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Memory phenotype CD8⁺ T-cells with innate function selectively develop in the absence of active Itk

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Summary

T cells with a memory like phenotype and possessing innate immune function have been previously identified as CD8⁺CD44^{hi} cells. These cells rapidly secrete IFN γ upon stimulation with IL-12/IL-18 and are involved in innate responses to infection with *Listeria monocytogenes*. The signals regulating these cells are unclear. The Tec kinase Itk regulates T cell activation and we report here that a majority of the CD8⁺ T cells in Itk null mice have a phenotype of CD44^{hi} similar to memory like innate T cells. These cells are observed in mice carrying an Itk mutant lacking the kinase domain, indicating that active Tec kinase signaling suppresses their presence. These cells carry preformed message for and are able to rapidly produce IFN γ upon stimulation in vitro with IL-12/IL-18, and endow Itk null mice the ability to effectively respond to infection with *L. monocytogenes* or exposure to LPS by secretion of IFN γ . Transfer of these cells rescues the ability of IFN γ null mice to reduce bacterial burden following *L. monocytogenes* infection indicating that these cells are functional CD8⁺CD44^{hi} T cells previously detected in vivo. These results indicate that active signals from Tec kinases regulate the development of memory like CD8⁺ T cells with innate function.

Keywords

Listeria monocytogenes; Itk; IFN γ ; IL-12

Introduction

Itk, a Tec family kinase, regulates signals emanating from the T cell Receptor [1–4]. Mice lacking Itk have reduced numbers of CD4⁺ T cells, and these cells exhibit reduced proliferation and IL-2 production in vitro and in vivo [3–5]. Itk also modulates the differentiation of helper T cells into Th2 cells, or alternatively, controls their production of Th2 cytokines such as IL-4 [6–8]. Indeed, infection of Itk deficient mice on a Balb/c background with parasites such as *S. mansoni* or *L. donovani*, results in the generation of a predominant Th1 response [4,6,7]. In agreement with these findings, Itk null mice are resistant to developing Th2 mediated allergic

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asthma [9,10]. By contrast, these mice are more susceptible to infection with the intracellular parasite *T. gondii* [2], as well as infection with VSV and LCMV [11,12].

T cells are generally characterized as naïve cells, generally identified as CD62L^{hi} and/or CD44^{lo} or memory cells, generally identified as CD62L^{lo} and/or CD44^{hi} (for review see [13, 14]). Recently, a population of CD8⁺ T cells with “innate” like function expressing a similar memory like phenotype has been identified [15,16]. These cells are CD8⁺CD44^{hi}, can rapidly secrete IFN γ upon stimulation with IL-12 and IL-18, and play important roles in the innate response against infections such as *L. monocytogenes*, or chronic infections with viruses such as γ Herpes virus [16–20] (for review see [21]). The origin of these cells or the regulation of their function is poorly understood.

During analysis of mice lacking Itk, it has been noted that these mice have increased percentage of memory like CD4⁺ as well as CD8⁺ T cells [22–24]. Most recently, the memory like CD8⁺ T cells were shown to be CD44^{hi}/CD122⁺, and to be restricted by MHC class 1b [22–25]. Whether these cells represent the previously described innate type memory T cells is unclear [16–20]. We report here that a majority of the CD8⁺ T cells in Itk null mice have a phenotype similar to memory like CD8⁺ innate T cells. These cells do not require active Tec kinase signaling for their development, carry preformed message for IFN γ , which they are able to rapidly produce upon stimulation with IL-12/IL-18. Finally, we show that Itk null mice exhibit enhanced response to infection with *L. monocytogenes* in vivo and that transfer of Itk null CD8⁺CD44^{hi} reduces bacterial burden in *L. monocytogenes* infected IFN γ null mice. These results indicate that active signals from Tec kinases regulate the development and function of memory like T cells with innate function.

Results

Increased presence of CD8⁺ T cells with memory like phenotype in Itk null mice

CD8⁺CD44^{hi} T cells which resemble memory cells represent an increased percentage of total CD8⁺ T cells in Itk null mice. This increased population of cells are present in the youngest mice tested (age <1 month) and the increase persists out to >12 months of age (Fig. 1a, shown for the spleen). The percentage of these cells is increased in the lymph nodes, blood and spleen of mice lacking Itk, suggesting that this is not dependent on preferential enrichment of these cells in specific tissues (data not shown). Expression of CD122, the IL-2R β chain, has been reported to define this population of memory like CD8⁺ T cells [26], and we found that the CD8⁺CD44^{hi} population in both WT and Itk null mice expressed CD122, although the Itk null mice carry a larger percentage of these cells (Fig. 1b). Analysis of the number of CD8⁺CD44^{hi} T cells in 6–8 week old WT and Itk null mice indicated that the Itk^{-/-} mice had significantly more of these T cells in the spleen (Fig. 1c). These data suggest that signals from Itk negatively regulate the development of CD8⁺ T cells having a memory like phenotype. Alternatively, Itk signals are required for the development of conventional CD8⁺ T cells expressing low levels of CD44 and in their absence, CD8⁺CD44^{hi} T cells predominate.

Tec kinase activity suppresses the presence of CD8 memory like T cells

We and others have reported that the kinase domain and/or activity of Itk is dispensable for specific functions of Itk in vitro [27–29]. To determine if this domain or active signaling by Itk is required for the presence of the memory like T cells, we analyzed mice carrying a mutant Itk lacking the kinase domain, replaced with GFP, instead of WT Itk (Itk Δ Kin_{Tg}/Itk^{-/-} mice). These mice had the memory like CD8⁺ T cells similar to those seen in Itk null mice (Fig. 2a). WT mice had significantly lower percentage of CD8⁺CD44^{hi}CD122⁺ than those found in Itk^{-/-} and Itk Δ Kin/Itk^{-/-} mice (Fig. 2b). These data indicate that active signaling by Tec

kinases reduces the development of these cells or enhances the development of conventional CD8⁺CD44^{lo} T cells.

Itk^{-/-} memory like CD8⁺ T cells carry preformed message for IFN γ

To determine if these memory like CD8⁺ T cells exhibit similar functional behavior in vitro, we analyzed their ability to rapidly secrete IFN γ upon stimulation. Comparison of IFN γ production by PMA/Ionomycin stimulated CD8⁺CD44^{hi} T cells in vitro indicates that the Itk null cells behave similar to those from WT mice in rapidly (within 6 hours) secreting this cytokine, with a similar percentage of the CD8⁺CD44^{hi} T cells responding from both WT and Itk^{-/-} mice (Fig. 3a). By contrast, CD8⁺CD44^{lo} T cells did not secrete any appreciable IFN γ in this time period.

A role for memory like CD8⁺CD44^{hi} T cells in the early innate response following exposure to LPS or infection with the intracellular bacteria *L. monocytogenes* has been previously reported [18]. Infection of macrophages by *L. monocytogenes* results in the secretion of IL-12 and IL-18 [30], and these cytokines can stimulate CD8⁺CD44⁺ memory like T cells to secrete IFN γ , thus activating macrophages for TNF- α production and bacterial clearance [19]. We therefore determined if this population is the same population that is expanded in mice lacking Itk. Analysis of IFN γ production in cells from WT and Itk null mice stimulated in vitro with IL-12 and IL-18 revealed that WT and Itk null CD8⁺CD44^{hi} secreted significant levels of this cytokine, and again a similar percentage of Itk null CD8⁺CD44^{hi} T cells responded compared to WT cells (Fig. 3b). Similarly, incubation of purified CD8⁺CD44^{hi} T cells from WT and Itk revealed that they had similar proliferative responses to IL-2, in agreement with their expression of CD122, while the CD8⁺CD44^{lo} population did not proliferate to any significant extent (Fig. 3c). This population of cells also rapidly secretes IFN γ in response to exposure to LPS in vivo, as revealed by injection of mice with LPS, followed by analysis of intracellular cytokine in CD8⁺CD44^{hi} T cells from WT and Itk null mice (data not shown). Since these cells were able to rapidly secrete IFN γ , we evaluated freshly isolated unstimulated CD8⁺CD44^{hi} T cells and found that these cells carry high levels of preformed message for IFN γ , as well as high levels of the transcription factor T-Bet (compared to the CD8⁺CD44^{lo} T cell population) (Fig. 3d). Thus these memory like CD8⁺ T cells with innate function are poised to rapidly secrete IFN γ .

Itk^{-/-} mice exhibit enhanced clearance of *Listeria* dependent on CD8⁺ T cells

To determine if this population of memory like T cells is involved in the response against *L. monocytogenes*, we infected WT and Itk null mice with low numbers of *L. monocytogenes* (2 $\times$ 10³ CFU), and analyzed the spleens for bacterial numbers after three days. We recovered approximately 2 fold lower bacteria from Itk null mice than from WT mice, suggesting that Itk null mice were better able to mount an innate immune response against *L. monocytogenes* (Fig. 4a). This is most likely due to the fact that Itk null spleens contained significantly percentage as well as higher numbers of CD8⁺CD44^{hi} T cells compared to WT mice (see Fig. 1c). When analyzed at 7 days post infection, we could not detect any bacteria in either WT or Itk null mice indicating that eventual clearance is similar in these mice (data not shown). Macrophages have been shown to be largely responsible for bacterial clearance, and we found that stimulation of splenic cells with heat killed or live *L. monocytogenes* resulted in significantly enhanced TNF- α production by Itk^{-/-} splenocytes (Fig. 4b). Examination of the cells secreting TNF- α revealed that F4/80⁺ macrophages are the only population in splenocytes that secrete TNF- α in response to *L. monocytogenes* stimulation (data not shown), and that a significantly higher percentage of the Itk^{-/-} macrophages secreted TNF- α upon stimulation (Fig. 4b). By contrast, peritoneal macrophages from the same mice secreted similar levels of TNF- α upon stimulation with heat killed *L. monocytogenes* (Fig. 4b). Note that Itk^{-/-} and WT mice have similar numbers of macrophages in the spleen and peritoneum. These

data suggest that splenic macrophages from *Itk* null mice are more responsive to *L. monocytogenes* stimulation, and that this response may contribute to the advantage that these mice have upon infection.

***Itk*^{-/-} memory T cells reduce bacterial burden in IFN γ null mice**

We reasoned that an increased numbers of CD8⁺CD44^{hi} memory like T cells with innate function in *Itk* null mice may be able to secrete elevated levels of IFN γ , resulting in increased macrophage activation in the spleen, but not in the peritoneum, where there are significantly less T cells. We therefore determined if the CD8⁺CD44^{hi} population of T cells in *Itk* null mice could secrete IFN γ during infection with *L. monocytogenes*. We found that CD8⁺CD44^{hi} T cells actively secrete IFN γ in vivo 24 hours after infection with *L. monocytogenes* (Fig. 4c).

Mice lacking IFN γ cannot reduce bacterial burden when infected with *L. monocytogenes* due to their inability to produce IFN γ critical for this event [17]. Indeed, *Itk* null mice had elevated levels of IFN γ in the serum during infection (Fig. 4d). CD8⁺ T cells are critical producers of IFN γ , and we found that antibody-mediated depletion of CD8⁺ T cells resulted in significantly reduced ability to clear this infection in both WT and *Itk* null mice, indicating that CD8⁺ T cells play a critical role in the early innate response to the bacteria (Fig. 5a). The majority of CD8⁺ T cells in the ITK null mice are of the CD44^{hi} phenotype, and we had found that ITK null mice were better at clearing the infection with *L. monocytogenes*. We therefore wanted to determine if these T cells could secrete IFN γ and reduce bacterial burden in IFN γ null mice, and determine if the ITK null CD8⁺ T cells are more effective in the anti-listeria response. We did this by determining if transfer of cells from the WT and ITK null mice could rescue antibacterial responses in the mice lacking IFN γ . Sorted total CD8⁺ T cells from WT and *Itk*^{-/-} mice were transferred into IFN γ ^{-/-} mice, which were subsequently infected with *L. monocytogenes*. Analysis of bacterial burden in the spleen of these mice indicated that while transfer of WT CD8⁺ T cells could reduce their bacterial burden, the difference was not statistically significant ($p=0.092$ vs. control IFN γ null mice) (Fig. 5b). By contrast, transfer of total CD8⁺ T cells from ITK null mice was able to significantly reduce the bacterial burden on the IFN γ null mice (Fig. 5b). We next determine if the CD8⁺CD44^{hi} T cells in WT and ITK null mice were equivalent in their ability to secrete IFN γ and reduce bacterial burden in the IFN γ null mice. We therefore transferred sorted CD8⁺CD44^{hi} T cells from WT and *Itk* null mice into mice lacking IFN γ , then infected them with *L. monocytogenes*. Analysis of bacterial burden in the spleen of these mice showed that both WT and *Itk* CD8⁺CD44^{hi} T cells could reduce their bacterial burden, indicating that the CD8⁺CD44^{hi} T cells seen in *Itk* null mice represent the population of cells previously described ([17,21], Fig. 5b). Altogether, these data show that the CD8⁺CD44^{hi} T cells that develop in mice lacking signals from *Itk* possess innate like immune function in response to IL-12/IL-18, LPS or bacterial exposure in vitro and in vivo.

Discussion

A population of T cells having a memory like CD8⁺CD44^{hi} phenotype has been shown to develop in the absence of classical class I molecules, and these cells possess innate immune function in their ability to rapidly secrete IFN γ in response to signals from IL-12 and IL-18, and to infection with *L. monocytogenes* [19]. More importantly, these cells can reduce bacterial burden in *L. monocytogenes* infected IFN γ null mice in an antigen non-specific manner early in the innate response against this bacteria [17]. Our work here demonstrates that in the absence of Tec kinase signals the relative percentage of a population of CD8⁺CD44^{hi}CD122⁺ T cells increases. However, these cells behave in the same manner as WT CD8⁺CD44^{hi}CD122⁺ T cells in rapidly secreting IFN γ in response to stimuli from IL-12/IL-18 and infection with *L. monocytogenes*, suggesting that Tec kinase signals are not required for this function. We also

find that these cells carry preformed message for IFN γ as well as T-bet, suggesting a mechanism for their ability to rapidly secrete this cytokine.

The origin of T cells with a CD8⁺CD44^{hi} phenotype is controversial. Activation of naïve T cells in vitro generates a population of cells that have this phenotype, and the presence of the CD44 marker on T cells has been used to identify previously activated T cells [31–33]. In addition different T cell memory populations have been identified based on expression of CD44 and CD62L, including effector memory and central memory T cells [34]. Thus the expression of the CD44 marker on CD8⁺ T cells seem to represent those cells that have been previously activated and may have effector or memory function.

Similar populations of CD8⁺CD44^{hi} T cells have been reported in mice lacking classical class I molecules and T cells with this phenotype appear to be class Ib restricted [16,22,35]. These T cells are reported to be thymic-independent in origin and carry CD122, which may distinguish them from memory T cells, although this distinction is not always clear since older studies on memory T cells did not examine this marker [36]. While it has been suggested that these cells appear due to stimulation by normal flora, they are also present in germ-free mice [37]. H2-M3 restricted T cells can have a similar CD8⁺CD44^{hi} phenotype and can rapidly respond to *L. monocytogenes* infection compared to antigen specific T cell responses, although this H2-M3 specific expansion of these cells peaks at around 7 days, while the secretion of IFN γ by cells with a similar phenotype in response to IL-12/IL-18 induced by LPS activated macrophages is much quicker [19,38–41].

However, transfer of naïve T cells into lymphopenic mice can also generate T cells bearing the CD44 marker, and these cells can take on an effector function. Similarly, individuals who have had a bone marrow transplant, and elderly individuals and mice have elevated percentages of T cells with a similar phenotype [42–45]. In addition, viral and other infections can lead to expanded populations of T cell bearing this phenotype [20]. The appearance of these cells may represent homeostatic expansion of T cells to fill niches left empty by irradiation (during bone marrow transplant), reduced T cell output (in elderly individuals), viral exhaustion of antigen specific T cells, expansion of class Ib restricted T cells, or may represent “true memory” cells. It is possible that the immune system uses such a system of expanding CD8⁺ T cells with this phenotype (i.e. during homeostatic expansion, cells take on an “innate-effector” phenotype) to ensure that time of lymphopenia does not result in an immunocompromised position.

It is possible that there are several distinct population of CD8⁺CD44^{hi} T cells that contain pools of true memory T cells as well as cells that have undergone rapid homeostatic expansion such as those seen in class Ib restricted T cells, in lymphopenic mice, in cases of infections or class Ib restricted T cells. The increased relative percentage of these cells in mice lacking Tec kinase signals may be the result of entry of CD8⁺ T cells into a lymphopenic compartment early in the development of these mice, leading to homeostatic expansion and the acquisition of this CD44^{hi}CD122⁺ phenotype. These cells would then have “innate-like” effector function with preformed IFN γ message and the ability to rapidly secrete this cytokine upon stimulation with IL-12/IL-18 or infection with *L. monocytogenes*. Alternatively, these cells may be selected on class Ib molecules, which result in homeostatic expansion of these T cells and the acquisition of the CD8⁺CD44^{hi}CD122⁺ phenotype.

Recently, Dubois et al, Broussard et al and Atherly et al reported finding similar populations of CD8⁺CD44^{hi}CD122⁺ T cells in mice lacking Itk [24] or Itk/Rlk [22,23], however, these investigators did not determine if these cells were functional in vivo, or related to the population of CD8⁺CD44⁺ T cells previously reported to rapidly respond to bacterial infection, LPS or IL-12/IL-18 in vitro and in vivo [16–19,21]. It is unlikely that these CD8⁺CD44^{hi}CD122⁺ T cells are memory T cells as the latter are usually CD122^{-/lo} [36]. Our data presented here suggest

that the memory-like CD8⁺CD44^{hi} T cell populations are equivalent in WT and *Itk* null mice, and represent an innate like population of T cells.

Our data also indicates that the increased serum IFN γ observed in *L. monocytogenes* infected *Itk*^{-/-} mice is likely to be due to the increased numbers of these CD8⁺CD44^{hi} T cells in these mice, since transfer of these cells into IFN γ null mice can reduce bacterial burden in these mice, indicating that T cell-derived IFN γ is sufficient for this event. This increase in IFN γ could lead to more effective macrophage activation and provide the *ITK* null mice with an advantage in the macrophage response to listeria. Interestingly, while the absence of Tec kinase signals results in an enhanced relative percentage of these cells, the “innate-type” function of these cell does not seem to be affected by the absence of *Itk* in that similar percentage of these cells produce IFN γ upon stimulation in vitro or in vivo compared to their WT counterparts. In addition, when equal numbers of WT and *Itk*^{-/-} CD8⁺CD44^{hi} T cells are transferred into IFN γ null mice, they behave similarly in being able to reduce bacterial burden. Given that T cell receptor mediated stimulation is reduced in the absence of *Itk*, these data suggest that the “innate” response of these cells is *Itk* independent.

Taken together our data indicate that in the absence of signals from Tec kinases such as *Itk*, there is an increased population of CD8⁺ T cells with a memory like phenotype and innate function that can rapidly secrete cytokine in vivo in response to bacterial infection as well as LPS.

Materials and Methods

Mice and bacterial infections

WT, *Itk* null [3], *Itk* Δ KinT_g/*Itk*^{-/-} and IFN γ ^{-/-} mice used were 1 week to greater than 12 months of age and were kept in specific pathogen free conditions. *Itk* Δ KinT_g/*Itk*^{-/-} were generated by cloning a mutant *Itk* with the kinase domain replaced with EGFP [29], into a transgenic expression cassette driven by the *Lck* proximal promoter and CD2 enhancer (kind gift of Dr. Anuradha Ray, University of Pittsburgh). These mice were backcrossed to the C57BL/6 background >10 generations. All other mice were on the same background. Experiments were approved by the IACUC at Pennsylvania State University. Mice (age matched 6–8 weeks old) were infected with erythromycin-resistant *L. monocytogenes* (2 $\times$ 10³ CFU, kind gift of Dr. Hao Shen, University of Pennsylvania) and sacrificed after 72 hours (similar results were observed after 24 and 48 hours). Bacterial CFU were determined by plating splenic extracts on BHI agar containing 5 μ g/ml erythromycin. To analyze T cell secretion of cytokine ex vivo following infection, mice were infected with *L. monocytogenes* (2 $\times$ 10³ CFU) for 24 hrs, and splenocytes isolated and incubated in vitro with Brefeldin A (10 μ g/ml) for a further 6 hours, followed by analysis of intracellular IFN γ as described below. Analysis of secretion of IFN γ in vivo was analyzed as previously described [46]. CD8⁺ T cells were depleted by injection of 1 mg. anti-CD8 mAb (YTS168.4, kind gift of Dr. Eric Harvill, Penn State University) and 24 hours later, infected with bacteria as described above, followed by analysis 3 days later. In experiments where CD8⁺CD44^{hi} T cells were transferred into IFN γ null mice, 3 $\times$ 10⁶ sorted CD8⁺CD44^{hi} T cells were transferred into these mice, then the mice were infected with 2 $\times$ 10³ CFU *L. monocytogenes*. Mice were analyzed at 3 days post infection.

Real-Time PCR analysis

CD8⁺CD44^{lo} or CD8⁺CD44^{hi} T cells were sorted from the spleens of WT and *Itk* null mice using a Cytopeia inFlux Cell Sorter (Cytopeia Inc., Seattle, WA), and quantitative RT-PCR performed using primer/probe sets for IFN- γ and T-bet, with GAPDH as a housekeeping gene as described [10]. Data was analyzed using the Comparative CT (threshold cycle) method and

normalized to GAPDH. The relative gene expression levels were then determined by comparing to the expression found in the WT CD8⁺CD44^{lo} populations, which were set at 1.

In vitro analysis of cytokine secretion and proliferation

Splenocytes were stimulated with 50 ng/ml PMA/0.5 μ M Ionomycin for 6 hours, or IL-12 (5 ng/ml, R&D Systems, Minneapolis, MN) and IL-18 (10 ng/ml, MBL, Watertown, MA) overnight, in the presence of Brefeldin (10 μ g/ml) and analyzed for intracellular IFN γ and cell surface CD8 and CD44 by flow cytometry using specific antibodies. Splenocytes or peritoneal macrophages were incubated with heat killed *L. monocytogenes* for 6 hours, and analyzed for TNF- α using a specific ELISA (R&D Systems) or intracellular TNF α in F4/80⁺ cells by flow cytometry. To analyze responses to IL-2, sorted CD8⁺CD44^{hi} from WT and Itk^{-/-} mice were stimulated at 1 $\times$ 10⁵/well in triplicate with recombinant murine IL-2 (250 U/ml) for 3 days and proliferation measured by H³-thymidine incorporation.

Data analysis

Statistical evaluation was conducted by using the Student *t* test with a probability value of *P* $\leq$.05 considered statistically significant.

Abbreviations Used

Itk, Interleukin-2 Inducible T cell kinase; Rlk, Resting Lymphocyte Kinase; T-bet, T-box expressed in T cells.

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Figure 1.

Increased percentage of CD8⁺CD44^{hi} memory phenotype T cells in mice lacking Itk. (a) Spleens from WT and Itk^{-/-} mice were analyzed for the percentage of CD8⁺CD44^{hi} cells over the indicated time frame. (b) Analysis of CD44 and CD122 expression on CD8⁺ T cells in WT and Itk null mice. (c) Number of CD8⁺CD44^{hi} T cells in the spleens of WT and Itk^{-/-} mice. (*p=0.0007, n=5).

Figure 2.

Active Tec kinase signaling regulates the presence of CD8⁺CD44^{hi} memory like T cells. (a) Spleens from WT, *Itk*^{-/-} and *Itk* Δ Kin_{Tg}/*Itk*^{-/-} mice were analyzed for the percentage of CD8⁺CD44^{hi}CD122⁺ cells. (b) Percentage of CD8⁺CD44^{hi} T cells that carry CD122 in the indicated mice (*p<0.05; NS. Not statistically significant vs. WT mice).

Figure 3.

CD8⁺CD44^{hi} T cells rapidly secrete IFN γ upon activation with IL-12/IL-18. (a) Splenocytes from WT and Itk^{-/-} mice were stimulated with PMA/Ionomycin followed by analysis for intracellular IFN γ in gated CD8⁺ T cells. (b) Similar experiments as described in (a) except that cells were stimulated with IL-12 and IL-18. Percentages shown in (a) and (b) indicate those CD8⁺CD44^{hi} that respond to stimulation (upper right) and those that did not respond (lower right). (c) Purified CD8⁺CD44^{hi} from WT and Itk^{-/-} mice were stimulated with IL-2 for 3 days and proliferation measured by H³-thymidine incorporation. (d) CD8⁺CD44^{hi} and CD8⁺CD44^{lo} T cells from WT and Itk^{-/-} mice were sorted by flow cytometry and message for IFN γ (left) and T-bet (right) analyzed by QRT-PCR. Data are corrected for GAPDH

expression and expressed as fold over the WT CD8⁺CD44^{lo} populations, which was set at 1.
*p<0.05, NS, not statistically significant vs. WT mice.

Figure 4.

CD8⁺CD44^{hi} T cells respond to infection with *L. monocytogenes* in vivo. (a) WT and *Itk*^{-/-} mice were infected with 2×10^3 CFU *L. monocytogenes* for 3 days and the number of bacteria in the spleen determined (* $p < 0.05$ vs. WT mice). (b) Splenocytes (left panels) or peritoneal macrophages (right panels) from WT and *Itk*^{-/-} mice were stimulated in vitro with HKLM and cells analyzed for the percentage of TNFα⁺F4/80⁺ macrophages by flow cytometry (top panels) or supernatants analyzed for TNFα by ELISA (bottom panels). (c) WT and *Itk*^{-/-} mice were infected with 2×10^3 CFU *L. monocytogenes*, and 24 hrs later, splenocytes harvested and CD8⁺CD44^{hi} T cells analyzed for intracellular IFNγ. Percentages shown indicate those CD8⁺CD44^{hi} that respond to stimulation (upper right) and those that did not respond (lower

right). (* $p < 0.05$ vs. WT mice). (d) WT and *Itk*^{-/-} mice were infected 2×10^4 CFU *L. monocytogenes* for 2 days and serum IFN γ determined by ELISA (* $p < 0.05$ vs. WT mice).

Figure 5.

CD8⁺CD44^{hi} T cells reduce bacterial burden in IFN γ null mice infected with *L. monocytogenes*. (a) CD8⁺ T cells were depleted from WT and Itk^{-/-} mice, and the mice infected with *L. monocytogenes* 24 hours later. Mice were then analyzed as described in figure 4. (b) Sorted total CD8⁺ T cells from WT and Itk^{-/-} mice were transferred into IFN γ ^{-/-} mice, which were subsequently infected with 2×10^3 CFU *L. monocytogenes* for 3 days and the number of bacteria in the spleen determined as in figure 4 (*p=0.092 for recipients of WT^{-/-} T cells vs. IFN γ ^{-/-} mice; **p<0.05 for recipients of ITK^{-/-} T cells vs. IFN γ ^{-/-} mice; **p<0.01 for recipients of WT T cells vs. ITK^{-/-} T cells). (c) Sorted CD8⁺CD44^{hi} T cells from WT and Itk^{-/-} mice were transferred into IFN γ ^{-/-} mice, which were subsequently infected with

2×10^3 CFU *L. monocytogenes* for 3 days and the number of bacteria in the spleen determined as in figure 4 (* $p < 0.05$ vs. $\text{IFN}\gamma^{-/-}$ mice).