ITK and IL-15 support two distinct subsets of CD8$^+$ T cells

Sigrid Dubois, Thomas A. Waldmann*, and Jürgen R. Müller*

Metabolism Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Contributed by Thomas A. Waldmann, June 22, 2006

CD8$^+$ T cells are commonly divided into naïve CD44^{lo}CD122^{lo} and "memory phenotype" CD44^{hi}CD122^{hi} cells. Here we show data suggesting that these two cell populations represent independent CD8$^+$ T cell subsets. Whereas IL-15$^{-/-}$ mice lack CD44^{hi}CD122^{hi} CD8$^+$ T cells, mice deficient in the kinase ITK lack CD44^{lo}CD122^{lo} cells among CD8$^+$ T cells. The same defects were observed during thymus development. CD44^{hi}CD122^{hi} cells were found among double-positive thymocytes and increased in frequency during CD8 development in wild-type mice. At the mature stage, IL-15$^{-/-}$ mice harbored virtually no CD44^{hi}CD122^{hi} CD8$^+$ thymocytes. In contrast, ITK$^{-/-}$ mice lacked CD44^{lo}CD122^{lo} CD8$^+$ cells at this stage. We generated mice with genetic deletions in both IL-15 and ITK and observed a severe reduction of all CD8$^+$ T cells. The two CD44^{lo}CD122^{lo} and CD44^{hi}CD122^{hi} CD8$^+$ T cell subsets differed in the periphery in that natural killer (NK) receptor expression was found only on CD44^{hi}CD122^{hi} CD8$^+$ T cells. This expression was paralleled by their ability to respond to both T cell receptor and NK receptor engagements. In contrast, CD44^{lo}CD122^{lo} CD8$^+$ T cells mounted stronger responses to T cell receptor stimulation but failed to recognize NK receptor ligands. Thus, whereas ITK-dependent CD44^{lo}CD122^{lo} CD8$^+$ T cells appear to represent conventional CD8$^+$ T cells, IL-15-dependent CD44^{hi}CD122^{hi} CD8$^+$ T cells may have functions in both adaptive and innate immunity.

thymic development | T cell activation | NK receptors

Distinct subsets of lymphocytes depend on IL-15 (1, 2). Mice with deletions in either IL-15 or its private receptor chain, IL-15Rα, harbor reduced numbers of natural killer (NK) cells, NKT cells, CD8$^+$CD44^{hi} T cells, T cell receptor (TCR)$\gamma^+\delta^+$ T cells, and intraintestinal CD8$\alpha^+\beta^-$ T cells (3, 4). In addition, IL-15$^{-/-}$ mice are unable to maintain antigen-specific CD8$^+$ memory T cells after immunization with viruses (5, 6). These defects point to functions of IL-15 in both adaptive and innate immunity.

Antigenic stimulation of naïve CD8$^+$ T cells induces the high expression of CD44 (7, 8), for which all CD44^{hi}CD8$^+$ T cells were termed "memory phenotype" cells. An injection of IL-15 into mice induces the expansion of these CD8$^+$CD44^{hi} T cells independent of antigenic stimulation (9, 10). It was concluded that IL-15 directly supports the maintenance of CD8$^+$ memory T cells. However, a number of other treatments also selectively increase the number of CD8$^+$CD44^{hi} T cells without antigenic stimulation that include "bystander" proliferation in response to poly I:C or LPS (11), proliferation after an injection of mature dendritic cells (12), or lymphopenia-induced proliferation after CD8$^+$ T cell transfer into irradiated hosts (13, 14). Because the presence of antigen should be a necessity for the generation of true memory cells, subsets of CD8$^+$CD44^{hi} T cells may have functions other than in CD8 memory.

Mice with a deletion in the tec kinase ITK have reduced numbers of both peripheral CD4$^+$ and CD8$^+$ T cells (15). Among the CD8$^+$ T cells that are present, the majority express high levels of CD44 and CD122 (16). The function of these cells has not been fully elucidated. Defects in CD8$^+$ T cells in ITK$^{-/-}$ mice include positive and negative selection, cytokine production, TCR engagement-induced proliferation, and reduced cytolytic activity against allogenic splenocytes and against virally infected cells (15).

Here we show that IL-15 and ITK support two distinct subsets of CD8$^+$ T cells that are present in both the thymus and the periphery. The two subpopulations of CD8$^+$ T cells appear to have independent functions in the periphery.

Results

CD8$^+$ T Cells in ITK$^{-/-}$ Mice Depend on IL-15. CD8$^+$ T cells in ITK$^{-/-}$ mice and in IL-15$^{-/-}$ mice are phenotypically different. In particular, CD8$^+$ T cells in ITK$^{-/-}$ mice express high levels of CD44 and CD122. In contrast, IL-15$^{-/-}$ CD8$^+$ T cells express low levels of CD44 and CD122. This difference could be caused by a dysregulation of both surface markers. Alternatively, ITK and IL-15 could support two distinct subsets of CD8$^+$ T cells. To distinguish between these possibilities, we determined whether the CD44^{hi}CD122^{hi} CD8$^+$ T cells in ITK$^{-/-}$ mice depend on IL-15. We initially injected the antibody Tmβ1, which inhibits the activity of transpresented IL-15 on the IL-2/15Rβ chain. One week after a 50-μg injection of Tmβ1, the percentage of peripheral blood CD8$^+$ T cells in ITK$^{-/-}$ mice was reduced to $<$15% compared with untreated wild-type mice (Fig. 1*A*). All of the remaining CD8$^+$ T cells expressed high levels of CD44 and CD122 (Fig. 1*A* and data not shown).

We then generated mice that are deficient in both ITK and IL-15. CD8$^+$CD44^{hi}CD122^{hi} T cells represent 10–30% of all CD8$^+$ T cells in young wild-type mice. As described previously (3) and as shown in Fig. 1*B* for blood, IL-15$^{-/-}$ mice had a strong reduction of CD8$^+$CD44^{hi}CD122^{hi} T cells. This reduction was accompanied by a decrease of the total peripheral CD8$^+$ T cell number by 40–50% (Table 1, which is published as supporting information on the PNAS web site). ITK$^{-/-}$ mice lacked CD8$^+$CD44^{lo}CD122^{lo} T cells, and their total CD8$^+$ T cell number was reduced to 20–30% compared with wild-type mice. These reductions point to the possibility of an independent regulation of the number of peripheral CD8$^+$CD44^{hi}CD122^{hi} T cells and CD8$^+$CD44^{lo}CD122^{lo} T cells. When mice with deficiencies in both ITK and IL-15 were analyzed, virtually no CD8$^+$ T cells were detected in peripheral tissues (Fig. 1*B* and Table 1). These data suggest that IL-15 and ITK support two distinct subpopulations of CD8$^+$ T cells with IL-15 supporting CD44^{hi}CD122^{hi} and ITK supporting CD44^{lo}CD122^{lo} CD8$^+$ T cells.

Generation of CD8$^+$CD44^{hi}CD122^{hi} T Cells in the Thymus. CD44^{lo}CD122^{lo} and CD44^{hi}CD122^{hi} CD8$^+$ T cells could represent different differentiation stages of the same naïve cell. Alternatively, both cell types could be derived independently during thymic development. To distinguish between these two possibilities we investigated whether the CD8$^+$ T cell defects in ITK$^{-/-}$ and IL-15$^{-/-}$ mice could be observed during thymic development.

We analyzed thymi from 3-week-old mice. The total number

Conflict of interest statement: No conflicts declared.

Abbreviations: SP, single-positive; DP, double-positive; TCR, T cell receptor; NK, natural killer.

*To whom correspondence may be addressed. E-mail: tawald@helix.nih.gov or muellerj@mail.nih.gov.

IMMUNOLOGY

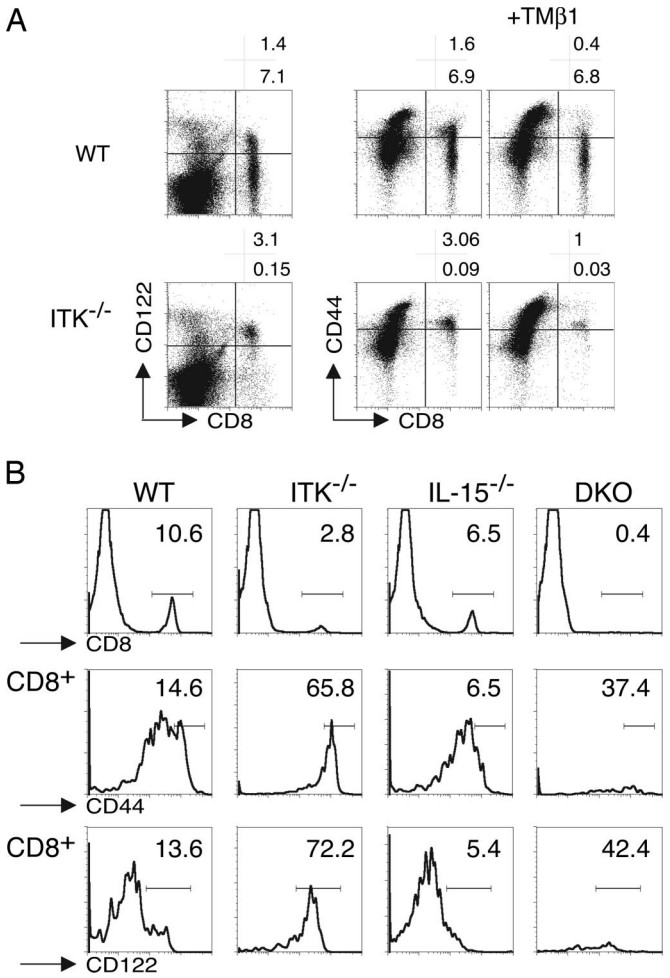

Fig. 1. $CD8^+$ T cells remaining in $ITK^{-/-}$ mice depend on IL-15. Cell samples were taken from blood and analyzed by FACS. (*A*) $ITK^{-/-}$ mice (*Lower*) harbor a slightly increased percentage of $CD44^{hi}CD122^{hi}$ $CD8^+$ T cells but lack $CD44^{lo}CD122^{lo}$ $CD8^+$ T cells compared with wild-type mice (*Upper*). Inhibiting IL-15 activity by injecting $ITK^{-/-}$ mice with Tmβ1 resulted in a reduction of the percentage of $CD8^+$ T cells (*Lower Right*). (*B*) Whereas both $ITK^{-/-}$ and $IL\text{-}15^{-/-}$ mice were characterized by reduced numbers of $CD8^+$ T cells (*Top*), mice with deficiencies in both genes (DKO) virtually lacked $CD8^+$ T cells. The expressions of CD44 and CD122 on $CD8^+$ T cells are shown in *Middle* and *Bottom* for each mouse. Numbers are shown as a percentages of gated cells. Data are representative of three mice each.

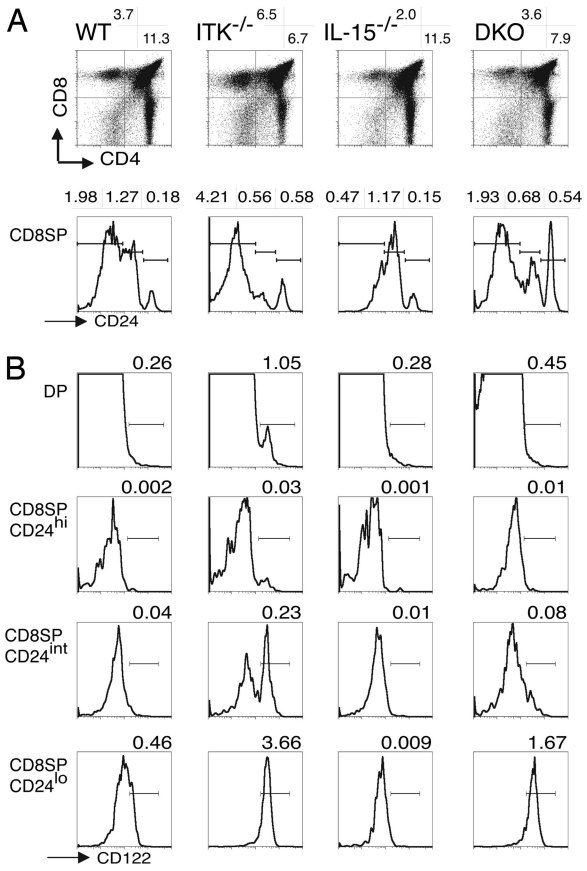

Fig. 2. Thymic developmental defects in $IL\text{-}15^{-/-}$ and $ITK^{-/-}$ mice. (*A*) Thymi from wild-type, $ITK^{-/-}$, $IL\text{-}15^{-/-}$, and $ITK^{-/-} \times IL\text{-}15^{-/-}$ (DKO) littermates were analyzed for their number of CD4- and CD8-expressing cells (*Upper*). *Lower* shows the $CD24^{hi}$, $CD24^{int}$, and $CD24^{lo}$ populations among CD8 SP thymocytes. All numbers represent percentages of thymocytes. (*B*) The expression of CD122 is shown for DP thymocytes and for the $CD24^{hi}$, $CD24^{int}$, and $CD24^{lo}$ populations of CD8 SP cells in samples from wild-type, $ITK^{-/-}$, $IL\text{-}15^{-/-}$, and $ITK^{-/-} \times IL\text{-}15^{-/-}$ mice. Data shown are representative of four independent analyses.

of cells differed slightly among wild-type, $ITK^{-/-}$, $IL\text{-}15^{-/-}$, and $ITK^{-/-} \times IL\text{-}15^{-/-}$ thymi (Table 1). As reported previously, thymi from $ITK^{-/-}$ mice contained more CD8 single-positive (SP) cells compared with wild-type mice (Fig. 2*A* and Table 1) (17). In contrast, IL-15 deficiency decreased the number of CD8 SP thymocytes. CD8 SP thymocytes are derived from CD4/CD8 double-positive (DP) cells and are commonly divided into $CD24^{hi}$-, $CD24^{int}$-, and $CD24^{lo}$-expressing cells, indicating successive maturation stages (18). Analyses of CD24 expression among CD8 SP thymocytes revealed a slight increase of the $CD24^{hi}$ population when $ITK^{-/-}$ thymi were compared with wild-type thymi (Fig. 2*A* and Table 1). In contrast, the absolute number of $CD24^{int}$ CD8 SP thymocytes was decreased to less than half in $ITK^{-/-}$ thymi compared with wild-type thymi and in $ITK^{-/-} \times IL\text{-}15^{-/-}$ compared with $IL\text{-}15^{-/-}$ thymi, suggesting a developmental defect at the $CD24^{hi}$-to-$CD24^{int}$ transition in the absence of ITK. Analyzing $CD24^{lo}$ CD8 SP thymocytes that are considered mature showed an increase in their number in $ITK^{-/-}$ mice compared with wild-type mice (Fig. 2*A* and Table 1).

To determine whether differences among $CD8^+$ T cells between $IL\text{-}15^{-/-}$ and $ITK^{-/-}$ mice that are seen in peripheral tissues are also found in the thymus, we studied the expression of CD122 during CD8 thymocyte development. In samples from wild-type mice, the percentage of $CD122^{hi}$ cells increased from 0.03% among DP thymocytes to 0.96%, 3.48%, and 21.5% among the $CD24^{hi}$, $CD24^{int}$, and $CD24^{lo}$ populations of CD8 SP cells, respectively (Fig. 2*B*, first column; numbers are not shown). Comparing $ITK^{-/-}$ mice with wild-type mice and comparing $ITK^{-/-} \times IL\text{-}15^{-/-}$ mice with $IL\text{-}15^{-/-}$ mice showed that ITK deficiency caused an increase in the absolute number of $CD122^{hi}$ cells at all stages of CD8 development (Fig. 2*B* and Table 1). At the mature $CD24^{lo}$ stage, $ITK^{-/-}$ thymi contained virtually no $CD122^{lo}$ CD8 SP cells. Comparisons of $IL\text{-}15^{-/-}$ mice with wild-type mice and comparisons of $ITK^{-/-} \times IL\text{-}15^{-/-}$ mice with $ITK^{-/-}$ mice revealed that IL-15 deficiency reduced the absolute number of $CD122^{hi}$ among CD8 SP cells at all stages of maturation (Fig. 2*B* and Table 1). At the mature $CD24^{lo}$ stage, $IL\text{-}15^{-/-}$ thymi contained virtually no $CD122^{hi}$ CD8 SP cells. Similar differences among wild-type, $ITK^{-/-}$, $IL\text{-}15^{-/-}$, and $ITK^{-/-} \times IL\text{-}15^{-/-}$ thymi were obtained if CD44 expression was analyzed instead of CD122 (data not shown).

To study whether the presence of $CD122^{hi}$ CD8 SP thymocytes was the result of $CD8^+$ memory cells that had migrated back to the thymus, we analyzed 1-week-old mice. All mice harbored few

peripheral CD8$^+$ T cells at this age (Fig. 5 and Table 2, which are published as supporting information on the PNAS web site). Thymi from 1-week-old mice also showed the presence of CD122^{hi} DP and CD8 SP cells that were comparable to those in older mice that were deficient in ITK or in IL-15. Taken together, ITK$^{-/-}$ mice manifest defects in the thymic development of CD44^{lo}CD122^{lo} CD8$^+$ T cells. In contrast, IL-15$^{-/-}$ mice are unable to generate mature CD44^{hi}CD122^{hi} CD8$^+$ thymocytes. The thymic presence of CD122^{hi} CD8 SP thymocytes at this early age is most consistent with the hypothesis that these cells are generated in the thymus rather than represent recirculated memory cells.

IL-15-Dependent CD44^{hi}CD122^{hi} CD8$^+$ T Cells Express NK Receptors. The presence of two subsets of CD8$^+$ T cells both in the thymus and in the periphery suggests different functions for CD44^{hi}CD122^{hi} and CD44^{lo}CD122^{lo} CD8$^+$ T cells. Functional differences between lymphocyte populations are often accompanied by changes in receptor expression patterns. We therefore determined the expression of additional surface markers that are known to characterize CD8$^+$ T cells at various stages of activation and differentiation. We used FACS to analyze total splenic CD8$^+$ T cells from ITK$^{-/-}$ and IL-15$^{-/-}$ mice and compared them with CD44^{hi}CD122^{hi} and CD44^{lo}CD122^{lo} CD8$^+$ T cells from wild-type mice.

As shown in Fig. 3, no expression difference was observed among wild-type, ITK$^{-/-}$, and IL-15$^{-/-}$ mice for CD25, CD69, and CD127 among splenic CD8$^+$ T cells. Whereas neither CD44^{hi}CD122^{hi} nor CD44^{lo}CD122^{lo} CD8$^+$ T cells expressed the activation markers CD25 and CD69, all CD8$^+$ T cells expressed CD127. In contrast, differences were found for both CD62L and CD45RB that are used to characterize memory cells (19, 20). Whereas CD44^{hi}CD122^{hi} and ITK$^{-/-}$ CD8$^+$ T cells contained a significant population that was negative for both markers, virtually all CD44^{lo}CD122^{lo} and IL-15$^{-/-}$ CD8$^+$ T cells expressed CD62L and CD45RB (Fig. 3).

The strongest difference between CD44^{hi}CD122^{hi} and CD44^{lo}CD122^{lo} CD8$^+$ T cells was found in the expression of NK receptors. None of the studied NK receptors, including NKG2A/C/E, NKG2D, Ly49C/F/H/I, and CD94, were detected on either CD8$^+$ T cells from IL-15$^{-/-}$ mice or wild-type CD44^{lo}CD122^{lo} CD8$^+$ T cells. In contrast, all analyzed NK receptors were expressed on subpopulations of ITK$^{-/-}$ and on wild-type CD44^{hi}CD122^{hi} CD8$^+$ T cells. The expression of selected surface markers shows that CD8$^+$ T cells from IL-15$^{-/-}$ mice are phenotypically similar to CD44^{lo}CD122^{lo} CD8$^+$ T cells from wild-type mice. In contrast, CD8$^+$ T cells from ITK$^{-/-}$ mice resemble normal CD44^{hi}CD122^{hi} CD8$^+$ T cells.

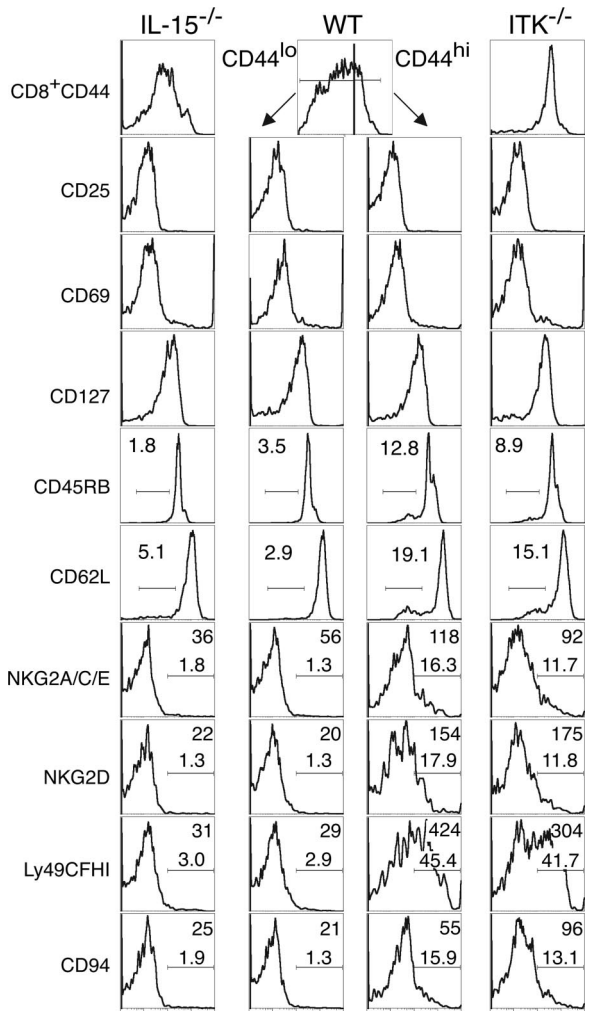

Fig. 3. Phenotypical characterizations of CD8$^+$ T cells from ITK$^{-/-}$ and IL-15$^{-/-}$ mice. Total spleen CD8$^+$ T cells from IL-15$^{-/-}$ (first column) and ITK$^{-/-}$ (fourth column) mice were compared with sorted CD44^{lo}CD122^{lo} (second column) and CD44^{hi}CD122^{hi} (third column) CD8$^+$ T cells from wild-type mice. Differences in the expression of CD45RB, CD62L, and various NK receptors indicate that whereas IL-15$^{-/-}$ CD8$^+$ T cells are phenotypically similar to wild-type CD44^{lo}CD122^{lo} CD8$^+$ T cells, CD8$^+$ T cells from ITK$^{-/-}$ mice resemble normal CD44^{hi}CD122^{hi} CD8$^+$ T cells. Numbers shown for CD45RB and CD62L are percentages of gated cells. For NK receptors, mean fluorescence intensities (upper numbers) and percentages of gated cells (lower numbers) are indicated.

IL-15-Dependent CD8$^+$CD44^{hi}CD122^{hi} T Cells Respond to both TCR and NK Receptor Engagement. Differences between CD44^{hi}CD122^{hi} and CD44^{lo}CD122^{lo} CD8$^+$ T cells that were observed in their NK receptor expression may indicate functional differences. To investigate, we compared responses to TCR and NK receptor engagement in CD8$^+$ T cells from ITK$^{-/-}$ and IL-15$^{-/-}$ mice to wild-type CD44^{hi}CD122^{hi} and CD44^{lo}CD122^{lo} CD8$^+$ T cells that had been sorted by FACS. As reported previously, TCR engagement via CD3 cross-linking induced an only weak proliferation in CD8$^+$ T cells from ITK$^{-/-}$ mice compared with IL-15$^{-/-}$ CD8$^+$ T cells (Fig. 4*A*) (17). This difference was less pronounced if cells were costimulated with anti-CD28. When CD44^{hi}CD122^{hi} CD8$^+$ T cells were compared with CD44^{lo}CD122^{lo} CD8$^+$ T cells from wild-type mice, differences similar to ITK$^{-/-}$ and IL-15$^{-/-}$ CD8$^+$ T cells in their proliferation response to TCR engagement were observed (Fig. 4*A*).

Similar results were obtained when CD25 induction was measured as a marker of activation after stimulation via CD3/CD28 cross-linking (Fig. 4*A*). Whereas more than half of IL-15$^{-/-}$ CD8$^+$ T cells and CD44^{lo}CD122^{lo} CD8$^+$ T cells showed an activated phenotype, <5% of either ITK$^{-/-}$ or CD44^{hi}CD122^{hi} CD8$^+$ T cells expressed CD25 12 h after CD3/28 cross-linking. These data suggest that CD44^{hi}CD122^{hi} CD8$^+$ T cells exhibit a more limited response to TCR engagement than do CD44^{lo}CD122^{lo} CD8$^+$ T cells.

To investigate responses to NK receptor engagement we used two *in vitro* systems. An antibody to NKG2D has been reported to activate NK cells (21). Fig. 4*B* shows that the activation of ITK$^{-/-}$ CD8$^+$ T cells and wild-type CD44^{hi}CD122^{hi} CD8$^+$ T cells was increased by a 16-h incubation with plate-bound anti-NKG2D in addition to anti-CD3. In a second *in vitro* system we studied the response of CD8$^+$ T cells to the melanoma cell line B16. These cells do not express MHC class I unless treated with IFN-γ. An additional expression of the NKG2D ligand Raet1 was achieved via transient transfection. When wild-type CD8$^+$ T cells were exposed to B16 for 12 h, a significant CD69

IMMUNOLOGY

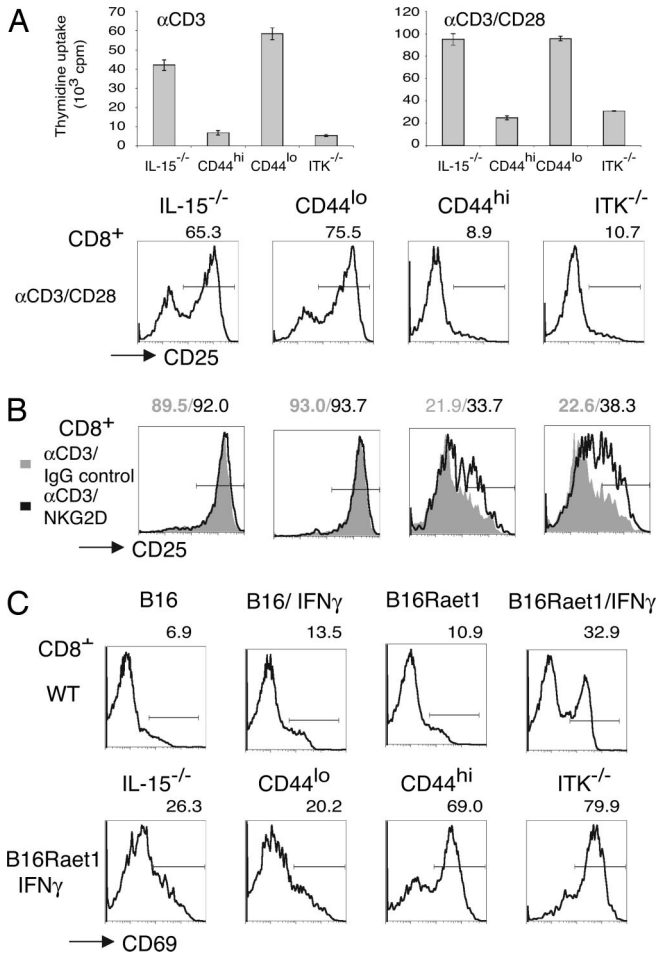

Fig. 4. Activation of CD8$^+$ T cells by TCR and NK receptor engagement. (*A*) Thymidine uptake was determined as a measure of proliferation in response to plate-bound anti-CD3 or anti-CD3/CD28 by using CD8$^+$ T cells from ITK$^{-/-}$ or IL-15$^{-/-}$ mice and CD44^{hi}CD122^{hi} and CD44^{lo}CD122^{lo} CD8$^+$ T cells from wild-type mice (*Upper*). Whereas strong responses were observed for IL-15$^{-/-}$ and wild-type CD44^{lo}CD122^{lo} CD8$^+$ T cells, the proliferation response was decreased in ITK$^{-/-}$ and wild-type CD44^{hi}CD122^{hi} CD8$^+$ T cells. *Lower* shows the CD25 induction as a measure of activation of IL-15$^{-/-}$, wild-type CD44^{lo}CD122^{lo}, wild-type CD44^{hi}CD122^{hi}, and ITK$^{-/-}$ CD8$^+$ T cells 12 h after anti-CD3/CD28 stimulation. (*B*) The CD25 induction in response to plate-bound anti-CD3 (gray areas) and anti-CD3/anti-NKG2D (solid lines, 16 h) is shown for the same CD8$^+$ T cells. (*C*) Wild-type CD8$^+$ T cells were coincubated with B16 cells for 12 h. Significant activation as measured by CD69 induction was detected if B16 expressed both MHC class I (induced by IFN-γ) and the NKG2D ligand Raet1 (induced by transfection) (*Upper*). *Lower* shows that significant activation in response to MHC class I/Raet1-expressing B16 cells was observed in ITK$^{-/-}$ and wild-type CD44^{hi}CD122^{hi} CD8$^+$ T cells but not in IL-15$^{-/-}$ and wild-type CD44^{lo}CD122^{lo} CD8$^+$ T cells after a 12-h coincubation.

induction was observed only in the presence of both IFN-γ-induced MHC class I and Raet1 on B16 (Fig. 4*C*). When CD8$^+$ T cells from different sources were coincubated with IFN-γ-treated and Raet1-transfected B16 cells, the majority of ITK$^{-/-}$ CD8$^+$ T cells and wild-type CD44^{hi}CD122^{hi} CD8$^+$ T cells showed an activated phenotype. In contrast, only ≈20% of IL-15$^{-/-}$ CD8$^+$ T cells or wild-type CD44^{lo}CD122^{lo} CD8$^+$ T cells expressed CD69. Taken together, CD44^{hi}CD122^{hi} CD8$^+$ T cells are able to respond to both TCR and NK receptor engagement. In contrast, CD44^{lo}CD122^{lo} CD8$^+$ T cells exhibit a stronger response to TCR engagement than do CD44^{hi}CD122^{hi} CD8$^+$ T cells. These response patterns suggest functional differences between the two subsets of CD8$^+$ T cells.

Discussion

IL-15$^{-/-}$ mice are characterized by a lack of CD8$^+$CD44^{hi}CD122^{hi} T cells (4), and ITK$^{-/-}$ mice have reduced numbers of CD8$^+$CD44^{lo}CD122^{lo} T cells (15). In this study we show data suggesting that CD44^{hi}CD122^{hi} and CD44^{lo}CD122^{lo} cells represent two independent subpopulations of CD8$^+$ T cells. This conclusion is based on several observations. First, the two populations of CD44^{lo}CD122^{lo} and CD44^{hi}CD122^{hi} CD8$^+$ T cells can be detected during normal thymic development. Defects of ITK$^{-/-}$ and IL-15$^{-/-}$ mice that are described for peripheral CD8$^+$ T cells are also found in the thymus: ITK$^{-/-}$ thymocytes do not include mature CD44^{lo}CD122^{lo} CD8 SP cells in parallel, and few mature CD44^{hi}CD122^{hi} CD8 SP thymocytes can be detected in IL-15$^{-/-}$ mice. This finding suggests the thymic generation of two lineages of CD44^{lo}CD122^{lo} and CD44^{hi}CD122^{hi} CD8$^+$ T cells that depend on ITK and IL-15, respectively.

The thymic presence of CD44^{hi}CD122^{hi} CD8$^+$ T cells has been reported previously (22). Urdahl *et al.* (22) argued that the activated phenotype of MHC class Ib-restricted thymocytes was a consequence of thymic rather than peripheral interactions. The data shown here also suggest the conclusion that the expression of CD44 and CD122 can be acquired in the thymus. (*i*) CD122^{hi} CD8 SP cells were detected among CD24-expressing cells, and peripheral CD8$^+$ T cells lack CD24 expression. (*ii*) Three-week-old ITK$^{-/-}$ mice harbor few peripheral CD44^{lo}CD122^{lo} CD8$^+$ T cells or even mature CD44^{lo}CD122^{lo} CD8 SP thymocytes. If the presence of CD44^{hi}CD122^{hi} CD8 SP thymocytes had resulted from a migration of CD8 memory cells into the thymus, the presence of CD44^{lo}CD122^{lo} CD8$^+$ T cells would be a prerequisite for the generation of memory cells. (*iii*) CD44^{hi}CD122^{hi} CD8 SP thymocytes were detected in 1-week-old mice that had few peripheral CD8$^+$ T cells. (*iv*) CD8 SP thymocytes were detected in ITK$^{-/-}$ × IL-15$^{-/-}$ mice that virtually lack peripheral CD8$^+$ T cells. This finding suggests the thymic generation of CD44^{hi}CD122^{hi} CD8 SP thymocytes and their subsequent loss after thymic egress in ITK$^{-/-}$ × IL-15$^{-/-}$ mice. Collectively these data suggest that CD44^{hi}CD122^{hi} thymocytes are not the result of recirculated memory cells but rather represent a naïve CD8$^+$ T cell lineage.

Peripheral CD8$^+$ T cells from IL-15$^{-/-}$ and ITK$^{-/-}$ mice are phenotypically different. IL-15$^{-/-}$ CD8$^+$ T cells are indistinguishable from wild-type CD44^{lo}CD122^{lo} CD8$^+$ T cells, and CD8$^+$ T cells from ITK$^{-/-}$ mice resemble CD44^{hi}CD122^{hi} CD8$^+$ T cells. Of particular interest is the expression of NK receptors in IL-15-dependent CD44^{hi}CD122^{hi} CD8$^+$ T cells that was not observed in CD8$^+$CD44^{lo}CD122^{lo} T cells. The detection of these receptors actually classifies at least a subpopulation of IL-15-dependent CD44^{hi}CD122^{hi} CD8$^+$ T cells as NKT cells. Among the NK receptors, we were able to detect expression of NKG2D, NKG2A/C/E, CD94, and Ly49 proteins. Based on their ligands, the expression of these receptors implies a sensitivity of CD44^{hi}CD122^{hi} CD8$^+$ T cells to stress-induced NKG2D ligands as well as a sensitivity to changes in the levels of MHC class I that is recognized by both CD94/NKG2A/C/E heterodimers and Ly49 proteins.

CD44^{lo}CD122^{lo} and CD44^{hi}CD122^{hi} CD8$^+$ T cells differ in their activation patterns. Both CD8$^+$ T cells from IL-15$^{-/-}$ mice and wild-type CD44^{lo}CD122^{lo} CD8$^+$ T cells responded more strongly to TCR engagement by proliferation and by CD25/69 up-regulation than did CD8$^+$ T cells from ITK$^{-/-}$ mice and wild-type CD44^{hi}CD122^{hi} CD8$^+$ T cells. In contrast, responses to NK receptor activation were detected only in CD8$^+$ T cells from ITK$^{-/-}$ mice and in wild-type CD44^{hi}CD122^{hi} CD8$^+$ T cells. These *in vitro* data suggest functional differences between the two cell types.

Our data suggest that the effect of ITK deficiency on $CD8^+$ T lymphocytes is predominantly caused by defects in thymic development rather than in peripheral cells. A number of differences between peripheral $CD8^+$ T lymphocytes from wild-type and $ITK^{-/-}$ mice have been described that include TCR engagement-induced proliferation, cytokine production, ERK, and nuclear factor of activated T cells activation, among others (15). We repeated several of these experiments and observed that the responses were identical if $ITK^{-/-}$ cells were compared with $CD44^{hi}$ $CD8^+$ T cells from wild-type mice (unpublished observations). Together with data presented in this article, this finding seems to indicate that most of the described differences between wild-type and $ITK^{-/-}$ $CD8^+$ T cells appear to be caused by comparisons of different cell types rather than represent true intrinsic defects caused by a lack of ITK in peripheral $CD8^+$ T cells.

Our data suggest different functions for $CD44^{hi}CD122^{hi}$ and $CD44^{lo}CD122^{lo}$ $CD8^+$ T cells. We believe that the ITK-dependent subpopulation of $CD44^{lo}CD122^{lo}$ $CD8^+$ T cells represents conventional $CD8^+$ T cells that are involved in adaptive immunity. In contrast, IL-15-dependent $CD44^{hi}CD122^{hi}$ $CD8^+$ T cells are able to respond to both TCR and NK receptor stimulation. As such, they may be part of both adaptive and innate immunity. These $CD44^{hi}CD122^{hi}$ $CD8^+$ T cells may be identical to NKT cells that have been described in autoimmune diseases such as celiac disease in humans (23, 24). Their less vigorous negative selection that was observed in $ITK^{-/-}$ mice may explain their involvement in autoimmune reactions (25). A similar cell type was described in cultures that use high concentrations of IL-2 or IL-15 (26, 27). Similar to our results, these cultured cells responded to cells that expressed NK receptor ligands. These $CD44^{hi}CD122^{hi}$ $CD8^+$ T cells may have functions in the immunosurveillance of tumor cells. Teague *et al.* (28) described the ability of IL-15-cultured $CD8^+$ T cells to inhibit tumor growth. In their interpretation, IL-15 overcame immunotolerance mechanisms. As an alternative, a culture in IL-15 may selectively propagate $CD44^{hi}CD122^{hi}$ $CD8^+$ T cells that, based on their less vigorous negative selection, may recognize tumor-specific epitopes that are not recognized by conventional $CD8^+$ T cells.

In summary, our data suggest that two distinct subpopulations of $CD8^+$ T cells exist that depend on either ITK or IL-15 for their development. Whereas ITK supports conventional $CD44^{lo}CD122^{lo}$ $CD8^+$ T cells, IL-15-dependent $CD44^{hi}CD122^{hi}$ $CD8^+$ T cells appear to have functions in both adaptive and innate immunity.

Materials and Methods

Mice. C57BL/6 and C57BL/6-IL-$15^{-/-}$ mice were provided by Taconic (Hudson, NY). C57BL/6-$ITK^{-/-}$ mice were kindly provided by P. Schwartzberg (National Institutes of Health, Bethesda, MD). Tmβ1 (BD Biosciences, San Diego, CA) treatment was done by i.p. injection, and mice were analyzed 1 week after treatment. Comparisons among wild-type, $ITK^{-/-}$, IL-$15^{-/-}$, and $ITK^{-/-} \times$ IL-$15^{-/-}$ mice were done with littermates. All mice were cared for under protocols approved by the National Cancer Institute Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

Cytometry and Cell Sorting. Antibodies that were used for cytometry were from BD Biosciences, except antibodies recognizing CD25, CD122, CD127, and NKG2D, which were purchased from eBioscience (San Diego, CA). Blood cells were analyzed after removing erythrocytes by using Ficoll centrifugation. Erythrocytes were removed from spleen cell suspensions by lysis in ACK. For cytometry analyses, cells were blocked with a mixture of rat IgG1, IgG2a, and IgG2b, mouse IgG1, and hamster IgG1 for 15 min on ice that was followed by a 30-min incubation on ice with the specific antibody. $CD8^+$ cells were sorted from spleen and lymph node by using the $CD8^+$ T cell isolation kit (negative sorting; Miltenyi Biotec). $CD44^{lo}$ and $CD44^{hi}$ $CD8^+$ T cells were sorted by FACS.

Proliferation Assay. Antibodies were bound to 96-well plates (10 μg/ml each in PBS at 4°C for 24 h). $CD8^+$ T cells were plated into 96-well plates at 5×10^4 per well. Cells were incubated for 48 h. [^{3}H]Thymidine [1 μCi (1 Ci = 37 GBq); Amersham, Piscataway, NJ] was present during the final 12 h of the assay.

Activation Assays. To detect activation by antibody-mediated receptor cross-linking, antibodies (5 μg/ml each) were bound to 12-well plates as described above. $CD8^+$ T cells were plated into 12-well plates at 2×10^5 per well in RPMI medium 1640 supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and antibiotics. The surface expression of CD25 and CD69 was determined on $CD8^+$ T cells 12 or 16 h later.

For activation induced by the syngeneic melanoma cell line B16, the NKG2D ligand Raet1 was amplified by RT-PCR, cloned, sequenced, and inserted into pCDNA3.1 (Invitrogen, Carlsbad, CA). This mammalian expression vector and a control vector were transfected into B16 by using Lipofectamine 2000 (Invitrogen). Transfected cells were selected for 2 days in G418 (600 μg/ml) and plated into 12-well plates at 1×10^5 per well. Murine IFN-γ (20 ng/ml; Peprotech, Rocky Hill, NJ) was added after 24 h. After 48 h, IFN-γ was removed by three washes in PBS, and 2×10^5 $CD8^+$ T cells were added. The surface expression of CD25 and CD69 was determined on $CD8^+$ T cells after 12-h coincubations.

We thank Dr. S. Sharrow for cell sortings and Dr. P. Schwartzberg for providing $ITK^{-/-}$ mice. This work was supported by the intramural research program of the National Cancer Institute, National Institutes of Health.

1. Fehniger, T. A., Cooper, M. A. & Caligiuri, M. A. (2002) *Cytokine Growth Factor Rev.* **13,** 169–183.
2. Waldmann, T. A., Dubois, S. & Tagaya, Y. (2001) *Immunity* **14,** 105–110.
3. Lodolce, J. P., Boone, D. L., Chai, S., Swain, R. E., Dassopoulos, T., Trettin, S. & Ma, A. (1998) *Immunity* **9,** 669–676.
4. Kennedy, M. K., Glaccum, M., Brown, S. N., Butz, E. A., Viney, J. L., Embers, M., Matsuki, N., Charrier, K., Sedger, L., Willis, C. R., *et al.* (2000) *J. Exp. Med.* **191,** 771–780.
5. Becker, T. C., Wherry, E. J., Boone, D., Murali-Krishna, K., Antia, R., Ma, A. & Ahmed, R. (2002) *J. Exp. Med.* **195,** 1541–1548.
6. Schluns, K. S., Williams, K., Ma, A., Zheng, X. X. & Lefrancois, L. (2002) *J. Immunol.* **168,** 4827–4831.
7. Budd, R. C., Cerottini, J. C., Horvath, C., Bron, C., Pedrazzini, T., Howe, R. C. & MacDonald, H. R. (1987) *J. Immunol.* **138,** 3120–3129.
8. Budd, R. C., Cerottini, J. C. & MacDonald, H. R. (1987) *J. Immunol.* **138,** 1009–1013.
9. Zhang, X., Sun, S., Hwang, I., Tough, D. F. & Sprent, J. (1998) *Immunity* **8,** 591–599.
10. Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J. & Marrack, P. (2000) *Science* **288,** 675–678.
11. Lodolce, J. P., Burkett, P. R., Boone, D. L., Chien, M. & Ma, A. (2001) *J. Exp. Med.* **194,** 1187–1194.
12. Dubois, S. P., Waldmann, T. A. & Muller, J. R. (2005) *Proc. Natl. Acad. Sci. USA* **102,** 8662–8667.
13. Goldrath, A. W., Bogatzki, L. Y. & Bevan, M. J. (2000) *J. Exp. Med.* **192,** 557–564.
14. Cho, B. K., Rao, V. P., Ge, Q., Eisen, H. N. & Chen, J. (2000) *J. Exp. Med.* **192,** 549–556.
15. Berg, L. J., Finkelstein, L. D., Lucas, J. A. & Schwartzberg, P. L. (2005) *Annu. Rev. Immunol.* **23,** 549–600.
16. Lucas, J. A., Atherly, L. O. & Berg, L. J. (2002) *J. Immunol.* **168,** 6142–6151.
17. Liao, X. C. & Littman, D. R. (1995) *Immunity* **3,** 757–769.

18. Sprent, J. & Kishimoto, H. (2002) *Immunol. Rev.* **185,** 126–135.
19. Ernst, D. N., Weigle, W. O., Noonan, D. J., McQuitty, D. N. & Hobbs, M. V. (1993) *J. Immunol.* **151,** 575–587.
20. Gourley, T. S., Wherry, E. J., Masopust, D. & Ahmed, R. (2004) *Semin. Immunol.* **16,** 323–333.
21. Jamieson, A. M., Diefenbach, A., McMahon, C. W., Xiong, N., Carlyle, J. R. & Raulet, D. H. (2002) *Immunity* **17,** 19–29.
22. Urdahl, K. B., Sun, J. C. & Bevan, M. J. (2002) *Nat. Immunol.* **3,** 772–779.
23. Meresse, B., Chen, Z., Ciszewski, C., Tretiakova, M., Bhagat, G., Krausz, T. N., Raulet, D. H., Lanier, L. L., Groh, V., Spies, T., *et al*. (2004) *Immunity* **21,** 357–366.
24. Hue, S., Mention, J. J., Monteiro, R. C., Zhang, S., Cellier, C., Schmitz, J., Verkarre, V., Fodil, N., Bahram, S., Cerf-Bensussan, N. & Caillat-Zucman, S. (2004) *Immunity* **21,** 367–377.
25. Schaeffer, E. M., Broussard, C., Debnath, J., Anderson, S., McVicar, D. W. & Schwartzberg, P. L. (2000) *J. Exp. Med.* **192,** 987–1000.
26. Dhanji, S., Teh, S. J., Oble, D., Priatel, J. J. & Teh, H. S. (2004) *Blood* **104,** 2116–2123.
27. Dhanji, S. & Teh, H. S. (2003) *J. Immunol.* **171,** 3442–3450.
28. Teague, R. M., Sather, B. D., Sacks, J. A., Huang, M. Z., Dossett, M. L., Morimoto, J., Tan, X., Sutton, S. E., Cooke, M. P., Ohlen, C. & Greenberg, P. D. (2006) *Nat. Med.* **12,** 335–341.