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Naive and Innate Memory Phenotype CD4⁺ T Cells Have Different Requirements for Active Itk for Their Development

Jianfang Hu*† and Avery August²‡

The Tec family kinase Itk regulates the development of conventional and innate CD8⁺ T cells. However, little is known about the role of Itk in the development of CD4⁺ T cell lineages, although the role of Itk in the T cell activation and function is well defined. We show in this study that Itk null mice have increased percentage of CD62L⁺CD44⁺ memory phenotype CD4⁺ T cells compared with wild-type mice. These cells arise directly in the thymus, express high levels of transcripts for the T-bet and IFN-γ and are able to produce IFN-γ directly ex vivo in response to stimulation. Itk deficiency greatly decreases the number of CD4⁺ T cells with CD62L⁺CD44⁻ naive phenotype, but has no effect on the number of memory phenotype CD4⁺ T cells, indicating that the development of memory phenotype CD4⁺ T cells is Itk-independent. We further show that the development of the naive phenotype CD4⁺ T cells is dependent on active Itk signals and can be rescued by expression of Itk specifically in T cells. Our data also show that Itk is required for functional TCR signaling in these cells, but not for the innate function in response to IL-12/IL-18 or Listeria monocytogenes stimulation. These results indicate that CD62L⁺CD44⁻ “naive” CD8⁺ and CD62L⁺CD44⁺ “innate memory phenotype” CD4⁺ T cells may be independent populations that differ in their requirement for Itk signals for development. Our data also suggest that CD4⁺CD62L⁺CD44⁺ memory phenotype T cells have innate immune function. The Journal of Immunology, 2008, 180: 6544–6552.

Mature CD4⁺ single positive (SP) and CD8⁺ SP T cells arise from CD4⁺CD8⁻ double positive T cell precursors in the thymus. The development of CD4⁺ and CD8⁺ T cells can be influenced by the strength and duration of signals received through the TCR in double positive thymocytes (1, 2). In addition to conventional T cell lineages, double positive thymocytes also give rise to some other lineages of mature T cells, such as regulatory T cells and NKT cells (3–5), which are called nonconventional T cells. Studies of many knockout mice have identified proteins required for regulatory T cells and NKT cells, but less is known about signaling pathways leading to the specific development of CD4⁺ and CD8⁺ T cells.

The IL-2 inducible T cell kinase Itk is the predominant Tec kinase expressed in T cells and is activated downstream of the TCR (6–12). Specifically, Itk seems to act as an amplifier of TCR signals, and is required for the full activation of phospholipase-γ1, Ca²⁺ mobilization, and activation of transcription factors such as NFAT, NF-κB, and AP-1. These transcription factors activate a number of genes, including cytokine and other genes involved in cytokine signaling, survival, and differentiation (8–10, 13, 14). Thus, Itk can affect multiple processes important for T cell development, activation, and effector function (8). These affected processes include impaired positive selection (15, 16), altered CD4/CD8 commitment, defects in TCR-induced proliferation, IL-2 production, and reduced activation-induced cell death in the absence of Itk (8–10, 17).

We and others have recently shown that Itk is required for the development of conventional CD44⁺ “naive” CD8⁺ T cells but not CD44⁺CD44⁺ “innate memory phenotype” CD8⁺ T cells (18–21). In the absence of Itk, CD8⁺ T cells resemble activated or memory cells, express memory markers, carry high levels of preformed messages for IFN-γ and T-bet, the T-box expressed in T cells, and rapidly produce IFN-γ ex vivo in response to stimulation (18, 19, 21). These CD8⁺ T cells develop as a result of interaction with MHC molecules expressed on hematopoietic cells in the thymus (18). These properties suggest that these cells share properties with innate T cells such as H2-M3-specific CD8⁺ T cells, mucosal-associated invariant MAIT cells, or CD1d-specific NKT cells (5, 22–24). Although conventional CD8⁺ T cell development is abolished in the absence of Itk, the development of CD4⁺ T cell lineage seems less affected (18, 19), suggesting that Itk may play different roles in the development of CD4⁺ and CD8⁺ T cell lineage. In this study, we report that a higher percentage of CD4⁺ T cells in Itk null mice have a CD62 ligand (CD62L⁺CD44⁺ memory phenotype (MP)) and show effector function ex vivo in response to stimulation. Itk deficiency greatly decreases the number of CD4⁺ T cells with naive phenotype (NP), but has no effect on the number of NP MP CD4⁺ T cells. We also specifically show that active Itk signaling is required for these effects, and that Itk is required for function through the TCR signaling of these cells, but not for the innate function in response to IL-12/IL-18 or Listeria monocytogenes stimulation. These results indicate that CD62L⁺CD44⁺ naive and CD62L⁺CD44⁺ innate MP CD4⁺ T cells may include independent populations that differ in their requirement for Itk signals for development.

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3 Abbreviations used in this paper: SP, single positive; MP, memory phenotype; NP, naive phenotype; WT, wild type; CD62L, CD62 ligand.

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Materials and Methods

**Mice**

Wild-type (WT) and Itk-deficient (Itk−/−) mice used were 2 days to more than 12 mo of age and were kept in specific pathogen-free conditions. Tg(Leu-ItkΔKin)Itk−/− mice were generated previously in our lab and were backcrossed to the C57BL/6 background over 10 generations (21). Tg(CD2-IleItk)Itk−/− mice were generated by cloning a human Itk cDNA into a transgenic expression cassette driven by the CD2 promoter and CD2 enhancer. These mice were backcrossed over five generations. In both cases, the expression level of the transgene was roughly 30% of endogenous Itk as determined by quantitative RT-PCR. All mice were on the C57BL/6 background. All experiments were approved by the Institutional Animal Care and Use Committee at Pennsylvania State University.

**Abs and flow cytometry**

Cells were incubated for 30 min at 4°C with Abs in 100 μl of PBS/0.2% FBS, followed by two washes in PBS/0.2% FBS. The following Abs were purchased from BD Pharmingen and used as suggested by the manufacturer: anti-CD8α-FITC, CD44-CyChrome, CD122-PE, IFN-γ-FITC, CCR7-PE, CD127-PE, NK1.1-PE, and BrdU-FITC. Anti-CD62L-allophycocyanin Alexa Fluor 750 was purchased from eBioscience. Cells were analyzed using a FCS500 from Beckman Coulter.

**Quantitative real-time PCR analysis**

CD4+CD62Llow or CD4+CD62Lhigh T cells were sorted from the spleens of WT and Itk null mice using a Cytopeia Cell Sorter. Total RNA was prepared from sorted cells using RNAse Mini kit (Qiagen). cDNA was generated using You Prime First-Strand beads (GE Healthcare), and quantitative PCR was performed using primer/probe sets for IFN-γ, T-bet, and Eomesodermin (Applied Biosystems), with GAPDH as a housekeeping gene. Data were analyzed using the comparative threshold cycle ΔACT method and normalized to GAPDH and relative to a calibrator sample. The relative gene expression levels were then determined by comparing to the expression found in the WT CD4+CD62Llow populations, which were set as 1 or as indicated in the respective experiment.

**In vitro analysis of cytokine secretion**

Splenocytes were stimulated with 50 ng/ml PMA/0.5 μM ionomycin (PMA/ionomycin) for 6 h, or IL-2 (5 ng/ml) and IL-18 (10 ng/ml; MBL) overnight, in the presence of Brefeldin A (10 μg/ml) and analyzed for intracellular IFN-γ and cell surface CD4 and CD44 by flow cytometry.

**BrdU incorporation**

Mice were treated with BrdU in drinking water (dissolved at 0.8 mg/ml) for 9 days, with mice given fresh BrdU-containing drinking water daily. Splenocytes were collected and stained for surface marker using appropriate Abs, followed by washing in PBS and resuspending in ice-cold 0.15 M NaCl. The cells were fixed and permeabilized in ice-cold 95% ethanol for 30 min on ice, then washed with PBS and fixed again in 1% paraformaldehyde for 30 min at room temperature. To detect BrdU, the cells were spun down and resuspended in 1 ml of DNase I solution (50 U/ml DNase I in 4.2 mM MgCl2/0.15 M NaCl (pH = 5)) for 10 min at room temperature. The cells were then washed and resuspended in 100 μl of a 1/10 dilution of anti-BrdU-FTTC for 30 min at room temperature. The cells were washed and resuspended in 500 μl of PBS and analyzed by flow cytometry. For turnover analysis, the mice were treated with BrdU-containing water for 9 days and then treated with normal water for indicated days, and analyzed by the procedure described.

**Fetal thymic organ culture**

Fetal thymi were harvested from E16 pregnant females. Thymic lobes were placed in Transwell plates (Costar) with 2 ml of DMEM (containing 15% FCS) for the indicated time periods.

**Bone marrow chimeras**

Bone marrow was isolated from femurs and tibia of Thy1.1 WT and Itk−/− mice. A total of 1 × 10⁸ cells were injected into lethally irradiated congenic WT (Thy1.2) mice. Mice were analyzed 8 wk after reconstitution. To determine whether Itk−/− T cells can compete with WT T cells during development, a 1:1 mixture (5 × 10⁶ cells each) of bone marrow from Thy1.2/CD45.1 congenic WT and Thy1.2/CD45.2 congenic Itk−/− mice were injected into irradiated Thy1.1/CD45.2 WT mice. Analysis of donor-derived WT (Thy1.2/CD45.1) T cells and Itk−/− (Thy1.2/CD45.2) T cells followed by 6 wk of reconstitution.

**Proliferation**

Purified CD4⁺CD62Llow and CD4⁺CD62Lhigh T cells from WT and Itk−/− mice were stimulated at 2 × 10⁵ cells/well in triplicate with 1 μg/ml anti-CD3 or 1 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 for 3 days. Proliferation was measured by [3H]thymidine incorporation over the final 18 h.

**Bacterial infection**

To analyze T cell secretion of cytokine ex vivo following infection, mice were infected with 2 × 10⁵ CFU *L. monocytogenes* for 24 h, and splenocytes isolated and incubated in vitro with Brefeldin A (10 μg/ml) for a further 6 h, followed by analysis of intracellular IFN-γ as previously described (21).

**Statistical analysis**

Data were analyzed by Student’s t test, with a value of *p* < 0.05 considered statistically significant.

**Results**

**Increased percentages of CD4⁺CD62LlowCD44high T cells in Itk null mice**

We and others have recently shown that Itk is required for the development of conventional CD44high naive CD4⁺ T cells but not a CD44lowinhome population of CD4⁺ T cells (18–21). Similarly, Li and Berg (25) have observed that there is a higher proportion of CD4⁺ T cells with a MP in mice lacking Itk. To determine whether Itk affects the development of the CD4⁺ T cell lineage, we examined the CD4⁺ T cell lineages in the spleens of Itk−/− and WT mice by characterizing the expression of surface maturation markers, CD62L and CD44. We found that Itk−/− mice show a higher percentage of MP (for simplicity, we refer to cells carrying CD62Llow, CD44high, or CD62LlowCD44high as MP cells because similar results were observed using these markers) CD4⁺ T cells (Fig. 1A). We also found that CD4⁺ T cells with MP were present in the youngest mice (<1 mo) and the increase persists until over 12 mo of age (Fig. 1B). These data indicated that Itk regulates the development of CD4⁺ T cell lineage by altering the ratio of NP to MP CD4⁺ T cells.

**Homeostasis of naive and MP CD4⁺ T cells does not contribute to the increased percentage of MP cells in Itk null mice**

Maintenance of the size of the lymphocyte pool is critical for proper immune responses. When NP (for simplicity, we refer to cells carrying CD62Lhigh, CD44low, or CD62LlowCD44low as NP cells because similar results were observed using these markers) T cells are introduced into a lymphopenic compartment, they undergo homeostatic expansion, converting to a phenotype that resembles MP cells (26). It is possible that Itk null mice have altered T cell homeostasis, leading to increased percentage of MP T cells. To test this hypothesis, the turnover of CD4⁺ T cells in Itk−/− and WT mice was determined by examining CD4⁺ T cell incorporation of BrdU. Itk−/− and WT mice were fed with BrdU-containing water for 9 days, after which their CD4⁺ T cells in the spleen were analyzed for incorporation of BrdU. We found that the CD4⁺CD62Lhigh T cells incorporated little BrdU during this period, and CD4⁺CD62Llow cells from Itk−/− mice incorporated slightly less BrdU compared with cells from WT mice (Fig. 1C). We also found that the amount of BrdU incorporated into CD4⁺CD62Llow cells from both Itk−/− and WT mice was similar (Fig. 1C), indicating that a similar percentage of Itk−/− and WT CD4⁺CD62Llow T cells were actively incorporating BrdU over this time period. These data suggest that Itk null T cells do not have increased proliferation in vivo. Indeed, transferring NP WT
percentages of CD4 stained for CD4, CD44, CD62L expression and analyzed by FACS. The cells in WT and Itk null mice are shown.

The percentage of BrdU analyzed for CD4 and CD62L expression along with analysis for BrdU. BrdU-containing water for 9 days, and splenocytes were collected and CD4CD62L expression because the latter is not phosphonic environment (data not shown). To determine whether these MP CD4 T cells exhibit effector function ex vivo, we examined their ability to secrete IFN-γ upon stimulation. We found that a large proportion of the CD4CD44high population produces IFN-γ in response to PMA/ionomycin stimulation, whereas the CD4CD44low population did not secrete any IFN-γ during this period. CD4CD44high T cells from Itk/−/− mice behave similar to those from WT mice by rapid production of IFN-γ, with a similar percentage of WT as well as Itk/−/− CD4CD44high T cells making this cytokine (Fig. 2A). These results indicate that MP CD4 T cells exhibit effector function to secrete effector cytokines ex vivo.

We and others have found that MP CD8 T cells with innate function rapidly secrete IFN-γ upon stimulation because they carry large amounts of preformed messenger for this cytokine as well as the IFN-γ regulator T-bet (18, 19, 21). The ability of CD4CD44high but not CD4CD44low T cells to rapidly produce IFN-γ when stimulated suggests that these two subsets may differ in the expression of preformed IFN-γ message. We therefore analyzed mRNA from freshly isolated unstimulated CD4CD62Llow and CD4CD62Lhigh T cells from Itk/−/− and WT mice for preformed mRNA for IFN-γ and T-bet by real-time quantitative RT-PCR. We found that CD4CD62Llow T cells carry significantly higher levels of preformed messenger for IFN-γ, as well as higher levels of the transcription factor T-bet compared with the CD4CD62Lhigh T cells, although there was no difference between WT and Itk/−/− CD4CD62Llow T cells (Fig. 2B). Analysis of mRNA for Eomesodermin, a T-bet-related transcription factor, also revealed both WT and Itk/−/− CD4CD62Llow T cells expressed 3- to 4-fold more Eomesodermin than CD4CD62Lhigh cells (data not shown). These data indicated that the MP CD4 T cells have higher levels of T-bet and IFN-γ transcripts, which may contribute to the ability of these cells to rapidly secrete IFN-γ upon stimulation.

Phenotypic characterization of NP and MP CD4 T cells in Itk/−/− mice

To further characterize these two T cell populations, we examined them for expression of a variety of surface markers. As shown in Fig. 3, WT and Itk/−/− CD4CD44highCD62Llow T cells expressed similar levels of CD122, CCR7, and CD127, suggesting that these two populations were the same in the two strains of mice. There were differences in expression of specific markers between NP and MP T cells in the expression of CD122, CCR7, and CD127. Of interest is that the MP cell subset, but not the NP subset, also expresses low levels of NK1.1, the marker for NK and NKT cells. Also Itk/−/− mice have a smaller percentage of these cells than WT mice as previously suggested (27, 28)(data not shown). The small percentage of the MP CD4 T cells that are NK1.1 or α-galactosylceramide/CD1d tetramer-positive rules out the possibility that MP CD4 T cells are NK or NKT cells although these cells can
also carry preformed message for IFN-γ and rapidly secrete cytokine upon stimulation (28 and data not shown).

**MP CD4+ T cells develop in the thymus**

Our data show that Itk−/− mice have increased percentage of MP CD4+ T cells. One potential explanation for these results is that T cells that develop in the thymus migrated into a lymphopenia-like environment in the Itk−/− mice, during which they proliferated and up-regulated CD44 and down-regulated CD62L. This observation could result in the finding of higher percentages of MP CD4+ T cells in periphery of Itk−/− mice because a higher percentage of NP T cells would undergo lymphopenia-induced proliferation in these mice. However, we have already shown that Itk−/− T cells do not undergo increased homeostatic expansion. Another potential explanation is that these MP CD4+ T cells develop in the thymus and migrate out into the periphery, and that in the absence of Itk, more of these cells develop, or alternatively, less NP cells develop in the thymus resulting in the observed increased percentage of these cells in these mice. We therefore wanted to determine whether these cells originated in the thymus during T cell development. To examine this issue, we first analyzed CD4+ T cells in newborn mice from birth through the first week. We found that MP CD4+ T cells were present at 2 days after birth in both WT and Itk−/− mice (Fig. 4A). Furthermore, an increased percentage of CD4+CD44+CD62Llow was detected in Itk−/− mice compared with WT controls. The percentage of MP CD4+ T cells decreased at days 4 and 7 after birth in WT mice, whereas in Itk−/− mice, this percentage also decreased but remained elevated compared with WT mice. This finding suggests that MP CD4+ T cells develop in the thymus. We also characterized the expression of specific surface maturation markers that identify these cells. We found that CD4+ SP thymocytes in 6- to 8-wk-old Itk−/− mice exhibited a higher percentage of CD44high and CD122high populations than cells in WT mice, which suggests that Itk−/− mice contained a higher percentage of MP CD4+ SP thymocytes than found in WT mice (Fig. 4B). As previously reported, almost all CD8+ SP thymocytes in Itk−/− mice exhibit a MP (CD44highCD122high) (18–21). To further confirm this, we evaluated fetal thymic organ cultures from WT and Itk−/− mice in which T cell development occurs in vitro, ruling out potential re-circulation of already developed cells back into the thymus as would occur in the animal. Our results show that a higher percentage of CD4 SP T cells develop in the CD44highCD122high phenotype in Itk null fetal thymic organ cultures than in the WT cultures (Fig. 4C).
These data suggest that the MP CD4⁺ and CD8⁺ T cell compartments differ in some aspects of development because the percentage of the CD4⁺ compartment that had this MP was lower than the percentage seen in the CD8⁺ compartment, but they have similar phenotypes. These data also suggest that the increased percentage of MP CD4⁺ T cells observed in the absence of Itk reflects either enhanced development of these cells, or reduced development of NP CD4⁺ T cells.

Altered CD4⁺ lineage development in the absence of Itk is intrinsic to bone marrow-derived cells

To better understand whether the altered development of Itk⁻/⁻ CD4⁺ T cells was due to defects intrinsic to the developing T cells, we generated bone marrow chimeric mice in which WT and Itk⁻/⁻ bone marrow was injected into lethally irradiated WT congenic mice. After reconstitution, the percentage of CD4⁺ CD44highCD62Llow T cells in the spleen was clearly higher in mice reconstituted with Itk⁻/⁻ bone marrow compared with mice reconstituted with WT bone marrow (Fig. 5A). However, when we compared the number of CD4⁺ CD44highCD62Llow T cells in the spleen, we found similar numbers regardless of whether the mice received WT or Itk⁻/⁻ bone marrow (Fig. 5B). To further determine whether CD4⁺ CD44highCD62Llow MP T cells are indeed able to develop independently of Itk expression, we performed competitive mixed bone marrow chimera analyses to determine whether Itk null cells can effectively compete with WT cells in the same host for development to these two cell populations. Our results confirm that although development of Itk null CD4⁺ CD44highCD62Lhigh NP T cells was reduced compared with cells found in WT counterparts, development of CD4⁺ CD44highCD62Llow MP T cells was not affected, and an equal number of WT and Itk⁻/⁻ cells developed (Fig. 5, C and D). This finding indicates that Itk is not required for the development of CD4⁺ CD44highCD62Llow MP T cells, but is required for the development of CD4⁺ CD44lowCD62Lhigh NP T cells, and that the increased percentage observed in the absence of Itk is due to reduced development of the latter population. These data also suggest that these two populations of T cells are distinct and have distinct requirements for their development.

Tec kinase activity is required for the presence of NP CD4⁺ T cells, but not for the MP CD4⁺ T cells

Our data shows that Itk regulates the development of CD4⁺ T cells by changing the ratio of NP to MP CD4⁺ T cells, and suggest that these represent unique and separate populations of CD4⁺ T cells. Our data also shows that their development is intrinsic to bone marrow-derived cells. To further determine whether this process was intrinsic to the T cells and whether the Tec kinase signaling...
was involved, we examined the percentage and absolute number of these two CD4^+ T cell lineages in transgenic mice carrying Itk expressed in a T cell-specific manner (driven by the CD2 promoter, Tg(CD2-Itk)^+ mice), as well as transgenic mice carrying a mutant Itk lacking its kinase also expressed in a T cell-specific manner (Tg(Lck-ItkΔKin)^+ mice) (21). Tg(CD2-Itk)^- mice expressed low levels of Itk under the CD2 promoter (~25–30%); however, this was sufficient to significantly rescue the development of NP CD4^+ T cells, but did not have any effect on the number of MP CD4^+ T cells (Fig. 6). By contrast, analysis of the NP and MP CD4^+ T cells in Tg(Lck-ItkΔKin)^+ mice carrying the mutant Itk lacking its kinase domain instead of WT Itk revealed that these mice had NP and MP CD4^+ T cell populations similar to those seen in Itk null mice (Fig. 6). These data indicate that active signaling by Itk enhances the development of NP CD4^+ T cells, but has little effect on the development of MP CD4^+ T cells.

**FIGURE 6.** The development of MP CD4^+ but not NP CD4^+ T cells is independent of active Itk signaling. A. Splenectomy from WT, Itk^−/−, Tg(CD2-Itk)^+ and Tg(Lck-ItkΔKin)^+ mice were stained for CD4, CD44, CD62L expression and analyzed by FACS, with gating on CD4^+ populations. The percentage of CD4^+CD62L^lowCD44^hi and CD4^+CD62L^hiCD44^lo populations is shown. Data are representative of six mice from experiments with the same result. B. Total number of splenic CD4^+CD62L^lowCD44^hi and CD4^+CD62L^hiCD44^lo T cell populations from the same mice as in A (n = 3). **, p < 0.05 vs Itk^−/− CD4^+ CD62L^hi CD44^lo T cells; ***, p < 0.05 vs Itk^−/− CD4^+ CD62L^hi CD44^lo T cells.

Itk is required for TCR-induced but not innate signal-induced elaboration of MP CD4^+ effector function

Itk regulates signals emanating from the TCR (6–12). To determine whether the MP CD4^+ T cells require Itk for their proliferation through the TCR, we analyzed purified CD4^+CD62L^low and CD4^+CD62L^hi T cells for proliferative responses to anti-CD3 or anti-CD3/28 stimulation. The results show that both CD4^+CD62L^low and CD4^+CD62L^hi populations from Itk^−/− mice had less proliferation in response to anti-CD3 and anti-CD3/28 stimulation (Fig. 7A), which indicated that both populations of cells are dependent on Itk for TCR-induced proliferation. Similar results were found when we examined IFN-γ secretion, with only WT CD4^+CD62L^low but not Itk^−/− CD4^+CD62L^hi cells making this cytokine, although at much lower levels than seen with Fig. 2A (data not shown).

A role for MP CD8^+ T cells in the early innate response following infection with *L. monocytogenes* has been reported (29–31). Infection of macrophages with *L. monocytogenes* results in the secretion of IL-12 and IL-18, which together can induce the rapid
four hours later, splenocytes were harvested and CD4 WT and Itk null CD4 in the last 18 h (n for 3 days. Thymidine uptake was determined as a measure of proliferation by rapidly secreting IFN-cytogenes.

FIGURE 7. TCR activation, but not innate activation, of MP CD4+ TCR is Itk-dependent. A, CD4+CD62Llow and CD4+CD62Lhigh T cells from WT or Itk−/− mice were stimulated with anti-CD3 and anti-CD3/28 for 3 days. Thymidine uptake was determined as a measure of proliferation in the last 18 h (n = 3 mice). *, p < 0.05. B, Splenocytes from WT and Itk−/− mice were stimulated with IL-12 and IL-18 followed by analysis for intracellular IFN-γ on gated CD4+ T cells. Percentages shown indicate those CD4+ T cells secreting IFN-γ upon stimulation. Data are representative of six mice from experiments with the same results. C, WT and Itk−/− mice were infected with 2 × 10^7 CFU L. monocytogenes. Twenty-four hours later, splenocytes were harvested and CD4+CD44high T cells were analyzed for intracellular IFN-γ. Percentages shown indicate those CD4+ T cells secreting IFN-γ upon infection. Data are representative of six mice from experiments with the same results.

secretion of IFN-γ from MP CD8+ T cells. We have shown that MP CD8+ T cells respond to IL-12/IL-18 stimulation by rapidly secreting IFN-γ, and can also respond to infection with L. monocytogenes by rapidly secreting IFN-γ (21). We therefore determined whether MP CD4+ T cells could also respond to IL-12/IL-18 stimulation to secrete IFN-γ. Analysis of IFN-γ production in cells from WT and Itk−/− mice revealed that MP CD4+ T cells produced significant levels of IFN-γ, and similar percentages of WT and Itk null CD4+CD44high cells responded (Fig. 7B). Of note, PMA/ionomycin-stimulated cells, shown in Fig. 2A, reveal similar responses. Analysis of MP CD4+ T cells revealed that these cells (and a similar percentage in WT and Itk−/− mice) could also rapidly secrete this IFN-γ during infection with L. monocytogenes (Fig. 7C). These data suggest that Itk is required for TCR stimulation of MP CD4+ T cells, but not for their development or elaboration of innate immune function.

Discussion

In this study, we show that development of a population of CD4+ T cells that carry memory markers CD44highCD62Llow is independent of Itk expression, whereas the naive population of CD4+ T cells, CD44lowCD62Lhigh, are dependent on Itk for their development. Our data also suggest that these MP CD4+ T cells develop very early in the thymus and are not dependent on active Itk-mediated signals. These MP CD4+ T cells carry preformed message for IFN-γ and T-bet, and rapidly secrete this cytokine upon stimulation with IL-12 and IL-18. More importantly, this population rapidly secretes IFN-γ upon infection with L. monocytogenes, suggesting that they may participate in the early innate immune response. These data suggest that these cells represent an apparently separate subpopulation of CD4+ T cells from those with the NP.

Our data provide compelling evidence to support the existence of two independent subpopulations of CD4+ T cells: CD62LhighCD44low, which are an apparent NP, and CD62LlowCD44high, which are an apparent MP. We use the term apparent because these markers have traditionally been used to refer to these two populations. However, it is clear that they include distinct populations that have different requirements for development. The CD4+CD62LhighCD44low T cells resemble the NP T cell population, and lack preformed message for IFN-γ and T-bet, and do not rapidly secrete IFN-γ upon stimulation with PMA/ionomycin, IL-12/IL-18, or Listeria infection. This population is dependent on Itk expression and activity for its development. By contrast, the CD4+CD62LlowCD44high T cell population develops in an Itk-independent manner. Our data also show that the development of these two populations of CD4+ T cells is T cell-intrinsic because Itk null bone marrow also gave rise to these two populations in a cell-intrinsic manner when transferred into WT mice. The independence of these two cell populations is supported by the fact that they appear very early in T cell development. These two populations of cells were not significantly different in their expression of TCR Vβ3, Vβ5, Vβ6, or Vβ8, suggesting that they are not oligoclonally selected or expanded in vivo (data not shown), as in the case of NKT cells (32).

These populations also carry different cell surface markers that separate them phenotypically. Some of these markers can be clearly tied to their function, such as the expression of CD122 in cell lineages that can develop effector function before Ag encounter (33–35). Indeed, IL-15 has been shown to be critical for the elaboration of innate immune function. The CD4+CD62LlowCD44high T cells resemble the NP, but not for their development or elaboration of innate immune function. These features are shared by some nonconventional T cell lineages that can develop effector function before Ag encounter such as NKT cells (38). We note that it is unlikely that the...
cytokine secretion response that we observe is due to NKT cells because these cells comprise at most 10% of this MP CD4+ T cell population, but we get up to 45% of these MP CD4+ T cells secreting IFN-γ upon stimulation. In addition, the Itk−/− have a reduced percentage and number of NKT cells (27, 28 and data not shown). On the basis of these characteristics, CD62LlowCD44high CD4+ T cells should likely be included among these types of innate T cells, and we suggest the term innate MP CD4+ T cells.

The CD4+CD62LlowCD44high T cells with innate function are also distinct from the CD4+CD62LhighCD44low T cells in that they have different intracellular signaling requirements for development. As we show in this study, Itk deficiency greatly decreases the number of CD4+CD62LhighCD44high T cells, with no effect on the number of CD4+CD62LlowCD44high T cells, suggesting that Itk is not required for the development of the CD4+CD62LlowCD44high T cells. Although the development of CD4+CD62LlowCD44low high T cells is Itk-independent, our data also show, however, that Itk is still required for functional TCR signaling in these cells. Thus development and functional TCR activation of these cells have different signaling requirements.

We and others have previously reported that the development of “conventional” CD8+CD44lowCD122low CD4+ T cells is abolished in the absence of Itk (18–21). Our data show that the development of a similar population of CD4+ T cells is also affected in the absence of Itk, suggesting that Itk plays a critical role in the development of both CD8+ as well as CD4+ T cells that have NP CD44low. We have also shown that the MP CD8+ T cells observed in the absence of Itk share the same properties with a population in normal WT mice, and more importantly can function in an innate manner to rapidly secrete IFN-γ until naive T cells.

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Disclosures
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