiate upon antigen recognition and activation, converting into long-lived antigen specific memory CD8$^+$ T cells that reside in part in bone marrow (Becker et al., 2005). It is therefore possible that the cells we observed are due to homeostatic expansion of a small number of naïve T cells and/or proliferation of memory T cells in the transferred donor bone marrow. However, we found that depletion of T cells prior to BMT did not affect the development of IMP CD8$^+$ T cells (**Figure 2-2**). This suggests that IMP CD8$^+$ T cells observed in WT→MHCI$^{-/-}$ chimeric model are the result of hematopoietic MHCI selection and development rather than homeostatic expansion of T cells from donor bone marrow.

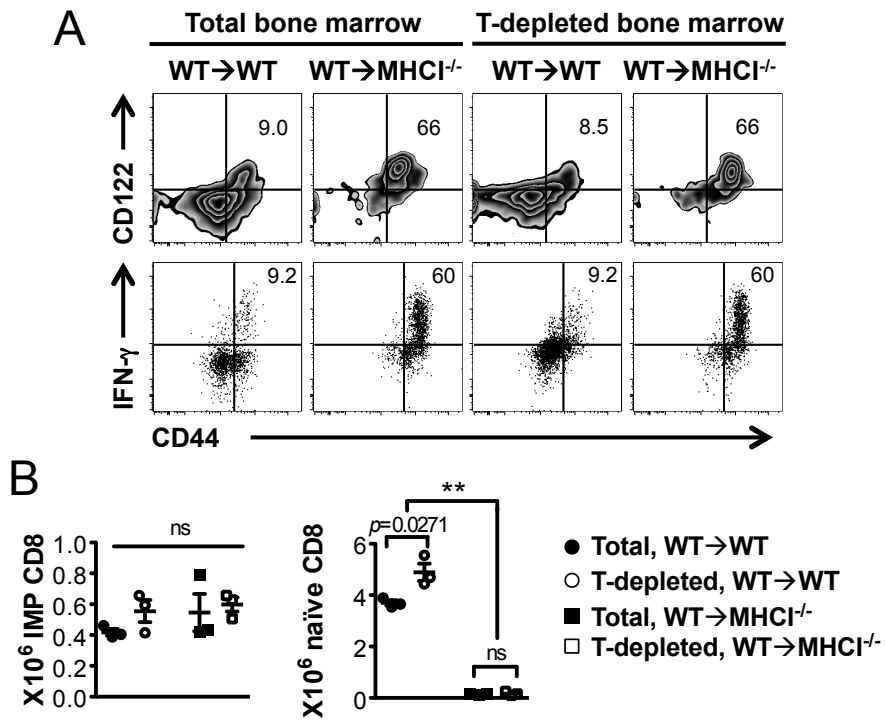
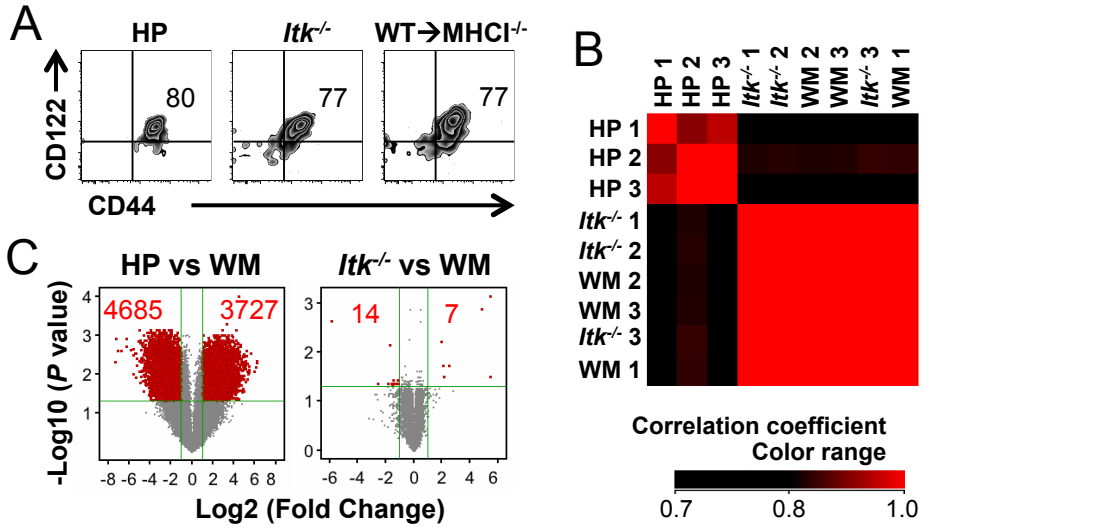


Figure 2-2. IMP CD8$^+$ T cells in WT→MHCI$^{-/-}$ chimeras develop despite depletion of T cells from donor bone marrow. WT bone marrow was either left intact or depleted of T-cells, and used as donors to generate "WT→WT" and "WT→MHCI$^{-/-}$" chimeras. Donor TCRβ^+CD8α^+ cells were analyzed. (**A**) Flow cytometric analysis for CD44 and CD122, and percentage of CD44^{hi} IFN-γ producing CD8$^+$ T cells in response to P/I. (**B**) Percentages and numbers of IMP and naïve CD8$^+$ T cells, and percentage of P/I induced CD44^{hi} IFN-γ^+ CD8$^+$ T cells. n = 3 in each group. *$p < 0.05$, ns = not significant, by Student's *t* test.

To confirm that the IMP $CD8^+$ T cells in the WT→$MHCI^{-/-}$ chimeras are not the result of homeostatic expansion, we analyzed whole genome gene expression profiles of sorted IMP $CD8^+$ T cells from WT→$MHCI^{-/-}$ chimeras (WM), *Itk*$^{-/-}$ mice, and $CD8^+$ T cells derived from homeostatic expansion of naïve $CD8^+$ T cells in *Rag1*$^{-/-}$ recipients (**Figure 2-3** A). Correlation coefficient matrix shows that gene expression between WM IMP $CD8^+$ T cells and *Itk*$^{-/-}$ IMP $CD8^+$ T cells are highly correlated (Correlation coefficient > 0.98), while both are less correlated with HP $CD8^+$ T cells (Correlation coefficient: 0.66 ~ 0.87) (**Figure 2-3** B). Among 27800 genes, compared to WM $CD8^+$ T cells, there are ~ 4000 up regulated and > 4000 down regulated by more than 2 fold in HP $CD8^+$ T cells ($P < 0.05$, P values were generated by asymptotic computation with Benjamini-Hochberg false discovery rate correction), while there are only 21 of these in *Itk*$^{-/-}$ $CD8^+$ T cells (**Figure 2-3** C). We examined a few meaningful genes that were changed between these cells by q-RT-PCR and consistent with the differential expression profile identified by microarray (**Figure 2-3** D), we found up-regulation of TNFR (*Tnfarsf1a*) and NFκB2 (*Nfkb2*), and down-regulation of Eomesodermin (*Eomes*) and Bim (*Bcl2l11*) (**Figure 2-3** E), in HP $CD8^+$ T cells but not *Itk*$^{-/-}$ $CD8^+$ T cells compared to WM IMP $CD8^+$ T cells. This whole genome expression analysis strongly suggests that IMP $CD8^+$ cells generated in WT→$MHCI^{-/-}$ chimeras highly resemble IMP $CD8^+$ T cells in *Itk*$^{-/-}$ mice, and are distinct from those derived from homeostatic expansion.



D

Name	HP	*Itk*$^{-/-}$
Tnfrsf1a	5.89	0.97
Nfkb2	3.91	1.16
Eomes	0.32	1.68
Bcl2l11	0.12	1.36

WM values were set as 1.

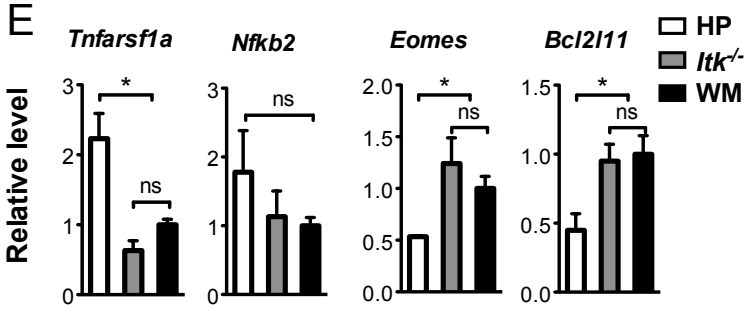


Figure 2-3. Hematopoietic MHCI dependent CD8$^+$ T cells resemble innate memory CD8$^+$ T cells in *Itk*$^{-/-}$ mice, but are distinct from HP cells. Comparison of HP, *Itk*$^{-/-}$ and WM (WT→MHCI$^{-/-}$) CD8$^+$ T cells. (**A**) HP, *Itk*$^{-/-}$ and WM CD8$^+$ T cells share expression of CD44 and CD122. (**B**) *Itk*$^{-/-}$ and WM CD8$^+$ T cells show extremely high correlation in whole genome gene expression, which is distinct from HP CD8$^+$ T cells. Samples were clustered based on the hierarchy of correlation. (**C**) HP but not *Itk*$^{-/-}$ CD8$^+$ T cells, exhibit significantly higher number of differentially expressed genes compared to WM CD8$^+$ T cells. Genes with significant change (fold change > 2, corrected $P < 0.05$) are shown in red. Numbers in red indicate numbers of gene that significantly up- or down- regulated. (**D**) Relative *Tnfrsf1a, Nfkb2, Eomes and Bcl2l11* expressional level in HP and *Itk*$^{-/-}$ CD8$^+$ T cells compared to WM IMP CD8$^+$ T cells as determined by microarray analysis. Quantile normalized gene expressional values were normalized to mean values of WM IMP CD8$^+$ T cells. n = 3 in each group. (**E**) Relative mRNA expression for *Tnfrsf1a, Nfkb2, Eomes and Bcl2l11* expressional level in HP and *Itk*$^{-/-}$ CD8$^+$ T cells compared to WM IMP CD8$^+$ T cells, determined by qRT-PCR. Gene expressional level was first normalized to GAPDH value of each sample, than normalized to the average value of WM IMP CD8$^+$ T cells. n = 3 in each group. *$p < 0.05$, ns = no significance by Student's *t* test.

Antigen-naïve OVA specific IMP CD8$^+$ T cells promptly respond to antigen in the absence of primary antigen exposure

The BMT approach suggests a method to generate antigen-naïve, antigen specific IMP CD8$^+$ T cells. To study the *primary* response of antigen specific IMP CD8$^+$ T cells, we utilized OTI mice (expressing OVA specific T cell receptor on CD8$^+$ T cells) as bone marrow donors, to generate antigen-naïve OVA specific IMP CD8$^+$ T cells in MHCI$^{-/-}$ recipients. Reciprocally, OTI transgenic mice that lack MHCI$^{-/-}$ (MHCI$^{-/-}$OTI) were used as donors to generate OVA specific naïve CD8$^+$ T cells in WT recipients, with OTI→WT chimeras as controls. Similar to the results of BMTs in non-transgenic backgrounds, OTI→MHCI$^{-/-}$ chimeras had predominantly TCR transgene positive OVA specific CD44^{hi}CD122$^+$ IMP CD8$^+$ T cells that rapidly produced IFN-γ. Analogously, MHCI$^{-/-}$OTI→WT chimeras had predominantly TCR transgene positive OVA specific CD44^{lo}CD122$^-$ naïve CD8$^+$ T cells (**Figure 2-4** A&B). Using this model, we examined whether antigen-naïve, antigen specific IMP CD8$^+$ T cells can respond to antigen in the absence of prior antigenic exposure. OTI transgenic IMP and naïve CD8$^+$ T cells were directly stimulated *in vitro* with antigen presenting cell/OVA or OVA$_{257-264}$ epitope of OVA recognized by the OTI TCR, without prior primary antigen exposure *in vivo*. We found that OVA/OVA$_{257-264}$ specific IFN-γ and TNF-α production by CD44^{hi} CD8$^+$ T cells peaked on the third day post stimulation in OTI IMP CD8$^+$ T cells (OTI→MHCI$^{-/-}$) (**Figure 2-4** C), suggesting that these cells were highly functional IFN-γ/TNF-α double producers (**Figure 2-4** C), and indicating that antigen naïve OVA specific IMP CD8$^+$ T cells can rapidly respond in a highly functional manner to specific antigen in the absence of primary antigenic

exposure. By contrast, naïve OTI cells (MHCI$^{-/-}$OTI→WT) did not show significant cytokine response > 6 days of stimulation as would be expected *in vivo*.

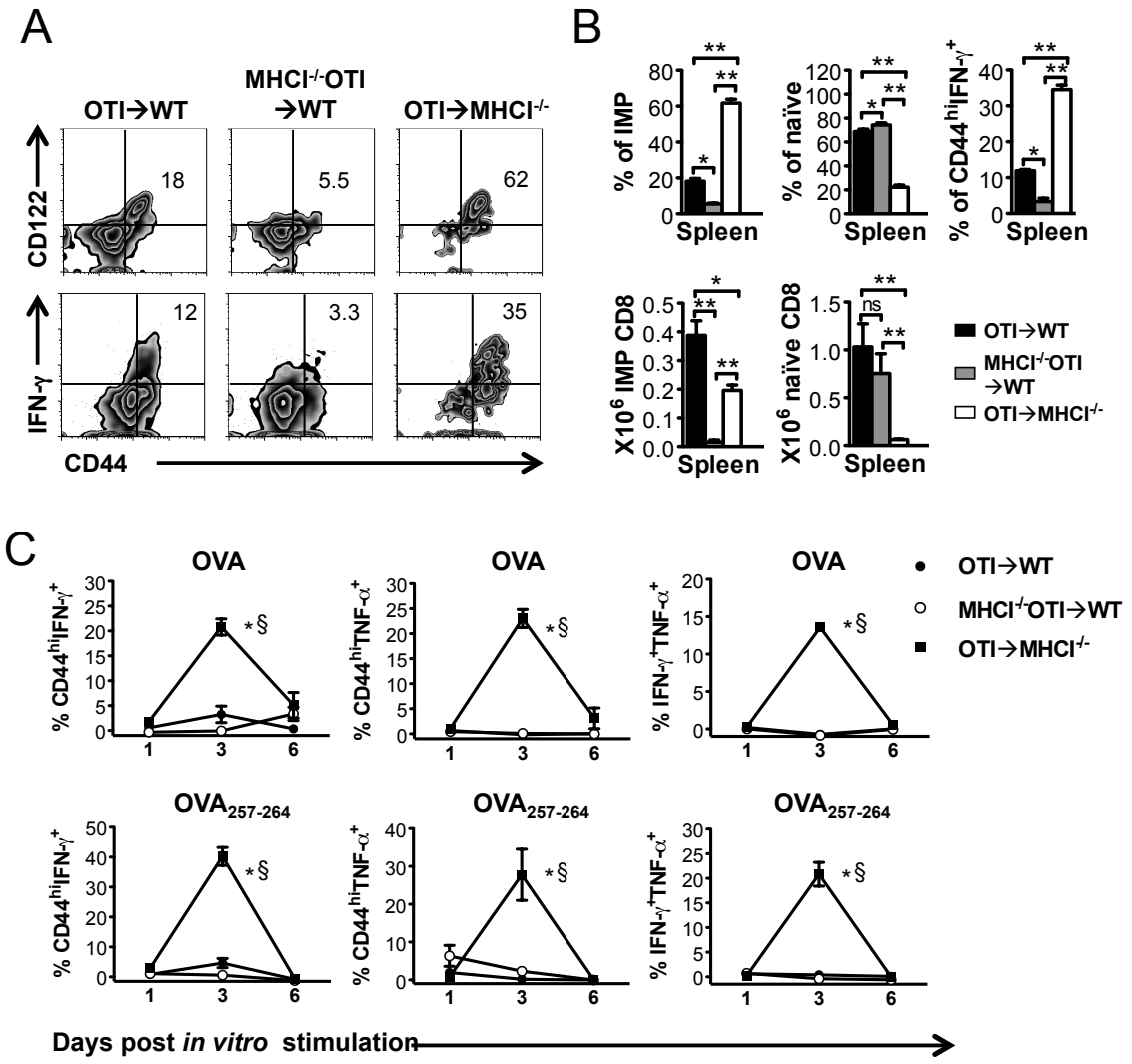


Figure 2-4. Hematopoietic MHCI dependent OTI IMP CD8$^+$ T cells exhibit prompt and potent antigen specific response *in vitro* without primary antigen exposure. BMTs were done as indicated and donor TCR transgene positive CD8α^+ cells from OTI→WT (CD45.2$^+$MHCI$^+$), MHCI$^{-/-}$OTI→WT (CD45.2$^+$MHCI$^-$) and OTI→MHCI$^{-/-}$ (CD45.2$^+$MHCI$^+$) chimeras analyzed. (**A**) Flow cytometric analysis of CD8$^+$ T cells for CD44 and CD122 and P/I induced IFN-γ production in chimeric spleens. (**B**) Percentages and numbers of CD44^{hi}CD122$^+$ IMP CD8$^+$ T cells, CD44^{lo}CD122$^-$ naïve CD8$^+$ T cells, and proportion of CD44^{hi} IFN-γ producing cells in response to P/I. *$p < 0.05$, ns = not significant, by Student's *t* test. Data represent results from three independent experiments. (**C**) Percentages of CD44^{hi} IFN-γ$^+$, CD44^{hi} TNF-α^+ and IFN-γ$^+$/TNF-α^+ double positive donor CD8$^+$ T cells in response to OVA and $OVA_{257-264}$ peptide along the time course. All values were corrected for the response of non-stimulated controls. *$p < 0.05$, compared to "OTI→WT" chimeras, $^{\S}p < 0.05$, compared to "MHCI$^{-/-}$OTI→WT" chimeras, by two-way ANOVA. Data represent results from two independent experiments.

Discussions

The function of IMP $CD8^+$ T cells, and the mechanism behind their development, has elicited significant interest. Recently, Rafei *et. al.* showed that antigen-naïve OTI transgenic $CD44^{hi}CD8^+$ thymocytes selected on thymic MHCIa and that have never left thymus, can promptly respond to OVA-peptide with cytokine secretion (Rafei et al., 2011). In addition, Haluszczak *et. al.* showed that a proportion of functional antigen-specific peripheral $CD8^+$ T cells in germ-free mice have a memory-like phenotype, although one cannot rule out that these cells are antigen-experienced (Haluszczak et al., 2009). Our findings that endogenous $CD8^+$ T cells in nude mice have a partial innate memory phenotype suggests that perhaps other signals may be required for the complete development and/or maturation of these cells, which may be indirectly dependent on the thymic structure or the *Foxn1* gene. This could involve a precursor that traffics through the thymus prior to our BMT. In the absence of ITK or the transcription factor KLF2, the increase in innate memory-like $CD8^+$ T cells have been suggested to be due to the influence of IL-4 produced by NKT-like cells in these mice (Weinreich et al., 2010). What role IL-4 signaling plays in the development of these hematopoietic MHCI-dependent IMP $CD8^+$ T cells remains to be determined. Our preliminary analysis indicates that IMP $CD8^+$ T cells differentially express the TNFR (lower than HP cells) and Bim and the transcription factor Eomesodermin (higher than HP cells), the latter previously observed in IMP $CD8^+$ T cells (Lee et al., 2011). Future experiments will distinguish the role of such factors in the development of these cells.

Using bone marrow chimeras and transgenic approaches, we show for the first

time that we can develop significant numbers of antigen-naïve, antigen specific IMP $CD8^+$ T cells that are similar to those that develop in the absence of ITK, but different from those that develop due to homeostatic expansion, although they have similar phenotypes. Our work provides a model, and opens the door for more detailed analysis of the development and more importantly, function of such cells.

In the immune system, innate immune cells initiate a relatively antigen non-specific response to primary infection, allowing cells of the adaptive immune system to develop more specific and exquisite antigen responses, along with the generation of immune memory (Iwasaki and Medzhitov, 2010; Zinkernagel, 2002). In particular, antigen specific memory T cell responses are thought to be generated only after antigenic exposure (Janeway CA, 2005). However, our work suggests that antigen naïve innate memory $CD8^+$ T cells can generate potent memory-like antigen specific responses. We suggest that such IMP $CD8^+$ T cells evolved to rapidly respond early in infections in an antigen specific fashion, assisting the innate immune response and allowing priming of the antigen specific adaptive immune response. These cells may occupy an essential niche in the early immune response to primary infection**.**

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CHAPTER THREE

ITK tunes IL-4 induced development of innate memory CD8$^+$ T cells in a γδ T cell independent manner[cd]

[c] Weishan Huang, Arun Kumar Kannan, Qian Qi, Jianfang Hu, and Avery August. ITK tunes IL-4 induced development of innate memory CD8$^+$ T cells in a γδ T cell independent manner. *Submitted.*

[d] Weishan Huang, and Avery August. Editorial: Jack of all trades? The versatility of IL-4 in CD8$^+$ T cell polarity. *Journal of Leukocyte Biology.* Invited review, *in press*.

Abstract

The development of different types of memory $CD8^+$ T cells has been intensely studied. True memory $CD8^+$ T cells develop in response to antigenic exposure, and can provide life-long protection against reinfection. More recently other types of memory $CD8^+$ T cells have been described. In mice lacking ITK, memory-like $CD8^+$ T cells spontaneously arise, referred to as innate memory phenotype (IMP, $CD44^{hi}/CD122^+$) cells, suggested to develop under the influence of IL-4 secreted by NKT-like γδ T cells found in *Itk*$^{-/-}$ mice. However, we report here that while IMP $CD8^+$ T cell development in *Itk*$^{-/-}$ mice is dependent on IL-4/STAT6 signaling, it is not dependent on γδ T cells, including NKT-like γδ T cells. Moreover, our experiments suggest a model of IMP $CD8^+$ T cell development where ITK tunes the $CD8^+$ T cell response to exogenous IL-4, leading to the innate memory phenotype. This work challenges the current model of IMP $CD8^+$ T cell development, illustrating a γδ T cell-independent, cell-intrinsic role for ITK in memory $CD8^+$ T cell development through synergy between IL-4 and the TcR. These findings furthermore suggest that some naïve $CD8^+$ T cells may be pre-programmed by weak homeostatic T cell receptor signals, to become memory phenotype cells with the ability to rapidly elaborate effector function. The role of ITK in this process suggests a mechanism by which IMP $CD8^+$ T cells can be rapidly generated in response to infection.

Introduction

The development of different types of memory $CD8^+$ T cells has been intensely studied. True memory $CD8^+$ T cells develop in response to antigenic exposure, and can provide life-long protection against reinfection (Harty and Badovinac, 2008; Lau et al., 1994). More recently other types of memory $CD8^+$ T cells have been described, including those generated during homeostatic proliferation in lymphopenic environments (or HP cells), virtual memory (or VM cells) or innate memory phenotype (IMP cells) (Akue et al., 2012; Broussard et al., 2006; Cheung et al., 2009; Haluszczak et al., 2009; Huang et al., 2013). The routes by which these cells develop seem to vary, and with the exception of true memory cells, their provenance is to date unclear. IMP $CD8^+$ T cells (characterized by the phenotype $CD44^{hi}/CD122^+$) express the transcription factor Eomesodermin (Eomes), carry preformed mRNA for and rapidly produce IFN-γ upon stimulation (Broussard et al., 2006; Hu et al., 2007; Huang et al., 2013).

The Tec family non-receptor tyrosine kinase ITK plays an important role in T cell activation and differentiation, and its absence leads to defects in positive selection in the thymus, as well as alterations in Th2 and Th17 cytokine secretion, with reduced Th2/Th17 responses to infection or diseases such as allergic asthma (Au-Yeung et al., 2006; Ferrara et al., 2006; Gomez-Rodriguez et al., 2009; Lucas et al., 2002; Miller et al., 2004; Mueller and August, 2003; Schaeffer et al., 2001). In addition, *i*NKT cells in *Itk*$^{-/-}$ mice exhibit defective maturation and reduced ability to produce effector cytokines, including IL-4 (Au-Yeung and Fowell, 2007; Felices and Berg, 2008; Qi et al., 2012; Qi et al., 2011). However, while the absence of ITK results in reduced Th2

responses, they paradoxically carry elevated serum IgE, suggestive of an ongoing Th2 response (Miller et al., 2004; Schaeffer et al., 2001). While, *Itk*$^{-/-}$ mice also have elevated numbers of NKT-like γδ T cells that express the transcription factor PLZF and secrete IL-4 (Felices et al., 2009; Qi et al., 2009; Yin et al., 2013). We and others have shown that the elevated serum IgE, observed in *Itk*$^{-/-}$ mice is dependent in part on NKT-like γδ T cells that secrete IL-4 in these mice (Felices et al., 2009; Qi et al., 2009).

Mice lacking ITK also develop a population of IMP CD8^{+} T cells, shown to be dependent on IL-4 and PLZF (Lai et al., 2011; Weinreich et al., 2010), and it has been proposed that IMP CD8^{+} T cell development in the absence of ITK is due to the PLZF-dependent NKT-like γδ cells producing IL-4, which acts on by-stander cells in a cell-extrinsic manner (Lai et al., 2011). Indeed, IL-4 can directly act on naïve CD8^{+} T cells to induce IFN-γ and Eomes expression (Oliver et al., 2012), providing support for this model. We have tested this model and find that while IMP CD8^{+} T cell development in *Itk*$^{-/-}$ mice is dependent on IL-4/STAT6 signaling, it is surprisingly not dependent on γδ T cells, including NKT-like γδ T cells. Furthermore, results from WT and *Itk*$^{-/-}$ mixed bone marrow chimeric models and partial ITK re-expression in *Itk*$^{-/-}$ background model suggest a cell-autonomous role for ITK in this process. We also show that that ITK negatively tunes the differentiation of CD44^{hi}CD122^{+} CD8^{+} T cells from naïve precursors. This work challenges the current model of IMP CD8^{+} T cell development, illustrating a γδ T cell-independent cell-intrinsic role of ITK in memory CD8^{+} T cell development through synergy between IL-4 and the TcR. Furthermore, the role of ITK in this process suggests a mechanism by which IMP CD8^{+} T cells can

be rapidly generated in response to infection.

Materials and Methods

Mice.

All mice were on a C57BL/6 background. WT, *Tcrd*$^{-/-}$ (B6.129P2-*Tcrd*^{tm1Mom}/J), Thy1a (B6.PL-*Thy1*^{a}/CyJ) and CD45.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) mice were from the Jackson Laboratory (Bar Harbor, ME). *Itk/Tcrd* DKO mice were generated by crossing *Itk*$^{-/-}$ and *Tcrd*$^{-/-}$ mice. Thy1a *Itk*$^{-/-}$ mice were generated by crossing Thy1a and *Itk*$^{-/-}$ mice. *Il4ra*$^{-/-}$ mice were a kind gift from Dr. Frank Brombacher (University of Cape Town, South Africa), via Dr. Fred Finkelman (University of Cincinnati) (Herbert et al., 2008). *Itk*$^{-/-}$*Il4ra*$^{-/-}$ mice were generated by crossing *Itk*$^{-/-}$ and *Il4ra*$^{-/-}$ mice. *Stat6*$^{-/-}$ (generated by Grusby's group (Kaplan et al., 1996)) and *Il13*$^{-/-}$ (from Dr. Alfred Bothwell at Yale University) mice were kindly provided by Dr. Margaret S. Bynoe (Cornell University). *Itk*$^{-/-}$*Stat6*$^{-/-}$ and *Itk*$^{-/-}$*Il13*$^{-/-}$ mice were generated by crossing *Itk*$^{-/-}$ with *Stat6*$^{-/-}$ or *Il13*$^{-/-}$ mice. ITK$_{tg}$*Itk*$^{-/-}$ (Tg(hCD2-*Itk*)*Itk*$^{-/-}$) mice were as previously described (Hu and August, 2008). OTI *Rag*$^{-/-}$ (B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1) mice were purchased from Taconic (Hudson, NY). *Itk*$^{-/-}$ OTI *Rag*$^{-/-}$ mice were generated by crossing *Itk*$^{-/-}$ and OTI *Rag*$^{-/-}$ mice. All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at Cornell University.

Antibodies and reagents.

All fluorescent antibodies are listed in "fluorochrome-target" format as follows: eFluor 450-CD122, FITC-TCRγδ, PerCP-eFluor 710-Eomes, PE-Cy7-IFN-γ

(eBioscience, San Diego, CA); V500-CD44, FITC-TCRβV5, FITC-Thy1a, PE-TCRαV2, PE-T-bet, Alexa Fluor 647-IL-4, Alexa Fluor 647-T-bet, Allophycocyanin-CD45.2, Alexa Fluor 700-CD4, Alexa Fluor 700-Ki67, PE-Cy7-CD62L, Allophycocyanin-Cy7-TCRβ, Allophycocyanin-Cy7-TCRαV2, Allophycocyanin-Cy7-Thy1a (BD Biosciences, San Jose, CA); PE-Texas Red-CD8α (Invitrogen, Carlsbad, CA); Allophycocyanin-NK1.1 (BioLegend, San Diego, CA). Fc blocking antibody was from eBioscience. Biotin-conjugated anti- Gr-1, DX5, TER119, CD122 and CD11b were from BD Biosciences, and anti- B220, F4/80, CD25, CD4 and CD8α were from eBioscience. Purified anti-CD3ε and CD28 antibodies used in stimulation were from BD Biosciences. PE-PBS57 loaded CD1d tetramer was from the National Institute of Allergy and Infectious Diseases Tetramer Facility. Anti-IL-4 monoclonal antibody (clone 11B11) was from ATCC (Manassas, VA). Recombinant murine IL-4 and IL-13 were from PeproTech (Rocky Hill, NJ) and IL-5 was a kind gift from Dr. Judith Appleton (Cornell University).

T cells stimulation for flow cytometry.

Live cells were blocked and stained immediately after collection. For IMP $CD8^+$ T cell response, thymocytes and splenocytes were stimulated with PMA/Ionomycin (P/I: 100 ng/ml/0.5μM) and Brefeldin A (10μg/ml) (Sigma-Aldrich, St. Louis, MO) for 6 hours, followed by fixation/permeabilization and staining with indicating antibodies using FoxP3 staining buffer Kit (eBioscience). For *i*NKT cell response, 2 μg/mouse α-GalCer (KRN 7000, Cayman Chemical, Ann Arbor, MI) was injected i.p. to mice 2

hours prior to collection of splenocytes, followed by 4-hour incubation with Brefeldin A and staining with surface markers, fixation with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), permeabilization and staining of cytokine antibodies using PBS containing 0.4% saponin (Sigma). All flow cytometry data were acquired on LSRII (BD Biosciences), and analyzed in FlowJo (Tree Star, Ashland, OR).

Mixed bone marrow chimerism.

Bone marrow chimeras were generated as previously described (Huang et al., 2013), with bone marrow composed of 1:1-mixed WT and *Itk*$^{-/-}$ cells.

Cell purification and real time q-PCR.

CD8^{+} and CD4^{+} T cells were purified by magnetic negative selection. Cells were stained with biotin-conjugated antibodies against Gr-1, DX5, TER119, CD11b, B220, F4/80, CD25, CD122 and CD4, followed by anti-biotin MicroBeads (Miltenyi Biotec, MA). Naïve CD8^{+} T cells from OTI *Rag1*$^{-/-}$ or *Itk*$^{-/-}$ OTI *Rag1*$^{-/-}$ with purity higher than 92% (CD8α^{+}TCRβV5^{+}CD44^{lo}CD122^{-}) were used. Purified cells were used to extract RNA for real time q-PCR as previously described (Qi et al., 2012), or used for *in vitro* stimulation.

IL-4 stimulation in vitro and in vivo.

Naïve CD8^{+} T cells cells were treated *in vitro* with IL-4 (PeproTech, Rocky Hill, NJ; 40 ng/ml) and/or anti-CD3ε/28 antibodies (1 μg/ml of each, unless otherwise

specified) for 5 days for flow cytometry analysis. Cells with IL-4 in culture had more than 90% viability by the time of analysis, based on trypan blue (Sigma) staining. For *in vivo* stimulation, 0.4 μg of IL-4 was incubated in room temperature with 4 μg of IL-4 antibody (11B11) for 5 minutes to form stable IL-4/antibody complex, then given to mice via retro-orbital injection every 24 hours. 24 hours post the 6^{th} dose, mice were euthanized for analysis.

Statistical analysis.

Un-paired two-tailed Student's *t* test was performed using GraphPad Prism v5.00 (GraphPad, San Diego, CA), with $p < 0.05$ considered statistically significant.

Results

γδ T cells are dispensable for development of IMP CD8$^+$ T cells in Itk$^{-/-}$ mice

Itk$^{-/-}$ mice have elevated development of IMP CD8$^+$ T cells, which is thought to be dependent on IL-4 (Weinreich et al., 2010). *i*NKT cells have been proposed to be the source of IL-4, however, *i*NKT cells in *Itk*$^{-/-}$ mice have impaired ability to produce IL-4 production ((Qi et al., 2012), and see **Figure 3-2**). By contrast, these mice have increased NKT-like γδ T cells that are able to produce IL-4 (Felices et al., 2009; Qi et al., 2009; Yin et al., 2013). Given these findings, we explored whether γδ T cells serve as producers of IL-4 in the place of the defective *i*NKT cells for induction of IMP CD8$^+$ T cell development in the absence of ITK. We generated *Itk*$^{-/-}$ mice that lack the ability to make γδ T cells by removing the *Tcrd* gene. We then analyzed the thymi (**Figure 3-1** A) and spleens (**Figure 3-1** B) of WT, *Tcrd*$^{-/-}$, *Itk*$^{-/-}$ and *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice for CD8$^+$ T cells that carry the markers of IMP T cells, CD44 and CD122, their ability to rapidly produce IFN-γ, and their expression of transcription factors T-bet and Eomes. To our surprise, we found that the absence of γδ T cells on the *Itk*$^{-/-}$ background does not affect the development of IMP CD8$^+$ T cells (**Figure 3-1** A&B, upper panels), their capacity to rapidly produce IFN-γ (**Figure 3-1** A&B, middle panels), or their expression of Eomes (**Figure 3-1** A&B, lower panels). This suggests that although γδ T cells with enhanced potential for IL-4 production are increased in *Itk*$^{-/-}$ mice, they are dispensable for the excessive development of IMP CD8$^+$ T cells in these mice. This is different from their requisite role in the hyper-IgE syndrome in *Itk*$^-$

$^{-/-}$ mice (Felices et al., 2009; Qi et al., 2009).

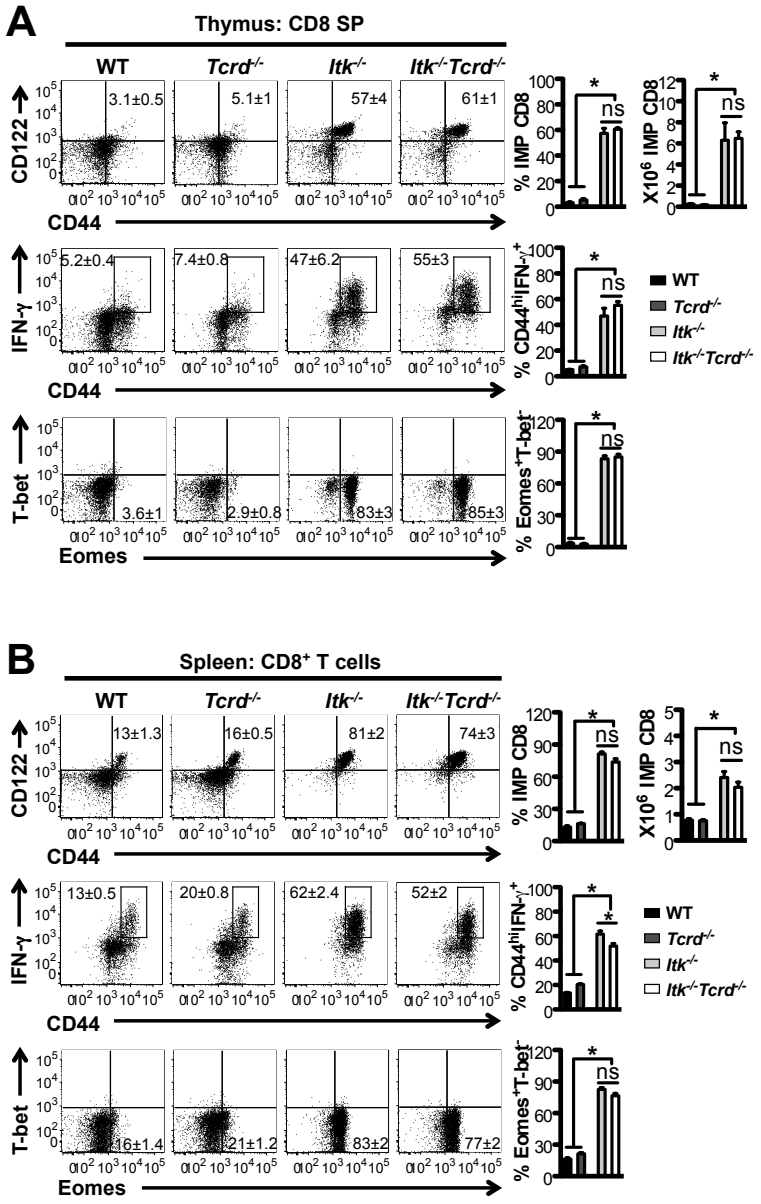

Figure 3-1. γδ T cells are dispensable for development of IMP CD8$^+$ T cells in *Itk*$^{-/-}$ mice. Expression of memory markers CD44 and CD122 (top panel), P/I induced IFN-γ (middle panel) and transcription factors Eomes/T-bet (bottom panel) in (**A**) CD8 single positive thymocytes and (**B**) CD8$^+$ splenocytes from WT, *Tcrd*$^{-/-}$, *Itk*$^{-/-}$ and *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice. *$p < 0.05$, "ns" = no significance, by Student's *t* test. n ≥ 6 from 3 independent experiments.

Persistent deficiency in iNKT development and function Itk$^{-/-}$ mice lacking γδ T cells

In *Itk*$^{-/-}$ mice, there is enhanced development of a subpopulation of γδ T cells that have features of NKT cells, albeit with features of incomplete differentiation (Yin et al., 2013). It has been suggested that the absence of ITK favors the development of γδ NKT cells over *i*NKT cells, due to potential competition between the two populations, although recent studies have suggested that such competition may be between the most mature subsets of each cell type (Felices and Berg, 2008; Yin et al., 2013). However, this evidence is indirect, and since we did not observe any effect of the absence of γδ T cells on the development of IMP CD8$^+$ T cells in *Itk*$^{-/-}$ mice, we wondered whether the blockade of γδ NKT cell development in *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice would result in an enhancement of *i*NKT cell development, potentially rescuing their ability to produce IL-4. This could also have the effect of influencing development of IMP CD8$^+$ T cells in these mice. We therefore analyzed development and function of *i*NKT cells in the *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice. As previously reported, in the absence of ITK, there is reduced maturation and numbers of the CD1d tetramer positive *i*NKT cell population, and these cells are defective in secreting IL-4 and IFN-γ upon stimulation with the *i*NKT cell ligand α-GalCer (Felices and Berg, 2008; Qi et al., 2012; Yin et al., 2013). Similar to what was observed for *Itk*$^{-/-}$ *i*NKT cells, there was reduced percentage, number (**Figure 3-2** A&B, upper panels), maturation (**Figure 3-2** A&B, lower panels) and α-GalCer induced IL-4 and IFN-γ production (**Figure 3-2** C&D) by *i*NKT cells in *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice. Thus the *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice present an interesting

condition for enhanced IMP $CD8^+$ T cell development in which ITK and γδ T cells are absent, and *i*NKT cells are impaired in ability to produce IL-4. Thus possible explanations for the continued presence of IMP $CD8^+$ T cells in *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice include alternative source(s) of IL-4 and/or a cell-autonomous role of ITK.

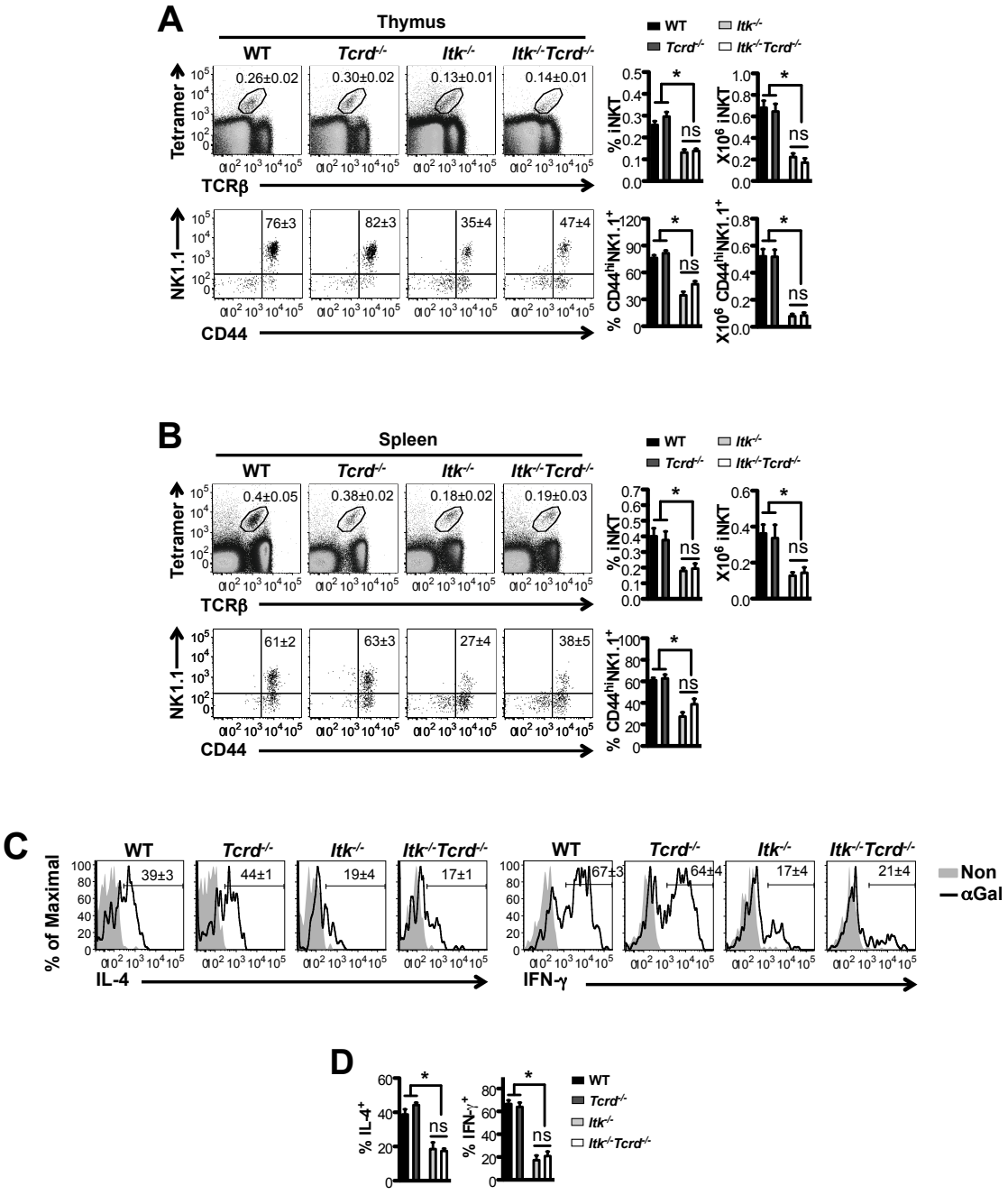

Figure 3-2. Impaired development of *i*NKT cells and cytokine production in *Itk*$^{-/-}$ mice lacking γδ T cells. Proportion of *i*NKT cells and expression of maturation markers by *i*NKT cells in the (**A**) thymus and (**B**) spleens of WT, *Tcrd*$^{-/-}$, *Itk*$^{-/-}$ and *Itk*$^{-/-}$ *Tcrd*$^{-/-}$ mice. n ≥ 6 from 3 independent experiments. (**C**) α-GalCer induced IL-4 and IFN-γ production by *i*NKT cells in the spleens of WT, *Tcrd*$^{-/-}$, *Itk*$^{-/-}$ and *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice. *$p < 0.05$, "ns" = no significance, by Student's *t* test. n ≥ 5 from 3 independent experiments.

ITK negatively tunes IMP CD8$^+$ T cell development in an IL-4/STAT6 dependent cell-intrinsic manner

Confirming the role for IL-4 in this process, removal of either the IL-4Rα or STAT6 resulted in complete loss of IMP CD8$^+$ T cells in the absence of ITK in the thymus and spleen (**Figure 3-3** A&B). Furthermore, although the IL-4 related cytokine IL-13 shares the IL-4Rα to transduce its signals and can also activate STAT6, the loss of IL-13 along with ITK does not affect the development of IMP CD8$^+$ T cells, indicating that the requirement is uniquely associated with IL-4/STAT6 signaling axis. Indeed, *in vitro* stimulation revealed that naïve CD8$^+$ T cells uniquely respond to IL-4, but not the other two canonical Th2 cytokines, IL-5 and IL-13, with increased proliferation, capacity for IFN-γ expression and expression of STAT6 (**Supplemental Figure 3-1**). However, IMP CD8$^+$ T cells require the presence of other T cells (or B cells) as a source for IL-4 as illustrated in the following experiment. We generated mice lacking ITK along with all T (and B cells), except for antigen specific T cell receptor transgenic cells (Ovalbumin specific OTI T cells, OTI/*Rag1*$^{-/-}$/*Itk*$^{-/-}$), and determined the phenotype of the resulting CD8$^+$ T cells. We found that the resulting *Itk*$^{-/-}$ CD8$^+$ T cells did not have the IMP phenotype (**Figure 3-4** A), suggesting that the absence of ITK requires the corporation of IL-4 produced by other T (or B cells) to drive the development of IMP CD8$^+$ T cells.

We have so far failed to identify alternative sources of IL-4 in *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice, and so we focused on the possibility that ITK plays cell-intrinsic function in the development of IMP CD8$^+$ T cells. Our previous work identified ITK as a cell-

intrinsic suppressor of NKT-like γδ T cells using "WT + *Itk*$^{-/-}$" 1:1-mixed bone marrow chimeras (Qi et al., 2009). Using these chimeric models, we further found that despite a lower ratio of total CD8^{+} T cells derived from *Itk*$^{-/-}$ donor, a high percentage of the CD8^{+} T cells of *Itk*$^{-/-}$ origin developed the memory phenotype, while the WT CD8^{+} T cells did not develop the same phenotype, despite being in the same environment during development (**Figure 3-3** C, lower panel). Furthermore, mice expressing ITK on the *Itk*$^{-/-}$ background to ~35% of WT levels (ITK$_{tg}$*Itk*$^{-/-}$ mice) develop intermediate levels of IMP CD8^{+} T cells, suggesting an inverse correlation between ITK expression levels and development of IMP CD8^{+} T cells (**Figure 3-3** D). These data suggest a cell-intrinsic regulatory role of ITK in IMP CD8^{+} T cell differentiation, dependent on IL-4 signaling.

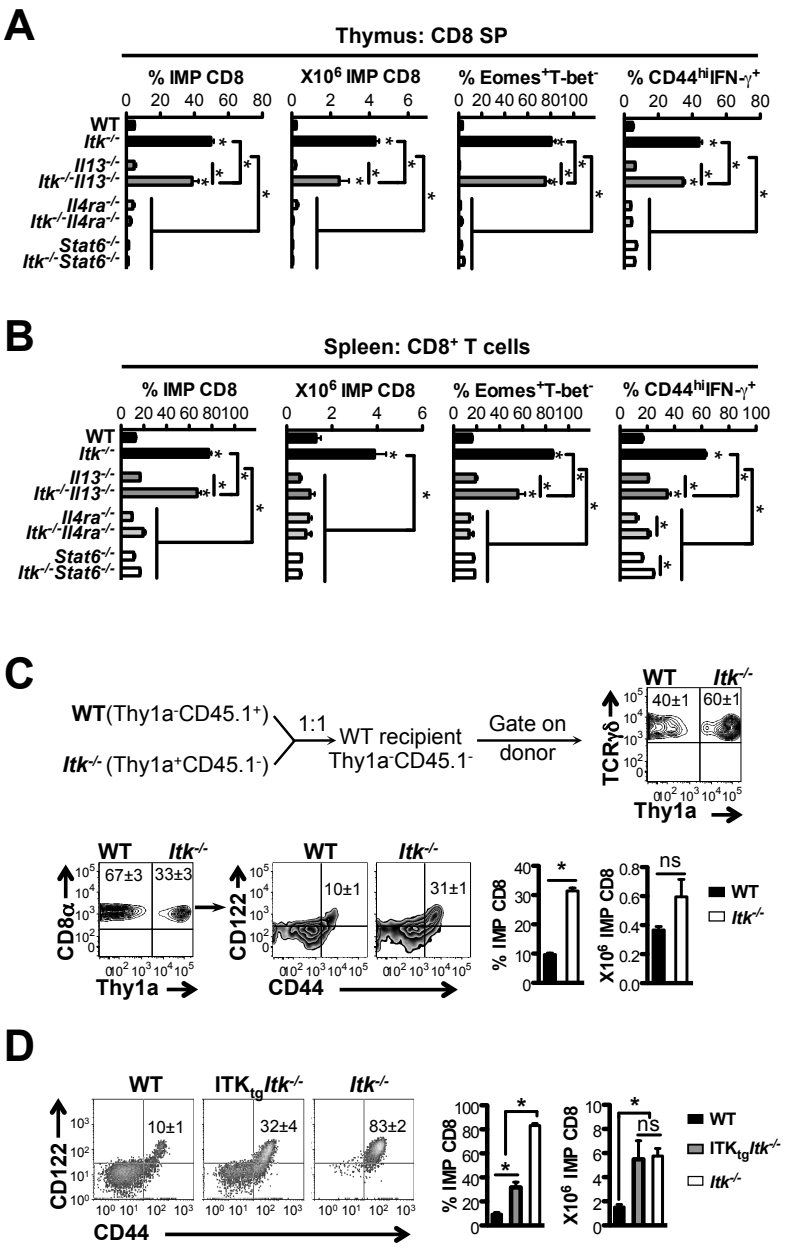

Figure 3-3. IL-4/STAT6 signaling axis is required and ITK plays a cell-autonomous role in development of IMP T cells. Percentage, number of $CD44^{+}CD122^{hi}$ IMP $CD8^{+}$ T cells, percentage of $Eomes^{+}$ cells and percentage of P/I induced IFN-γ producing cells of (**A**) CD8 single positive thymocytes and (**B**) $CD8^{+}$ T splenocytes from WT, *Itk*$^{-/-}$, *Il13*$^{-/-}$, *Itk*$^{-/-}$*Il13*$^{-/-}$, *Il4ra*$^{-/-}$, *Itk*$^{-/-}$*Il4ra*$^{-/-}$, *Stat6*$^{-/-}$, *and Itk*$^{-/-}$ *Stat6*$^{-/-}$ mice. n ≥ 3, combined from 3 independent experiments. (**C**) WT ($Thy1a^{-}$ $CD45.1^{+}$) and *Itk*$^{-/-}$ ($Thy1a^{+}CD45.1^{-}$) bone marrow cells were mixed in 1:1 ratio and used as donors. Donor originated splenocytes of chimeric mice were analyzed. Ratio of γδ T cells of WT and *Itk*$^{-/-}$ origin were used to confirm functional reconstitution and

donor $CD8^+$ T cells analyzed for percentages and number of IMP T cells. n = 3 shown, data represent results of 2 independent experiments. (**D**) Percentage and number of IMP $CD8^+$ T cells in WT (100% ITK expression), $ITK_{tg}Itk^{-/-}$ (~35% ITK expression) and $Itk^{-/-}$ (0% ITK expression) splenocytes. n = 3. Data represent result of more than 3 independent experiments. *$p < 0.05$, "ns" = no significance, by Student's *t* test.

We next investigated the potential role of ITK downstream of IL-4 and the TcR, using naïve $CD8^+$ T cells isolated from WT or *Itk*$^{-/-}$ mice in OTI *Rag*$^{-/-}$ background, which avoids complicating factors when using the $CD8^+$ T cells from the Itk$^{-/-}$ mice (**Figure 3-4** A). When cultured *in vitro* in the presence of IL-4 for 5 days, *Itk*$^{-/-}$ naïve $CD8^+$ T cells required the presence of TcR signals to expand to the WT levels, however, a higher proportion of these cells took on an IMP-like state ($CD44^{hi}CD122^+$), and these $CD44^{hi}CD122^+$ $CD8^+$ T cells are induced to a significantly higher level when both IL-4 and TcR signals are present (**Figure 3-4** B). These data suggest that *in vitro*, ITK inversely tunes the response to naïve $CD8^+$ T cells to IL-4 to develop into IMP-like cells.

We also tested whether this was the case *in vivo*, administering IL-4 (in the form of the stabilized IL-4/α-IL-4 complex) (Finkelman et al., 1993), to further determine the function of ITK in regulating naïve $CD8^+$ T cell responses to IL-4. We found that 6 days post *in vivo* IL-4/α-IL-4 administration, both WT and *Itk*$^{-/-}$ CD8 single positive thymocytes and $CD8^+$ T cells in the spleens exhibited an IMP-like state, with elevated percentage and number of $CD44^{hi}CD122^+$ cells compared to controls. In addition, the percentage and number of these cells were significantly higher in the absence of ITK (**Figure 3-4** C). More interestingly, although naïve *Itk*$^{-/-}$ $CD8^+$ T cells had far higher levels of Eomes mRNA compared to WT, it is not translated into Eomes protein until IL-4 is provided, suggesting that the naïve *Itk*$^{-/-}$ $CD8^+$ T cells exist in a primed state for IL-4 signals (**Figure 3-4** D).

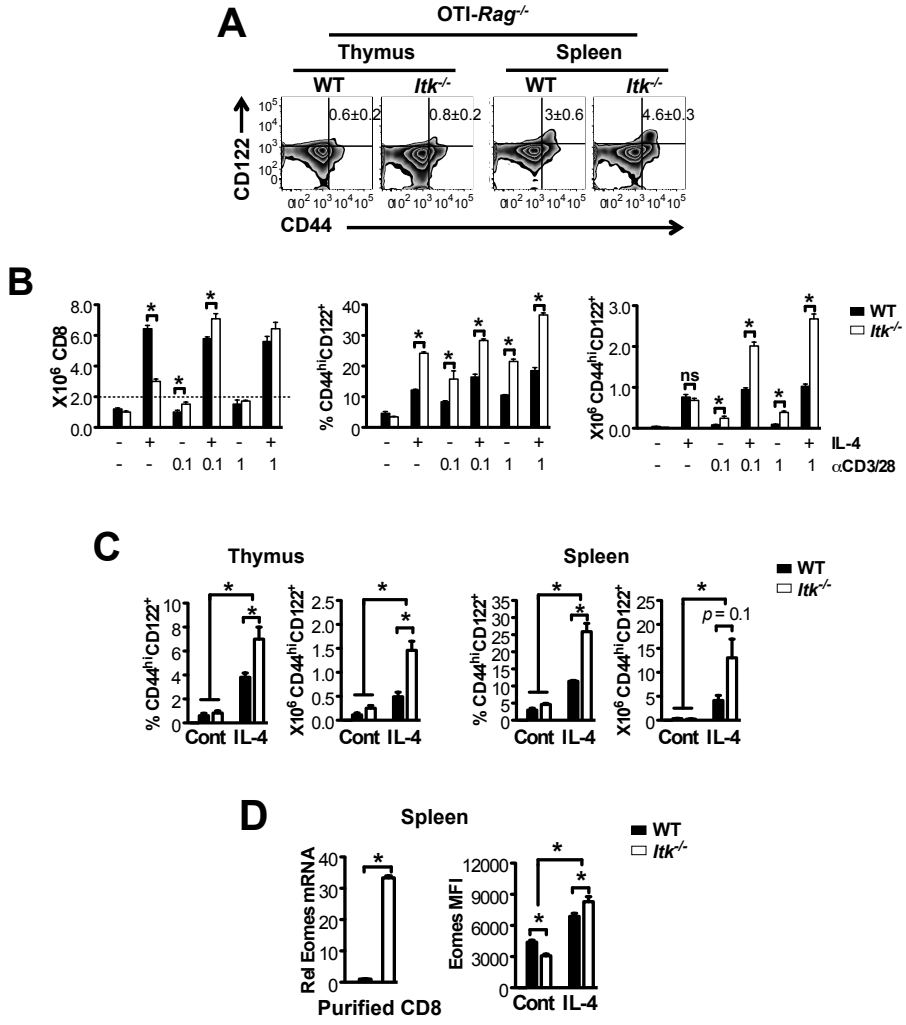

Figure 3-4. ITK negatively tunes IL-4/TcR synergy in IMP CD8$^+$ T cell development. All cells used in the experiments shown in this figure were on the OTI *Rag1*$^{-/-}$ background. (**A**) IMP marker expression by CD8 single positive thymocytes and CD8$^+$ T splenocytes in WT and *Itk*$^{-/-}$ mice. Data represent results from more than 10 mice each. (**B**) Purified WT and *Itk*$^{-/-}$ naïve CD8$^+$ T cells were stimulated as indicated for 5 days, and total CD8$^+$ T cell number, percentage and number of CD44^{hi}CD122$^+$ CD8$^+$ T cells analyzed. n = 3 in each. Data represent result of 2 independent experiments. (**C**) WT and *Itk*$^{-/-}$ mice that received 6 doses of IL-4/α-IL-4 Ab complexes were analyzed for CD44^{hi}CD122$^+$ proportion of CD8 single positive thymocytes and CD8$^+$ splenocytes. n ≥ 3. (**D**) Eomes mRNA expression of purified WT and *Itk*$^{-/-}$ naïve CD8$^+$ T cells with WT level set as 1 and mean fluorescent intensity (MFI) for Eomes protein expression in CD8$^+$ T cells from splenocytes of mice in (**C**). n ≥ 3. *$p < 0.05$, by Student's *t* test.

Discussions

Taken together, our data suggest that in naive $CD8^+$ T cells, ITK acts as a cell-intrinsic tuner for IL-4 signals. Downstream of the TcR ITK acts to negatively tune the IL-4 signals since in its absence, naïve $CD8^+$ T cells exhibit better differentiative responses. It is possible that, as has been recently reported for IL-7, TcR signals tune cytokine signals that cells receive, in the example of IL-7 by regulating expression receptor expression (Kimura et al., 2013). In the case of IL-4, reduced TcR signals in the absence of ITK allows enhanced responses to IL-4 signals. Furthermore, this may be combined with the ability of ITK signals to suppress the expression of Eomes, which may be involved in the development of the IMP state. In the absence of ITK, Eomes mRNA is highly upregulated in naïve $CD8^+$ T cells, but IL-4 is required to drive the translation of this message.

While the development of IMP $CD8^+$ T cells in *Itk*$^{-/-}$ mice is dependent on the IL-4/STAT6 signaling axis, this work challenges the prevailing notion that development of these cells is due primarily to IL-4 production by NKT-like γδ T cells (Hu et al., 2007; Yin et al., 2013). Indeed our experiments suggest that these cells develop in response to a combination of exogenous IL-4 signals, but with intrinsic tuning by ITK. Weinreich *et al.* (Weinreich et al., 2010) previously suggested that an *in vivo* environment created by the absence of ITK can influence WT $CD8^+$ T cells to develop an IMP-like state in the presence of *Itk*$^{-/-}$ cells (using a "WT minority + *Itk*$^{-/-}$ majority" bone marrow chimerism strategy), and indeed, WT $CD8^+$ T cells can be skewed towards the IMP-state in the presence of IL-4. However, we have found that when the ratio of WT:*Itk*$^{-/-}$ bone marrow is 1:1, the WT cells are not influenced, while

the *Itk*$^{-/-}$ cells retain the ability to develop into IMP cells, suggesting that there may be a threshold for the effects of IL-4 to induce IMP CD8^{+} T cell differentiation, and that the absence of ITK tunes that threshold such that it is lower in its absence. If analogous to TcR tuning of IL-7 signals, the proposal that ITK tunes IL-4 signals would suggest an IL-4 receptor-mediated mechanism. However IL-4Rα expression is similarly maintained between WT and *Itk*$^{-/-}$ and γC was slightly lower in *Itk*$^{-/-}$ naïve CD8^{+} T cells (**Supplemental Figure 3-2**), and so it is possible that ITK regulates the intracellular signaling sensitivity to IL-4. Indeed, a role for TcR signals in modifying IL-4 signaling has been previously suggested. In CD4^{+} T cells, TcR signals can positively modify the IL-4Rα signaling complexes, via the ERK/MAPK and Calcium/Calcineurin pathways (Yamashita et al., 2000; Yamashita et al., 1999). By contrast, it has also been suggested that TcR signals transiently desensitize IL-4R signaling via these two pathways (Zhu et al., 2000). Moreover, the presence of IL-4 during the development of CD8^{+} T cells results in reduced TcR-induced calcium responses (Lewis et al., 1991). It should be noted that ITK positively regulates TcR induced activation of both the ERK/MAPK and calcium pathways (Andreotti et al., 2010), suggesting that perhaps ITK tunes IL-4 signaling in CD8^{+} T cells via these pathways.

The cell-autonomous role of ITK in suppressing IMP CD8^{+} T cell differentiation was further supported by the fact that in *Itk*$^{-/-}$*Tcrd*$^{-/-}$ mice where γδ T cells are absent and *i*NKT cells are defective in IL-4 production, CD8^{+} T cells largely have the memory phenotype in the absence of ITK. The synergy of IL-4 and TcR signaling pathways in CD44^{hi}CD122^{+} phenotype differentiation provides a potential

explanation for the excess of IMP $CD8^+$ T cell in the absence of ITK. Indeed, the fact that Eomes mRNA is significantly increased in *Itk*$^{-/-}$ naïve $CD8^+$ T cells provides strong evidence for an intrinsic role for ITK. Furthermore this elevated Eomes mRNA is not translated unless IL-4 is provided, indicating a requisite role of IL-4 in IMP $CD8^+$ T cell generation, and suggests that ITK suppresses Eomes expression and in its absence, mRNA is transcribed, priming for expression upon a signal for translation, and conversion to the memory phenotype. We suggest that under WT conditions, $CD8^+$ T cells that have received weak signals (such as those mimicked by the absence of ITK) may transcribe Eomes mRNA, and be primed to generate memory phenotype cells under inductive conditions such as IL-4. These findings furthermore suggest that some naïve $CD8^+$ T cells may be pre-programmed by virtue of weak signals they received either during development or during homeostatic expansion upon leaving the thymus, to become memory phenotype cells with ability to rapidly respond with effector function.

We have previously reported that IMP $CD8^+$ T cells can rapidly respond to primary antigens by producing IFN-γ and TNF-α (Huang et al., 2013), which can be critical in developing rapid response or vaccination strategies for emerging pathogens. ITK serves as a $CD8^+$ T cell-autonomous tuner for IMP differentiation, and targeting ITK may enhance selection or expansion of IMP $CD8^+$ T cells. This would be of tremendous benefit in dealing with emerging infectious diseases.

Acknowledgements

I thank Dr. Arun Kannan for help in T cell isolation and mouse injection, Dr. Fei Huang for generating *Itk*$^{-/-}$ OTI-*Rag*$^{-/-}$ mouse, Drs. Qian Qi and Jianfang Hu for characterizing the ITK$_{tg}$*Itk*$^{-/-}$ mouse phenotype, and Dr. Avery August for overall supervision and thoughtful discussion. I also thank Drs. Frank Brombacher and Fred Finkelman for *Il4ra*$^{-/-}$ mice, Dr. Margaret S. Bynoe for *Stat6*$^{-/-}$ and *Il13*$^{-/-}$ mice, and Dr. Judith A. Appleton for murine recombinant IL-5, Yoko Yoda, Misty S. Pocwierz, Tina Chew, Hana Kim, Omar Hasan Nijem and Dr. Rod Getchell for technical support, and Amie Wood for animal care.

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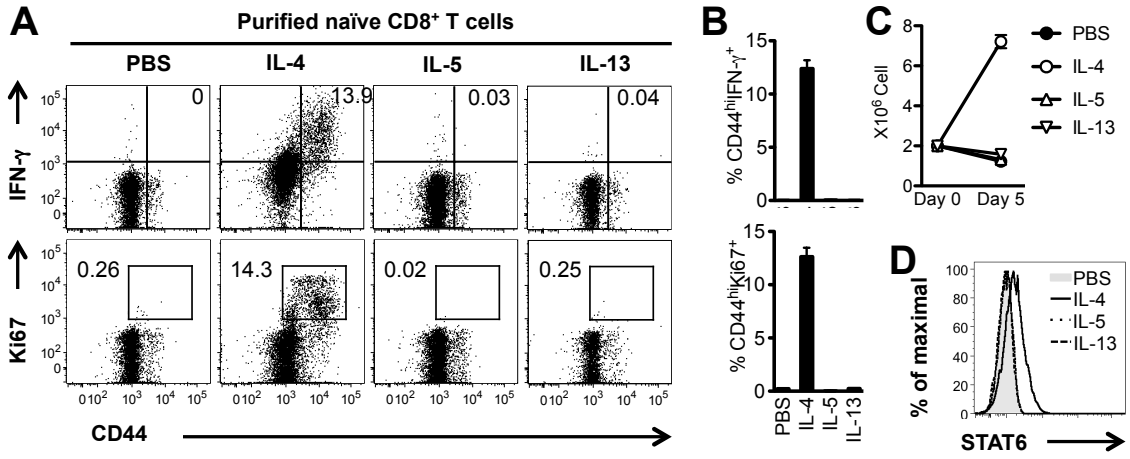
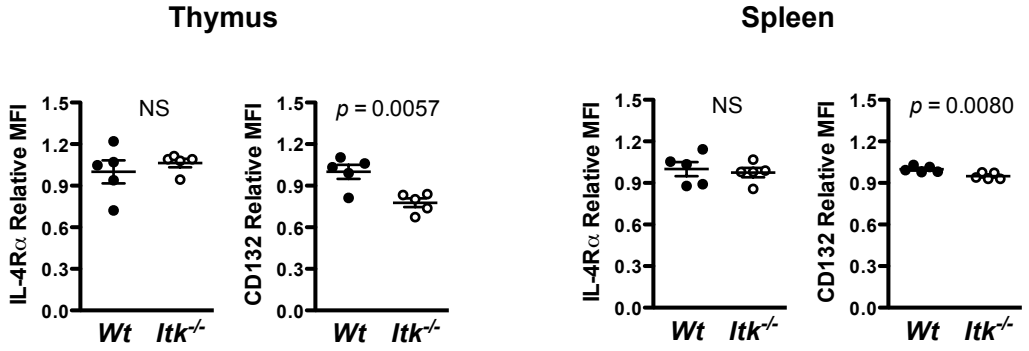


Supplemental Figure 3-1. Naïve CD8$^+$ T cells respond uniquely to IL-4 of the classical Th2 cytokines. Purified WT naïve CD8$^+$ T cells were stimulated with IL-4 (40ng/ml, PeproTech), IL-5 (50ng/ml, gift from Dr. Judith Appleton at Cornell University) or IL-13 (100ng/ml, PeproTech) for 5 days, then cells analyzed for (**A**) Ki67, or (**B**) expression of IFN-γ following stimulation with P/I. (**C**) Number of cells recovered. (**D**) Expression of STAT6 following stimulation with the indicated cytokines. n ≥ 3 shown, data represents results of more 3 independent experiments.



Supplemental Figure 3-2. Relative expression of IL-4Rα and γC expression on naïve CD8$^+$ T cells in WT and *Itk*$^{-/-}$ OTI-*Rag*$^{-/-}$ mice. CD8$^+$ T cells from the thymus and spleen of WT and *Itk*$^{-/-}$ OTI-*Rag*$^{-/-}$ mice were stained with IL-4Rα and γC antibodies. WT mean fluorescence intensity (MFI) average was set as 1. n = 5, combined from 2 independent experiments. *p* values were generated by Student's *t* test, NS: not significant.

CHAPTER FOUR

ITK suppresses CD8$^+$ T cell homeostatic proliferation and anti-tumor immunity[e]

[e] Weishan Huang, Junjie Luo, Lu Huang, Fei Huang, and Avery August. ITK suppresses CD8$^+$ T cell homeostatic proliferation and anti-tumor immunity. *Manuscript in preparation.*

Abstract

Loss of ITK perturbs $CD8^+$ T cell homeostasis and leads to spontaneous acquisition of innate memory/effector phenotype. Using naïve $CD8^+$ T cells obtained from OTI *Rag*$^{-/-}$ background, here we show that ITK regulates lymphopenia-induced $CD8^+$ T cell expansion in a T cell-autonomous manner. *Itk*$^{-/-}$ naïve $CD8^+$ T cells exhibit massive immediate expansion, accompanied with potent death programing and collapse right after. We show that this is independent of APC-MHCI, but dependent on the $CD8^+$ T cell-T cell interaction. The lack of ITK resulted in an enhanced effector program and anti-tumor immunity in homeostatically expanded $CD8^+$ T cells. These data suggest that ITK intrinsically regulates $CD8^+$ T cell homeostasis and ITK deficient homeostatically expanded T cells can generate potent anti-tumor immunity.

Introduction

$CD8^+$ memory T cells, typically characterized by high expression of CD44 and CD122, are involved in a broad spectrum of immune activities, such as parasitic infection (Cerwenka et al., 1999), immune disorders (Gluck et al., 2007) and tumorigenesis (Dobrzanski et al., 2004). Antigenic stimulation normally induces true memory $CD8^+$ T cells that can provide life-long protection against reinfection (Ahmed and Gray, 1996; Harty and Badovinac, 2008; Lau et al., 1994). In unimmunized condition, some other memory-like $CD8^+$ T cell types have been described, including those derived through homeostatic proliferation in lymphopenic environments (HP $CD8^+$ T cells), virtual memory cells found in normal homeostatic environment (VM $CD8^+$ T cells) and innate memory phenotype cells that can be selected through hematopoietic MHCI (IMP $CD8^+$ T cells) (Akue et al., 2012; Broussard et al., 2006; Cheung et al., 2009; Haluszczak et al., 2009; Hu et al., 2007; Huang et al., 2013). Among these exogenous antigen-independent $CD8^+$ T cells, VM cells carry markers and features of HP cells (Haluszczak et al., 2009), while IMP $CD8^+$ T cells are distinct from HP cells (Huang et al., 2013).

HP $CD8^+$ T cells are typically derived from naïve T cells proliferation in response to lymphopenia condition, dependent on cytokines (IL-7 and/or IL-15) and self-antigen-MHCI tonic interaction(Cho et al., 2000; Ernst et al., 1999; Goldrath and Bevan, 1999a; Kieper and Jameson, 1999; Le Campion et al., 2002). During lymphopenia-induced proliferation, $CD8^+$ T cells undergo clonal expansion (Haluszczak et al., 2009) and functional maturation and progressively acquire a memory-like phenotype, but carry several distinctions compared to antigen-induced

memory. HP $CD8^+$ T cells do not up-regulate antigen-induced early activation markers CD25 and CD69, do not shed CD62L like in true memory cells, and express higher CD44 and CD122 (Cho et al., 2000; Goldrath et al., 2000; Murali-Krishna et al., 1999). However, like antigen-induced true memory cells, HP $CD8^+$ require the presence of $CD4^+$ T cells during proliferation to acquire protective memory (Hamilton et al., 2006). These protective HP $CD8^+$ T cells have been shown to provide considerable protection against infection (Hamilton et al., 2006) and tumor growth (Dummer et al., 2002; Hu et al., 2002; Wrzesinski and Restifo, 2005), executed by IFN-γ mediated cytotoxicity. It is recently reported that, following initiation of homeostatic proliferation in a lymphopenic environment, IL-7 is essential in inducing mTOR activity which further induces T-bet expression in early proliferation, and this T-bet expression is essential for the protective HP $CD8^+$ T cell memory formation in later stage; in the later stage, IL-15 gets involved, which turns down mTOR activity and so T-bet expression, and turns on the reciprocal Eomes expression for HP-induced memory differentiation (Li et al., 2011).

Tec family kinase, IL-2 inducible T-cell kinase (ITK) is an essential signaling mediator downstream of T cell receptor and plays critical roles in T cell differentiation and activation (see review (Andreotti et al., 2010)). In the absence of ITK (and/or TXK), $CD8^+$ T cells spontaneously develop a memory-like phenotype independently of prior antigenic stimulation (Berg, 2007; Broussard et al., 2006; Hu et al., 2007). Similar phenotype have been observed in mice expressing a mutant of SLP-76 which is defective in interacting with ITK, and in those lacking transcription factors Krüppel-like factor 2, inhibitor of DNA binding 3 protein, CREB binding protein, and over-

expressing of T cell factor-1 and β-Catenin (Fukuyama et al., 2009; Jordan et al., 2008; Sharma et al., 2012; Verykokakis et al., 2010; Weinreich et al., 2009). IMP $CD8^+$ T cells express high level of Eomes, and can rapidly produce IFN-γ upon non-antigenic and antigenic stimulation (Broussard et al., 2006; Hu et al., 2007; Huang et al., 2013). The acquired innate memory phenotype in $CD8^+$ T cells in *Itk*$^{-/-}$ mice is dependent on IL-4 (Weinreich et al., 2010), and is genomically distinct from HP $CD8^+$ T cell memory (Huang et al., 2013). Nevertheless, the lack of ITK perturbs $CD8^+$ memory homeostasis, and ITK regulates the reciprocal expression of T-bet and Eomes, to regulate the IFN-γ promoter accessibility (Kannan et al., 2013). Whether ITK plays any functions in HP $CD8^+$ T cell proliferation, memory differentiation mediated by T-bet/Eomes, protective ability formation mediated by IFN-γ remains to be investigated.

Because of the pivotal role of ITK in T cell activation and function-mediated development of allergic diseases, targeting ITK kinase activity has been proposed for therapeutic purpose (see review (Sahu and August, 2009)). Here we show that, ITK suppresses $CD8^+$ T cell proliferation in a lymphopenic environment and associated anti-tumor immunity in a $CD8^+$ T cell manner, thus targeting ITK may have additional clinical benefit in cancer therapy.

Materials and Methods

Mice.

All mice were on a C57BL/6 background. OTI *Rag*$^{-/-}$ (B6.129S7-*Rag1*^{tm1Mom} Tg(TcraTcrb)1100Mjb N9+N1) mice were purchased from Taconic Labs (Hudson, NY). *Itk*$^{-/-}$ OTI *Rag*$^{-/-}$ mice were generated by crossing *Itk*$^{-/-}$ and OTI *Rag*$^{-/-}$ mice. CD45.1^{+} OTI *Rag*$^{-/-}$ mice were generated by crossing CD45.1^{+} (B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ, The Jackson Laboratory, Bar Harbor, ME) and OTI *Rag*$^{-/-}$ mice. *B2m*$^{-/-}$ *Rag*$^{-/-}$ mice were generated by crossing *B2m*$^{-/-}$ (B6.129P2-*B2m*^{tm1Unc}, Jackson) and *Rag*$^{-/-}$ mice. The *Rag*$^{-/-}$ mice were kind gifts from Drs. Judith Appleton, Eric Denkers and Margaret Bynoe (Cornell University). All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at Cornell University.

Antibodies.

All fluorochrome-conjugated antibodies used are listed in "fluorochrome-target" format as follows: eFluor 450-CD122 (IL-2Rβ/IL-15Rα), PE-T-bet, PerCP-Cy5.5-CD127 (IL-7Rα), PerCP-eFluor 710-Eomes, PE-Cy7-IFN-γ, and PE-Cy7-NKG2D were purchased from eBioscience (San Diego, CA); V500-CD44, FITC-CD45.1, FITC-CD45.2, FITC-Fas, FITC-TCRVβ5, PE-Annexin V, PE-IL-4Rα, PE-TCRVα2, PE-TNF-α, PE-CF594-CD8α, Cy5-Annexin V, Alexa Fluor 647-T-bet, Alexa Fluor 700-CD45.1, Alexa Fluor 700-CD45.2, Alexa Fluor 700-CD62L, Alexa Fluor 700-Ki67 and Allophycocyanin-Cy7-TCRVα2 were purchased from BD Biosciences (San

Diego, CA); PE-Texas Red-CD8α were purchased from Invitrogen (Carlsbad, CA); FITC-Bcl-2, FITC-KLRG1, Allophycocyanin-CD132 (γC) and PE-Cy7-CD62L were from Biolegend (San Diego, CA). Biotin-conjugated anti- Gr-1, DX5, TER119, CD122 and CD11b were from BD Biosciences, anti- B220, F4/80, CD11c, c-kit, CD25 and CD4 and CD8α were from eBioscience, and anti- CD44 was from Biolegend.

Cell purification and adoptive transfer.

$CD8^+$ naïve T cells were purified from age matching OTI *Rag*$^{-/-}$ (referred to as **WT**) and *Itk*$^{-/-}$ OTI *Rag*$^{-/-}$ (referred to as ***Itk***$^{-/-}$) littermates through magnetic negative selection. Splenocytes were stained by biotin-conjugated antibodies against Gr-1, DX5, TER119, CD11b, B220, F4/80, CD25, CD44, CD122 and CD4, followed by anti-biotin MicroBeads (Miltenyi Biotec, Cambridge, MA). Cells passed though MS column (Miltenyi) with purity ($CD8\alpha^+TCRV\beta5^+CD44^{lo}CD122^-$) higher than 95% were used for adoptive transfer into *Rag*$^{-/-}$ recipients, through retro-orbital injection (120,000 cell/mouse unless specified otherwise). Recipients were sacrificed for analysis at indicated time points.

in vitro stimulation.

For analysis of IFN-γ and TNF-α secretion by expanded cells, splenocytes were collected, plated at 2×10^6 cells/ml, and either left unstimulated, or stimulated with 100 ng/ml PMA/0.5 μM Ionomycin (Sigma) (referred to as **P/I**), 0.1μg/ml $OVA_{257-264}$ peptide (Peptides International, Louisville, KY), in the presence of 10 μg/ml Brefeldin

A (Sigma) for 6-7 hours, followed by staining and flow cytometric analysis.

Rapamycin treatment.

Rapamycin (**Rapa**) was purchase from LC Laboratories (Woburn, MA). *Rag*$^{-/-}$ hosts were weighted and 10 mg/kg (rapa/body weight) was delivered through intraperitoneal injection daily for 10 days consecutively following adoptive transfer of purified OTI-*Rag*$^{-/-}$ naïve CD8^{+} T cells.

Tumor inoculation and HP CD8^{+} T cell treatment.

EG7-OVA thymoma cells (American Type Culture Collection, Manassas, VA) were injected subcutaneously to the backside of CD45.1 mice. 2 days post tumor inoculation, rescuing cells (0.12×10^{6} WT or *Itk*$^{-/-}$ 65 day HP expanded CD8^{+} T cells) were adoptive transferred by retro-orbital injection. Tumor diameters (major and minor) were measured with calipers at indicated the time points, and used in an ellipse area formula: Volume = 0.524 × (major diameter/2) × (minor diameter/2)^{2}. At endpoint of the experiment, tumors were excised and weighed. Tumors, draining lymph nodes (axillary and brachial lymph nodes on the side of the tumor), and spleens were collected to determine donor HP CD8^{+} T cell counts. Cells from draining lymph nodes were stimulated with P/I or $OVA_{257-264}$ peptide to determine cytokine production.

Flow cytometric staining.

Cells were resuspended and blocked with anti mouse CD16/CD32 antibody (Fc block,

eBioscience) in 2% fetal bovine serum containing PBS (flow buffer), followed by surface staining by the indicated antibodies. After surface staining: cells subjected to cytokine and nuclear staining were fixed and permeablized with the FoxP3 staining buffer set (eBioscience), followed by incubation with antibodies; cells subjected to apoptotic assay were stained for Annexin V and PI/RNase as previously described (Qi et al., 2012). Stained cells were analyzed on LSRII system (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc., OR).

Statistical analysis.

Statistical graphs present data "mean ± s.e.m.". Unpaired two-tailed student's *t* test (referred to as ***t* test**) and two-way ANalysis Of VAriance between groups (referred to as **ANOVA**) were performed using GraphPad Prism version 5.00 for Macintosh (GraphPad, San Diego, CA). A difference with $p \leq 0.05$ is considered significant.

Results

ITK regulates lymphopenia-induced proliferation

Under normal condition, naïve $CD8^+$ T cells rarely divide, however, when T cell numbers are reduced to low levels, naïve $CD8^+$ T cells will expand to repopulate the host, generating a memory phenotype. This lymphopenia-induced homeostatic proliferation could be achieved by transferring naïve $CD8^+$ T cells into lymphopenic hosts such as *Rag*$^{-/-}$ mice (Cho et al., 2000; Goldrath and Bevan, 1999a; Goldrath and Bevan, 1999b; Marrack et al., 2000; Surh and Sprent, 2008). $CD8^+$ T cells in *Itk*$^{-/-}$ mice carry a spontaneous innate memory phenotype(Broussard et al., 2006; Hu et al., 2007) (**Figure 4-1** a**,** "Non-Tg"), which is distinct from the memory-like phenotype formed post homeostatic proliferation in lymphopenic environment (Huang et al., 2013). $CD8^+$ T cell memory acquisition in *Itk*$^{-/-}$ mice has been shown to be induced by an IL-4 secreting lymphocytes (Weinreich et al., 2010). In order to investigate the role of ITK in naïve $CD8^+$ T cells expansion in a lymphopenic environment, we generated *Itk*$^{-/-}$ mice carrying an OTI transgene (transgenic T cell receptor on only $CD8^+$ T cells, specific for chicken ovalbumin 257-264 presented by MHCI H-$2k^b$ (Hogquist et al., 1994)), in *Rag*$^{-/-}$ background where no other lymphocytes (Mombaerts et al., 1992) other than OTI-$CD8^+$ T cells can develop. $CD8^+$ T cells in *Itk*$^{-/-}$ OTI-*Rag*$^{-/-}$ mice are thus predominantly naïve unlike what is observed in the *Itk*$^{-/-}$ mice (**Figure 4-1** a). We used these OTI-*Rag*$^{-/-}$ models to purify WT and *Itk*$^{-/-}$ $CD8^+$ T cells and transferred them into lymphopenic *Rag*$^{-/-}$ recipients to examine homeostatic expansion of these cells. We found that *Itk*$^{-/-}$ naïve $CD8^+$ T cells underwent rapid and massive expansion,

resulting a > 10 fold population in 10 days, compared to WT cells (**Figure 4-1** b). This was followed by an early population collapse. The *Itk*$^{-/-}$ HP population decline was comparable to the level of WT T cells, 40 days post transfer, while the WT HP population keep on rising to the peak level around 60 days post transfer (**Figure 4-1** b). Thus ITK regulates the dynamics of CD8^{+} T cell homeostatic proliferation in a lymphopenic environment, as ITK deficiency results in loss of control in early expansion and alter sustenance of this population.

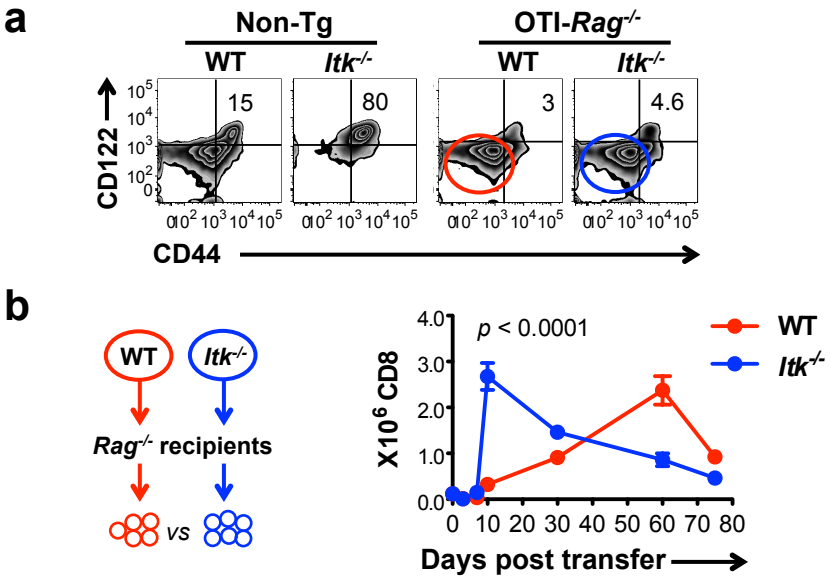


Figure 4-1. ITK deficiency in naïve CD8$^+$ T cells results to massive early expansion followed by collapse during homeostatic expansion. (**a**) Representative plots of CD44 and CD122 expression of CD8$^+$ T cells from non-transgenic (Non-Tg) or OTI-*Rag*$^{-/-}$ transgenic WT and *Itk*$^{-/-}$ mice. (**b**) Model and dynamics of WT (red circle as in **a**) vs. *Itk*$^{-/-}$ (blue circle as in **a**) naïve CD8$^+$ T cell expansion in lymphopenic environment: numbers of Vα2$^+$CD8α$^+$ expanded from 0.12×10^6 naïve CD8$^+$ T cells along the time course. n = 3 -14, from more than 2 independent experiments for each time point. *p* values was generated by ANOVA.

ITK regulates CD8$^+$ T cell proliferation and apoptosis during expansion induced by lymphopenia

The relative pace of cell proliferation and death determines the rate of CD8$^+$ T cell repopulation. To provide more insight into the altered dynamics of the CD8$^+$ T cell lymphopenic expansion by the lack of ITK, we examined the proliferative capacity and apoptotic tendency of the HP CD8$^+$ T cells. The CD8$^+$ T cell in total splenocytes taken directly from WT or *Itk*$^{-/-}$ OTI *Rag*$^{-/-}$ mice were used to examine the phenotype at initial time point (day 0). Purified WT and *Itk*$^{-/-}$ OTI *Rag*$^{-/-}$ CD8$^+$ T cells transferred into *Rag*$^{-/-}$ mice and expanded in spleen of the recipients were analyzed at early (day 10) and late (day 75) phases of the lymphopenic expansion. Based on analysis of markers of proliferation (Ki67$^+$) and programed cell death (Annexin V$^+$), we found that *Itk*$^{-/-}$ CD8$^+$ T cells exhibited significantly higher signs of proliferation in the early phase, and retained a higher proliferative ability into the later phase of the lymphopenic expansion compared to WT CD8$^+$ T cells (**Figure 4-2** a, left). On the other hand, *Itk*$^{-/-}$ CD8$^+$ T cells exhibited significantly higher apoptotic program much earlier than the WT cells (**Figure 4-2** a, right). Expression of the pro-apoptotic Fas and anti-apoptotic Bcl-2 genes has been shown to regulate CD8$^+$ T cell apoptosis through distinct mechanisms in regulating T cell homeostasis (Fortner et al., 2010; Strasser et al., 1995). We thus examined the expression of these and found that *Itk*$^{-/-}$ CD8$^+$ T cells significantly up-regulated Fas and down-regulated Bcl-2 in the process of lymphopenia-induced proliferation (**Figure 4-2** b, top row), suggesting a more potent and rapid-acting cell programed cell death and reduced survival ability, compared to WT cells. Lymphopenia-induced T cell expansion has been reported to be extrinsically

regulated by the common γ chain (γC) cytokines such as IL-2, IL-7 and IL-15, and recently reports suggest that IL-4 may also play a role in regulating $CD8^+$ T cell expansion (Oliver et al., 2012). We found, very interestingly, that the robustly expanding *Itk*$^{-/-}$ $CD8^+$ T cell expressed lower levels of IL-2Rβ/IL-15Rα (shared subunit by IL-2 and IL-15 receptor complexes), IL-4Rα, IL-7Rα and γC compared to the WT cells during lymphopenia-induced proliferation (**Figure 4-2** b, bottom row). The reduced expression of cytokine receptors in *Itk*$^{-/-}$ cells may be the result of downregulation of these cytokines following the immediate hyper-proliferation in lymphopenic environment, and may further contribute to the observed failure in sustaining cell survival at the later stages of HP. It is therefore likely that ITK functions as "cruise control" through some environment-sensing, growth/death-regulating machinery.

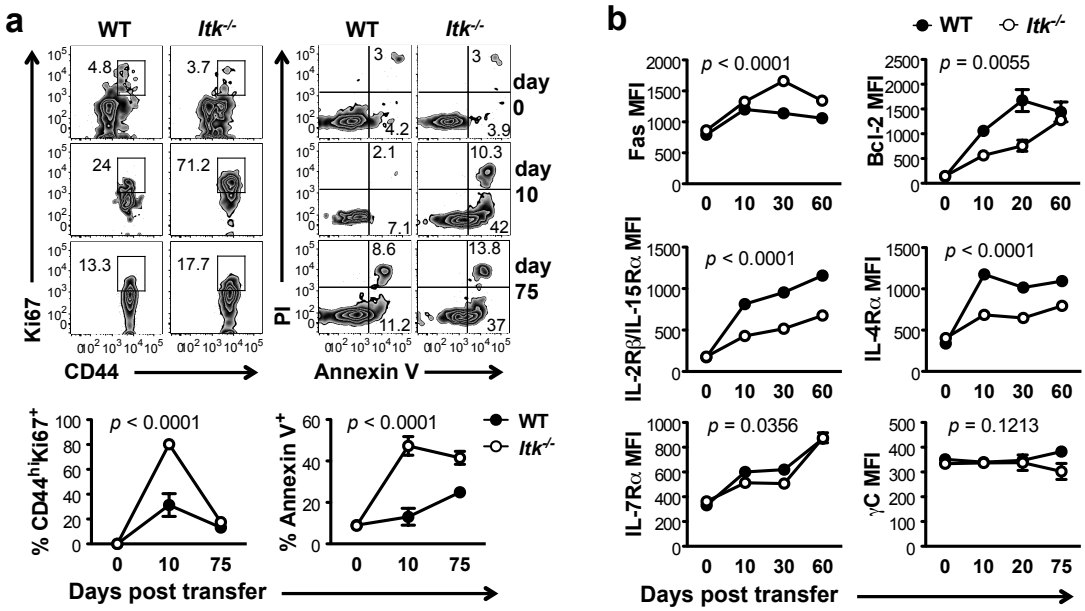


Figure 4-2. ITK regulates CD8$^+$ T cell proliferation and death in lymphopenic environment. Analysis of Vα2$^+$CD8α$^+$ T cells as indicated. (**a**) Representative plots and percentages of proliferative (Ki67$^+$) and apoptotic (Annexin V$^+$) CD8$^+$ T cells at initial (day 0), early (day 10) and late (day 75) phase of lymphopenia-induced expansion. n ≥ 6, from more than 2 independent experiments for each time point. (**b**) Dynamics of expression (Mean Fluorescent Intensity, MFI, mean ± s.e.m.) of Fas, Bcl-2, IL-2Rβ/IL-15Rα, IL-4Rα, IL-7Rα, γC expression CD8$^+$ T cell during lymphopenia-induced expansion. n ≥ 3, from more than 2 independent experiments for each time point. *p* values were generated by ANOVA.

ITK deficiency enhances CD8$^+$ T cell effector function during lymphopenia-induced proliferation

CD8$^+$ T cells undergoing lymphopenia-induced proliferation progressively acquire a memory phenotype, however, antigen-inducible early activation and effector markers are not typically expressed (Cho et al., 2000). For example, they display high levels of CD44 and CD122, but do not shed CD62L (Goldrath et al., 2000; Murali-Krishna et al., 1999). However, we found that in the absence of ITK, such cells acquire a strong effector memory phenotype characterized by CD44^{hi}CD62L^{lo} (Wherry et al., 2003), from very early during HP, which persisted (**Figure 4-3** a), suggesting that the lack of ITK signals resulted in a bias or enhancement in effector memory CD8$^+$ T cells. This effector memory phenotype is accompanied by expression of NKG2D, which can regulate TcR-independent, cytotoxic killing of other cells, including CD8$^+$ T cells (Chu et al., 2013). T-bet, a potent transcriptional factor that regulates driving IFN-γ producing antigen-induced CD8$^+$ effector T cells and their function, including cytotoxicity (Sullivan et al., 2003), was transiently up-regulated in *Itk*$^{-/-}$ CD8$^+$ T cells undergoing lymphopenia-induced proliferation at the very early stage (**Figure 4-3** b). In CD8$^+$ T cell differentiation, T-bet expression is induced by mTOR activation and is essential for effector formation; while Eomes expression as a result of low mTOR activity contributes to memory differentiation (see review (Chi, 2012)). In non-transgenic *Itk*$^{-/-}$ mice, CD8$^+$ T cells exhibit a predominant memory phenotype, with significantly up-regulated Eomes expression (Atherly et al., 2006). In contrast, we did not observe an increase but rather a slight decrease in Eomes expression in *Itk*$^{-/-}$ HP CD8$^+$ T cells, compared to WT counterpart (**Figure 4-3** b).

When stimulated by PMA/Ionomycin, a similar proportion of WT and *Itk*$^{-/-}$ HP CD8^{+} T cells secreted IFN-γ and TNF-α, but *Itk*$^{-/-}$ HP CD8^{+} T cells secreted more cytokine per cells as shown by higher mean fluorescent intensity (**Figure 4-3** b).

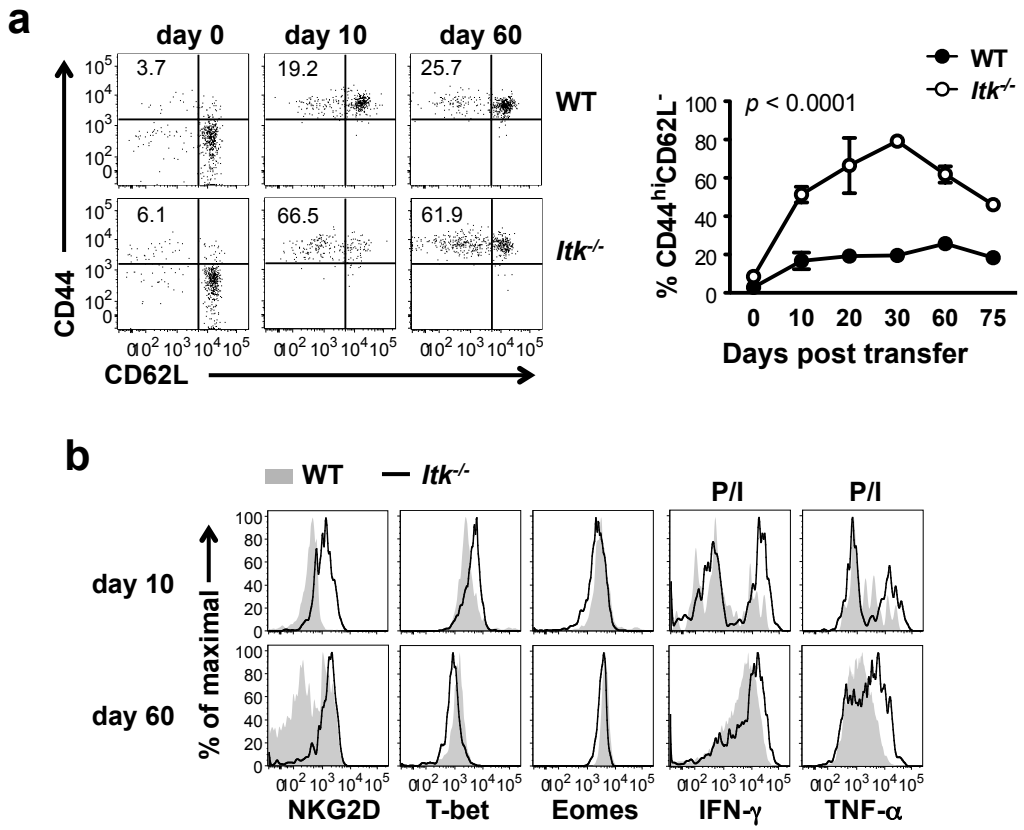


Figure 4-3. ITK deficiency promotes CD8$^+$ T cell effector memory development in lymphopenia-induced proliferation. (**a**) Representative plots and summary of CD44^{hi}CD62L^{lo} effector memory phenotype CD8$^+$ T cells derived from CD8$^+$ T cells from the lymphopenic environment. n ≥ 3, from at least 2 independent experiments for each time point. *p* value was generated by ANOVA. (**b**) Representative (of n ≥ 10) plots of NKG2D, T-bet and Eomes expression and P/I induced IFN-γ and TNF-α in CD8$^+$ T cells in early (day 10) and late (day 60) phase of lymphopenic proliferation.

The hyperactive lymphopenia-induced proliferation in the absence of ITK requires mTOR activation

During $CD8^+$ T cell activation and differentiation, CD62L shedding, increased T-bet expression, and reduced expression of Eomes, KLF2, FoxO3 and Bim expression are hallmarks of mTOR activity (see review (Chi, 2012)). We observed these changes in the *Itk*$^{-/-}$ HP $CD8^+$ T cells, (including reduction in Eomes, KLF2, FoxO3 and Bim expression at the mRNA level (**Supplemental Figure 4-1**)), suggesting the possibility that mTOR is hyperactive in the absence of ITK during this process. To further examine this hypothesis, we blocked mTOR activation using Rapamycin during the early phase of $CD8^+$ T cell lymphopenic expansion (i.p. injection, 10 consecutive days). We found that, blocking mTOR activation significantly shrunk the increased $CD8^+$ T cell population in the absence of ITK: *Itk*$^{-/-}$ $CD8^+$ naïve T cells only gave rise to a very limited numbers of cells, comparable to those of WT origin (**Figure 4-4** a). More importantly, blocking mTOR activation prevented the enhanced CD62L shedding in *Itk*$^{-/-}$ HP $CD8^+$ T cells undergoing lymphopenic expansion (**Figure 4-4** b). The lack of mTOR activity also alters the expression of IL-2 and IL-15 shared receptor and reduced the expression of proliferative marker Ki67 to levels similar to WT cells (compared to PBS treated group) (**Figure 4-4** c, top row). In support of a role for mTOR in this process, we also observed reduced expression of T-bet in Rapamycin treated recipients of *Itk*$^{-/-}$ $CD8^+$ T cells, while Eomes remained at a lower level compared to WT (**Figure 4-4** c, middle row). These data suggest that mTOR activity is required, in the absence of ITK, for the hyperactive proliferation and effector phenotype generation in $CD8^+$ T cells

undergoing lymphopenia-induced proliferation. However, the impaired survival ability indicated by increased expression of Fas and decreased expression of Bcl-2 in the absence of ITK is an mTOR independent phenotype (**Figure 4-4** c, bottom row).

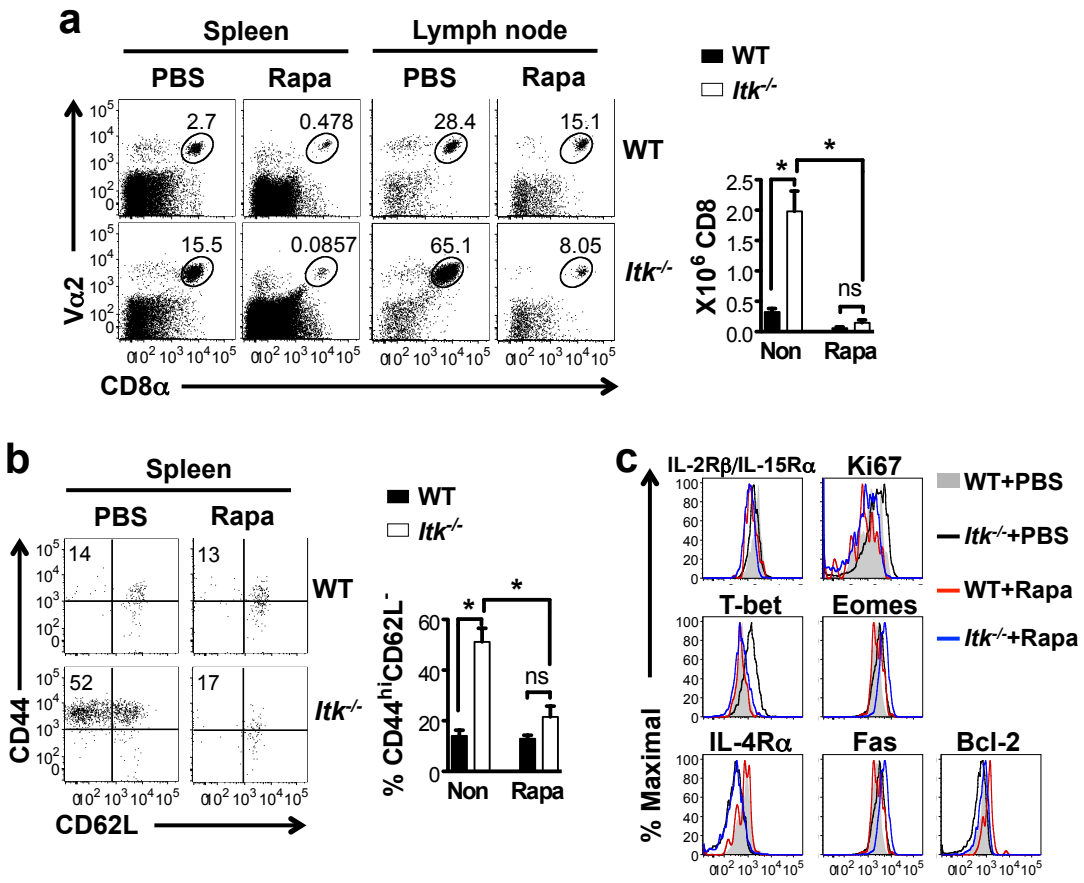


Figure 4-4. ITK regulates CD8$^+$ T cell proliferation and effector memory development but not programmed cell death through mTOR. *Rag*$^{-/-}$ recipients of naïve WT or *Itk*$^{-/-}$ OTI-*Rag*$^{-/-}$ CD8$^+$ T cells were treated for 10 days with PBS or Rapamycin (Rapa). (**a**) Representative plots and number (sum of spleen and lymph node) of WT and *Itk*$^{-/-}$ CD8$^+$ T cells expanded 10 days post transfer. (**b**) Representative plots and percentage of CD44^{hi}CD62L^{lo} effector memory phenotype CD8$^+$ T cells. (**c**) Representative plots of IL-2Rβ/IL-15Rα, Ki67, IL-4Rα, Fas, Bcl-2, T-bet and Eomes in expanded CD8$^+$ T cells. n ≥ 5, from 4 independent experiments. *$p < 0.05$; ns = "not significant", by *t* test.

CD8$^+$ T cell-intrinsic regulation by ITK in lymphopenia-induced proliferation and memory differentiation

Although WT and *Itk*$^{-/-}$ naïve CD8$^+$ T cells were transferred and initiated lymphopenia-induced proliferation in exactly the same environmental setting, *Itk*$^{-/-}$ cells turned on an immediate massive expansion resulting in a significantly larger population compared to WT cells. The large *Itk*-/- HP CD8$^+$ T cell population at the early phase of lymphopenia-induced proliferation may have significant influence on the conditions *in vivo*, for example, in cytokine abundance and cell-cell proximity/interaction, which may further lead to the altered later phenotype observed in *Itk*$^{-/-}$ proliferation, death and memory differentiation compared to WT cells. To test this possibility, we utilized naive T cells from congenic CD45.1$^+$ WT and CD45.2$^+$ *Itk*$^{-/-}$ mice on the OTI-*Rag*$^{-/-}$ background as donors, and performed an equally mixed donor transfer (**Figure 4-5** a). This co-transfer model allows WT and *Itk*$^{-/-}$ CD8$^+$ T cells to proliferate in exactly the same *in vivo* environment along the time course. We found that, *Itk*$^{-/-}$ CD8$^+$ T cells still exhibited the hyperactive expansion, forming a significantly higher fraction (**Figure 4-5** b&c), and number (**Figure 4-5** d) among total CD8$^+$ T cells expanded in lymphopenic recipients, independently of the presence of co-expanding WT cells. Compared to WT cells, the down-regulation of IL-2/IL-5, IL-4 and IL-7 receptor expression, enhanced proliferative property (Ki67), up-regulated Fas and down-regulated Bcl-2 expression was T cell intrinsic in *Itk*$^{-/-}$ HP cells, as the co-presence of WT cells in the same lymphopenic recipients did not change these previously observed differences. Differences in effector memory markers such as reduced CD62L, increased CD44, KLRG1, and NKG2D also persisted in this co-

expansion model. In addition, transient up-regulation of T-bet transient was also maintained with no change in Eomes (**Figure 4-5** f). These data suggest that the T cell sensitivity to growth driving signals and cytokines, along with survival potential is T cell-intrinsically regulated by ITK.

However interestingly, we noted that the presence of WT cells prevented the collapse *of the Itk*$^{-/-}$ population as previously observed upon single transfer (shown in **Figure 4-1)**. Furthermore we observed that there was a similar potential of apoptotic program in WT and *Itk*$^{-/-}$ cells, suggesting that the previously enhanced apoptotic potential observed upon single transfer (shown in **Figure 4-2**) is a cell-extrinsic effect, likely due to reduced availability of vital resources per cell under those conditions. Note that, when co-expanded with *Itk*$^{-/-}$ cells, WT HP CD8^{+} T cells exhibit higher apoptotic potential starting at the early stage (day 10 in **Figure 4-5** e, ~ 30%) compared to when expanding alone (day 10 in **Figure 4-2** a, ~ 10%). It is possible that, in the co-transfer model, WT and *Itk*$^{-/-}$ T cells compete for the growth factors and cytokines, and *Itk*$^{-/-}$ T cells can better compete, driving more proliferation and survival of these cells, despite the increases in pro-apoptotic Fas and decreased anti-apoptotic Bcl-2 expression.

Evaluation of cytokine producing capability of WT and *Itk*$^{-/-}$ T cells expanded for 10 and 60 days by stimulation with PMA/Ionomycin (P/I) revealed that they had similar percentage of cells secreting IFN-γ and TNF-α (**Figure 4-5** g). ITK is downstream of TcR and is critical for cytokine production to antigenic stimulation (see review (Andreotti et al., 2010)). Although *Itk*$^{-/-}$ cells responded to OVA peptide stimulation with lower percentage of cells secreting these cytokines, this percentage

rose over the time during the expansion (day 60 vs. day 10, is 1.2 fold in WT, but 3 fold in *Itk*$^{-/-}$), suggesting that *Itk*$^{-/-}$ HP T cells adjusted their sensitivity to TcR signaling during homeostatic proliferation, leading to subsequent increases in cytokine production upon antigenic stimulation.

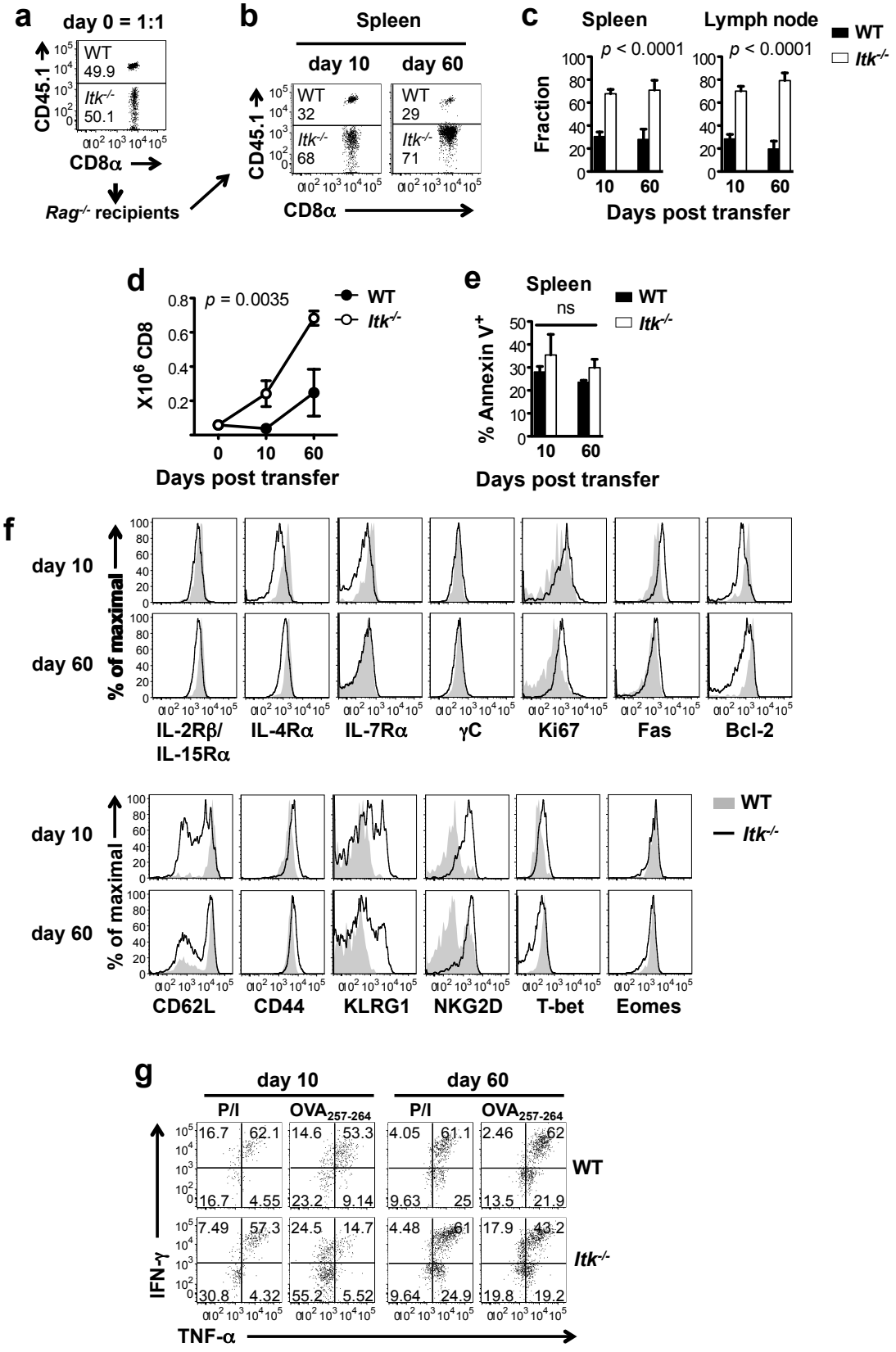

a
day 0 = 1:1
CD45.1
WT
49.9
Itk-/-
50.1
CD8α
Rag-/- recipients
b
Spleen
day 10
day 60
WT
32
Itk-/-
68
WT
29
Itk-/-
71
CD45.1
CD8α
c
Spleen
Lymph node
p < 0.0001
p < 0.0001
Fraction
10
60
Days post transfer
WT
Itk-/-
d
p = 0.0035
X10^6 CD8
WT
Itk-/-
Days post transfer
e
Spleen
ns
% Annexin V+
Days post transfer
f
day 10
day 60
% of maximal
IL-2Rβ/
IL-15Rα
IL-4Rα
IL-7Rα
γC
Ki67
Fas
Bcl-2
CD62L
CD44
KLRG1
NKG2D
T-bet
Eomes
WT
Itk-/-
g
day 10
day 60
P/I
OVA257-264
16.7
62.1
16.7
4.55
14.6
53.3
23.2
9.14
4.05
61.1
9.63
25
2.46
62
13.5
21.9
WT
7.49
57.3
30.8
4.32
24.5
14.7
55.2
5.52
4.48
61
9.64
24.9
17.9
43.2
19.8
19.2
Itk-/-
IFN-γ
TNF-α

Figure 4-5. ITK intrinsically regulates $CD8^+$ T cell proliferation, death and effector memory development in lymphopenia-induced homeostatic proliferation. (**a**) Purified congenic naïve WT ($CD45.1^+$) and *Itk*$^{-/-}$ ($CD45.1^-$) $CD8^+$ T cells were mixed 1:1, and a total of 120,000 T cells were transferred to *Rag*$^{-/-}$ lymphopenic recipients. (**b**) Representative plots and (**c**) summary of fractions of WT and *Itk*$^{-/-}$ $V\alpha2^+CD8\alpha^+$ T cells expanded in lymphopenic environment for 10 and 60 days. *p* values were generated by *t* test, comparing WT and *Itk*$^{-/-}$ at each time point. (**d**) Number of expanded WT and *Itk*$^{-/-}$ $CD8^+$ T cells. *p* value was generated by ANOVA. (**e**) Percentage of Annexin V^+ WT and *Itk*$^{-/-}$ $V\alpha2^+CD8\alpha^+$ T cells expanded in lymphopenic environment for 10 and 60 days. ns = "not significant", by *t* test. (**f**) Representative plots for expression of indicated markers by WT and *Itk*$^{-/-}$ $CD8^+$ T cells expanded for 10 and 60 days. (**g**) IFN-γ and TNF-α expression induced by P/I and $OVA_{257-264}$ peptide. $n \geq 6$, from more at least 3 independent experiments for each time point. ns = "not significant".

MHCI expression in recipients is not required to trigger hyperactive proliferation and effector memory program by donor Itk$^{-/-}$ HP CD8^{+} T cells

Lymphopenia-induced CD8^{+} T cell proliferation has been defined to be the cell cycling in response to space, although independent of cognate antigenic simulation, it has been suggested that tonic interaction with self-antigen-MHC is critical (Ernst et al., 1999; Goldrath and Bevan, 1999a). However, CD8^{+} T cell memory maintenance seems to be independent of host MHC- T cell TcR interaction, as memory CD8^{+} T cells can maintain active division in MHCI deficient recipients (Murali-Krishna et al., 1999). Thus, we wondered whether the lymphopenic recipient required MHCI expression to trigger the hyper-proliferation of *Itk*$^{-/-}$ CD8^{+} T cells. To investigate this, we generated lymphopenic *Rag*$^{-/-}$ mice with additional loss of a key component of MHCI, β2m resulting in lack of MHCI, (*B2m*$^{-/-}$*Rag*$^{-/-}$), and used them as lymphopenic recipients. Co-transfer of equal numbers of WT and *Itk*$^{-/-}$ naïve CD8^{+} T cells into *B2m*$^{-/-}$*Rag*$^{-/-}$ recipients gave rise to predominantly *Itk*$^{-/-}$ HP CD8^{+} T cells (**Figure 4-6** a&b), leading to significantly larger *Itk*$^{-/-}$ HP population (**Figure 4-6** c). These data suggest that tonic interaction between recipient-MHCI and HP CD8^{+} T cell receptor is not required for *Itk*$^{-/-}$ HP CD8^{+} T cell hyper-proliferation. Furthermore, the expression of cytokine receptors and effector memory markers resembled that of the case when recipient MHCI was available (**Figure 4-6** e), suggesting that recipient MHCI is not required for the T cell intrinsic function regulated by ITK during CD8^{+} T cell homeostatic proliferation in lymphopenic condition. Quite interestingly, *Itk*$^{-/-}$ HP cells expanded in the absence of lymphopenic recipient expression of MHCI exhibited had equal ability to produce IFN-γ and TNF-α, and adjusted their sensitivity to cognate

antigen/TcR stimulation at a much early stage. This was evidenced by the fact that on day 10, *Itk*$^{-/-}$ HP cells could readily respond to OVA peptide stimulation with more 40% cells secreting high level of IFN-γ and TNF-α (**Figure 4-6** f). This number in the case when recipient MHCI was available was around 15% on day 10, and required much longer times (60 days) to rise to around 40%. These data suggest that the absence of host MHCI enhances the tuning of TcR for increased sensitivity in the absence of ITK.

Also notable was that the lack of recipient MHCI allowed a much larger fold expansion (over initial number) by *Itk*$^{-/-}$ CD8^{+} T cells during HP (33-fold expansion by day 10 in the absence of MHCI (**Figure 4-6** c), compared to 3-fold expansion in the presence of MHCI (**Figure 4-5** d)). This 10-fold higher expansion suggests a regulatory function for recipient MHCI in controlling the homeostasis of *Itk*$^{-/-}$ HP CD8^{+} T cells. Also interestingly, when the *Itk*$^{-/-}$ CD8^{+} T cells were the predominant population in lymphopenic conditions, the population collapse in later phase of HP was similar to the case when *Itk*$^{-/-}$ cells was expanding in single transfers (**Figure 4-1** b, *Itk*$^{-/-}$ dynamics). On day 60 under MHCI deficient conditions where the *Itk*$^{-/-}$ CD8^{+} T cells underwent expansion, WT cells exhibited significantly higher apoptotic potential (55.6% vs. 23.6% Annexin V^{+}) (**Figure 4-6** d), accompanied by significantly higher expression of Fas (MFI ratio = 1:1.65) and lower expression of Bcl-2 (MFI ratio = 1:0.65), suggesting that WT cells competed poorly for survival signals in the presence of the overwhelming response of the *Itk*$^{-/-}$ HP CD8^{+} T cells.

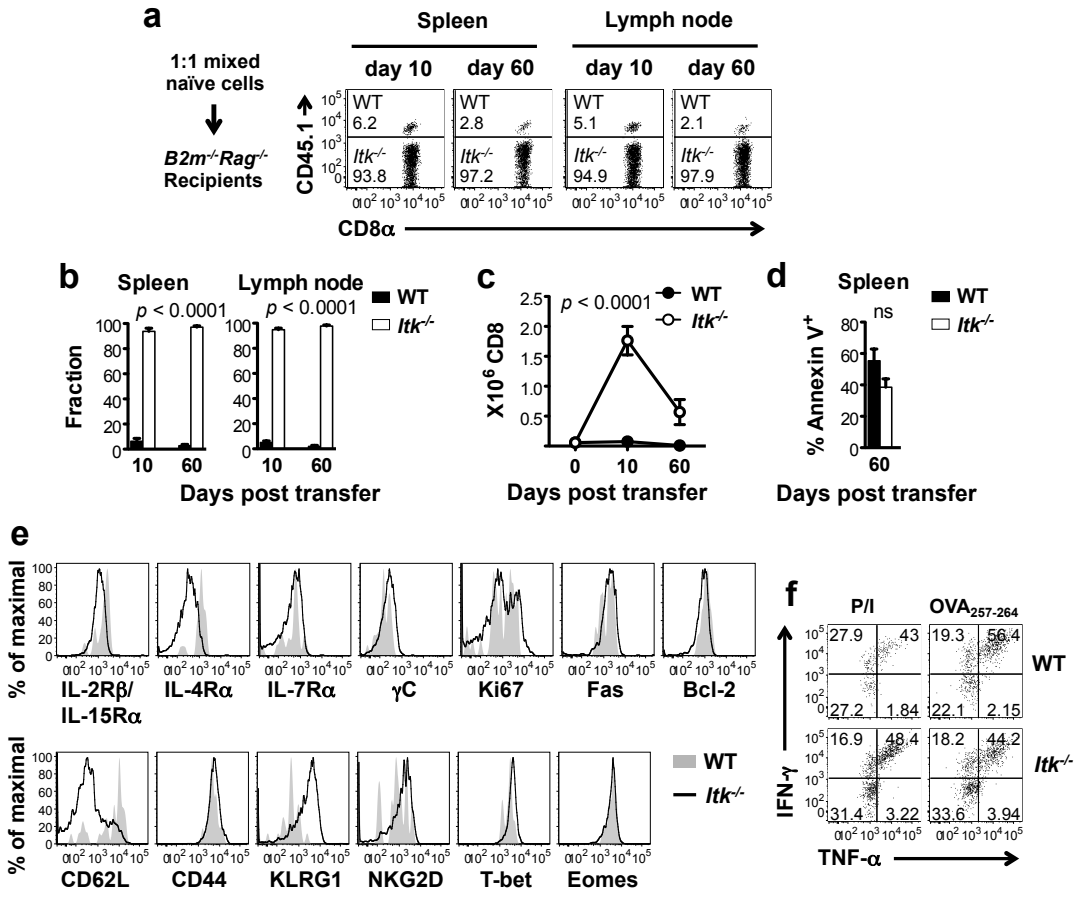


Figure 4-6. The CD8$^+$ T cell-intrinsic function of ITK in lymphopenia-induced proliferation is independent of recipient MHCI expression. (**a**) 1:1 mixture (total 120,000) of WT and *Itk*$^{-/-}$ naïve OTI-*Rag*$^{-/-}$ CD8$^+$ T cells were transferred into *B2m*$^{-/-}$ *Rag*$^{-/-}$ recipients: representative plots and (**b**) summary of fractions of WT and *Itk*$^{-/-}$ Vα2$^+$CD8α$^+$ T cells expanded in lymphopenic environment for 10 and 60 days. *p* values were generated by *t* test, comparing WT and *Itk*$^{-/-}$ at each time point. (**c**) Number of expanded WT and *Itk*$^{-/-}$ CD8$^+$ T cells. *p* value was generated by ANOVA. (**d**) Percentage of Annexin V$^+$ WT and *Itk*$^{-/-}$ Vα2$^+$CD8α$^+$ T cells expanded in lymphopenic environment for 60 days. ns = "not significant", by *t* test. (**e**) Representative plots for expression of indicated markers by WT and *Itk*$^{-/-}$ CD8$^+$ T cells expanded for 60 days. (**f**) IFN-γ and TNF-α expression induced by P/I and OVA$_{257-264}$ peptide in cells expanded for 10 days. n ≥ 5, from at least 2 independent experiments for each time point.

T cell-T cell interactions promote Itk$^{-/-}$ HP CD8^{+} T cell hyper-proliferation

As CD8^{+} T cell also express MHCI and T cell-T cell (T-T) interactions can drive the proliferation and effector development of CD8^{+} T cells (Gerard et al., 2013), we examined the role of T cell-T cell interaction in lymphopenia-induced CD8^{+} T cell proliferation. Since transfer of 120,000 T cells could potentially allow T-T interaction, we reduced the number of transferred cells to reduce the incidence of T-T interaction after transfer. WT and *Itk*$^{-/-}$ naïve CD8^{+} were equally mixed at a very low density (2000 cells in total), and transfer into *B2m*$^{-/-}$*Rag*$^{-/-}$ recipients and monitored for expansion. Ten days post transfer we found that WT cells hardly proliferated, while *Itk*$^{-/-}$ cells did, thus *Itk*$^{-/-}$ formed the predominant HP population (**Figure 4-7** a&b) when the T-T proximity was reduced, suggesting that the hyperactive proliferation in the absence of ITK is T cell intrinsic, with these cells more sensitive compared to WT cells.

It was however interesting that in the relatively smaller compartment, the lymph node, some WT cells were seen, suggesting that this environment may be able to generate the proximity required for responses by WT cells. Indeed, when we compared the fold expansion over initial numbers of transferred CD8^{+} T cells, we found that when the T cell-T cell proximity was reduced, the significantly higher fold expansion of *Itk*$^{-/-}$ cells in *B2m*$^{-/-}$*Rag*$^{-/-}$ recipients was reduced to a similar level of that seen in *Rag*$^{-/-}$ recipients, (**Figure 4-7** c). This data suggest that while recipient-MHCI acts to suppress homeostatic expansion of *Itk*$^{-/-}$ cells, MHCI is required for this expansion, and T cell-T cell interaction is sufficient to allow this hyper-expansion.

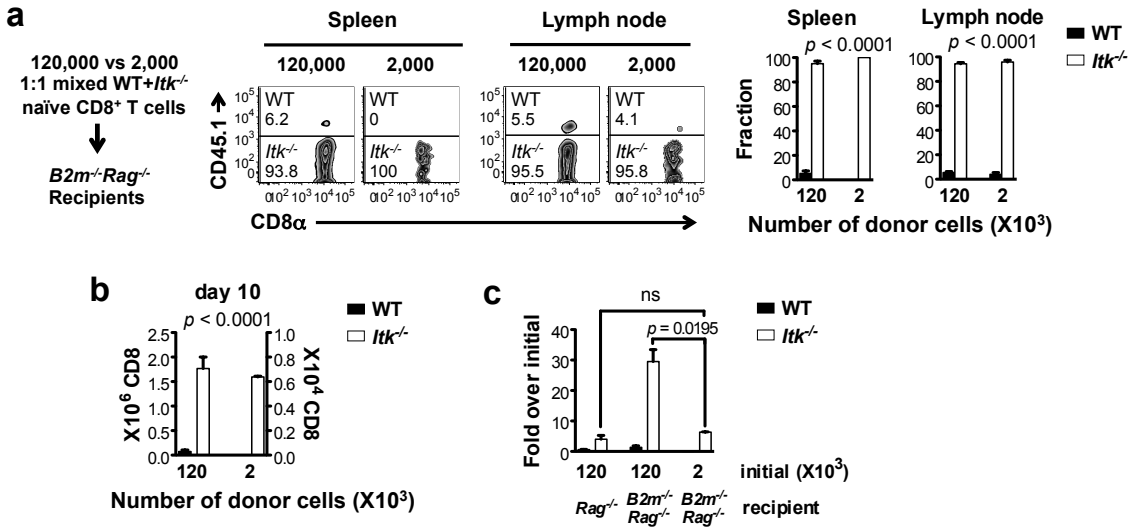


Figure 4-7. T cell-T cell interaction promotes hyperactive proliferation of *Itk*$^{-/-}$ CD8^{+} T cells. (**a**) 1:1 mixture (total 120,000 or 2000) WT and *Itk*$^{-/-}$ naïve OTI-*Rag*$^{-/-}$ CD8^{+} T cells were transferred into *B2m*$^{-/-}$*Rag*$^{-/-}$ recipients: representative plots and summary of fractions of WT and *Itk*$^{-/-}$ Vα2^{+}CD8α^{+} T cells expanded in lymphopenic environment for 10. (**b**) Number of WT and *Itk*$^{-/-}$ CD8^{+} T cells expanded from 120,000 (left y axis) or 2000 (right y axis) initial cells. (**c**) Fold expansion of WT and *Itk*$^{-/-}$ CD8^{+} T cells in the same environment: 120,000 initial cells in *Rag*$^{-/-}$ recipients, 120,000 initial cells in *B2m*$^{-/-}$*Rag*$^{-/-}$ recipients, and 2000 initial cells in *B2m*$^{-/-}$*Rag*$^{-/-}$ recipients. *p* values were generated by *t* test; ns = "not significant". n ≥ 2, combined from 2 independent experiments.

ITK suppresses anti-tumor immunity in lymphopenia-induced proliferated CD8$^+$ T cells

Early transient expression of T-bet regulated by mTOR activation is required for the effector memory T cells that give rise to anti-tumor memory CD8$^+$ T cells. This transition is control by down-regulating mTOR activity and thus T-bet expression (Li et al., 2011). We have observed, in *Itk*$^{-/-}$ CD8$^+$ T cells, a transient hyper-expression of T-bet in early HP that was significantly reduced at later stages (**Figure 4-3** b), suggesting that *Itk*$^{-/-}$ HP CD8$^+$ T cells might exhibit an anti-tumor effector immunity. WT HP CD8$^+$ cells can develop a protective cytotoxic memory function with the help of CD4$^+$ T cells during HP process (Hamilton et al., 2006). In our model, naïve CD8$^+$ T cells expanding in lymphopenic recipients would fail to generate protective HP CD8$^+$ T cells due to the absence of CD4$^+$ T cells. However, the fact that we observed a strong effector phenotype in *Itk*$^{-/-}$ HP CD8$^+$ T cells, which was not seen in WT cells, suggested the possibility that the absence of ITK in during HP of CD8$^+$ T cells may result in enhanced anti-tumor effector in the expanded cells. To test this hypothesis, we inoculated WT mice with EG7-OVA 0.5 million lymphoma cells, and 2 days post tumor inoculation, we transferred WT or *Itk*$^{-/-}$ HP CD8$^+$ T cells that had been separately expanded for 70 days in *Rag*$^{-/-}$ recipients. Consistent to previous reports (Hamilton et al., 2006), WT HP CD8$^+$ T cells expanded in the absence of CD4$^+$ T cells did not exhibit any anti-tumor immunity. By contrast, *Itk*$^{-/-}$ HP CD8$^+$ T cells suppressed tumor size by more than 50% (**Figure 4-8** a&b). We did not observe significant hyper-proliferation of *Itk*$^{-/-}$ HP CD8$^+$ T cells in tumor recipients, however, there was a trend towards increased numbers of these cells at the tumor site,

suggesting increased accumulation of these cells in the site of the tumor (**Figure 4-8** c).

We have described the increased sensitivity of *Itk*$^{-/-}$ HP CD8^{+} T cells responding to antigenic stimulation over the time of expansion (**Figure 4-5**). Thus we examined whether these cells could secrete cytotoxic cytokines IFN-γ and TNF-α in response to antigen specific stimulation given the presence of this tumor antigen environment. We found that *Itk*$^{-/-}$ HP CD8^{+} T cells from draining lymph nodes readily responded to P/I stimulation with increased IFN-γ and TNF-α production (**Figure 4-8** d, P/I). More importantly, these cells responded to tumor antigen $OVA_{257-264}$ with increased IFN-γ and TNF-α (**Figure 4-8** d). The anti-tumor immunity in HP CD8^{+} T cell in the absence of ITK gain, in the absence of CD4^{+} T cell help suggests that ITK is a CD8^{+} T cell-intrinsic suppressor for the development of effector function during homeostatic proliferation.

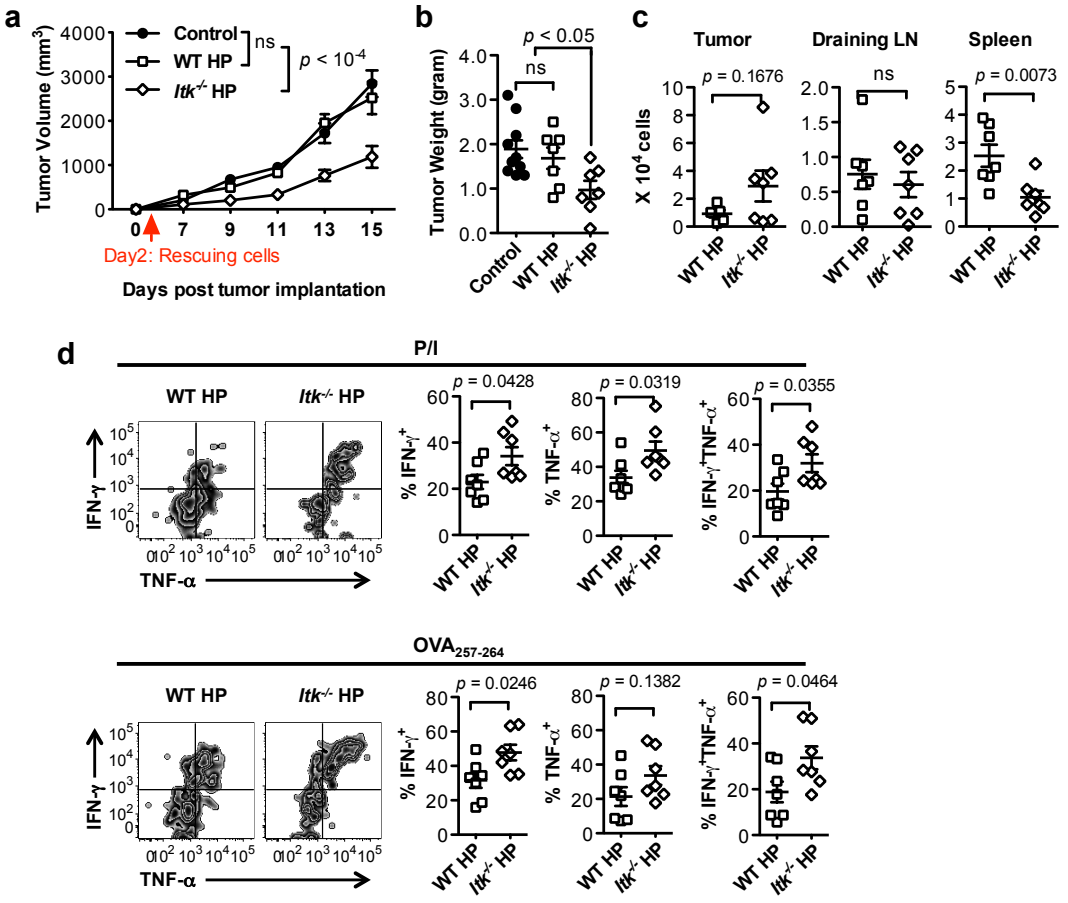


Figure 4-8. ITK suppresses CD8$^+$ T cell homeostatic proliferation induced anti-tumor immunity. CD45.1$^+$CD45.2$^-$ WT mice were inoculated (s.c.) with EG7-OVA thymoma cells, and received CD45.1$^-$CD45.2$^+$ WT or *Itk*$^{-/-}$ HP CD8$^+$ T cells 2 days later. (**a**) Tumor size of tumor recipients that received no (control), WT HP or *Itk*$^{-/-}$ HP CD8$^+$ T cells along the time course post tumor inoculation. *p* values were generated by ANOVA. (**b**) Tumor weight on day 15. (**c**) Numbers of CD45.2$^+$ CD8$^+$ T cells in tumor site, draining lymph nodes, and spleen on day 15. (**d**) P/I and OVA$_{257-264}$ induced IFN-γ and TNF-α production by CD45.2$^+$ CD8$^+$ T cells in draining lymph nodes of tumor recipients on day 15. *p* values in (**b-d**) were generated by *t* test. n ≥ 7, ns = "not significant".

Discussions

We have shown in our previous work that in the absence of ITK, although $CD8^+$ T cells spontaneously acquire a memory-like phenotype, they are distinct from memory-like cells derived from lymphopenia-induced homeostatic proliferation (Huang et al., 2013). It was suggested that the memory-like $CD8^+$ T cells generated in *Itk*$^{-/-}$ mice were dependent on a T cell-extrinsic IL-4 mediated effect (Weinreich et al., 2010). Here we show a $CD8^+$ T cell-intrinsic regulatory function of ITK in lymphopenia-induced proliferation and anti-tumor immunity development.

In lymphopenia, due to, for example, chemotherapy or irradiation or viral infection, naïve $CD8^+$ T cells initiate homeostatic proliferation in response to cytokines and self-antigen-MHC tonic interaction (Cho et al., 2000; Ernst et al., 1999; Goldrath and Bevan, 1999a). As a result, naïve $CD8^+$ T cells derive the phenotypic characteristics of antigen-induced memory, such as high expression of CD44 and CD122, rapid production of IFN-γ and cytotoxicity in response to antigenic stimulation; but vary in expression of early T cell activation (eg. CD25 and CD69) and effector markers (eg. CD62L) (Goldrath et al., 2004; Marleau and Sarvetnick, 2005; Stockinger et al., 2004). The absence of ITK in naïve $CD8^+$ T cells results in a progressively rapid expansion triggered by lymphopenic environment. Unlike WT HP $CD8^+$ T cells that retain CD62L expression, and lack the expression of effector memory markers in early stage of lymphopenia-induced proliferation (Cho et al., 2000), *Itk*$^{-/-}$ HP $CD8^+$ T cells significantly down-regulate CD62L, giving rise to a majority of $CD44^{hi}CD62L^{lo}$ effector memory-like population, together with up-regulation of effector markers NKG2D and KLGR1. This is accompanied by an

mTOR activity dependent up-regulated expression of T-bet, a critical transcriptional factor in activating $CD8^+$ T cell effector memory differentiation (Li et al., 2011). Inhibiting mTOR suppresses the hyper-proliferation, up-regulated of T-bet expression and massive shedding of CD62L in *Itk*$^{-/-}$ homeostatically expanded $CD8^+$ T cells, suggesting that ITK is a regulatory factor in lymphopenia induced $CD8^+$ T cell proliferation and effector memory differentiation, and it functions through regulating the activity of mTOR. The hyper-proliferative activity and effector memory formation induced by the lack of ITK in this process is $CD8^+$ T cell intrinsic, as the co-presence of WT $CD8^+$ T cells in lymphopenic recipients fails to rescue these *Itk*$^{-/-}$ HP $CD8^+$ T cell phenotype.

We have observed a consistent down-regulation of IL-2/IL-15, IL-4 and IL-7 receptors from early phase of lymphopenia-induced $CD8^+$ T cell proliferation in the absence of ITK. These cytokines have potent effect on $CD8^+$ T cell proliferation and/or homeostasis (Cho et al., 2007; Oliver et al., 2012; Sprent and Surh, 2011). The down-regulation of these cytokine receptors on *Itk*$^{-/-}$ HP $CD8^+$ T cells compared to WT cells, may be the result of early responsiveness, which functions as a regulatory machinery to compensate the hyper-active early response. The early competitiveness of these cytokine receptors in the absence of ITK may be, in part, the reason of the early massive expansion.

In comparison to the naïve $CD8^+$ T cell homestasis in static state, HP homeostasis share the same requirement of self-antige/MHC tonic stimulation (Marrack et al., 2000), but differ in that HP memory sustention is independent of co-stimulatory signaling (Prlic et al., 2001). It has been proposed that the recipient MHCI

expression (or antigen presenting cell (APC) -expressed MHCI) is a prerequisition for self-antigen presentation to trigger the lymphopenia-induced proliferation (Cho et al., 2000; Seddon and Zamoyska, 2002), however, we found that in the absence of ITK, recipient MHCI actually provides a regulatory system to try to compromise the hyper-active prliferation of *Itk*$^{-/-}$ HP CD8^{+} T cells. As *Itk*$^{-/-}$ naïve CD8^{+} T cells expanded 10-fold more in the absence of recipient MHCI. Then who provides the proliferation-promoting "tonic interaction"? CD8^{+} T cells express MHCI, and in antigen-induced memory differentiation, CD8^{+} T cell-T cell synaptic interation plays a major role in the development of protective memory, beyond the role of APC (Gerard et al., 2013). T-T synapse can be disrupted by blocking the interaction mediator (integrin LFA-1 component CD11a), or by extensive dilution on T cell density to prevent the accessible proximity among T cells (Gerard et al., 2013). In the absenc of APC-MHCI, when T-T interaction was interupted in *Itk*$^{-/-}$ CD8^{+} T cells, we observed a significantly reduced fold expansion, suggesting that T-T interation gives the major contribution to the hyper-proliferation seen in *Itk*$^{-/-}$ HP CD8^{+} T cells. However, in the absence APC-MHCI with reduced T-T interaction, *Itk*$^{-/-}$ cells can still proliferate significantly more than the WT cells, which may be contributed by the cytokine effects.

One profound function of HP CD8^{+} T cells is anti-tumor activity, which benefits tumor therapy following immunocompromiation due to chemo- or radio-therapy (Dummer et al., 2002; Hu et al., 2002; Wrzesinski and Restifo, 2005). The inherent difficulty in using this strategy in cancer therapy is autoimmunity caused by the proliferating T cells, especially when the TGF-β signaling intensity is reduced (Dudley et al., 2002; Zhang and Bevan, 2012). The occcurance of this autoimmune

complication is dependent on the co-presence of $CD4^+$ and $CD8^+$ T cells (Zhang and Bevan, 2012). Naïve $CD4^+$ T cells alone cause severe autoimmune inflammatory bowel disease in lymphopenia host mediated by $CD4^+$ effector cells (Morrissey et al., 1993), although this effect can be suppressed by addition of memory $CD4^+$ T cell (Mottet et al., 2003). Due to the fundamental role of $CD4^+$ T cell help in inducing protective immunity in $CD8^+$ cells during lymphopenia-induced proliferation (Hamilton et al., 2006), anti-tumor WT $CD8^+$ T cell clonal expansion may have to include $CD4^+$ T cell in the initial infusion. This creates a dilemma of having both $CD8^+$ T cell anti-tumor immunity, and $CD4^+$ T cell autoimmunity in lymphopenic recipients.

We report here that, in the absence of ITK, $CD8^+$ T cell clonal expansion in lymphopenia gives rise to significant anti-tumor protection. Although ITK is downtrean of TcR, *Itk*$^{-/-}$ $CD8^+$ T cells seemed to adujust their sensitivity in sensing associared clonal antigen, with increasing ability in IFN-γ and TNF-α cytokine production. This $CD8^+$ T cell-intrinsic training is executable in the absence of ITK. To date, no evidence has shown autoimmunity mediated by $CD8^+$ T cells expanded in lymphopenia, and the rapidly expanded *Itk*$^{-/-}$ HP $CD8^+$ T cells described in our model didn't induced mouse wasting or other overt problems, suggesting that these cells target and perform cytotoxicity in an antigen specific manner. Our discovery suggests a potential way of targeting ITK to selectively skew anti-tumor immunity in HP $CD8^+$ T cells in the absence of $CD4^+$ T cell help. This menthod can be significantly benefitial when tumor antigen can be identified.

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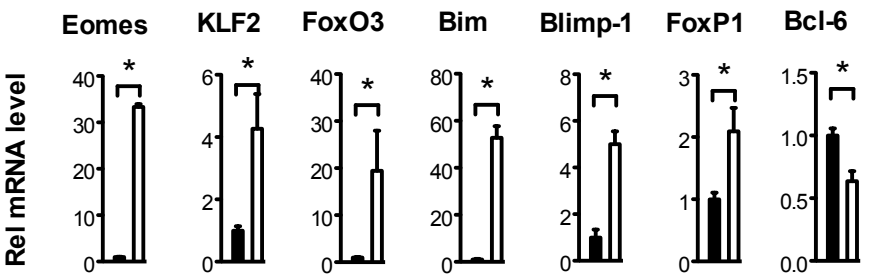

Supplemental Figure 4-1. Altered expression of transcriptional factor in *Itk*$^{-/-}$ naïve CD8^{+} T cells. Purified WT and *Itk*$^{-/-}$ OTI-*Rag*$^{-/-}$ naïve CD8^{+} T cells were analyzed for gene expression by quantitative real-time PCR. Values were normalized to internal GAPDH values first, and then normalized to WT average. n = 3, *$p < 0.05$ by Student's *t* test.

CHAPTER FIVE

Innate Memory CD4$^+$ T cells suppress autoimmune graft-versus-host disease in mice[f]

[f] Weishan Huang, Qian Qi, Jianfang Hu, Fei Huang, Terri M. Laufer, and Avery August. Innate Memory CD4$^+$ T cells suppress autoimmune graft-versus-host disease in mice. *Submitted.*

Abstract

Chronic graft-versus-host disease (cGVHD) is the major complication post bone marrow transplant (BMT). Major histocompatibility complex class II (MHCII) influenced $CD4^+$ T cell differentiation and function play critical roles in cGVHD, and the lack of hematopoietic MHCII causes cGVHD in syngeneic recipients. Using murine models of BMT, we find that $MHCII^{-/-} \rightarrow$WT BMTs exhibited defective development of hematopoietic MHCII-dependent innate memory phenotype (IMP, $CD44^{hi}/CD62L^{lo}$) $CD4^+$ T cells, and developed cGVHD. Transferring IMP $CD4^+$ T cells, but not conventional T regulatory cells (Treg), abrogates pathogenesis, while dendritic cells (DC) expressing MHCII rescues IMP $CD4^+$ T cell development and prevents pathogenesis. Furthermore the absence of Tec kinase IL-2-inducible T-cell kinase (Itk) in $MHCII^{-/-}$ donors leads to preferential development of IMP $CD4^+$ T cells and partially prevents pathogenesis. We conclude that IMP $CD4^+$ T cells, regulated by Itk and DC-MHCII, can be suppressive during cGVHD. This work provides a mechanism for the development of, and an approach to suppress, cGVHD.

Introduction

Bone marrow transplant (BMT) is a widely used therapeutic approach however graft-versus-host disease (GVHD) affects post-transplant recovery (Li and Sykes, 2012). GVHD can be categorized as acute GVHD (aGVHD) or chronic (cGVHD). aGVHD is typically mediated by direct cytotoxic effect of donor-derived T cells towards the host, where both $CD4^+$ and $CD8^+$ T cells are involved (Schroeder and DiPersio, 2011). In contrast, only $CD4^+$ T cells are necessary and cytotoxic $CD8^+$ T cells are dispensable for the development of cGVHD (Rolink and Gleichmann, 1983; Rolink et al., 1983; Zhang et al., 2006). Syngeneic transplantation in mice has revealed that MHCII-CD4 interactions play critical roles in the establishment of successful graft acceptance(Marguerat et al., 1999; Teshima et al., 2003). Transplantation of bone marrow lacking expression of MHCII into lethally irradiated syngeneic WT recipients ($MHCII^{-/-}\rightarrow$WT) evolves into systemic cGVHD, with lethal colitis, suggesting an impaired hematopoietic-MHCII mediated peripheral regulatory mechanism (Marguerat et al., 1999), which is to date unclear.

We and others have previously described a subset of $CD4^+$ T cells that have a memory-like phenotype, which we refer to as innate memory phenotype (IMP, $CD44^{hi}CD62L^{lo}$) $CD4^+$ T cells (Hu and August, 2008; Qiao et al., 2012). Memory-like cells have been better described in the $CD8^+$ T cell compartment, with elevations observed in a variety of genetically manipulated mice such as those lacking Itk, CBP, Id3 or KLF2 (Berg, 2007; Broussard et al., 2006; Fukuyama et al., 2009; Horai et al., 2007; Hu et al., 2007; Verykokakis et al., 2010; Weinreich et al., 2010) or expressing a Slp-76 mutant that is defective in Itk binding (Jordan et al., 2008), and those over-

expressing TCF-1, β-catenin (Sharma et al., 2012) or PLZF (Kovalovsky et al., 2010). *Itk*$^{-/-}$ mice exhibit an increased percentage of IMP CD4^{+} T cells that rapidly produce IFN-γ upon stimulation (Hu and August, 2008). Here we have generated mice with predominantly naïve or IMP CD4^{+} T cells, and show that such IMP CD4^{+} T cells are transcriptomically similar to those that develop in WT mice. We further show that the lack of IMP CD4^{+} T cells in the MHCII$^{-/-}$→WT BMT chimeras is associated with significant weight loss, active inflammatory intraepithelial lymphocytes (IEL) in the intestine, and a systemic collapse of regulatory CD4^{+} T cell function. IMP CD4^{+} T cells produced IL-10, and unlike conventional T regulatory cells, could prevent weight loss, gut inflammation and activation of inflammatory CD8αβ IEL in MHCII$^{-/-}$→WT chimeras. Furthermore MHCII expression on dendritic cells (DCs) completely rescues the development of IMP CD4^{+} T cells, which suppressed the pathogenesis of cGVHD in such chimeras. Finally, we show that Itk plays a role in the development of these IMP cells.

Materials and Methods

Mice.

All mice were on a C57BL/6 background. MHCII$^{-/-}$ (B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J), Thy1.1 (B6.PL-*Thy1*^{a}/CyJ) and CD45.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) mice were from The Jackson Laboratory (Bar Harbor, ME). CD11c promoter driven MHCII re-expressing *Tg(CD11c-MHCII)/MHCII*$^{-/-}$ (referred to as **MHCII$_{DC}$**) mice were previously described (Lemos et al., 2003). Itk$^{-/-}$MHCII$^{-/-}$ mice were generated by crossing Itk$^{-/-}$ and MHCII$^{-/-}$ mice. All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at The Pennsylvania State University and Cornell University.

Bone marrow chimeras and body weight.

Bone marrow chimeras were generated as previously described (Hu and August, 2008), and weighed at indicated time points post transplantation the same time each day.

Antibodies and flow cytometric staining.

All fluorochrome-conjugated antibodies used are listed in "fluorochrome-target" format as follows: PE-FoxP3, Allophycocyanin-CD4, PerCP-Cy5.5-TNF-α, PE-Cy7-Thy1.1, PE-Cy7-CD62L and PE-Cy7-IFN-γ were from eBioscience (San Diego, CA); FITC-CD45.1, V500-CD44, FITC-TCRβ, PE-CD8β, PE-CD25, Alexa Fluor 700-CD45.2, Alexa Fluor 700-CD62L, Alexa Fluor 700-IL-10, PE-Cy5-CD44, PerCP-

Cy5.5-CD8α, PE-Cy7-CD4 and Allophycocyanin-Cy7-TCRβ were from BD Biosciences (San Diego, CA); PE-Texas Red-CD4 and PE-Texas Red-CD8α were from Invitrogen (Carlsbad, CA). Cells were stained for flow cytometric analysis as previously described(Huang et al., 2013), followed by analysis on a FC500 (Beckman Coulter, Brea, CA) or LSRII system (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc., OR).

Cell sorting and adoptive transfer.

WT naïve ($CD44^{lo}CD62L^{hi}$) and WT IMP ($CD44^{hi}CD62L^{lo}$) $TCR\beta^{+}CD4^{+}$ T cells from WT mice, chimeric naïve ($CD45.2^{+}$ $MHCII^{-/-}\rightarrow CD45.1^{+}$ WT: $CD45.2^{+}CD44^{lo}CD62L^{hi}$) and chimeric IMP ($CD45.1^{+}$ WT$\rightarrow CD45.2^{+}$ $MHCII^{-/-}$: $CD45.2^{-}CD44^{hi}CD62L^{lo}$) $TCR\beta^{+}CD4^{+}$ T cells of donor source from bone marrow chimeras were sorted on a Cytopeia Influx Cell Sorter (Cytopeia, Seattle, WA), and cells with purity higher than 95% were used for all experiments. For regulatory cell transfer experiments, conventional regulatory T cells ($TCR\beta^{+}CD4^{+}CD25^{hi}$) and IMP $CD4^{+}$ T cells ($TCR\beta^{+}CD4^{+}CD44^{hi}CD62L^{lo}$) were sorted from WT mice ($Thy1.1^{+}$) on a FACSAria Cell Sorter (BD Biosciences). $0.2\text{-}0.3\times10^{6}$ cells per injection was used if not specified. Intraepithelial lymphocytes (IEL) were isolated as described (Denning et al., 2007).

Microarray analysis.

Total RNA was isolated from sorted WT naïve, WT IMP, chimeric naïve and chimeric IMP $CD4^{+}$ T cells using a RNeasy Plus Mini Kit (Qiagen, Valencia, CA), amplified

using MessageAmp™ Premier RNA Amplification Kit (Life Technologies, Grand Island, NY), followed by examination on Affymetrix Mouse 430.2 array (Affymetrix, Santa Clara, CA). Microarray data were processed, analyzed and rendered using Genespring Version 12 (Agilent, Santa Clara, CA) as previously described (Huang et al., 2013). All values were further normalized to the average of WT naïve $CD4^+$ T cells. Data have been deposited into the National Center for Biotechnology Information's Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/gds) under accession number GSE46892.

T cell stimulation and cytokine assay.

Splenocytes were left unstimulated, or stimulated as previously described (Hu and August, 2008; Hu et al., 2007) prior to staining and flow cytometric analysis. To examine T cell-derived cytokine production, total splenocytes were stimulated with 1 μg/ml anti-CD3ε and anti-CD28 antibodies (eBioscience) for 3 days. Supernatants were examined for cytokines on a Milliplex multiplex system (EMD Millipore, Billerica, MA); cells were re-stimulated with PMA/Ionomycin/Brefeldin A as previously described for flow cytometric analysis (Hu and August, 2008).

Statistical analysis.

Unpaired two-tailed student's *t* test and two-way ANOVA were performed using GraphPad Prism (GraphPad, San Diego, CA). Group average was plotted with error bar indicating s.e.m.. Differences with probability $P < 0.05$ were considered statistically significant.

Results

IMP CD4$^+$ T cells develop dependent on hematopoietic MHCII expression and independent of thymic positive selection

Naïve or conventional CD4$^+$ T cells are characterized as CD44^{lo}/CD62L^{hi} (Surh and Sprent, 2008), and require the expression of MHCII on thymic epithelium for development (Laufer et al., 1996; Markowitz et al., 1993). The memory αβ T cell population (CD44^{hi} and/or CD62L^{lo}) is heterogeneous and is composed of both genuine antigen-specific memory T cells, as well as memory phenotype cells that arise independently of antigen stimulation (MacLeod et al., 2009; Surh and Sprent, 2008). Hematopoietic MHCI is required for the development of innate memory CD8$^+$ T cells (Horai et al., 2007; Huang et al., 2013). To investigate this in the CD4$^+$ T cell compartment, we generated bone marrow chimeras to allow T cell development in the absence of either hematopoietic or thymic epithelial MHCII. Compared to the WT bone marrow control, transfer of MHCII$^{-/-}$ bone marrow into WT mice (MHCII$^{-/-}$ →WT chimeras) gave rise to predominantly naïve CD4$^+$ T cells, with defects in the development of functional IMP CD4$^+$ T cells (CD44^{hi}CD62L^{lo} IFN-γ producers). By contrast, transfer of WT bone marrow into MHCII$^{-/-}$ deficient mice (WT → MHCII$^{-/-}$ chimeras) gave rise to predominantly IMP CD4$^+$ T cells (**Figure 5-1** a&b). The lack of hematopoietic MHCII in the MHCII$^{-/-}$→WT chimeras led to defects in the development of IMP CD4$^+$ T cells, while the absence of thymic MHCII in the WT → MHCII$^{-/-}$ chimeras significantly diminished naïve CD4$^+$ T cell development and allowed for IMP CD4$^+$ development, suggesting that IMP CD4$^+$ can bypass thymic

selection. To confirm this, we utilized athymic Nude mice in similar experiments. In Nude mice, only a few dysfunctional memory-like $CD4^+$ T cells (with memory markers but not IFN-γ producing ability) were seen (**Supplemental Figure 5-1** a&b). However, Nude recipients of WT bone marrow (WT→Nude chimeras) gave rise to functional IMP $CD4^+$ T cells, suggesting that these cells can develop in the absence of a thymus (**Supplemental Figure 5-1** c&d).

To compare naïve and IMP $CD4^+$ T cells derived via this process to those from WT mice, we sorted $MHCII^{-/-}$→WT (**MW**) naïve and WT→$MHCII^{-/-}$ (**WM**) IMP $CD4^+$ T cells and compared their gene expression profiles to non-manipulated **WT** counterparts. We found few genes differentially expressed in cells from the chimeric mice compared to WT cells (**Figure 5-1** c). Hierarchical clustering of genes with > 2 fold change between at least two groups, also classified chimeric naïve and IMP $CD4^+$ T cells as similar to their non-grafted naïve and IMP $CD4^+$ T cells counterparts respectively (**Figure 5-1** d). Thus the bone marrow chimeras can be considered as models to generate separate populations of naïve and IMP $CD4^+$ T cells.

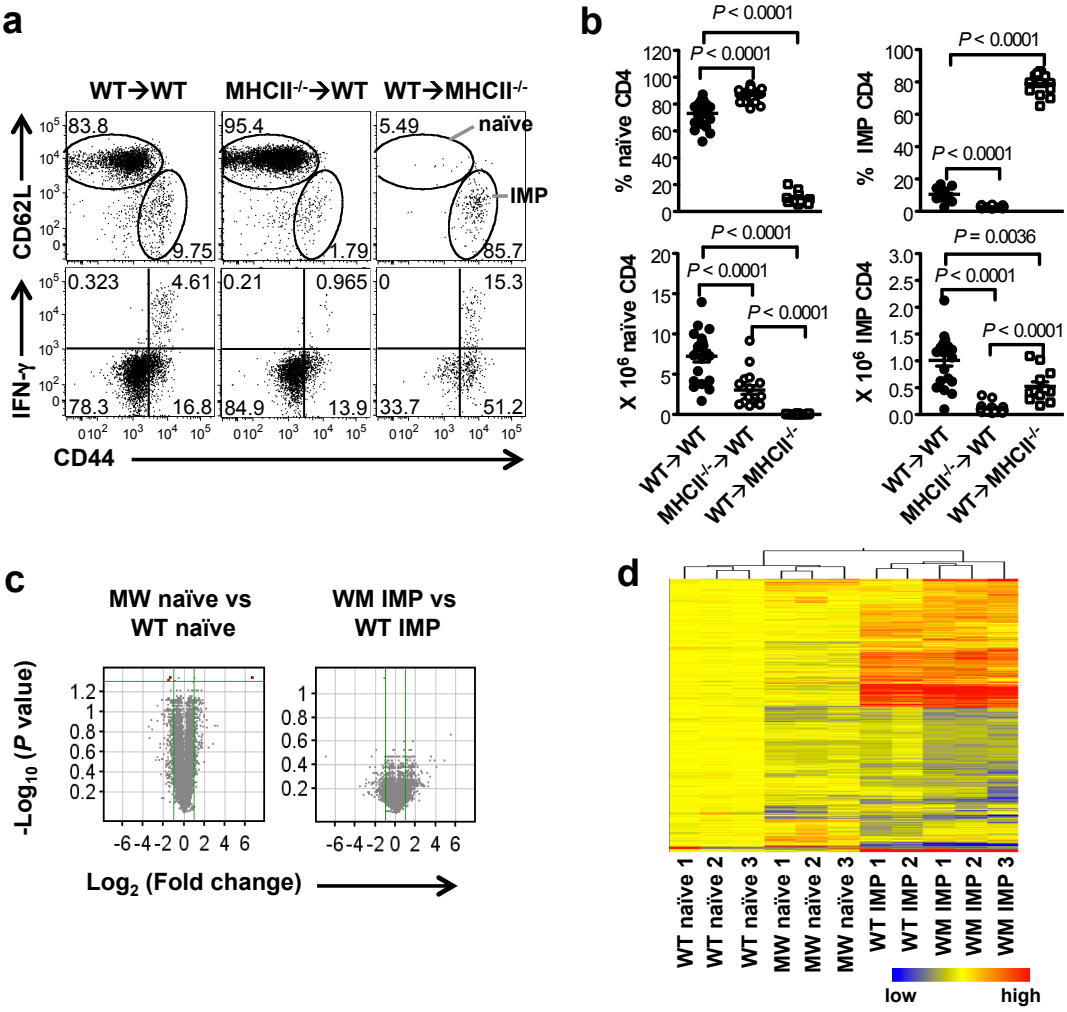

Figure 5-1. Generation of chimeric mice with predominant naïve or IMP CD4$^+$ T cells. Bone marrow chimeras were generated and analyzed as indicated. (**a**) Flow cytometric analysis of expression of CD44, CD62L and IFN-γ by donor CD4$^+$ T cells. Data represent results from more than 5 independent experiments. (**b**) Percentages (upper) and numbers (lower) of naïve (left) and IMP (right) CD4$^+$ T cells in indicated chimeras. Data were combined from more than 3 independent experiments. *P* values were generated by Student's *t* test. (c) Volcano plot of genes differentially expressed in CD4$^+$ T cells from MW naïve, WM IMP and WT IMP CD4$^+$ T cells. Genes with > 2 fold change, $P < 0.05$, compared to WT naïve CD4$^+$ T cells were included. (**d**) Hierarchical clustering of genes expressed by naïve and IMP CD4$^+$ T cells from chimeric (MHCII$^{-/-}$→WT: MW; WT→MHCII$^{-/-}$: WM) and WT mice. Quantile normalized gene expression (to average level of WT naïve cells) were filtered for > 2 fold change between at least 2 groups.

Autoreactive pro-inflammatory IELs develop in the absence of hematopoietic MHCII

It has been reported that transfer of MHCII$^{-/-}$ bone marrow into syngeneic WT recipients results in fulminating autoimmune graft-versus-host disease, with deteriorating intestinal inflammation and subsequent mortality (Marguerat et al., 1999; Teshima et al., 2003). Consistently, we observed that MHCII$^{-/-}$→WT chimeras carrying predominantly naïve CD4^{+} T cells exhibited gradually decreasing body weight, while WT→MHCII$^{-/-}$ chimeras carrying predominantly IMP CD4^{+} T cells were able to maintain normal body weight (**Figure 5-2** a). Upon further analysis, we found that as a measure of the severity of intestinal inflammation, the proportion of the weight of the small intestine over body weight is highly correlated with the observed body weight loss (**Figure 5-2** b). Furthermore, IELs in MHCII$^{-/-}$→WT chimeras exhibited a significantly higher percentage of CD8αβ and lower percentage of CD4/CD8αα αβ T cells than WT→WT controls, while WT→MHCII$^{-/-}$ chimeras maintained comparable composition of these cell types (**Figure 5-2** c&d). Decreases in CD4/CD8αα IELs during intestinal inflammation has been attributed to a lack of regulatory T cell function in murine models of inflammatory bowel disease (Cheroutre and Lambolez, 2008; Das et al., 2003; Yu et al., 2008). CD4^{+} and CD8^{+} αβ IELs have been reported to be producers of IFN-γ in MHCII$^{-/-}$→WT bone marrow chimeras (Marguerat et al., 1999), and we found that these cells produce both IFN-γ and TNF-α, two highly inflammatory cytokines, which was not the case with these cells from WT→MHCII$^{-/-}$ chimeras (**Figure 5-2** e&f). These data suggest that the severe weight loss observed in the MHCII$^{-/-}$→WT chimeras is associated with increased

proinflammatory cytokines by $CD4^+$ and $CD8^+$ αβ IELs.

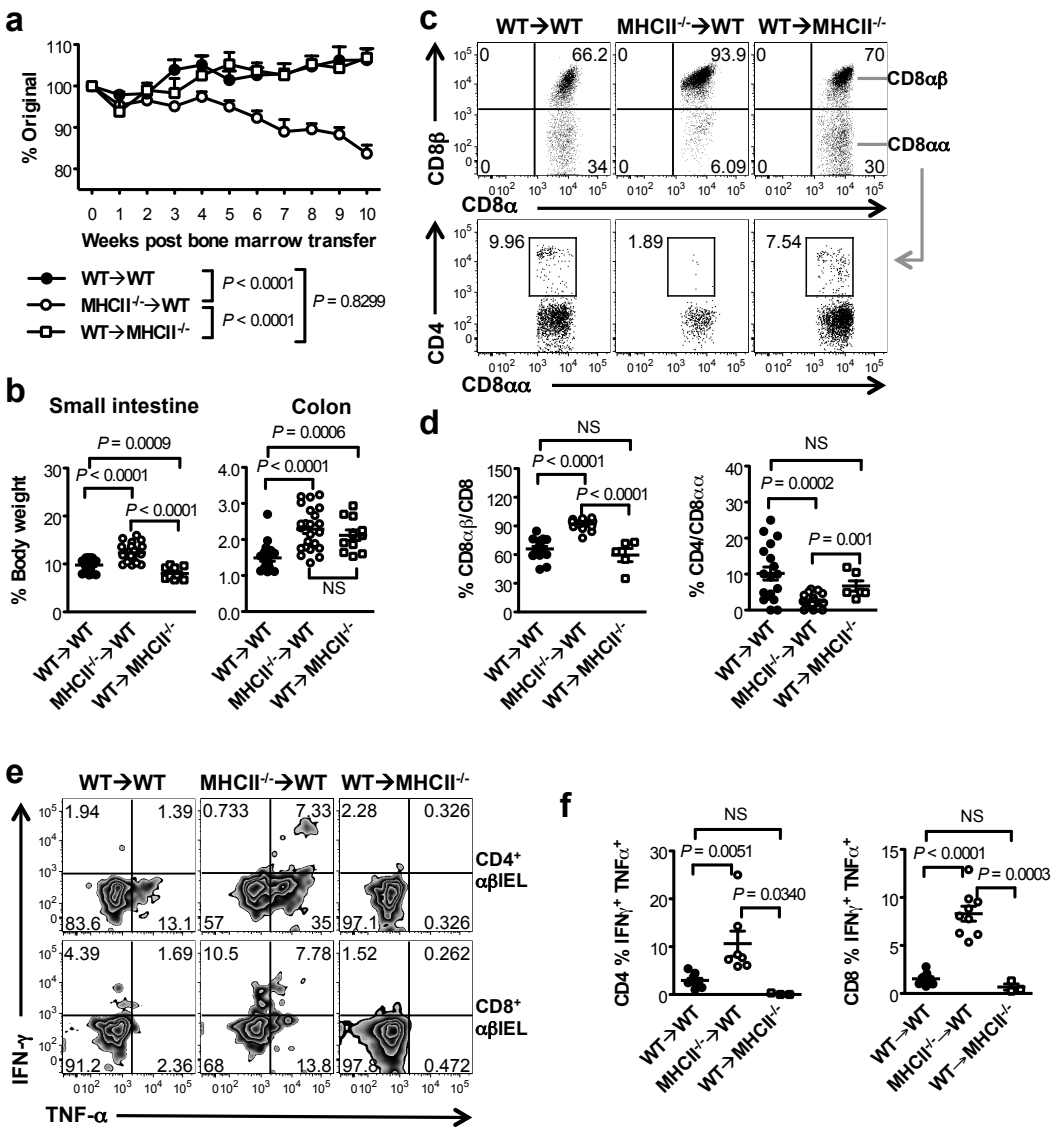

Figure 5-2. Gut inflammation and pro-inflammatory IELs in mice lacking IMP CD4$^+$ T cells. Bone marrow chimeras were generated as indicated, and analyzed 8-10 weeks post transplantation. (**a**) Significant weight loss by chimeric mice lacking IMP CD4$^+$ T cells. n = 5. Data represent results of > 3 independent experiments. *P* values were generated by two-way ANOVA, comparing groups connected. (**b**) Percent of small intestine or colon weight as a fraction of body weight. Data were combined from 3 independent experiments. (**c**) Expression of CD8αα and CD8αβ (upper) and CD4/CD8αα (lower) by TcR αβ IELs. (**d**) Percentages of CD8αβ and CD4/CD8αα TcR αβ IELs. (**e**) Expression of IFN-γ and TNF-α by CD4$^+$ and CD8$^+$ αβ IEL. (**f**) Percentages of IFN-γ/TNF-α producing CD4$^+$ and CD8$^+$ αβ IELs. In (**b-f**), data were representative or combined results of 3 independent experiments; *P* values were generated by Student's *t* test; NS = not significant.

Defective T cell regulatory function in the absence of IMP CD4$^+$ T cells

To investigate potential regulatory function of these T cell populations, we compared the composition and regulatory function of T cells in the spleens of WT→WT, MHCII$^{-/-}$→WT and WT→MHCII$^{-/-}$ bone marrow chimeras. Conventional regulatory CD4$^+$ T (Treg) cells expressing FoxP3 are well known suppressors of multiple autoimmune diseases in both human and mouse (Brusko et al., 2008; Hori et al., 2003; Sakaguchi et al., 2008). We found that MHCII$^{-/-}$→WT chimeric mice with severe gut inflammation had significant reductions in FoxP3$^+$ Treg cells as a percentage of total CD4$^+$ T cells, while this percentage was significantly higher in WT→MHCII$^{-/-}$ chimeras (**Figure 5-3** a). Surprisingly however, despite the higher percentage of FoxP3$^+$ CD4$^+$ T cells, the number of Treg cells in the WT→MHCII$^{-/-}$ chimeras was the same as that seen in MHCII$^{-/-}$→WT chimeras that developed disease (**Figure 5-3** b). Furthermore, the IMP CD4$^+$ T cells in the WT→MHCII$^{-/-}$ chimeras produced IL-10, a critical immunosuppressive cytokine (Barnes and Powrie, 2009) (**Figure 5-3** c&d). We also examined other T cell-derived cytokines that may be altered between the MHCII$^{-/-}$→WT chimeras and the WT→MHCII$^{-/-}$ chimeras, compared to WT→WT controls, and that correlated with disease occurrence. However, aside from the significant reduction in IL-10 and increase in TNF-α production in MHCII$^{-/-}$→WT chimeras (**Figure 5-3** e), we did not find any patterns among Th1/Th2/Th17 type cytokines examined (**Supplemental Figure 5-2**). These data suggest that in the MHCII$^{-/-}$→WT chimeras, there is a collapse in the regulatory compartment, however, the type of immunosuppressive T cell defect or collapse in these mice is unclear. Indeed, while the MHCII$^{-/-}$→WT chimeras have reduced

percentage and number of FoxP3^{+} Treg cells compared to WT mice, the number of these cells is comparable to that seen in the WT→MHCII$^{-/-}$ chimeras which do not develop disease, suggesting alternate or additional regulatory T cell components responsible for prevention of cGVHD.

We further analyzed the transcriptomes of IMP CD4^{+} T cells and found that they exhibit elevated expression of multiple master transcription factors for T helper sub-lineages, including AhR/c-MAF (driving IL-10 expression in Tr1), T-bet (Tbx21, Th1), FoxP3/Helios (Foxp3/Ikzf2, nTreg), and PU.1 (Sfpi-1, Th9), and slight increase in GATA3 (Th2) and RORγt (Rorc, Th17) (**Figure 5-3** f). Correspondingly, IMP CD4^{+} T cells carry a mosaic T helper cytokine profile at the transcriptional level, with equal expression of IL-4 (Th2) and IFN-γ (Th1), slightly enhanced IL-17A/IL-21 (Th17), and significantly increased IL-10 (Tr1), albeit in the absence of TGF-β (**Figure 5-3** g). Thus the IMP population of CD4^{+} T cells likely includes memory and memory-like cells for a number of different lineages, but prominent among them, are cells that have a regulatory phenotype, suggesting a mechanism for their effect *in vivo*.

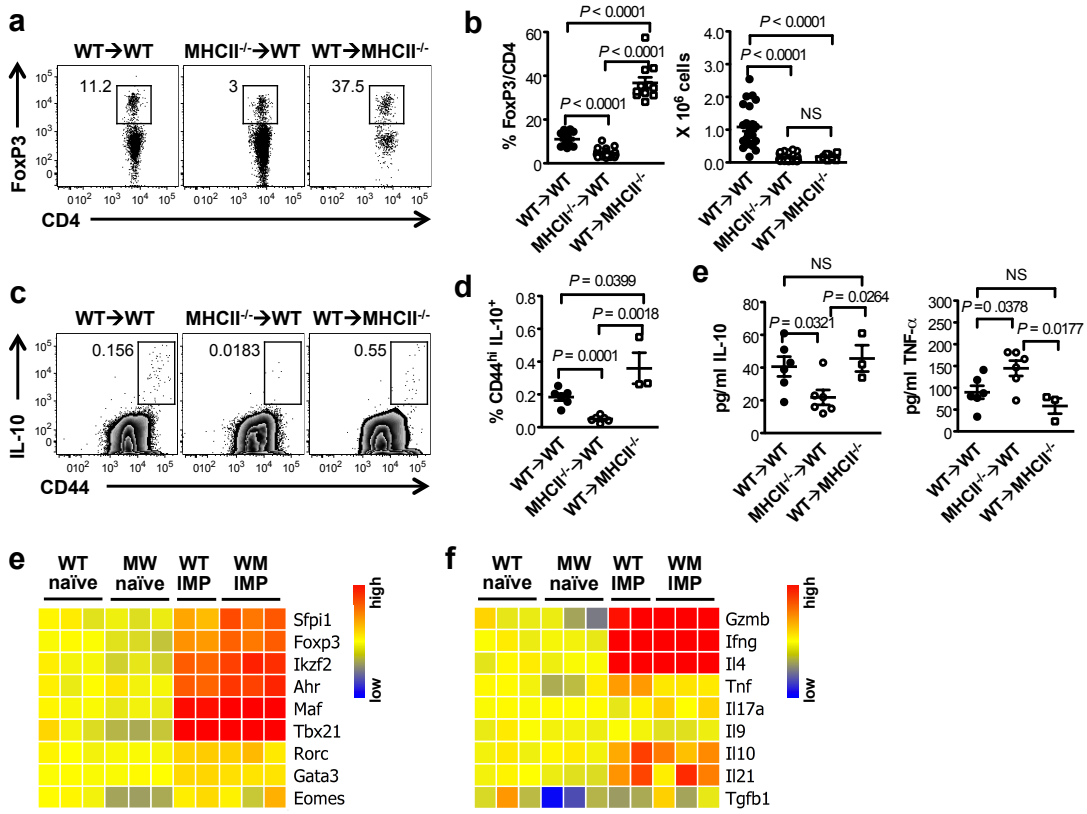

Figure 5-3. Systemic collapse of regulatory T cell function in mice lacking IMP CD4$^+$ T cells. Bone marrow chimeras were as described in Figure 2. (**a**) FoxP3 expression by donor CD4$^+$ T cells in spleens of indicated chimeric mice. (**b**) Percentages and numbers of conventional FoxP3$^+$ regulatory T cells of donor origin. In (**c-e**), splenocytes were stimulated with anti-CD3ε/CD28 antibodies for 3 days, followed by PMA/Ionomycin/Brefeldin A treatment for 6-8 hours prior to analysis. (**c**) Expression of IL-10 by CD44^{hi} CD4$^+$ T cells. (**d**) Percentages of CD44^{hi} IL-10 producing CD4$^+$ T cells induced by activation. (**e**) IL-10 and TNF-α secreted by splenocytes from the indicated chimeric mice stimulated anti-CD3ε/CD28 antibodies for 3 days. Data represent results from more than 3 independent experiments; *P* values were generated by Student's *t* test; NS = not significant. (**f**) Expression of CD4$^+$ T helper subset master transcription factors. (**g**) Expression of CD4$^+$ T helper subset cytokines.

IMP CD4$^+$ T cells but not conventional T regulatory cells rescue MHCII$^{-/-}$→WT chimeras from cGVHD

As shown in Figure 5-1, IMP CD4$^+$ T cells make up the majority of the CD4$^+$ population in WT→MHCII$^{-/-}$ chimeras that do not develop disease, and there are significantly more IMP CD4$^+$ T cells in these mice compared to MHCII$^{-/-}$→WT chimeras that do develop disease. In addition, although the IMP CD4$^+$ T cell population includes cells that express FoxP3, suggesting the presence of conventional Treg cells, they also include features of a Tr1 phenotype. We therefore hypothesized that the lack of regulatory IMP CD4$^+$ T cells, rather than Treg cells, was responsible for the autoimmune disorder in the MHCII$^{-/-}$→WT chimeras. To investigate this, we sort-purified conventional Treg cells (CD4$^+$TCRβ$^+$CD25^{hi}) and IMP CD4$^+$ T cells (CD4$^+$TCRβ$^+$CD44^{hi}CD62L^{lo}) from WT mice and transferred them into MHCII$^{-/-}$→WT chimeras to determine whether they could affect disease progression. Transfer of IMP CD4$^+$ T cells one-week post BMT significantly reduced weight loss of MHCII$^{-/-}$→WT chimeras, confirmed by analysis ratio of small intestine weight:body weight. By contrast, transfer of equal numbers of Treg cells (from the same donors) did not (**Figure 5-4** a&b). However, a single transfer of IMP CD4$^+$ T cells at the early stage did not completely abrogate disease, as MHCII$^{-/-}$→WT chimeric recipients eventually started losing significant amounts of weight by eight weeks. Transfer of cells 4 weeks post bone marrow transplantation did not have any effect on the disease progression (**Supplemental Figure 5-3**). These data suggest that IMP CD4$^+$ T cells and not conventional Treg cells are likely regulators of disease progression in these mice, requisitely effective from early stage.

Our previous work indicated that IMP $CD4^+$ T cells have a half-life of ~14 days *in vivo* (Hu and August, 2008) and so it was possible that incomplete rescue was due to turnover of the transferred IMP $CD4^+$ T cells. We therefore transferred IMP $CD4^+$ T cells once a week consecutively for 8 weeks to ensure the continuous presence of IMP $CD4^+$ T cells in the $MHCII^{-/-}$→WT chimeras. We found that this protocol prevented the progression of disease in these mice (**Figure 5-4** c&d). Additionally, analysis of the proportion of active CD8αβ αβ IELs, which is strictly required for intestinal epithelial barrier disruption (Zufferey et al., 2009), in the recipient mice 9 weeks post bone marrow transplantation revealed that this was also reduced by the transfer of IMP $CD4^+$ T cells, although regulatory CD4/CD8αα IELs were not induced (**Figure 5-4** e&f). Of note, the ratio of CD8αβ to $CD8^+$ αβ IELs in $MHCII^{-/-}$→WT chimeric recipients was restored to levels seen in the WT→WT chimeras level in the continuous presence of IMP $CD4^+$ T cells (**Figure 5-4** f upper panel). More importantly, the transferred IMP $CD4^+$ T cells suppressed IFN-γ/TNF-α production by both αβ $CD4^+$ and $CD8^+$ IELs, even when a single transfer was performed during the early stage post BMT (**Figure 5-4** g&h). The complete rescue and reversion of the CD8αβ to $CD8^+$ αβ IEL ratio, along with suppression of IFN-γ/TNF-α production suggests an important role for IMP $CD4^+$ T cells in suppressing cGVHD, allowing for successful syngeneic bone marrow transplantation.

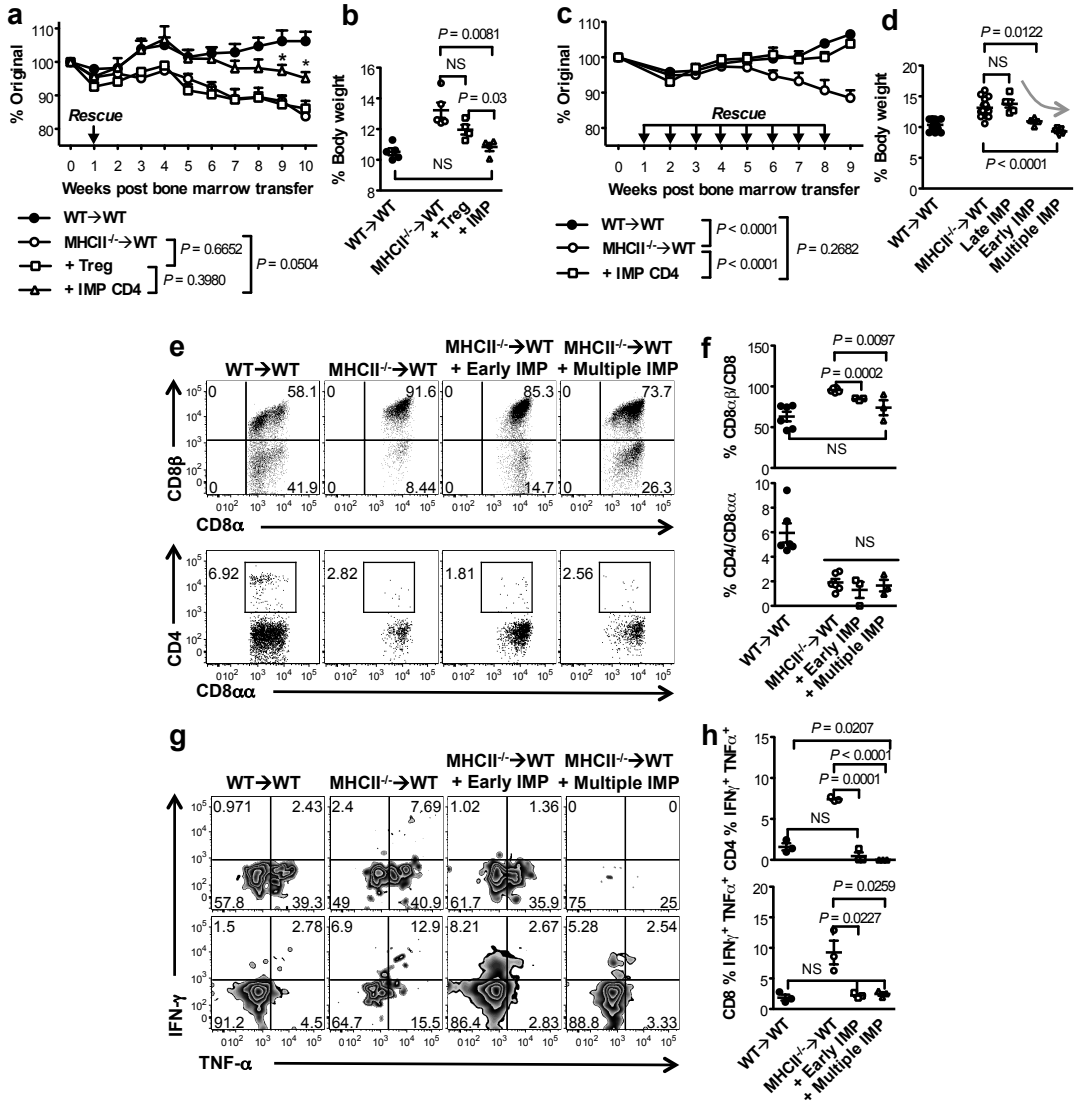

Figure 5-4. IMP CD4$^+$ but not conventional regulatory T cells suppress development of disease. (**a**) Bone marrow chimeras were generated as described, and 0.2 - 0.3 × 10^6 conventional regulatory T cells (+ Treg) or IMP CD4$^+$ T cells (+ IMP CD4) were retro-orbitally injected one week post bone marrow transplantation. n = 5. *P* values generated by two-way ANOVA. *$P < 0.05$ by Student's *t* test, comparing "+ IMP CD4" to non-rescue group of matched time point. (**b**) Percent of small intestine weight as a function of body weight at time of sacrifice of mice in (**a**). (**c**) Bone marrow chimeras were generated as described, and cell transfers carried out by weekly retro-orbital injection of 0.2 - 0.3 × 10^6 IMP CD4$^+$ T cells (one dose each week), until sacrifice. n=5. *P* values were generated by two-way ANOVA. (**d**) Percent of small intestine weight over body weight of MHCII$^{-/-}$→WT mice with different IMP CD4$^+$ rescue protocols. Late IMP: single dose 4 weeks post transfer (from **Supplemental Figure 5-3**); Early IMP: single dose 1 week post transfer (from **Figure 5-4** c);

Multiple IMP: one dose each week from 1 week post transfer. (**e**) Expression of CD8αα and CD8αβ (upper) and CD4/CD8αα (lower) by TcR αβ IELs. (**f**) Percentages of CD8αβ and CD4/CD8αα TcR αβ IELs. (**g**) Expression of IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ TcR αβ IEL. (**h**) Percentages of IFN-γ/TNF-α producing $CD4^+$ and $CD8^+$ TcR αβ IELs. In (**b,d,f&g**), *P* values were generated by Student's *t* test; NS = not significant.

Dendritic cell expression of MHCII is sufficient to allow IMP CD4$^+$ T cell development and prevents disease progression

Dendritic cells (DC) are the major expressers of MHCII among hematopoietic cells and enforce clonal deletion of self-reactive T cells, as well as the induction of T regulatory cells (Proietto et al., 2008). DCs defective in MHCII-CD4 interaction fail to maintain peripheral tolerance (Muth et al., 2012) and tolerogenic CD8α^+ plasmacytoid precursor DCs can enhance bone marrow engraftment through induction of regulatory T cells *in vivo* (Huang et al., 2011). It is possible that DC-MHCII also contributes to the development of IMP CD4$^+$ T cells, which are protective in syngeneic bone marrow chimeras. We therefore used a MHCII transgenic model where MHCII is re-expressed in MHCII$^{-/-}$ mice solely on CD11c$^+$ DCs (*Tg(CD11c-MHCII)/MHCII$^{-/-}$*, referred to as **MHCII$_{DC}$** hereafter) (Lemos et al., 2003), as donors to investigate whether DC-restricted MHCII is important for the development of IMP CD4$^+$ T cells and prevention of disease following bone marrow transplantation. We found that MHCII expression by DCs did not induce significantly more IMP-like CD4$^+$ T cells compared to complete MHCII deficiency, however, DC-restricted expression of MHCII licensed functional IMP CD4$^+$ T cells to become potent IFN-γ producers (**Supplemental Figure 5-4** a&b). Furthermore, transfer of bone marrow from DC-restricted MHCII transgenic mice to WT mice (MHCII$_{DC}$→WT), led to complete prevention of body weight loss (**Figure 5-5** c), reversion of ratios of intestine/body weight (**Figure 5-5** d), CD8αβ:CD8$^+$ αβ IEL ratio, CD4/CD8αα population (**Figure 5-5** e&f), and αβ IEL IFN-γ/TNF-α production (**Figure 5-5** g). This was associated with development of IMP CD4$^+$ T cells similar to that seen when MHCII is expressed in the complete

hematopoietic compartment (WT→MHCII$^{-/-}$ chimeras) (**Figure 5-5** a&b). In addition, a significantly higher percentage of CD4^{+} T cells in the MHCII$_{DC}$→WT chimeras were capable of producing IL-10 upon T cell receptor activation (**Figure 5-5** h). While there was some variation in T cell-derived TNF-α by cells from the MHCII$_{DC}$→WT chimeras, there was significantly enhanced IL-10 production (**Figure 5-5** j). Note that while DC-MHCII also rescued conventional FoxP3^{+} regulatory T cells to similar levels as in WT→WT controls (**Supplemental Figure 5-5** a&b), there were significantly higher numbers of IMP CD4^{+} T cells (**Figure 5-5** b, right panel). Combined with the inability of conventional Treg cells to prevent disease development, we conclude that the ability of CD4^{+} T cells from MHCII$_{DC}$→WT chimeras to produce IL-10 may be attributed to regulatory function of DC MHCII-licensed IMP CD4^{+} T cells.

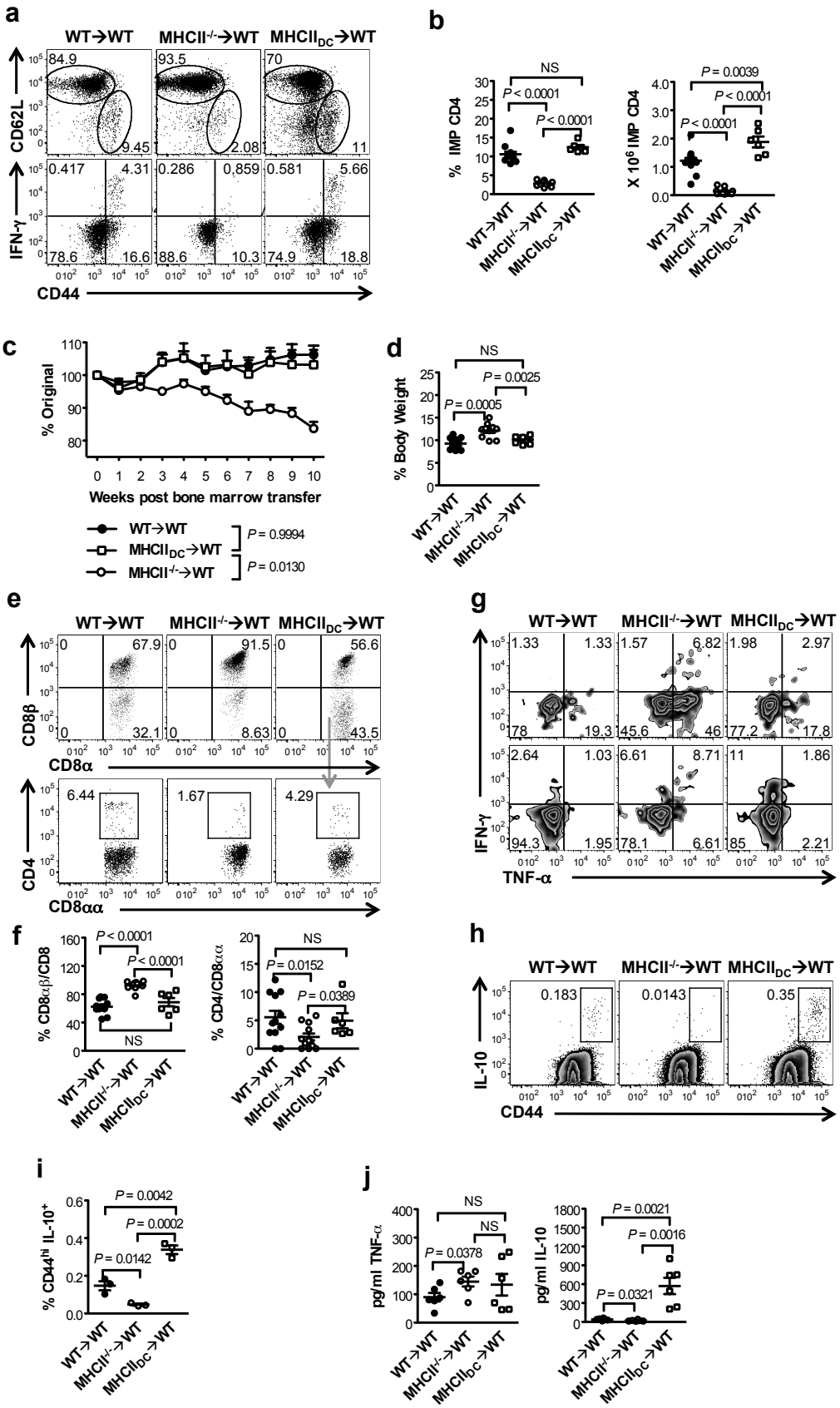
a
WT→WT
MHCII-/-→WT
MHCIIDC→WT
CD62L
IFN-γ
CD44
84.9
9.45
93.5
2.08
70
11
0.417
4.31
0.286
0.859
0.581
5.66
78.6
16.6
88.6
10.3
74.9
18.8
b
% IMP CD4
X 10^6 IMP CD4
NS
P < 0.0001
P < 0.0001
P = 0.0039
P < 0.0001
P < 0.0001
c
% Original
Weeks post bone marrow transfer
WT→WT
MHCIIDC→WT
MHCII-/-→WT
P = 0.9994
P = 0.0130
d
% Body Weight
NS
P = 0.0025
P = 0.0005
e
CD8β
CD8α
CD4
CD8αα
0
67.9
32.1
91.5
8.63
56.6
43.5
6.44
1.67
4.29
f
% CD8αβ/CD8
% CD4/CD8αα
P < 0.0001
P < 0.0001
NS
NS
P = 0.0152
P = 0.0389
g
IFN-γ
TNF-α
1.33
1.33
78
19.3
1.57
6.82
45.6
46
1.98
2.97
77.2
17.8
2.64
1.03
94.3
1.95
6.61
8.71
78.1
6.61
11
1.86
85
2.21
h
IL-10
CD44
0.183
0.0143
0.35
i
% CD44hi IL-10+
P = 0.0042
P = 0.0002
P = 0.0142
j
pg/ml TNF-α
pg/ml IL-10
NS
NS
P = 0.0378
P = 0.0021
P = 0.0016
P = 0.0321

Figure 5-5. DC-restricted expression of MHCII is sufficient to rescue IMP CD4$^+$ T cells and suppress development of disease. Bone marrow chimeras were generated as indicated, and were sacrificed for analysis 8-9 weeks post bone marrow transplantation. (**a**) Expression of CD44, CD62L and IFN-γ by CD4$^+$ T cells of donor origin. Data represent results from more than 2 independent experiments. (**b**) Percentages (left) and numbers (right) of IMP CD4$^+$ T cells in indicated chimeras. (**c**) DC-restricted expression of MHCII on donor bone marrow prevents weight loss. n = 5. Data represent 2 independent experiments. *P* values were generated by two-way ANOVA. (**d**) Percent of small intestinal weight as a function of body weight at time of sacrifice of mice in (**c**). (**e**) Expression of CD8αα and CD8αβ and CD4/CD8αα by TcR αβ IELs. (**f**) Percentages of CD8αβ and CD4/CD8αα TcR αβ IELs. (**g**) Expression of IFN-γ and TNF-α by CD4$^+$ (upper) and CD8$^+$ (lower) TcR αβ IEL. In (**h-j**), splenocytes were stimulated with anti-CD3ε/CD28 antibodies for 3 days, followed by PMA/Ionomycin/Brefeldin A for 6-8 hours prior to analysis. (**h**) Expression of IL-10 by CD44^{hi} CD4$^+$ T cells. (**i**) Percentages of CD44^{hi} IL-10 producing CD4$^+$ T cells induced by TcR activation. (**j**) IL-10 and TNF-α secretion. For (**b,d,f,i&j**), data represent results of 2 independent experiments. *P* values were generated by Student's *t* test; NS = no significance.

The absence of Tec kinase Itk leads to development of IMP CD4$^+$ T cells that can attenuate development of disease

In the absence of the non-receptor tyrosine kinase Itk, the percentage of functional (ability to produce IFN-γ) IMP CD4$^+$ is significantly increased (Hu and August, 2008) (**Supplemental Figure 5-4** c), and the combined absence of Itk and MHCII$^{-/-}$ also leads to significantly increased numbers of IMP CD4$^+$ T cells, however, these cells now have the capability to produce IFN-γ, unlike those seen in the absence of MHCII$^{-/-}$ (**Supplemental Figure 5-4** c&d). This suggests that Itk plays a role in regulating the development, licensing or function of IMP CD4$^+$ T cells. We therefore used these Itk$^{-/-}$MHCII$^{-/-}$ mice as bone marrow donors to determine the role of Itk in the development and function of IMP CD4$^+$ T cells, as well as on the development of disease due to the lack of hematopoietic MHCII. We found that the absence of Itk in MHCII$^{-/-}$ bone marrow rescued the development of IMP CD4$^+$ T cells (**Figure 5-6** a&b). In addition, the weight loss observed in the MHCII$^{-/-}$→WT mice is significantly attenuated in the WT mice that receive Itk$^{-/-}$MHCII$^{-/-}$ bone marrow (Itk$^{-/-}$MHCII$^{-/-}$→WT), and compared to WT→WT control, this weight loss is delayed (**Figure 5-6** c). Nevertheless, the intestine/body weight ratio and the ratio of CD8αβ to CD8$^+$ IELs were fully reversed to the control levels in the Itk$^{-/-}$MHCII$^{-/-}$→WT chimeric mice (**Figure 5-6** d, and **5-6** e, upper panel). However, the absence of Itk failed to rescue regulatory CD4/CD8αα IELs (**Figure 5-6** e, lower panel). Note that, Itk deficiency also restored the ability of splenic CD4$^+$ T cells to produce IL-10 (**Figure 5-6** g&h). Thus Itk plays a regulatory role in the development and function of IMP CD4$^+$ T cells.

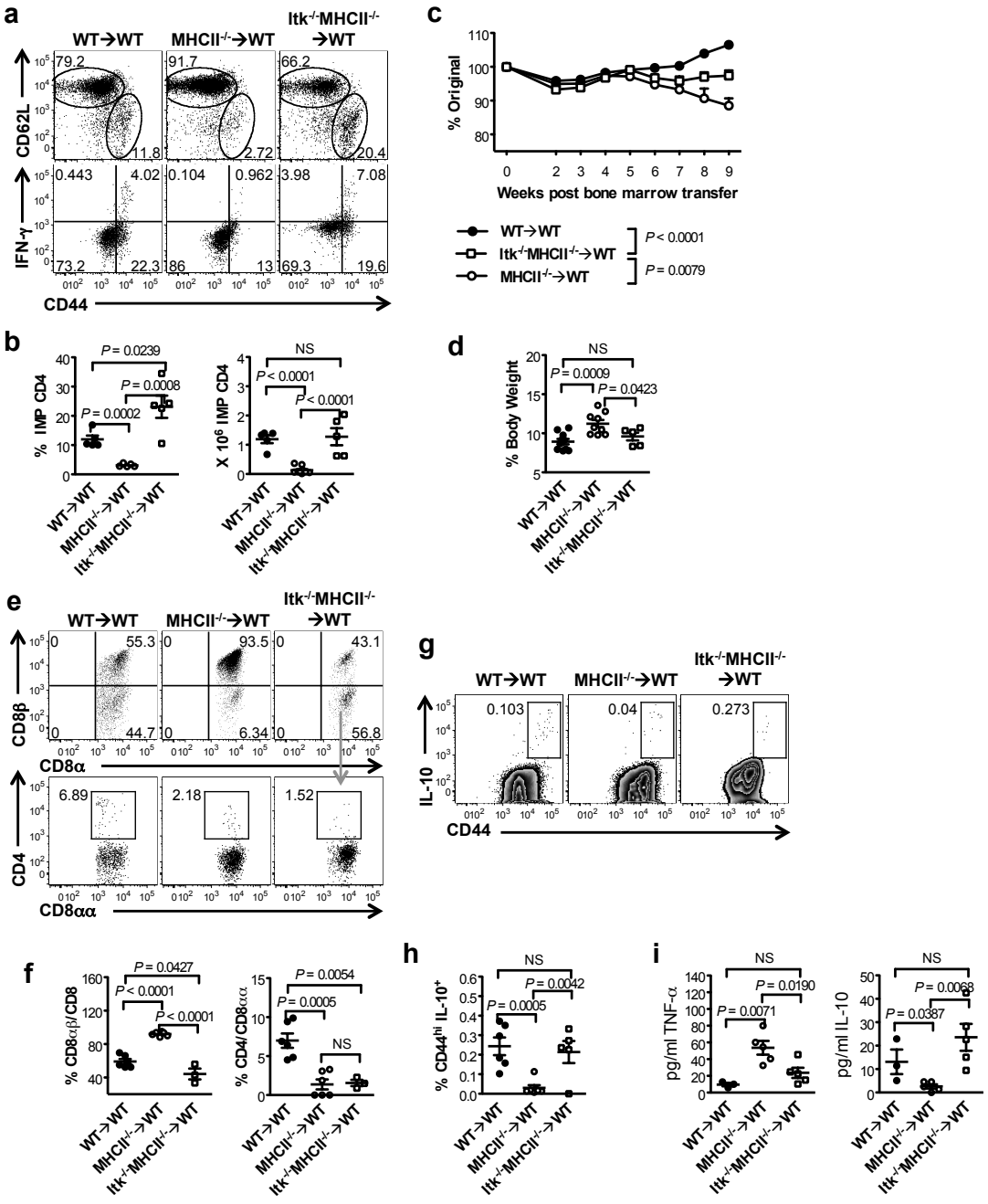

Figure 5-6. The absence of Itk rescues IMP CD4$^+$ T cell development and suppresses disease. Bone marrow chimeras were generated as indicated, and were sacrificed for analysis 9 weeks post bone marrow transplantation. (**a**) Expression of

CD44, CD62L and IFN-γ in $CD4^+$ T cells of donor origin. (**b**) Percentages (left) and numbers (right) of IMP $CD4^+$ T cells in indicated chimeras. (**c**) Absence of Itk in $MHCII^{-/-}$ bone marrow reduced severity of body weight loss of WT recipients. n = 5. *P* values were generated by two-way ANOVA. (**d**) Fraction of small intestinal weight over body weight at time of sacrifice of mice in (**c**). (**e**) Expression of CD8αα and CD8αβ and CD4/CD8αα in αβ IELs. (**f**) Percentages of CD8αβ and CD4/CD8αα αβ IELs. In (**g&h**), splenocytes were stimulated with anti-CD3ε/CD28 antibodies for 3 days, followed by PMA/Ionomycin/Brefeldin A for 6-8 hours prior to analysis. (**g**) Expression of IL-10 by $CD44^{hi}$ $CD4^+$ T cells. (**h**) Percentages of $CD44^{hi}$ IL-10 producing $CD4^+$ T cells induced by activation. (**i**) IL-10 and TNF-α secreted by splenocytes from the indicated chimeric mice stimulated anti-CD3ε/CD28 antibodies for 3 days. For (**b,d,f,h&i**), *P* values were generated by Student's *t* test; NS = no significance.

Discussions

BMT of bone marrow lacking expression of MHCII into sub-lethally irradiated syngeneic WT recipients results in systemic cGVHD with lethal colitis (Marguerat et al., 1999). The cellular mechanism of onset of this disease is not yet elucidated. Nevertheless, this syngeneic MHCII$^{-/-}$→WT transplant-induced cGVHD provides an excellent model to investigate factor(s) involved in the failure of "perfect-match" bone marrow grafts. Onset of disease may be due to reduced regulatory mechanisms in the periphery (Marguerat et al., 1999), or unfettered autoreactive T cells caused by failure of hematopoietic-MHCII mediated thymic negative selection (Teshima et al., 2003). The latter mechanism has been proposed since although WT→MHCII$^{-/-}$ chimeras fail to generate normal number of conventional T regulatory cells (Bensinger et al., 2001), they do not develop GVHD. Furthermore thymectomy of WT recipients, which eliminates positive selection, prevented GVHD induced by transplant of MHCII$^{-/-}$ bone marrow (Denning et al., 2003). However, these findings do not fully support the explanation that cGVHD in MHCII$^{-/-}$→WT chimeras is due to the lack of negative selection, and it is likely that this is coupled with a lack of regulatory system. Our data support the view that reduced development of the regulatory compartment significantly contributes to the development of cGVHD in MHCII$^{-/-}$→WT BMTs, and that IMP CD4^{+} T cells are dominant over Treg cells as a significant component of this regulatory system. This provides an explanation for the previous findings that in allogeneic BMT, donor naïve but not CD44^{hi}CD62L^{lo} IMP CD4^{+} T cells were responsible for the onset of GVHD (Zheng et al., 2008), and that chimeras with comparable number of Treg cells to healthy control chimeras, still developed severe

cGVHD (Sakoda et al., 2007).

Type 1 regulatory (Tr1) T cells are IL-10 producing $CD4^+$ T cells that play essential roles in preventing inflammation, autoimmunity and GVHD (for review see (Pot et al., 2011; Roncarolo et al., 2006)). Tr1 cells may be a mixture of multiple Th lineages producing IL-10, as IL-10 and IL-10-inducing factors have been found co-expressed with master regulators of Th1/2/17 as well as by conventional T regulatory cells (Tregs) (for review see (Zhu and Paul, 2010)). The ability of Tr1 cells to produce IL-10 relies on the expression of c-Maf and the Aryl hydrocarbon receptor (AhR) (Apetoh et al., 2010), and is orchestrated by IL-27 producing tolerogenic DCs (Toscano et al., 2011). Our finding that the IMP population expressed transcription factors that can regulate IL-10 suggests a mechanism for the function of these cells in preventing cGVHD, although it is clear that the IMP population includes other cell types as well, including Tregs, our data suggest that Tregs are insufficient to prevent cGVHD in this model.

Bone marrow derived DCs play critical roles in the development of self-tolerance (Hawiger et al., 2001; Probst et al., 2003), including inducing negative selection of developing thymocytes (Brocker et al., 1997), and the development of $Foxp3^+$ T regulatory cells (Sela et al., 2011). Ablation of DCs in donor marrow leads to $CD4^+$ T cell infiltration into multiple peripheral organs in syngeneic WT recipients (Ohnmacht et al., 2009), and bone marrow with DCs that are defective in cognate $CD4^+$ T-cell recognition resulted in impaired peripheral tolerance with fatal $CD8^+$ T cell cytotoxicity in syngeneic WT recipients (Muth et al., 2012). Moreover, we identified DCs as the primary hematopoietic cell expressing MHCII required for

selection and/or licensing of functional IMP $CD4^+$ T cells. Previous studies using a MHCII transgenic mouse model where MHCII is re-expressed in $MHCII^{-/-}$ background driven by CD4 promoter and enhancer showed that MHCII expression on CD4 expressing cells can contribute to selection of $CD4^+$ T cells with a similar memory-like phenotype (e.g., $CD44^{hi}$ (Li et al., 2005; Qiao et al., 2012)). These memory-like $CD4^+$ T cells have been defined as thymocyte-selected $CD4^+$ T cells, however, it is difficult to rule out that the CD4 promoter/enhancer does not also result in expression of MHCII on at least a subset of DCs since DCs have been reported to also express CD4 (Vremec et al., 2000). The properties of these cells in BMT have not been evaluated and so it is not clear if these cells are the same population of IMP $CD4^+$ T cells described here, as it is quite possible that there are alternative pathways for the development of IMP $CD4^+$ T cells. Nevertheless, we showed that DC expression of MHCII is requisite and sufficient for IMP $CD4^+$ T cell development, T-cell derived IL-10 production, and prevention of cGVHD in $MHCII^{-/-}\rightarrow$WT BMT model. Donor T cell derived TNF-α is detrimental and required in the development of GVHD (Borsotti et al., 2007; Schmaltz et al., 2003), whereas in humans, IL-10 promoter polymorphisms are associated with protection in transplantation between HLA-identical siblings (Lin et al., 2003). Our data recapitulated this reciprocal relationship. Thus MHCII expression by DCs is required for IMP $CD4^+$ T cell functional development, and the ability for $CD4^+$ T cells in the transplanted mice to produce IL-10, suggesting that DCs may function through antigen presentation to license $CD4^+$ T cells with an innate regulatory program, allowing them to suppress T cell-mediated GVHD.

The absence of Itk leads to reduced naïve $CD4^+$ T cells, but preserved numbers of IMP $CD4^+$ T cells (Hu and August, 2008). When Itk is deleted from $MHCII^{-/-}$ donor bone marrow, we observed rescued IMP $CD4^+$ T cell development, along with partial suppression of cGVHD. This partial response may be related to the role of Itk in T cell receptor signaling (Andreotti et al., 2010), trafficking (Xia et al., 2010) and/or survival (Qi et al., 2012). Nevertheless, targeting Itk can potentially serve as an *in vivo* supplement to generate IMP $CD4^+$ T cells, or *in vitro* to enrich IMP $CD4^+$ T cells for infusion therapy, thus reducing cGVHD.

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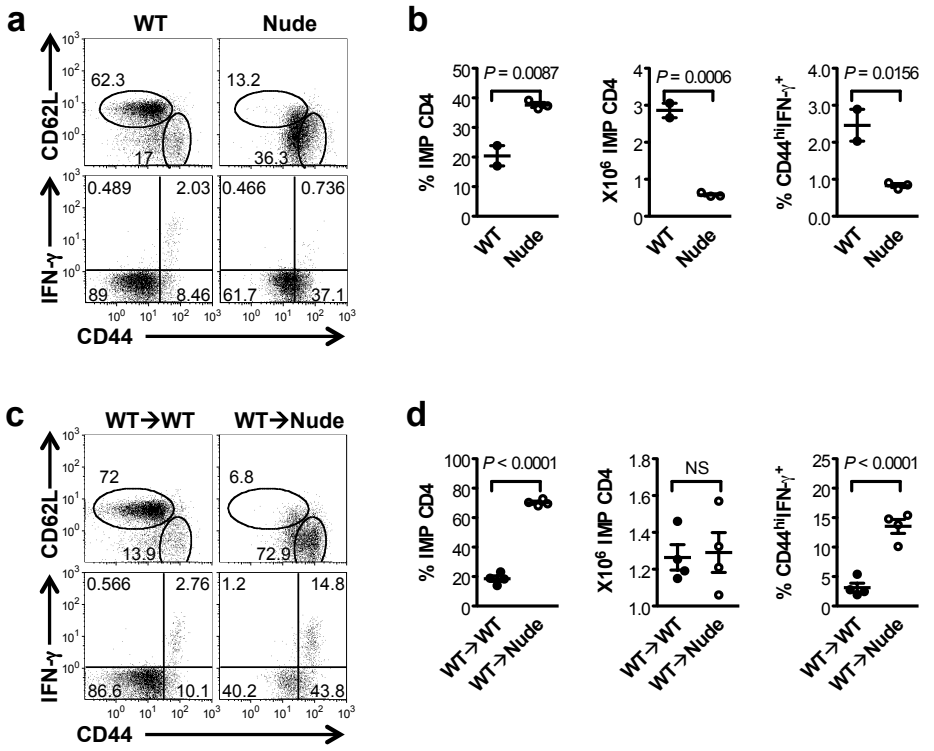
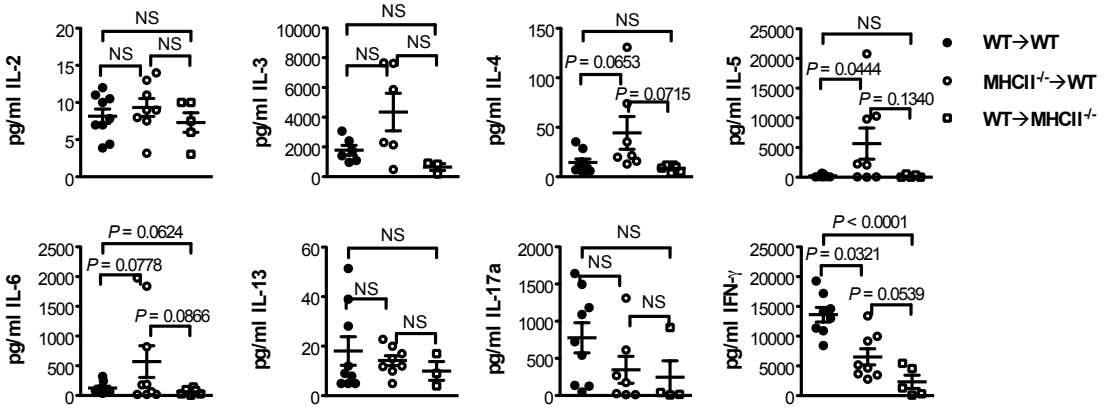
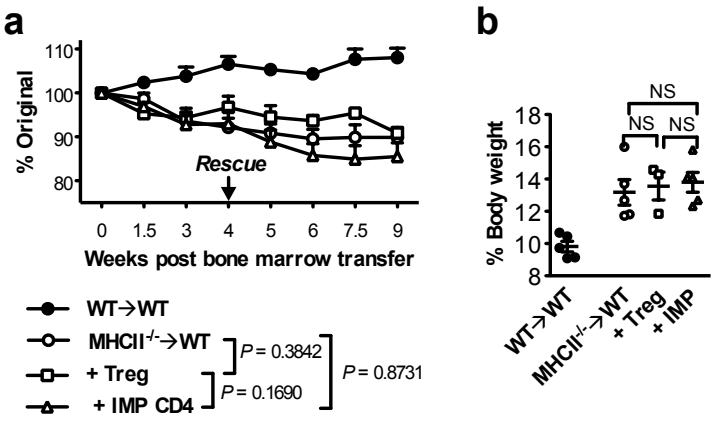


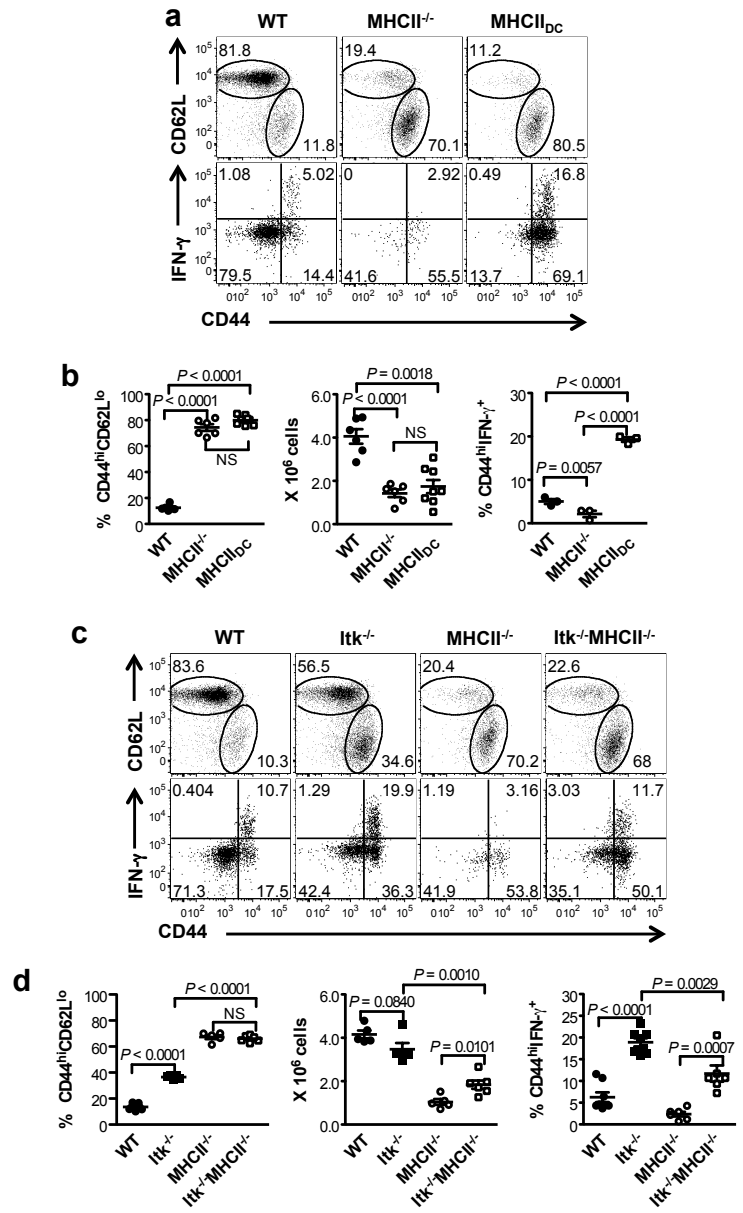
Supplemental Figure 5-1. Functional development of IMP CD4$^+$ T cells is independent of the thymus. (**a**) Expression of CD44, CD62L and IFN-γ by WT and Nude splenocytes. (**b**) Percentages (left) and number (middle) of IMP CD4$^+$ T cells and percentage of CD44^{hi} IFN-γ producing CD4$^+$ T cells (right) in WT and Nude mice splenocytes. (**c**) Bone marrow chimeric mice were generated as indicated, and splenocytes of 9-week old chimeras were analyzed for CD44, CD62L and IFN-γ expression. (**d**) Percentages (left) and number (middle) of IMP CD4$^+$ T cells and percentage of CD44^{hi} IFN-γ producing CD4$^+$ T cells (right) in indicated chimeric mice. *P* values were generated by Student's *t* test; NS = no significance.



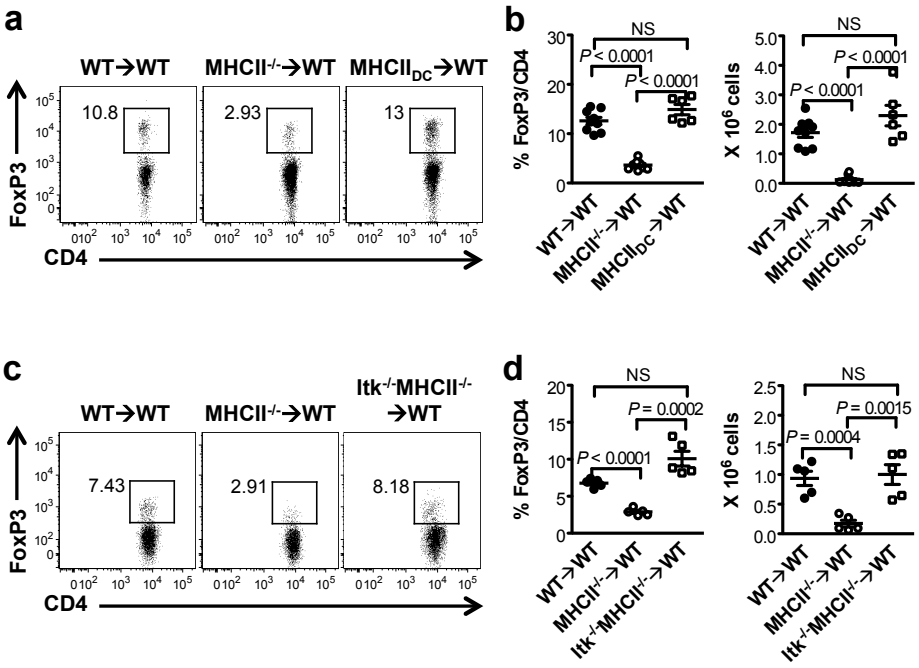
Supplemental Figure 5-2. Secretion of multiple T cell-derived cytokines by TcR stimulated splenocytes lacking either naïve or IMP CD4$^+$ T cells. Splenoctes from indicated chimeric mice were stimulated by anti-CD3e/CD28 antibodies for 3 days, and supernatants were subjected to multiplex analysis to determine level of secreted cytokines. Data combined from 2 independent experiments, n ≥ 5, *P* values were generated by Student's *t* test; NS = no significance.



Supplemental Figure 5-3. Pathogenesis due to lack of IMP CD4$^+$ T cells is irreversible. Bone marrow chimeras were generated as indicated, and 4 weeks post bone marrow transplantation, 0.2-0.3×10^6 conventional regulatory T cells (+ Treg) or IMP CD4$^+$ T cells (+ IMP CD4) were injected retro-orbitally. (**a**) Body weight curves of indicated groups. *P* values were generated by two way-ANOVA. (**b**) Small intestinal weight over body weight at the time of sacrifice of mice in (**a**). NS = no significance by Student's *t* test. n ≥ 3 in each group.



Supplemental Figure 5-4. DC-restricted MHCII expression or Itk deficiency promotes IMP CD4$^+$ T cell functional development in the absence of MHCII$^{-/-}$. (**a**) Expression of CD44, CD62L and IFN-γ by WT, MHCII$^{-/-}$ and MHCII$_{DC}$ splenocytes. (**b**) Percentages (left) and number (middle) of IMP CD4$^+$ T cells and percentage of CD44^{hi} IFN-γ producing CD4$^+$ T cells (right) in WT, MHCII$^{-/-}$ and CD11c/$_{MHCII}$ splenocytes. (**c**) Expression of CD44, CD62L and IFN-γ by WT, Itk$^{-/-}$, MHCII$^{-/-}$ and Itk$^{-/-}$MHCII$^{-/-}$ splenocytes. (**b**) Percentages (left) and number (middle) of IMP CD4$^+$ T cells and percentage of CD44^{hi} IFN-γ producing CD4$^+$ T cells (right) in WT, Itk$^{-/-}$, MHCII$^{-/-}$ and Itk$^{-/-}$MHCII$^{-/-}$ splenocytes. Data represent results from ≥ 2 independent experiments. *P* values were generated by Student's *t* test; NS = no significance.



Supplemental Figure 5-5. DC-restricted MHCII re-expression or Itk deficiency rescues conventional regulatory T cell development in MHCII$^{-/-}$→WT chimeras. Bone marrow chimeric mice were generated as indicated. CD4^{+} T cells of donor origin were analyzed for expression of FoxP3 (**a&c**) to determine percentages and numbers of conventional regulatory T cells (**b&d**). Data represent results from 2 independent experiments. *P* values were generated by Student's *t* test; NS = no significance.

CHAPTER SIX

ITK and BTK negatively regulate LPS-induced mast cell-derived TNF-α production and septic hypothermia[g]

[g] Weishan Huang, J. Luis Morales, Victor P. Gazivoda, and Avery August. ITK and BTK negatively regulate LPS-induced mast cell-derived TNF-α production and septic hypothermia. *Manuscript in preparation.*

Abstract

Mast cells are indispensible for LPS induced septic hypothermia, in which TNF-α plays an essential role to initiate sepsis. Tec kinases ITK and BTK regulate mast cell-derived TNF-α in response to allergic antigen, but their role in LPS induced TNF-α production by mast cells and related pathology is unclear. We sought to investigate the role(s) of ITK and BTK in mast cell response to septic condition. We found that the absence of ITK and BTK leads to exacerbation of LPS induced septic hypothermia, and enhanced TNF-α production by peritoneal mast cells. *Sash* mice reconstituted with $Itk^{-/-}Btk^{-/-}$ BMMCs develop more severe LPS induced septic hypothermia than those reconstituted with WT BMMCs. $Itk^{-/-}Btk^{-/-}$ mast cells exhibit hyperactive preformed and LPS-induced TNF-α production, along with enhanced NF-κB, Akt and p38 activation, compared to WT cells. Blockage of PI3K, Akt or p38 downstream MNK1 activation significantly suppresses TNF-α hyper-production by $Itk^{-/-}Btk^{-/-}$ mast cells in response to LPS, and attenuated septic hypothermia in $Itk^{-/-}Btk^{-/-}$ mice. Thus we conclude: ITK and BTK regulate the recovery of thermal homeostasis post septic response through mast cell function in mice. They share regulatory function downstream of TLR4/LPS in mast cells, through regulating the activation of PI3K/Akt, canonical NF-κB and p38 signaling pathways.

Introduction

TNF-α plays a major role in the thermal and cardiovascular disorders associated with sepsis (Berry et al., 2004; Meldrum, 1998). Mast cell derived TNF-α is a critical initiator in heart failure (Levick et al., 2008) and production of pro-inflammatory cytokines in ischemia reperfusion (Gilles et al., 2003; Reil et al., 2007). Administration of LPS in mice or humans causes sepsis-like symptoms, whereas mast cell derived TNF-α rapidly triggered by LPS is indispensible for septic hypothermia in mice (Nautiyal et al., 2009). Injection of TNF-α resembles late phase pathogenesis of LPS administration (Fairchild et al., 2009). Steroids inhibiting cytokine synthesis can blunt, but not eliminate, the severity of endotoxemea, which might be due to the incomplete blocking of the initial increase of provoking cytokine(s), or the release of preformed cytokine independent of transcriptional control (Alvarez et al., 2007; Fairchild et al., 2009). However, mast cell derived TNF-α can also exhibit a protective role in reducing mortality in a model of acute septic peritonitis (cecal ligation and puncture, CLP) (Echtenacher et al., 1996), and in modulating neutrophil influx during bacterial clearance (Malaviya et al., 1996). The crucial role of mast cell derived TNF-α in local infection, sepsis and myocardial dysfunction is not a novel observation, however, how mast cell derived TNF-α production is modulated, remains largely unexplained.

Tec family kinases Tec, ITK and BTK are non receptor tyrosine kinases acting downstream of numerous receptors, and have been shown to modulate mast cell responses downstream of FcεRIα (Iyer and August, 2008; Iyer et al., 2011; Kawakami et al., 1994; Kawakami et al., 1995; Schmidt et al., 2009). In mast cells, ITK and BTK

have redundant functions, which might be attributed to structural homology and shared substrates (Hata et al., 1998). Neither ITK nor BTK is required for mast cell development, but the absence of both leads to impaired FcεRIα-mediated degranulation and cytokine secretion, including TNF-α (Iyer and August, 2008; Iyer et al., 2011; Kawakami et al., 1994; Kawakami et al., 1995; Schmidt et al., 2009). However, the roles of ITK and BTK in mast cell responses to septic condition are unclear.

In mast cells, LPS was shown to activate BMMCs through TLR4 to activate MAPKs (Masuda et al., 2002) and NF-κB (Nigo et al., 2006), and induced PI3K/Akt activation that regulates NF-κB activation. BTK is shown as a TLR4 binding protein involved in NF-κB activation (Jefferies et al., 2003). In monocytic cells BTK phosphorylates TLR4 downstream MyD88-adaptor like protein (MAL), leading to MAL degradation (Gray et al., 2006). Although some reports suggest that BTK is a positive mediator in TLR4 signaling (Doyle et al., 2005; Gray et al., 2006; Jefferies et al., 2003; Mansell et al., 2006; Semaan et al., 2011), others suggest that BTK is dispensable, as LPS induced TNF-α and IL-6 was slightly increased in 129/Sv BMMCs lacking BTK. Recently, BTK was further identified as a negative regulator in human primary hematopoietic cell TLR4 signaling through degradation of MAL (Marron et al., 2012). This suggests that the role of BTK in TLR4 signaling might be cell type specific or strain dependent (Zorn et al., 2009). The role of ITK in mast cell response to LPS is not yet investigated.

In this study, we investigated the function of ITK and BTK in LPS-induced mast cell-mediated septic hypothermic response, and report an overall regulatory

function for these kinases in suppressing this pro-inflammatory response though regulation of NF-κB, PI3K/Akt, p38 and MNK1 signaling activity.

Materials and Methods

Mice.

All mice were on a C57BL/6 background. *Itk*$^{-/-}$*Btk*$^{-/-}$ mice were generated as previously described (Iyer et al., 2011). *Tnfa*$^{-/-}$ (B6.129S-*Tnf*^{tm1Gkl}/J) and *Sash* (*Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ) mice were from The Jackson Laboratory (Bar Harbor, ME). All experiments were approved by the Office of Research Protection's IACUC at The Pennsylvania State and Cornell Universities.

Generation of BMMCs and reconstitution of BMMCs in Sash mice.

Bone marrow cells collected from mouse femur were culture in complete DMEM with 10 ng/ml recombinant murine (rm) IL-3 (Cell Sciences, Canton, MA) and 50 ng/ml rmSCF (Rocky Hill, NJ) in ~10^6 cells/ml, with medium changed every other day. After 4 to 6 weeks in culture, BMMCs with purity (c-Kit$^+$FcεRIα$^+$) > 96% were used. For *in vitro* stimulation, 2×10^6/ml BMMCs were starved in complete DMEM without IL-3/SCF overnight, followed by indicated stimulation for indicated time-points. *Sash* mice received 5×10^6 BMMCs through retro-orbital (intravenous, i.v.) injection and 5×10^6 BMMCs though intraperitoneal (i.p.) injection to reconstitute mast cells, 12 weeks prior to experiment.

LPS induced hypothermia.

Mice were i.p. injected with 1 mg/kg (compound weight/body weight) LPS, and core body temperature (non-invasive measure of central abdomen) was measured at

indicated time-points using an infrared thermometer (Reed ST-8812, Tequipment.NET, Long Branch, NJ).

Microarray analysis.

BMMCs were starved for overnight, and treated with PBS or 100 ng/ml LPS for 1 hour. mRNA from treated cells was extracted with RNeasy Plus Mini Kit (Qiagen, Valencia, CA), followed by amplification and array assay (Whole Mouse Genome Microarray Kit, 4x44K, Agilent Technologies, Clara, CA) at the Cornell University Life Sciences Core Laboratories Center. Microarray data were analyzed using GeneSpring GX software (Agilent). Quantile-normalized gene values were used to identify genes with significant change (≥ 2 fold change, $P < 0.05$) in between at one pair of "WT+PBS", "WT+LPS", "*Itk*$^{-/-}$*Btk*$^{-/-}$+PBS", and "*Itk*$^{-/-}$*Btk*$^{-/-}$+LPS" groups. The significantly changed genes are further classified based on the Gene Ontology annotation. Those identified as "immune cytokine/chemokine" associated genes were used to screen for NF-κB targets (listed http://bioinfo.lifl.fr/NF-KB/NM-mouse.txt, derived from (Pahl, 1999)). Selected genes were clustered and presented by GeneSpring GX software.

Antibodies and reagents.

FITC conjugated anti-c-Kit, Phycoerythrin conjugated anti-FcεRIα, and PerCP-eFluor 710 conjugated anti-TNF-α antibody were from eBioscience (San Diago, CA). Anti-phospho- or total p65, p38, ERK, JNK and AKT, anti-phospho-IκBα, anti-p100/p52 and GAPDH antibodies were from Cell Signaling (Danvers, MA). Anti-IκBα antibody

was from Santa Cruz. Horseradish peroxidase (HRP) conjugated anti mouse/rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were detected by ECL system (GE Healthcare Biosciences, Pittsburgh, PA). LPS (*Escherichia coli* 0111:B4) was from Sigma-Aldrich (St. Louis, MO). The protein transportation blocker Brefeldin A was from Sigma, and used at 10 μg/ml. ITK/BTK cross-reactive inhibitor CNX584 was a kind gift from Akt inhibitor Akti1/2 (Santa Cruz Biotechnology, Dallas, TX) was used at 10 or 20 μM *in vitro*, and 5.5 mg/kg *in vivo*. PI3K inhibitor LY294002 (Cayman Chemical, Ann Arbor, MI) was used at 10 or 20 μM *in vitro*, and 6.86 mg/kg *in vivo*. MAP-kinase interacting kinase-1 inhibitor CGP57380 (Tocris, Bristol, United Kingdom) was used in 20 μM *in vitro*, and 9.8 mg/kg *in vivo*.

Confocal microscopy and Flow cytometry.

To determine p65 nuclear translocation, BMMCs were stimulated with 1 μg/ml LPS for 15 minutes, fixed and permeabilized with FoxP3 staining buffer kit (eBioscience), and stained with Alexa Fluor 488 conjugated anti-p65 antibody (Santa Cruz Biotechnology Santa Cruz, CA), in the presence of Fc block (anti-CD16/32, eBioscience, San Diego, CA). Following staining, cells were treated with Prolong Gold anti-fade reagent with DAPI (Invitrogen, Carlsbad, CA), and imaged on a TCS SP5 (Leica Microsystems, Inc., Buffalo Grove, IL) confocal microscope within 24 hours. Images were processed by ImageJ software (NIH) to calculate the fraction of nuclear p65 fluorescence over total. To determine mast cell TNF-α production, cells were stained with surface markers in the presence of Fc block, fixation with 4%

paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), permeabilization and staining of cytokine antibodies using PBS containing 0.4% saponin (Sigma). All flow cytometry data were acquired on LSRII (BD Biosciences), and analyzed in FlowJo (Tree Star, Ashland, OR).

Detection of TNF-α mRNA and secretion.

RNA extracted using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) was converted to cDNA with You Prime First-Strand beads kit (GE Healthcare, Piscataway, NJ). Quantitative real-time PCR (qRT-PCR) was carried out using Taqman probe sets (Applied Biosystems, Foster City, CA). Secreted cytokine in supernatant of indicated cells was measured using a Millipore multiplex system (Millipore, Billerica, MA) following manufacturer's instruction.

Statistical analysis.

Two-tailed Student's *t* test (referred to as "*t* test") and two-way ANalysis Of VAriance between groups (ANOVA) were performed using GraphPad Prism v5.00 (GraphPad, San Diego, CA), with $p < 0.05$ considered statistically significant.

Results

ITK and BTK negatively regulate LPS induced hypothermia and mast cell-derived TNF-α

Mast cells have been shown to be responsible for the LPS induced hypothermic response, which is mediated by LPS induced TNF-α (Nautiyal et al., 2009). To determine whether ITK and BTK play roles in mast cell response to LPS and thus regulate this the hypothermic response to LPS-induced sepsis, we injected WT, *Itk*$^{-/-}$, *Btk*$^{-/-}$ and *Itk*$^{-/-}$*Btk*$^{-/-}$ mice with 1 mg/kg LPS to induce septic hypothermia. We found that *Itk*$^{-/-}$ and *Btk*$^{-/-}$ mice did not exhibit significantly worse hypothermic response compared to WT mice (**Supplemental Figure 6-1**), however, *Itk*$^{-/-}$*Btk*$^{-/-}$ mice suffered significantly exacerbated LPS-induced hypothermia (**Figure 6-1** A). Furthermore, primary mast cells from mouse peritoneum lavage (PLMCs) from these mice carry higher level of preformed TNF-α, and responded to LPS stimulation with significantly higher TNF-α production (**Figure 6-1** B). These data suggest that, ITK and BTK limit the drop in body temperature from the homeostatic levels, and contribute to the recovery of thermal homeostasis post exposure to bacterial endotoxin. In the absence of ITK and BTK, mast cells are hyperactive to LPS-induced TNF-α production, which may be responsible for the exacerbation of hypothermic response in murine models.

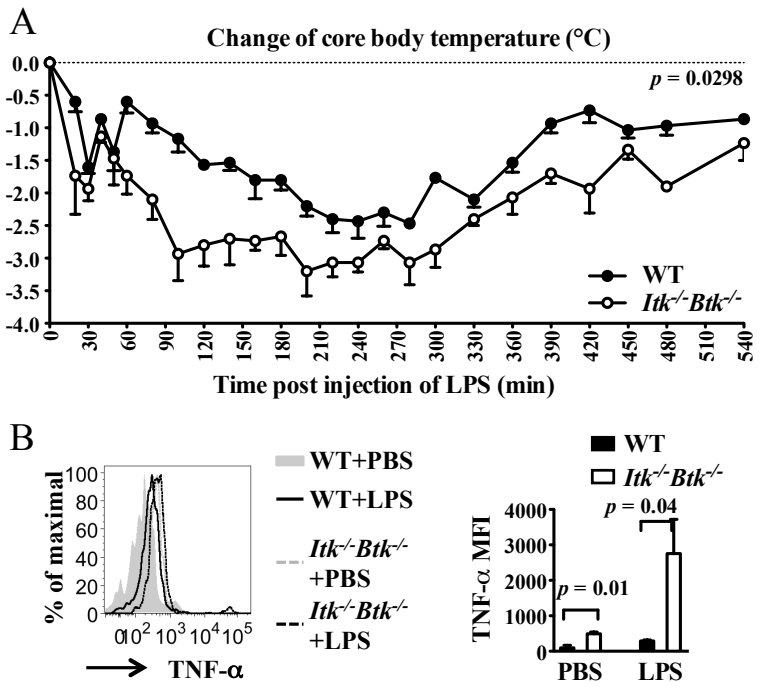


Figure 6-1. The absence of ITK and BTK enhances LPS-induced hypothermia and mast cell-derived TNF-α. (**A**) Change in core body temperature induced by LPS. n = 3, *p* value by ANOVA. (**B**) Representative plots and average MFI of TNF-α expression by peritoneal mast cells treated with PBS or LPS. n ≥ 3, *p* value by *t* test.

Itk$^{-/-}$Btk$^{-/-}$ BMMCs exhibit hyperactive TNF-α production and exacerbate LPS induced hypothermia

To confirm the role of mast cell-derived TNF-α in LPS-induced hypothermia in the absence of ITK and BTK, we aimed to generate a murine model with the loss of ITK and BTK only in mast cells. Reconstituting mast cell deficient *Sash* mice with bone marrow derived mast cells (BMMCs) is a potent tool to do so (Grimbaldeston et al., 2005). We characterized the effect of the absence of ITK and BTK in TNF-α production by the BMMCs. Very intriguingly, we found that in the absence of ITK and BTK, BMMCs carry higher amounts of preformed TNF-α transcripts (**Figure 6-2** A), and spontaneously generate and secrete low amounts of TNF-α (**Figure 6-2** B). When stimulated with LPS, *Itk$^{-/-}$Btk$^{-/-}$* BMMCs rapidly secreted significantly higher amounts of TNF-α compared to WT BMMCs, and the more LPS used, the more profound this effect was (**Figure 6-2** C). This hyperactive TNF-α producing phenotype in *Itk$^{-/-}$Btk$^{-/-}$* BMMCs is consistent with what we observed in primary PLMCs, suggesting that this was a conserved phenotype between mast cells differentiated *in vitro* and *in vivo*. We thus used these BMMCs to reconstitute *Sash* mice, and used the "BMMC+*Sash*" model to examine the function of ITK and BTK in LPS-induced mast-mediated hypothermic response. We found that *Sash* mice reconstituted with *Itk$^{-/-}$Btk$^{-/-}$* BMMCs exhibited more severe hypothermic response (**Figure 6-2** D), suggesting that the lack of ITK and BTK in mast cells only is sufficient to cause exacerbated hypothermic response in LPS-induced sepsis. Thus, ITK and BTK regulate murine thermal homeostasis in LPS-induced septic response through mast cell function.

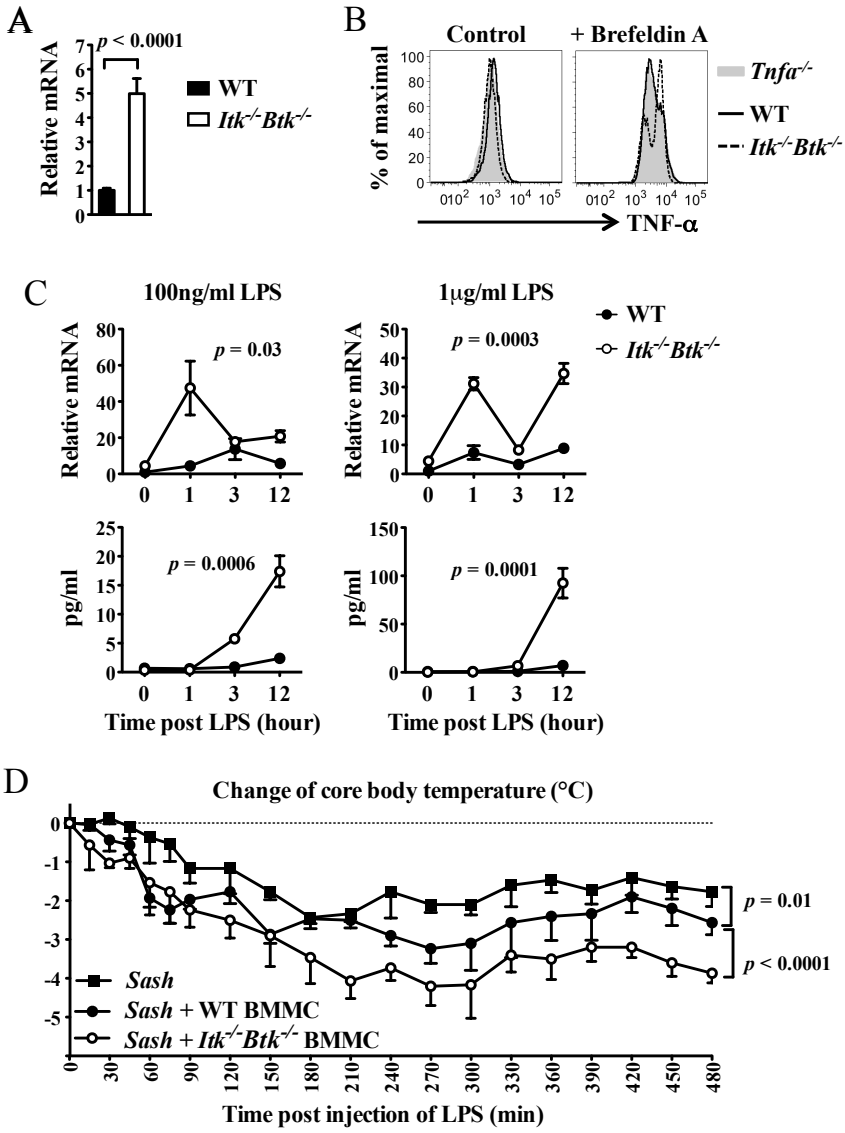


Figure 6-2. ITK and BTK negatively regulate LPS-induced hypothermia via mast cells. (**A**) Relative mRNA expression of preformed TNF-α mRNA in BMMCs. n =6, *p* value by *t* test. (**B**) Representative plots of TNF-α synthesis in the steady state by BMMCs, of 2 independent experiments. (**C**) LPS-induced TNF-α mRNA and secretion. *p* values by ANOVA, represent results of 3 experiments. (**D**) LPS-induced hypothermia in BMMC reconstituted *Sash* mice. Data represents result of 2 independent experiments. *p* by ANOVA.

ITK and BTK negatively regulate LPS induced canonical NF-κB activation in mast cells

LPS signaling emanating through TLR4 can trigger both canonical and non-canonical NF-κB pathways. The former is dependent on the phosphorylation and release of IκBα from p65 that can translocate into the nuclear compartment, while the latter triggers the processing of p100 to generate p52 that can form activating complexes to turn on target gene expression (see review (Oeckinghaus et al., 2011)). In order to find genes that are significantly altered by the absence of ITK and BTK in mast cell response to LPS, we stimulated WT and DKO BMMCs with PBS or LPS for 1 hour, and assay for gene expression using microarray. We screened for genes with significant change due to the lack of ITK and BTK function, and found those annotated by Gene Ontology database as immune response-associated. These genes were then compared to the reported NF-κB transcriptional targets (Pahl, 1999). We noticed from microarray analysis that, along with TNF-α, other NF-κB target genes are highly activated in the absence of ITK and BTK in mast cell response to LPS (**Figure 6-3** A). Thus we examined the activation status of BMMCs stimulation with LPS. We found that the canonical NF-κB signaling is highly active in the absence of ITK and BTK: IκBα exhibited higher basal phosphorylation, which was increased and persisted in response to LPS; meanwhile, p65 phosphorylation is significantly higher than that in WT BMMCs in response to LPS (**Figure 6-3** B, top four panels). In contrast, little conversion of p100 into p52 was observed in either WT or *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs in both steady state and LPS stimulated state (**Figure 6-3** B, top 5&6 panel). To further verify that p65 is activated and translocated to the nucleus, we examined

the subcellular translocation of p65 following LPS stimulation, and in comparing WT and *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs, we found that, LPS-induced p65 translocation is more efficient in the absence of ITK and BTK (**Figure 6-3** C). This suggests that ITK and BTK function in mast cells is to negatively regulate the activation of the canonical NF-κB signaling pathway, and accordingly suppress LPS-induced mast cell-derived TNF-α production.

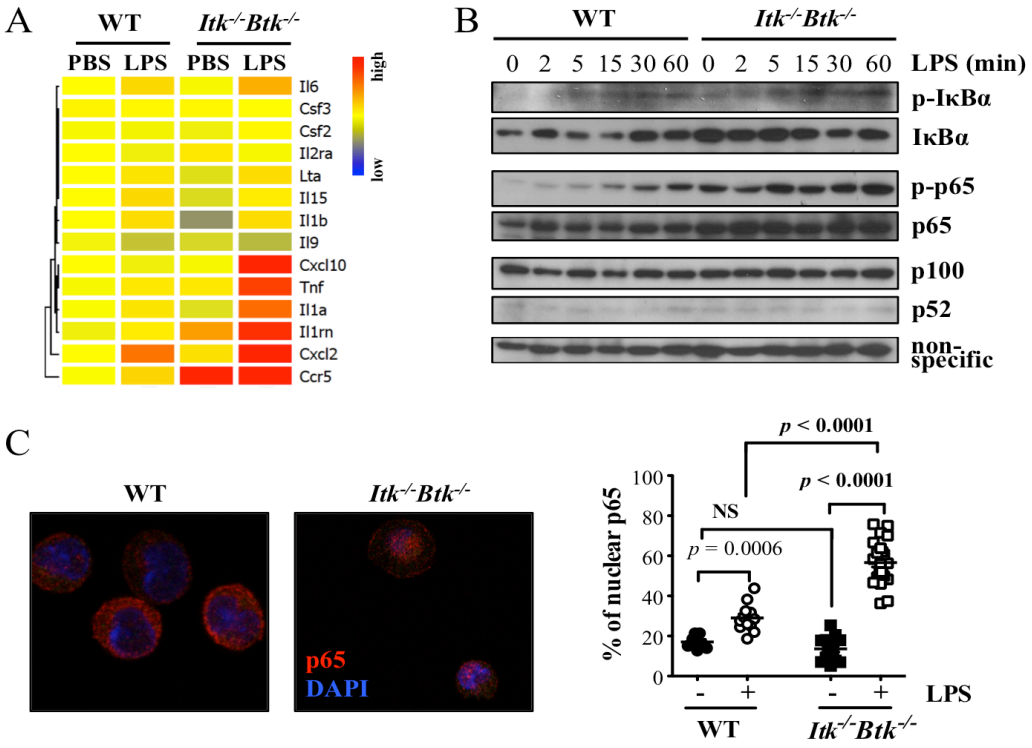


Figure 6-3. Hyperactive canonical NF-κB signaling in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMC response to LPS. (**A**) Significant change in of NF-κB targeted cytokine/chemokine associated gene expression by microarray. (**B**) LPS induced activation of key components of the NF-κB pathway. Data represent results of ≥ 2 experiments. (**C**) LPS-induced p65 nuclear translocation. *p* values by *t* test. NS: $p > 0.05$.

ITK and BTK regulate LPS induced Akt and p38 signaling activity in mast cells

LPS induced PI3K/Akt (Song et al., 2012) and MAPK (Avila et al., 2012) activation has been shown to regulate the activity of NF-κB pathway in mast cells. To determine whether the activation of these signaling pathways are altered in mast cell response to LPS in the absence of ITK and BTK, we examined the activation status of Akt and MAPKs (including p38, ERK and JNK). We found that Akt phosphorylation was enhanced in the absence of ITK and BTK in BMMCs stimulated with LPS along the time course (**Figure 6-4**). In mast cells lacking of ITK and BTK, p38 exhibited enhanced basal phosphorylation, and was further significantly induced by stimulation of LPS, in contrast, activation of ERK and JNK was impaired (**Figure 6-4**). This is consistent with very recent findings that p38 critically regulates LPS-induced TNF-α production in BMMCs, while ERK activation is dispensable (Hochdorfer et al., 2013). Thus, MAPKs are differentially regulated by ITK and BTK in the mast cell response to LPS. While ITK and BTK negatively regulate p38 activation, they are required for optimal activity of ERK and JNK in this process.

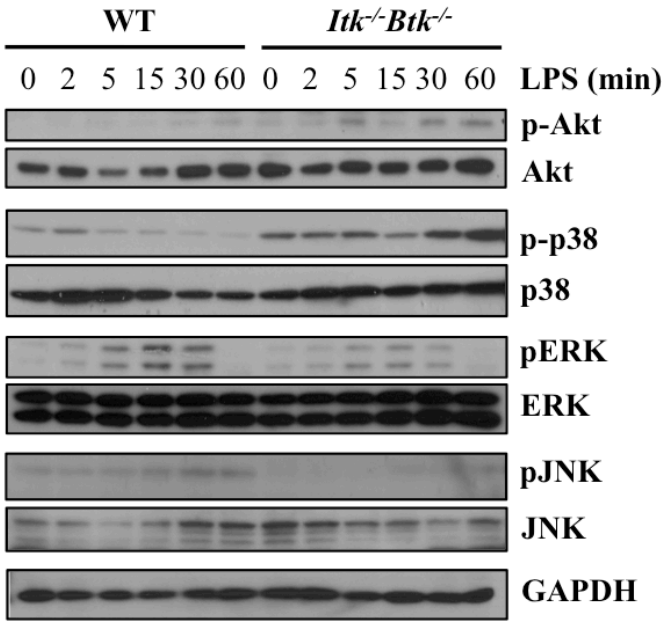


Figure 6-4. Hyperactive Akt and p38 signaling in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMC response to LPS. LPS induced activation of Akt and MAPKs. Data represent results from ≥ 2 independent experiments.

PI3K/Akt signaling activity is required for LPS induced TNF-α hyper-production and exacerbated hypothermia in the absence of ITK and BTK

The lack of ITK and BTK expression in BMMCs led to hyperactive Akt in response to LPS. PI3K has been well characterized as a major activator up-stream of Akt (Franke et al., 1997). To test whether PI3K and Akt can be attributed to the hyperactive TNF-α production and associated septic hypothermia due to the mast cells response to LPS in the absence of ITK and BTK, we used PI3K inhibitor LY294002 (Vlahos et al., 1994) and Akt inhibitor Akti1/2 (Barnett et al., 2005) to treat BMMCs and PLMCs, and measured the LPS-induced TNF-α production. Blocking PI3K or Akt activation resulted in impairment of LPS induced TNF-α mRNA production in both WT and $Itk^{-/-}Btk^{-/-}$ BMMCs, and of note, the $Itk^{-/-}Btk^{-/-}$ BMMCs TNF-α transcript level was severely blunted to the level of that observed in WT cells in response to LPS (**Figure 6-5** A). WT primary PLMCs exhibited weak dependence on PI3K and Akt activation in early TNF-α production in response to LPS stimulation (6 hours, **Figure 6-5** B), however, primary mast cells lacking the expression of ITK and BTK strongly depended on the PI3K and Akt activation to execute the hyperactive TNF-α production induced by LPS (**Figure 6-5** B). These data suggest that PI3K and Akt activation is required for the LPS-induced TNF-α production by mast cells, and contributes to the hyperactive TNF-α production in the absence of ITK and BTK. This hyperactive Akt responsiveness to LPS might be critical for the exacerbation of septic hypothermia induced by LPS in $Itk^{-/-}Btk^{-/-}$ mice observed (**Figure 6-1** A). We hence used Akti1/2 and LY294002 to pretreat $Itk^{-/-}Btk^{-/-}$ mice 30 minutes prior to LPS exposure, and found that the inhibition of Akt or PI3K activation significantly

attenuated LPS-induced hypothermia *Itk*$^{-/-}$*Btk*$^{-/-}$ mice (**Figure 6-5** C). The requisite role of PI3K and Akt activation in LPS-induced hyper-production of mast cell-derived TNF-α and exacerbated hypothermic response suggests that ITK and BTK regulate PI3K and Akt activity to regulate LPS-induced mast cell-mediated pro-inflammatory response and associated thermal homeostasis in mice. Furthermore, this is likely through the regulation on NF-κB target gene transcription.

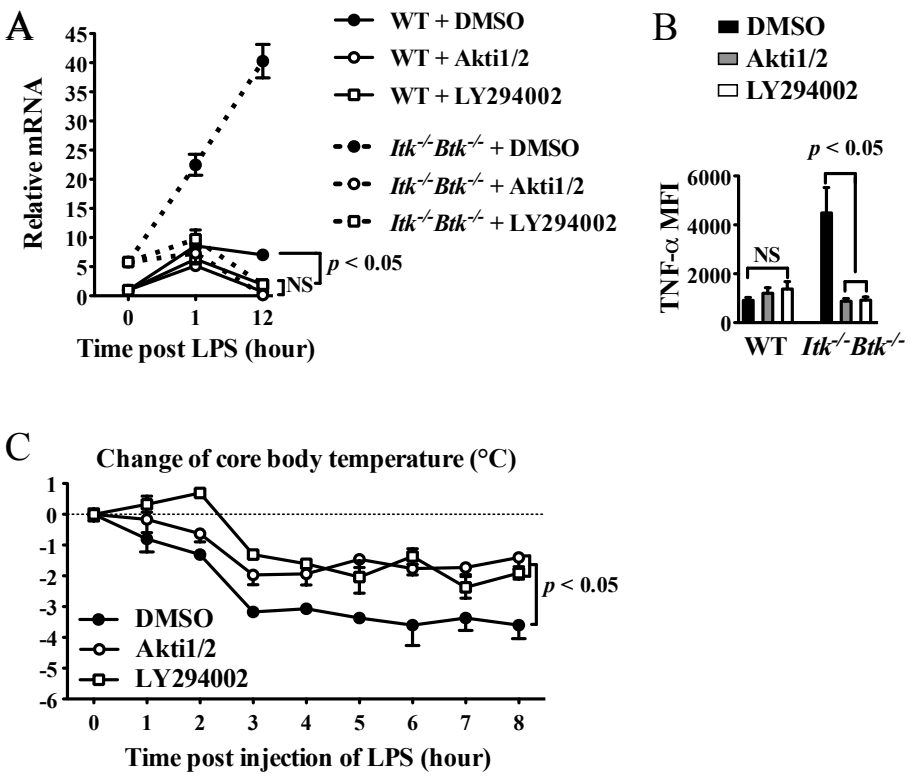


Figure 6-5. PI3K/Akt activity is required for hyperactive TNF-α production and enhanced hypothermia in the absence of ITK and BTK. Effects of Akt and PI3K inhibitors on LPS-induced (**A**) TNF-α mRNA in BMMCs and (**B**) TNF-α protein expression in PLMCs. (**C**) Akt and PI3K inhibitors attenuated LPS-induced hypothermia in the absence of ITK and BTK. n ≥ 3, *p* by *t* test between columns, by ANOVA between curves. NS: $p > 0.05$.

MNK contributes to the LPS induced TNF-α hyper-production and exacerbated hypothermia in the absence of ITK and BTK

Two major factors regulate the rate of its biosynthesis post TNF-α mRNA translation: the stability of TNF-α mRNA and rate of translation of TNF-α mRNA into protein, both of which were shown to be regulated by p38 activity. TNF-α mRNA contains an Adenylate-uridylate-rich element (ARE) in its 3', which can be bound be ARE-binding and -destabilizing factor tristetraprolin (TTP) (Lai et al., 1999). In macrophages, p38 activates MK2, which further phosphorylates TTP to reduce TTP binding affinity on the ARE, thus stabilizing TNF-α mRNA (Tiedje et al., 2012). However, in mast cells, LPS-induced TNF-α is dependent on p38 and independent of TTP (Hochdorfer et al., 2013; Kotlyarov et al., 1999), suggesting that TNF-α mRNA stabilization by p38/MK2/TTP signaling axis does not play a major role in TNF-α production by WT mast cells. If this p38/MK2/TTP axis played a role, then TNF-α mRNA should be significantly more stable in the *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs. We tested this by comparing the stability of LPS-induced TNF-α mRNA by blocking mRNA transcription/production using Actinomycin D, starting 1 hour after LPS-induced TNF-α mRNA production, and compared the rate of TNF-α mRNA degradation between WT and *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs. We found that this rate is significantly higher in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs indicated by a significantly shorter half-life (**Supplemental Figure 6-2**), suggesting that the hyperactive TNF-α production in the absence of ITK and BTK is not a result of p38 mediated TNF-α mRNA stabilization. It is therefore likely that the enhanced p38 activation in mast cells lacking ITK and BTK contributes to TNF-α production through mRNA translation. p38 mediated activation of MNK1

phosphorylates and reduces the binding affinity of eukaryotic initiation factor 4E (eIF4E) to the 5' cap structure of cytoplasmic mRNA, to ease eIF4E sliding and so facilitate cap-dependent translation (Scheper et al., 2002). This p38/MNK1 mediated initiation of TNF-α mRNA translation can be inhibited by > 75% by a MNK1 selective inhibitor CGP57380 (Buxade et al., 2005). To examine whether the hyperactive p38 signaling in LPS stimulated mast cells is responsible for the hyper-production of TNF-α through MNK1 mediated initiation of mRNA translation, we treated WT and *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs with MNK1 inhibitor CGP57380, found that, MNK1 inhibitor significantly reduced LPS-induced TNF-α mRNA production, and brought the level of TNF-α mRNA in the long run (12 hours) in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs similar to that observed in WT cells (**Figure 6-6** A), suggesting that, MNK1 mediated translation contributed to the hyperactive production of LPS-induced TNF-α in mRNA level in the absence of ITK and BTK. *Itk*$^{-/-}$*Btk*$^{-/-}$ primary mast cells treated with MNK1 inhibitor failed to turn on the LPS-induced hyperactive immediate synthesis of TNF-α protein (**Figure 6-6** B). These data suggest that MNK1 mediated TNF-α translation is required for the LPS-induced hyperactive TNF-α production observed in mast cell lacking the ITK and BTK, and is probably mediated by p38 hyperactivity. We also found that targeting MNK1 *in vivo* resulted in attenuation of the severe LPS-induced hypothermia in mice lacking ITK and BTK (**Figure 6-6** C), suggesting that MNK1 activity contributes to exacerbate LPS-induced mast cell mediated septic hypothermic response, likely through initiating TNF-α translation.

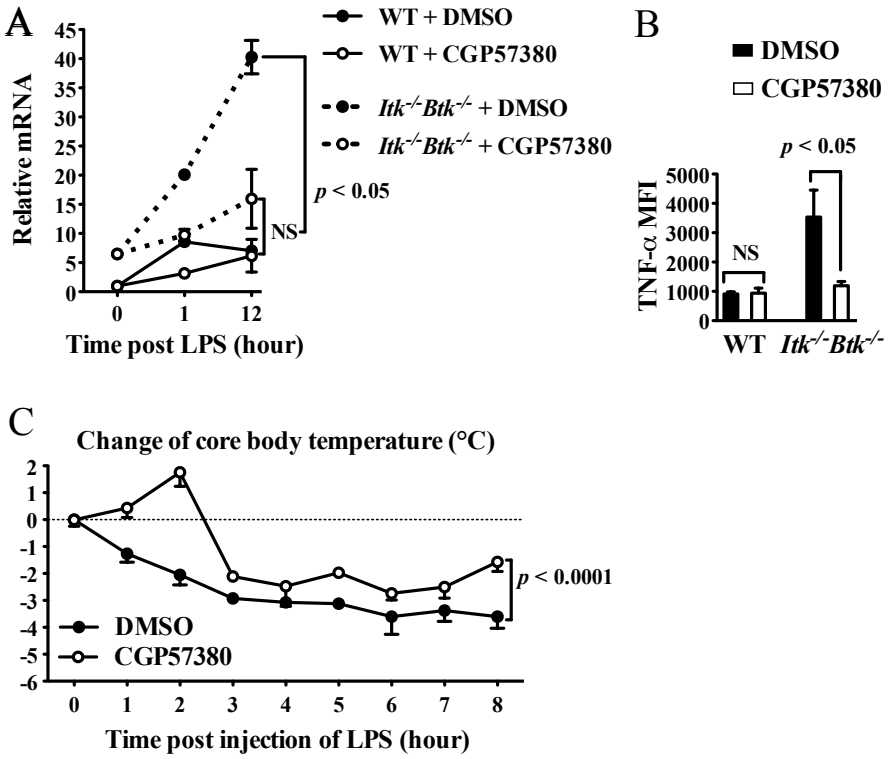


Figure 6-6. MNK is required for hyperactive TNF-α production and enhanced hypothermia in the absence of ITK and BTK. Effects of MNK inhibitor on LPS-induced (**A**) TNF-α mRNA in BMMCs and (**B**) TNF-α protein expression in PLMCs. (**C**) MNK inhibitor attenuated LPS-induced hypothermia in the absence of ITK and BTK. n ≥ 3, *p* by *t* test between columns, by ANOVA between curves. NS: $p > 0.05$.

Discussions

We show here that ITK and BTK share redundant role(s) in negatively regulating mast cell-mediated inflammatory response to gram-negative endotoxin LPS. In the absence of both ITK and BTK, mice suffered exacerbated LPS-induced hypothermia. Mast cells lacking ITK and BTK exhibited elevated preformed TNF-α and LPS-induced production, and contributed to enhance LPS-induced hypothermia in mice. ITK and BTK kinase activity is involved in executing this regulatory role. Mast cells lacking ITK and BTK also exhibited significantly enhanced LPS-induced signaling activity in canonical NF-κB, PI3K/Akt and p38 pathways. Blocking PI3K/Akt and p38-associated MNK activity dampened LPS-induced mast cell-derived TNF-α production and septic hypothermic response due to the absence of regulation by ITK and BTK.

Unlike macrophages that use both MAL/MyD88 and TRAM/TRIF adaptor complexes immediately downstream of TLR4, WT murine BMMCs use only MyD88 dependent signaling, with very low levels of TRIF and CD14 expression (Keck et al., 2011). We also excluded the possibility that TRAM/TRIF signaling complex is expressed in *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells to contribute to the observed hyperactive TNF-α production in response to LPS, as we did not observe similar hyper-production of IFNβ that lies downstream of TRAM/TRIF (Juang et al., 1998). BTK has been shown to be a regulatory mediator of MyD88/MAL signaling axis downstream of TLR4, by phosphorylating MAL for MAL degradation, in monocytic cell lines (Gray et al., 2006). However, it has been shown that in *Btk*$^{-/-}$ BMMCs, MAL stability is not altered (Zorn et al., 2009), and our observation is consistent with this: we did not find MAL

accumulation in *Itk*$^{-/-}$*Btk*$^{-/-}$ mast cells in the steady state nor in response to LPS (**Supplemental Figure 6-3**), suggesting that the hyperactive pro-inflammatory response in mast cells lacking ITK and BTK is not due to the MAL stabilization. Thus, ITK and BTK function as regulatory component of MyD88/MAL signaling axis downstream of TLR4/LPS in mast cells through an alternative mechanism other than MAL degradation. In the absence of BTK, LPS induced p38 activity is similar to that in WT cells, with moderate increase in TNF-α and IL-6 production (Zorn et al., 2009). However, the additional absence of ITK leads to a significant enhancement in LPS-induced p38 activation, and TNF-α and IL-6 (**Supplemental Figure 6-4**, as well as CXCL2 and IL-1β) production, suggesting that ITK and BTK share redundant function in negatively regulating LPS-induced p38 activation and associated pro-inflammatory cytokine production. Furthermore in mast cells lacking ITK and BTK, p38 may function to promote TNF-α protein translation, as its downstream effector MNK is an essential component in the hyper-production of TNF-α in LPS-stimulated mast cells. Our previous work suggested that the absence of ITK and BTK resulted in enhanced ERK activation in mast response to IgE-mediated antigen, without affect p38 activation (Iyer et al., 2011). While in the mast response to LPS, we observed impaired ERK and enhanced p38 activation, suggesting that the function of ITK and BTK in mast cells might be pathway specific.

ITK has been shown to interact with PI3K in T cells, and both ITK and BTK are directly downstream of PI3K, requiring PI3K activity for their activation (Fruman, 2004; Kuehn et al., 2008; Lu et al., 1998). However, BTK has been shown to differentially regulate B cell receptor signaling, distinct from PI3K immediately

downstream of the receptor, but share a common target in activating NF-κB further downstream (Suzuki et al., 2003). In IgE-mediated antigen induced signaling activity in mast cells, AKT activation, which lies downstream of PI3K, was not affected in the absence of BTK, while blocking PI3K activity by Wortmannin or LY294002 dampened BTK activation, in support of the previous findings that PI3K is upstream of BTK in mast cell antigenic response (Kuehn et al., 2008). However, the relationship between ITK/BTK and PI3K in cellular response to LPS remained largely undefined. Activation of PI3K pathway increases LPS-induced TNF-α production in mast cells (Hochdorfer et al., 2011). Our finding that LPS induced better activation of Akt in mast cells lacking ITK and BTK suggested a reciprocal regulation between ITK/BTK and PI3K.

The activation of lymphocytes and certain myeloid lineages in autoimmune diseases and hypersensitivity require the activation of ITK and BTK, making Tec kinase inhibitors promising selective targets for therapy (Di Paolo et al., 2010; Hendriks, 2011; Sahu and August, 2009). However, given the overall regulatory role of ITK and BTK in the mast cell response to LPS, these same inhibitors might exacerbate mast cell mediated diseases such as septic hypothermia. Due to the high homology structure of ITK and BTK, specific targeting may be difficult, hence mast cell function might be a critical issue in drug specificity and efficacy of those targeting Tec family kinases.

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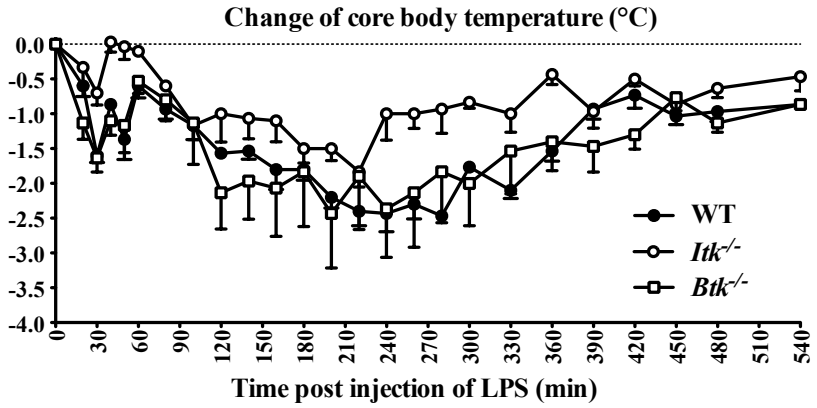
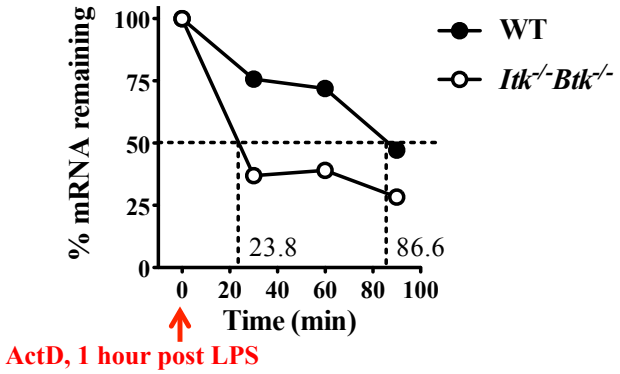
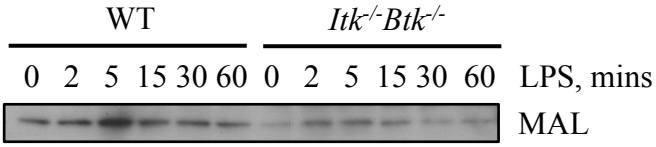


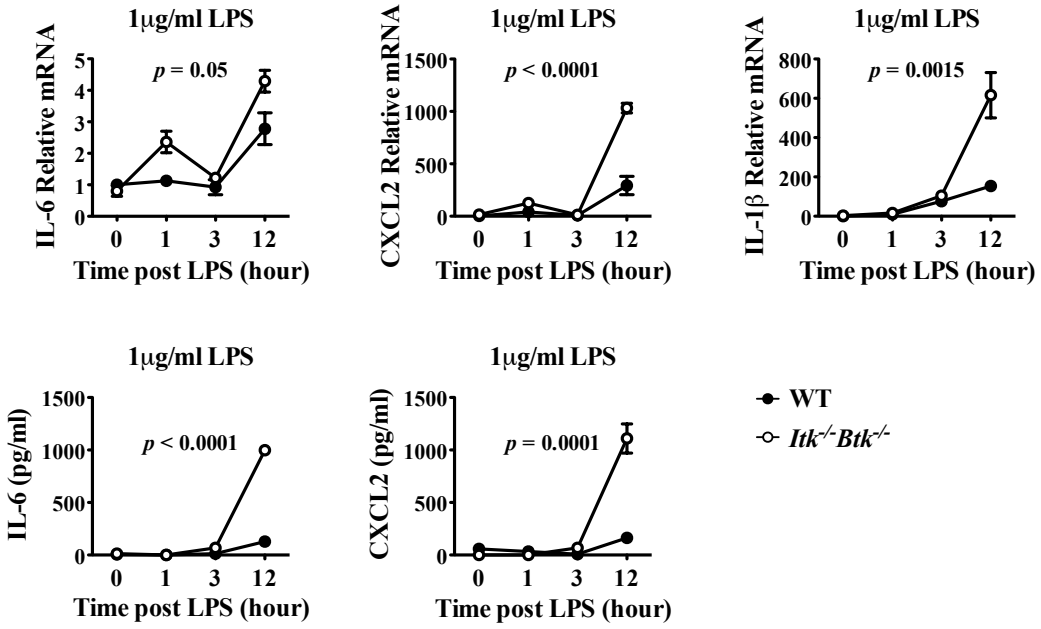
Supplemental Figure 6-1. The absence of ITK or BTK does not exacerbate LPS-induced hypothermia. (**A**) Change in core body temperature induced by LPS. n = 3.



Supplemental Figure 6-2. TNF-α mRNA is significantly less stable in *Itk*$^{-/-}$*Btk*$^{-/-}$ BMMCs compared to WT cells. BMMCs were stimulation with LPS for 1 hour, followed by addition of DMSO or Actinomycin D (ActD). TNF-α mRNA was measured every 0.5 hour, and normalized to the level in time 0 (1 hour post LPS). Half-life indicated was calculated based on the trend line generated by linear regression in the region where 50% mRNA falling in.



Supplemental Figure 6-3. No MAL accumulation is observed in the absence of ITK and BTK. The same membrane used in **Figure 6-4** was used to measure MAL expression. Control loading is GAPDH shown in **Figure 6-4**. Data represent results from ≥ 2 independent experiments.



Supplemental Figure 6-4. ITK and BTK negatively regulate LPS-induced pro-inflammatory cytokine expression. BMMCs were stimulated with 1 μg/ml LPS for indicated time. LPS-induced cytokine mRNA (upper panel) and secretion (lower panel) are presented. n = 2. *p* values by ANOVA.

CHAPTER SEVEN

Final discussion

Tec family kinase, ITK, has been one of significant interest during the past decade, in the fields of immunology and pharmacology, mainly because of its critical role in T cell development and function. In the absence of ITK, Th2- and Th17-mediated diseases such as allergic asthma and EAE are significantly attenuated (Douhan et al., 2008; Ferrara et al., 2006; Gomez-Rodriguez et al., 2009; Kannan et al., 2013a; Kannan et al., 2013b; Mueller and August, 2003), thus inhibition of ITK has been proposed as a therapeutic strategy against such inflammatory diseases. However, despite the defect in the development of pathogenic conventional Th2 and Th17 cells, innate memory phenotype (IMP) T cells are selectively developed in the absence of ITK (Hu and August, 2008; Hu et al., 2007). Hence it is urgent and necessary to understand the function of IMP T cells to achieve a comprehensive evaluation of the advantages and disadvantages of targeting ITK as a treatment strategy for inflammatory diseases.

Work presented in this dissertation shows that IMP $CD8^+$ T cells are very potent players in "primary" response to antigenic stimulation, with rapid IFN-γ and TNF-α secretion, which might be essential for clearance of infection ((Huang et al., 2013) as in chapter two); and that IMP $CD4^+$ T cells can suppress autoimmune graft-versus-host disease due to loss of donor MHCII function in syngeneic bone marrow transplantation (chapter five). These findings suggest that using inhibitors of ITK as a strategy can potentially increase the frequency of IMP T cells, to enhance IMP $CD8^+$ T cell-mediated immunity against infection and IMP $CD4^+$ T cell-mediated regulatory immune function, depending on the specific pathogenic conditions. One practical aim of interest in medicine would be to generate a protocol to enrich IMP T cells to be

used in, for example, infusion therapy. IL-4 was suggested to be responsible, *in vivo*, in inducing IMP $CD8^+$ T cells in the absence of ITK, in a T cell-extrinsic manner (Weinreich et al., 2010). However, we found that ITK intrinsically suppresses IMP-like $CD8^+$ T cell enrichment, in the presence of IL-4 (chapter three). Of note, the absence of ITK without IL-4 is not sufficient for development of IMP $CD8^+$ T cells. It is the combination of the absence of ITK and the presence of IL-4 that promotes IMP $CD8^+$ T cell enrichment. Mostly importantly, this combination significantly enhances IMP-like $CD8^+$ T cell enrichment *in vitro*, suggesting the possibility of using ITK inhibitors in the presence of IL-4 to differentiate IMP $CD8^+$ T cells *in vitro*, as a source of donor cells for infusion therapy. Similar cell-intrinsic effects of ITK on $CD4^+$ IMP T cell development have also been shown by a previous study (Hu and August, 2008). However, whether cytokines play any roles in collaboration with the lack of ITK to promote IMP $CD4^+$ T cell enrichment is not yet clear, and our work in progress (Huang and August, unpublished data) suggests that IL-4 is less critical in IMP $CD4^+$ T cell development, as $Itk^{-/-}Il4ra^{-/-}$ mice retain the high frequency of IMP $CD4^+$ T cells, whereas $Itk^{-/-}Stat6^{-/-}$ mice carry normal level (similar to WT) of IMP $CD4^+$ thymocytes. It is likely that other cytokines that utilize STAT6 signaling are involved in coupling with ITK deficiency to promote IMP $CD4^+$ T cell enrichment. It may be other Th2 cytokines such as IL-5 and IL-13 in the place of or in combination with IL-4 that can be candidates for future investigation. Nevertheless, the T cell-intrinsic function of ITK in suppressing IMP T cell differentiation, and potency of IL-4 in inducing $CD8^+$ T cell memory feature *in vitro*, suggest a promising cell culture protocol for clinical practice.

To a greater extent, the T cell-intrinsic function of ITK in suppressing $CD8^+$ T cell memory differentiation seems to be a general property, also applicable to memory-like $CD8^+$ T cell anti-tumor immunity development during lymphopenia-driven homeostatic proliferation (chapter four). This potentially expands the clinical spectrum for applications of ITK inhibitors. Infusion of tumor-reactive cells (derived and enriched against tumor antigen *in vitro*) following lymphodepletion induces marked tumor regression in metastatic melanoma patients, mainly because of the rapid repopulation of anti-tumor $CD8^+$ T cells in the lymphopenic condition (Dudley et al., 2002). In addition to the potential *in vitro* protocol in enriching memory-like $CD8^+$ T cells described above, ITK inhibition may also contribute to a more rapid expansion of the tumor-reactive infused cells *in vivo* through homeostatic proliferation in lymphopenic condition. More intriguingly, the lack of ITK function actually enhances HP $CD8^+$ anti-tumor immunity, independently of $CD4^+$ T cell help. $CD4^+$ T cells are critical helpers for $CD8^+$ T cells to acquire protective function in during homeostatic proliferation (Hamilton et al., 2006), but paradoxically, they also increase the of risk of developing autoimmunity in the lymphopenic or immunodeficient recipients (Dudley et al., 2002; Morrissey et al., 1993). In infusion therapy, inhibiting ITK may allow the exclusion of $CD4^+$ T cells in the donor composition (as $CD4^+$ T cells are not longer required to help the development of HP $CD8^+$ T cell anti-tumor immunity, in the absence of ITK), to develop effective HP $CD8^+$ T cell anti-tumor immunity against $CD4^+$ T cell-mediated autoimmunity.

Most of the ITK inhibitors that were reported or under investigation were designed to target the ITK kinase activity (Sahu and August, 2009; Vargas et al.,

2013). IMP T cell enrichment in the absence of ITK is mainly due to the lack of ITK kinase activity (Hu and August, 2008; Hu et al., 2007), thus ITK kinase inhibitors can serve to promote IMP T cell enrichment in the proposed protocol. Regarding HP $CD8^+$ T cell-related application, more research is required to determine whether ITK kinase activity is a proper target to ablate its T cell-intrinsic suppressive function in HP $CD8^+$ T cell expansion and anti-tumor immunity development. There are potential tools for investigating this, such as the murine model that expresses ITK kinase defective mutant (in T cells) on *Itk*$^{-/-}$ background. However, $CD8^+$ T cells developed in the absence of active ITK kinase function, acquire memory-like phenotype (Hu et al., 2007), which causes the same problem as *Itk*$^{-/-}$ mice do (insufficient number of naïve $CD8^+$ T cells can be isolated for related studies). Alternatively, naïve $CD8^+$ can be isolated from the *Itk*$^{-/-}$ OTI-*Rag*$^{-/-}$ mice, and transduced to re-express ITK mutants of the domain function of interest, including the kinase activity, although this approach is tedious and might introduce other interfering factors due to the transduction process. Currently, some work in progress is aiming to develop a murine model in which ITK kinase defect can be conditionally induced. Preliminary data show normal $CD8^+$ T cell composition in these mice, and operatable induction of ITK kinase defect to a satisfactory degree (Kannan and August, unpublished data). This model may serve as a critical tool in the near future to investigate the involvement of ITK kinase function in the development of various diseases, and to help better assess the value of targeting ITK as a treatment strategy.

In addition to the careful assessment of ITK kinase activity in T cell function and related diseases, developing ITK inhibitors with desired potency and specificity

against other kinases is also very important. Based on the ITK kinase domain crystal structure (bound with a potent broad-spectrum kinase inhibitor), it should be possible to design small molecules capable of distinguishing ITK from Src family kinases (SFKs), as both the size and shape of the ITK kinase pocket are distinct from those in SFKs (Brown et al., 2004). However, three of ITK kinase pocket-associated residues are highly conserved between ITK and BTK, while only one is unique for ITK (termed as ITK kinase gatekeeper or blocker) (Brown et al., 2004). Theoretically, this single gatekeeper would allow the development of ITK specific inhibitors (compared to BTK for example), however in fact, this is extremely difficult as other Tec kinases' covalent inhibitors may exploit ITK kinase pocket through hydrogen bonding interaction or hydrophobic binding (Brown et al., 2004; Lo, 2010; Sahu and August, 2009; Vargas et al., 2013). In addition, biological and biomedical tools to evaluate the specificity of ITK inhibitors are also quite limited, due to the high homology of Tec family kinases and their differential expression (in different cell types, as well as at different levels when expressed in the same cells).

ITK and BTK are expressed at similar levels in mast cells (Evans et al., 2009), and neither of them is required for mast cell differentiation *in vivo* and *in vitro*, making mast cell as an excellent model to evaluate kinase inhibitor specificity between ITK and BTK. We have used this system and described the regulatory function that is redundant between ITK and BTK in negatively regulating LPS induced TNF-α production and septic hypothermia (chapter six). Given the highly pro-inflammatory property of TNF-α and detrimental consequence of severe septic hypothermia, it is clearly important that kinase inhibitors developed are able to

distinguish ITK and BTK. However, with current progress in Tec kinase inhibitor development, it may be a bit much to expect the availability of inhibitors highly specific against ITK or BTK in a near future. Alternative strategies may be developed to compensate for the potential risk due to the simultaneous absence of ITK and BTK kinase activity. In mast cells, we showed that in the absence of ITK and BTK, PI3K, Akt and MNK1 inhibitors could reverse the LPS-induced TNF-α production and hypothermic response toward WT levels, and so might be considered as candidates to develop combinatorial treatment with ITK/BTK inhibitors to target T or B cell-mediated diseases.

It should be mentioned that we also have observed some distinct cell type-specific effects of the simultaneous absence of ITK and BTK in innate immune cell response to LPS (Huang and August, unpublished data), which may be due to the differential expression pattern of these kinases as well as the cell-specific signaling downstream of LPS/TLR4 ligation. For example, dendritic cells express BTK in significantly higher level compared to that of ITK (see Immunological Genome Project database: http://rstats.immgen.org/comparative/comparative_search.php) and use both MyD88 and TRIF-dependent signaling downstream of TLR4 (see review (Buer and Balling, 2003)); while mast cells carry ITK and BTK expression at similar level (Evans et al., 2009), and use only MyD88-dependent signaling (Keck et al., 2011). A more comprehensive understanding of the function of ITK and BTK in the innate immune system requires further investigation of other innate cells, such as macrophages, neutrophils, eosinophils and basophils.

Although ITK and BTK are highly conserved between mouse and human,

there could be differences in their associated immune functions in human compared to those in mice. Murine models are critical, but might not be sufficient to provide preliminary data to ensure an accurate clinical assessment on the therapeutic potential of ITK inhibition. For example, murine and human mast cells exhibit some species-specific differences (Bischoff, 2007; Bischoff and Kramer, 2007). Thus it is important to extend the study to human models to indicate whether pharmacological inhibition of ITK and/or other TFKs is of promise in clinical medicine.

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