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Jianhua Yu, ... , Susheela Tridandapani, Michael A. Caligiuri

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Research Article

Hematology

IL-15 may have a role in the development of T cell large granular lymphocyte (T-LGL) or NKT leukemias. However, the mechanisms of action and the identity of the cell subset that undergoes leukemic transformation remain elusive. Here we show that in both mice and humans, NKp46 expression marks a minute population of WT NKT cells with higher activity and potency to become leukemic. Virtually 100% of T-LGL leukemias in IL-15 transgenic mice expressed NKp46, as did a majority of human T-LGL leukemias. The minute NKp46<sup>+</sup> NKT population, but not the NKp46<sup>-</sup> NKT population, was selectively expanded by overexpression of endogenous IL-15. Importantly, IL-15 transgenic NKp46<sup>-</sup> NKT cells did not become NKp46<sup>+</sup> in vivo, suggesting that NKp46<sup>+</sup> T-LGL leukemia cells were the malignant counterpart of the minute WT NKp46<sup>+</sup> NKT population. Mechanistically, NKp46<sup>+</sup> NKT cells possessed higher responsiveness to IL-15 in vitro and in vivo compared with that of their NKp46<sup>-</sup> NKT counterparts. Furthermore, interruption of IL-15 signaling using a neutralizing antibody could prevent LGL leukemia in IL-15 transgenic mice. Collectively, our data demonstrate that NKp46 identifies a functionally distinct NKT subset in mice and humans that appears to be directly susceptible to leukemic transformation when IL-15 is overexpressed. Thus, IL-15 signaling and NKp46 may be useful targets in the treatment of patients with T-LGL or NKT leukemia.

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# NKp46 identifies an NKT cell subset susceptible to leukemic transformation in mouse and human

Jianhua Yu,<sup>1,2</sup> Takeki Mitsui,<sup>1,3</sup> Min Wei,<sup>1</sup> Hsiaoyin Mao,<sup>1</sup> Jonathan P. Butchar,<sup>4</sup> Mithun Vinod Shah,<sup>5</sup> Jianying Zhang,<sup>6</sup> Anjali Mishra,<sup>1</sup> Christopher Alvarez-Breckenridge,<sup>1</sup> Xingluo Liu,<sup>1</sup> Shujun Liu,<sup>1,2</sup> Akihiko Yokohama,<sup>1,3</sup> Rossana Trotta,<sup>1</sup> Guido Marcucci,<sup>1,2,7</sup> Don M. Benson Jr.,<sup>1,2,7</sup> Thomas P. Loughran Jr.,<sup>5</sup> Susheela Tridandapani,<sup>4</sup> and Michael A. Caligiuri<sup>1,2,7</sup>

<sup>1</sup>Department of Molecular Virology, Immunology, and Medical Genetics, and <sup>2</sup>Division of Hematology/Oncology, Department of Internal Medicine, The Ohio State University, Columbus, Ohio, USA. <sup>3</sup>Department of Medicine and Clinical Science, Graduate School of Medicine, Gunma University, Maebashi, Japan. <sup>4</sup>Division of Pulmonary, Allergy, Critical Care and Sleep Medicine, The Ohio State University, Columbus, Ohio, USA.

<sup>5</sup>Penn State Hershey Cancer Institute, Hershey, Pennsylvania, USA. <sup>6</sup>Center for Biostatistics, The Ohio State University, Columbus, Ohio, USA.

<sup>7</sup>The Ohio State University Comprehensive Cancer Center, The James Cancer Hospital and Solove Research Institute, Columbus, Ohio, USA.

**IL-15 may have a role in the development of T cell large granular lymphocyte (T-LGL) or NKT leukemias. However, the mechanisms of action and the identity of the cell subset that undergoes leukemic transformation remain elusive. Here we show that in both mice and humans, NKp46 expression marks a minute population of WT NKT cells with higher activity and potency to become leukemic. Virtually 100% of T-LGL leukemias in IL-15 transgenic mice expressed NKp46, as did a majority of human T-LGL leukemias. The minute NKp46<sup>+</sup> NKT population, but not the NKp46<sup>-</sup> NKT population, was selectively expanded by overexpression of endogenous IL-15. Importantly, IL-15 transgenic NKp46<sup>-</sup> NKT cells did not become NKp46<sup>+</sup> *in vivo*, suggesting that NKp46<sup>+</sup> T-LGL leukemia cells were the malignant counterpart of the minute WT NKp46<sup>+</sup> NKT population. Mechanistically, NKp46<sup>+</sup> NKT cells possessed higher responsiveness to IL-15 *in vitro* and *in vivo* compared with that of their NKp46<sup>-</sup> NKT counterparts. Furthermore, interruption of IL-15 signaling using a neutralizing antibody could prevent LGL leukemia in IL-15 transgenic mice. Collectively, our data demonstrate that NKp46 identifies a functionally distinct NKT subset in mice and humans that appears to be directly susceptible to leukemic transformation when IL-15 is overexpressed. Thus, IL-15 signaling and NKp46 may be useful targets in the treatment of patients with T-LGL or NKT leukemia.**

## Introduction

NKT cells are defined as a subset of T cells that share some characteristics with NK cells, particularly expression of the NK1.1 or DX5 antigen in mice and CD56 in humans (1). In both mice and humans, NKT cells are heterogeneous and are classified as either invariant NKT cells or NKT-like cells according to TCR and coreceptor expression (2). The invariant NKT cell subset is defined by CD1d dependence and invariant TCR expression (V $\alpha$ 14-J $\alpha$ 18 in mice and V $\alpha$ 24-J $\alpha$ 18 in humans) and is activated by  $\alpha$ -galactosylceramide. Invariant NKT cells are generally CD4<sup>+</sup> or double-negative (CD8<sup>-</sup>CD4<sup>-</sup>) TCR $\alpha\beta$ <sup>+</sup> T cells (with some CD8<sup>+</sup> in humans) and are highly biased toward either V $\beta$ 8.2, V $\beta$ 2, or V $\beta$ 7 in mice and V $\beta$ 11 in humans (2). Invariant NKT cells have strong associations with many diseases, including autoimmune diseases, cancer, and infection in both mice and humans (3, 4).

In contrast to invariant NKT cells, NKT-like cells are defined as CD1d-independent NK1.1<sup>+</sup> T cells in mice and CD1d-independent TCR $\alpha\beta$ <sup>+</sup> cells expressing NK-associated receptors in humans (2, 5). In C57BL/6 mice, the CD8<sup>+</sup>CD4<sup>-</sup> phenotype predominates the NKT-like cells, but CD8<sup>-</sup>CD4<sup>+</sup> and CD8<sup>-</sup>CD4<sup>-</sup> NK1.1<sup>+</sup> T cells can also be seen (6). In contrast to invariant NKT cells, which have been intensively studied, little is known about NKT-like cells in both mice and humans.

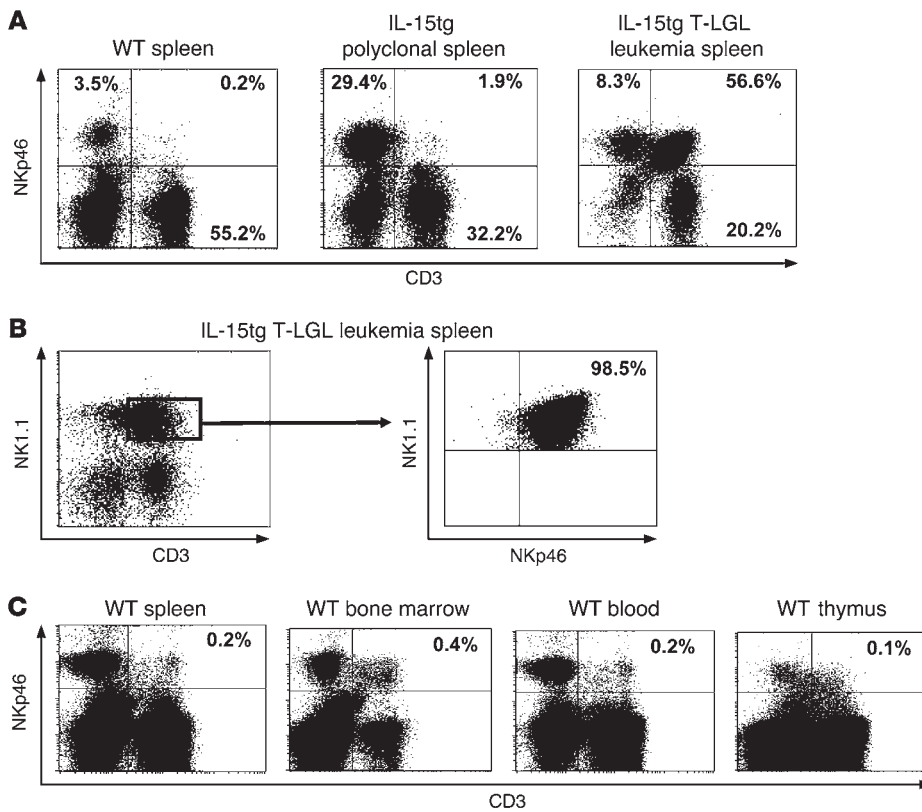
IL-15 is a pleiotropic cytokine that is important for both innate and adaptive immune cell homeostasis as well as peripheral immune function (7). IL-15 is required for the normal expansion and/or survival of CD8 memory T cells and NK cells (8–11). Both IL-15 and IL-15R $\alpha$  have been found to be expressed on LGL leukemia (also known as lymphoproliferative disease of granular lymphocytes), and IL-15 has also been shown to be overexpressed in patients with cutaneous T cell lymphomas (2, 12). Most recently, computer modeling studies have suggested that persistence of IL-15, together with PDGF, is sufficient to reproduce all known deregulations in T-LGL leukemia (13). We previously engineered an IL-15 transgenic mouse line, with global overexpression of the normally tightly regulated *Il15* gene product. In the first 10 weeks of life, these mice display significant, polyclonal expansions in their NK, NKT, and CD8 memory cell populations (referred to here as polyclonal IL-15tg mice). However, by the 15th–25th week of age, approximately 30% of these FvB/NJ mice develop drastically elevated white blood cell counts, consisting of a monoclonal population that is phenotypically NK (CD3<sup>-</sup>DX5<sup>+</sup>) or T-LGL (CD3<sup>+</sup>TCR $\beta$ <sup>+</sup>DX5<sup>+</sup>) leukemia (14, 15). These spontaneously arising NK or T-LGL leukemias are quite similar to the aggressive form of human NK or T-LGL leukemias (16).

Natural cytotoxicity receptors (NCRs) include NKp30 (NCR3), NKp44 (NCR2), and NKp46 (NCR1) molecules (17). NCRs can trigger NK-mediated lysis of various tumor cells through direct engagement of membrane ligands expressed by the tumor cells (17,

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**Figure 1**

Flow cytometric analysis of NKp46 expression on mouse T cells. **(A)** CD3 versus NKp46 staining of splenocytes isolated from WT FvB/NJ mice, IL-15tg polyclonal mice, and leukemic mice ( $n = 5$ /group). The T-LGL leukemic mice show a vastly expanded CD3<sup>+</sup>NKp46<sup>+</sup> fraction, with a total white blood cell count in peripheral blood of over  $1 \times 10^9$ /ml (normal white blood cell count =  $1-3 \times 10^6$ /ml). **(B)** A representative example of NKp46 expression on monoclonal NK1.1<sup>+</sup>CD3<sup>+</sup>T-LGL leukemic cells. NKp46 was expressed in all cases of mouse T-LGL leukemia examined ( $n = 10$ ) (data not shown). **(C)** CD3 versus NKp46 staining for WT FvB/NJ leukocytes from different organs. Results are summarized in Supplemental Table 1. A lymphocyte was used for these experiments, and percentages of cells in the respective quadrants are indicated in **A-C**.

18). NKp46 is a transmembrane type I glycoprotein, containing 2 immunoglobulin domains and a positively charged arginine residue in the transmembrane domain, the latter of which associates with the TCR $\zeta$  signaling molecule (19, 20). *NKp46* is conserved between mouse and human, whereas an *NKp44* homolog has not been identified anywhere in the mouse genome, and *NKp30* is a pseudogene in all examined mouse strains (21).

Several NK cell markers (e.g., CD56 in human and NK1.1 and DX5 [CD49b] in mouse) are expressed by T cell subsets (22, 23). Sivori et al. showed that the NKp46 molecule is specifically expressed by human CD56<sup>+</sup>CD3<sup>-</sup>HLA-DR<sup>-</sup> NK cells (18). Walzer et al. proposed that the phenotypic identification of NK cells across species is CD3<sup>-</sup>NKp46<sup>+</sup> (24, 25). However, NKp46 is also expressed on a minute fraction of CD3<sup>+</sup> cells in mice, while it is not expressed on CD1d-restricted NKT cells (24). To our knowledge, NKp46<sup>+</sup> NKT cells have not yet been identified in healthy humans, and they have not been characterized in either mice or humans.

In this report we characterize the minute population of NKp46<sup>+</sup> NKT cells in WT mice and identify a similar population in humans. We show that this small population is non-CD1d restricted and more closely resembles NK cells and is more activated, functionally more potent, and more responsive to IL-15 when compared with its NKp46<sup>-</sup> counterpart in WT mice and in healthy human donors. Indeed, cross-linking NKp46 receptor induces phosphorylation of Lck and Zap70, and NKp46<sup>+</sup> NKT cells have higher TYRO protein tyrosine kinase binding protein gene (*DAP12*, otherwise known as *Tyrbp*) expression, compared with their NKp46<sup>-</sup> counterparts. Further, we provide data that suggest that T-LGL leukemia appears to arise from the normal NKp46<sup>+</sup> subset of NKT-like cells when IL-15 is deregulated and

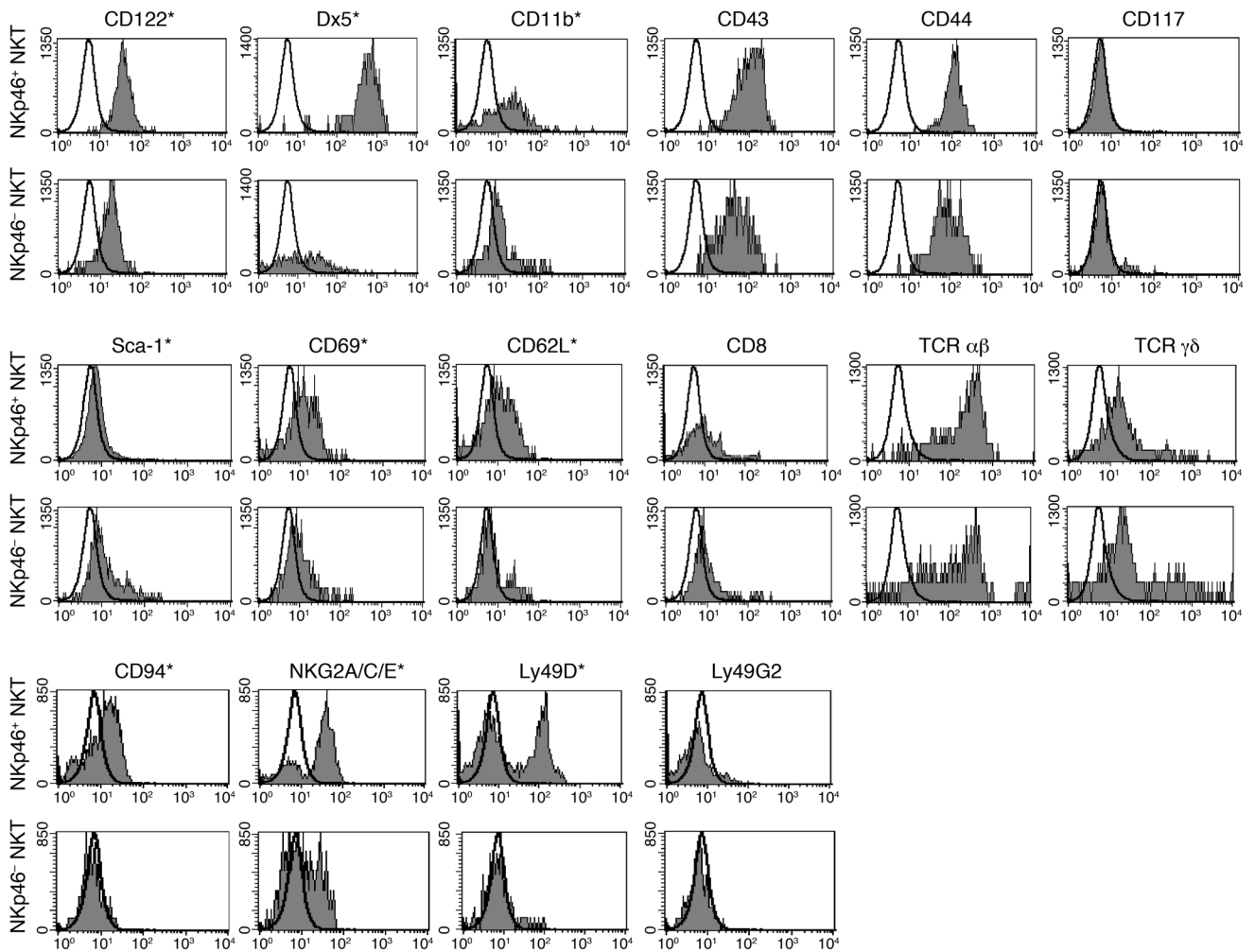
show that interruption of IL-15 signaling by TM- $\beta$ 1 mAb prevents the development of the NKp46<sup>+</sup> T-LGL leukemia.

## Results

**Cell surface expression of NKp46 on normal T cells and on T-LGL leukemic cells in mice.** We performed flow cytometric analyses to evaluate the expression of NKp46 on T splenocytes from 5 WT, 5 IL-15tg (non-leukemic), and 5 T-LGL leukemic FvB/NJ mice (Figure 1A). All WT mice had a minute fraction of T cells ( $0.18\% \pm 0.03\%$ ;  $n = 5$ ), which expressed NKp46 as previously described (24). We confirmed these data in SJL, DBA/2, NOD, NZW, and CBA/ca strains (data not shown). There was a significant expansion of the T cell population expressing NKp46 in the IL-15tg mice as compared with that in the WT mice ( $1.77\% \pm 0.11\%$  vs.  $0.18\% \pm 0.03\%$ ;  $P < 0.05$ ) (Figure 1A). In the IL-15tg mice with T-LGL leukemia, there was a distinct and dominant population of CD3<sup>+</sup>NKp46<sup>+</sup> cells ( $65.9\% \pm 15.8\%$ ;  $n = 5$ ). Gating on the malignant CD3<sup>+</sup>NK1.1<sup>+</sup> population showed that virtually all T-LGL leukemia cells in IL-15tg mice coexpress NKp46 (Figure 1B;  $n = 10$ ).

We examined the distribution of this NKp46<sup>+</sup> NKT population in the fresh spleen, bone marrow, peripheral blood, and thymocytes of WT FvB/NJ and C57BL/6 mice. We found an NKp46<sup>+</sup> NKT cell population present in a minute fraction in each of these organs or tissues and that the percentage was relatively higher in the bone marrow, regardless of mouse strain ( $P < 0.001$ ,  $n = 3$ ) (Figure 1C, data not shown, and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI43242DS1).

**Characterization of mouse NKp46<sup>+</sup> NKT cells.** We analyzed a variety of cell surface markers on both NKp46<sup>+</sup> and NKp46<sup>-</sup> fractions of CD3<sup>+</sup>NK1.1<sup>+</sup> NKT cells harvested from WT FvB/NJ mice. A rep-

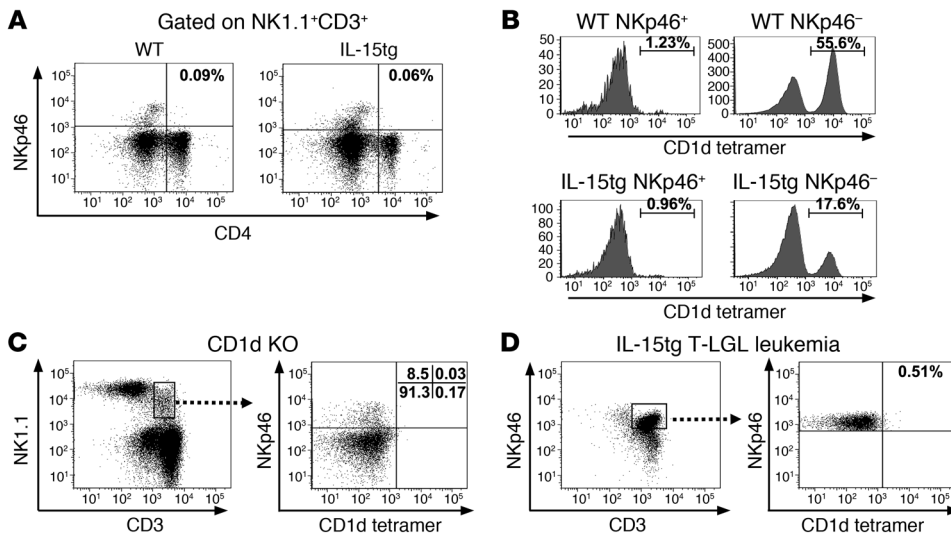


**Figure 2** Flow cytometric analysis to compare expression of surface markers in NKp46<sup>+</sup> and NKp46<sup>-</sup> mouse NKT cells. Freshly isolated WT FvB/NJ splenocytes of 3 mice were analyzed by flow cytometry. The NK1.1<sup>+</sup>CD3<sup>+</sup> gate was set for total NKT cells that were then divided between NKp46<sup>+</sup> and NKp46<sup>-</sup>. These 2 subsets were then assessed for surface expression of activation and differentiation markers as shown. Representative plots from 1 out of at least 3 independent experiments are shown. An asterisk indicates a significant difference of the mean surface marker expression when comparing NKp46<sup>+</sup> NKT cells to NKp46<sup>-</sup> NKT cells (\**P* < 0.05).

representative comparison is shown in Figure 2. The NKp46<sup>+</sup> NKT subset showed significantly higher expression of certain maturation (e.g., Dx5, Ly49D, CD11b, IL-15 receptor β chain [CD122], CD94, and NKG2A/C/E) (*P* < 0.05, *n* = 3 for each marker) and activation (e.g., L-selectin [CD62L] and CD69) (*P* < 0.05, *n* = 3 for both) NK markers. We also observed slightly (although statistically significant) lower expression of the stem cell marker Sca-1 in NKp46<sup>+</sup> NKT cells compared with that in NKp46<sup>-</sup> NKT cells (Figure 2), but the biological relevance of this difference is unknown and remains to be determined. Expression patterns did not differ between spleen and bone marrow NKT cells (data not shown). These results provide phenotypic evidence suggesting that NKp46<sup>+</sup> NKT cells more closely resemble NK cells and are more activated than NKp46<sup>-</sup> NKT cells and that the former might normally be more responsive to cytokines that promote activation in vivo.

*NKp46<sup>+</sup> NKT cells are CD4<sup>-</sup> non-CD1d-restricted NKT-like cells.* NKT cells have CD4<sup>+</sup> and CD4<sup>-</sup> subsets (2). To determine which CD4

subset NKp46<sup>+</sup> NKT cells belong to, we did a flow cytometric analysis. Figure 3A shows that NKp46<sup>+</sup> NKT cells were CD4<sup>-</sup> in both WT mice and IL-15tg mice. To determine whether NKp46<sup>+</sup> NKT cells are CD1d-dependent invariant NKT cells or CD1d-independent NKT-like NKT cells, we stained the NKp46<sup>+</sup> and NKp46<sup>-</sup> subsets of NK1.1<sup>+</sup>CD3<sup>+</sup> NKT cells with a CD1d tetramer loaded with the α-C-galactosylceramine analog PBS-57 (26). As shown in Figure 3B (top), in WT mice, NKp46<sup>+</sup> NKT cells had no CD1d tetramer staining, while NKp46<sup>-</sup> NKT cells contained a cell subset, which bound to the CD1d tetramer, and another subset, which did not bind to the CD1d tetramer. Similar staining results were found in IL-15tg mice (Figure 3B, bottom). Therefore, NKp46<sup>+</sup> NKT cells are non-CD1d-restricted NKT-like cells. We also noticed that IL-15tg mice selectively expanded non-CD1d-restrictive NKT cells rather than CD1d-dependent NKT cells, since a majority of NKT cells bound to the CD1d tetramer in WT mice but did not in IL-15tg mice (Figure 3B and data not shown). To further con-



**Figure 3**

NKp46<sup>+</sup> NKT cells are CD4<sup>-</sup> non-CD1d-restricted NKT-like cells. (A) Splenic cells from WT and IL-15tg mice were stained with NK1.1, CD3, CD4, and NKp46 mAbs. Cells were first gated on NK1.1<sup>+</sup>CD3<sup>+</sup>, followed by an assessment of NKp46 and CD4 expression. Percentages of cells are only shown for the top right quadrants. (B) Splenic cells from WT and IL-15tg mice were stained with the mAbs of NK1.1, CD3, NKp46, CD19, and CD1d tetramer loaded with the  $\alpha$ -C-galactosylceramine analog PBS-57. NK1.1<sup>+</sup>CD3<sup>+</sup>CD19<sup>-</sup> cells were first gated and then examined for NKp46 and CD1d expression. Percentages of cells positive for CD1d tetramer binding are shown. (C) Splenic cells from CD1d-deficient (CD1d KO) mice were stained with NK1.1, CD3, CD19, NKp46, and CD1d tetramer mAbs. CD19<sup>+</sup> cells (data not shown) were first gated out, and the remaining cells were examined for the NK1.1<sup>+</sup>CD3<sup>+</sup> population, followed by gating and an analysis of NKp46 and CD1d expression. Percentages of cells staining positive for all 4 quadrants are shown. (D) Splenic cells from IL-15tg T-LGL leukemia mice were stained with CD3, NKp46, CD19, and CD1d tetramer mAbs. CD19<sup>+</sup> cells (data not shown) were first gated out, and the remaining cells were examined for the NKp46<sup>+</sup>CD3<sup>+</sup> population, which was then assessed for CD1d expression. Data are representative of 1 out of at least 3 mice showing similar results. The percentage of cells staining positive in top right quadrant is shown for the right panel.

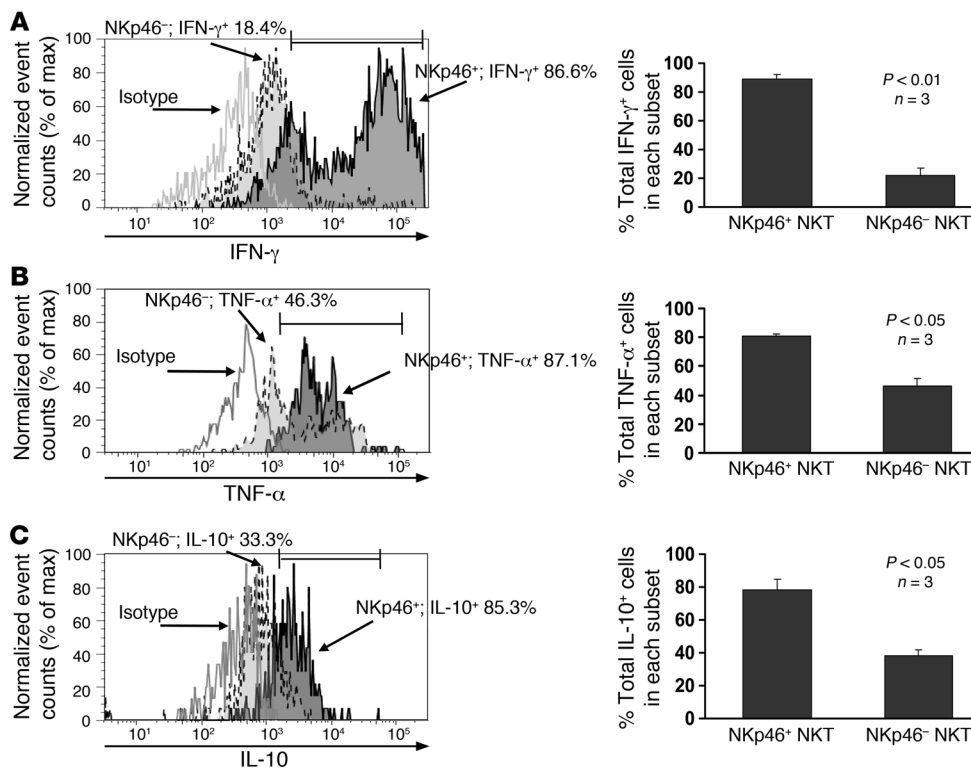
firm that NKp46<sup>+</sup> NKT cells are non-CD1d dependent, we stained splenic lymphocytes from CD1d-deficient (knockout) mice (Figure 3C). Results show that CD1d knockout mice had NKp46<sup>+</sup> NKT-like cells, while no CD1d-dependent cells were found. Finally, we found that T-LGL leukemia cells from IL-15tg mice, which we believe are derived from normal NKp46<sup>+</sup> non-CD1d-restricted NKT-like cells (see below), were entirely CD1d independent (Figure 3D).

**Evidence of functional activation in mouse NKp46<sup>+</sup> NKT cells.** The phenotyping results led us to hypothesize that NKp46<sup>+</sup> NKT-like cells might have functionally distinct properties compared with those of the NKp46<sup>-</sup> NKT cells. To test this hypothesis, we quantified cytokine secretion from populations of NKp46<sup>+</sup> and NKp46<sup>-</sup> NKT cells in freshly isolated splenocytes, after *in vitro* stimulation with a combination of IL-12 and IL-18. A larger fraction of NKp46<sup>+</sup> NKT cells produced IFN- $\gamma$  compared with that of NKp46<sup>-</sup> NKT cells (89.1%  $\pm$  3.5% vs. 22.1%  $\pm$  5.2%, respectively;  $P < 0.01$ ,  $n = 3$ ) (Figure 4A). Resting NKp46<sup>+</sup> NKT cells expressed nearly 2-fold higher TNF- $\alpha$  compared with that of NKp46<sup>-</sup> NKT cells (81.0%  $\pm$  1.4% vs. 46.5%  $\pm$  4.7%, respectively;  $P < 0.05$ ,  $n = 3$ ) (Figure 4B), and this difference remained despite activation by IL-2<sup>-</sup> or LPS (data not shown). The NKp46<sup>+</sup> NKT subset cells also produced significantly higher IL-10 than the NKp46<sup>-</sup> subset cells under LPS stimulation (78.5%  $\pm$  6.3% vs. 38.1%  $\pm$  3.6%, respectively;  $P < 0.05$ ,  $n = 3$ ) (Figure 4C), although no meaningful difference was found in the resting cells. These data suggest that NKp46<sup>+</sup> NKT-like cells are constitu-

tively more activated and functionally more potent than their NKp46<sup>-</sup> NKT counterparts.

*Mouse NKp46<sup>+</sup> NKT cells show a higher responsiveness to IL-15 *in vivo* and *in vitro*.* With higher expression of CD122 (Figure 2) and the seemingly selective *in vivo* expansion of the rare NKp46<sup>+</sup> NKT population in the IL-15tg mouse (Figure 1A), we assessed IL-15 responsiveness among WT NKp46<sup>+</sup> and NKp46<sup>-</sup> NKT populations *in vivo*. Freshly isolated spleen cells from 3 pairs of WT and polyclonal IL-15tg mice were prepared for this analysis. The percentage of NKp46<sup>+</sup> cells versus NKp46<sup>-</sup> cells among the NK1.1<sup>+</sup>CD3<sup>+</sup> NKT cells in WT FvB/NJ mice was 37.2%  $\pm$  8.9% versus 62.8%  $\pm$  8.9%, respectively ( $P < 0.05$ ,  $n = 3$ ). In polyclonal IL-15tg mice, the percentage of NKp46<sup>+</sup> cells versus NKp46<sup>-</sup> NKT cells was 55.8%  $\pm$  4.3% versus 44.2%  $\pm$  4.3% ( $P < 0.05$ ,  $n = 3$ ) (Figure 5A). Thus, with enhanced expression of endogenous IL-15, as is seen in the IL-15tg mouse, the frequency of NKp46<sup>+</sup> NKT cells increased by 50% over the frequency of NKp46<sup>+</sup> NKT cells in the WT mouse ( $P < 0.05$ ,  $n = 3$ ), while the frequency of NKp46<sup>-</sup> cells in the IL-15tg mouse decreased by 30% compared with the frequency of NKp46<sup>-</sup> NKT cells in the WT mouse ( $P < 0.05$ ,  $n = 3$ ). This inversion in the ratio of the frequency of NKp46<sup>+</sup> cells to the frequency of NKp46<sup>-</sup> cells in the presence of IL-15 was significant ( $P < 0.05$ ,  $n = 3$ ). A similar difference was observed in WT and IL-15tg mice with a C57BL/6 background (data not shown). These data suggest that compared with the NKp46<sup>-</sup> NKT cell population, the NKp46<sup>+</sup> NKT cell population is more responsive to endogenous IL-15 *in vivo* in terms of proliferation and/or survival.

To assess for differences in responsiveness to IL-15 between these 2 NKT subsets *in vivo*, we assessed their proliferative capacity by BrdU assay in IL-15tg mice. Five polyclonal IL-15tg mice received drinking water containing BrdU for 6 days and were then sacrificed and immediately analyzed for BrdU uptake in NKp46<sup>+</sup> and NKp46<sup>-</sup> subsets of NKT cells. As shown in Figure 5B (both the representative data and the summarized data), the NKp46<sup>+</sup> subset incorporated significantly more BrdU than the NKp46<sup>-</sup> subset in IL-15tg mice (19.7%  $\pm$  2.6% vs. 13.2%  $\pm$  1.8%;  $P < 0.05$ ,  $n = 5$ ). The NKp46<sup>+</sup> subset incorporated markedly more BrdU than the NKp46<sup>-</sup> subset in WT mice as well (data not shown). Since NKp46<sup>+</sup> NKT cells can develop into leukemia in IL-15tg mice but not in WT mice, we speculated that NKp46<sup>+</sup> NKT cells would have a greater proliferation in IL-15tg mice than in WT mice. To test this, a BrdU assay was performed on 4 pairs of IL-15tg and WT littermate mice. Results showed that NKp46<sup>+</sup> NKT cells incorpo-



**Figure 4**

Cytokine production of NKp46<sup>+</sup> and NKp46<sup>-</sup> mouse NKT cells. (A) Representative figure (left) and summary data (right) show that when activated by costimulation with IL-12 and IL-18, NKp46<sup>+</sup> NKT cells produce more IFN- $\gamma$  than NKp46<sup>-</sup> NKT cells (89.1%  $\pm$  3.5% vs. 22.1%  $\pm$  5.2%, respectively;  $P < 0.01$ ,  $n = 3$ ). (B) Representative figure and summary data show that resting NKp46<sup>+</sup> NKT cells have a higher capacity to produce TNF- $\alpha$  cytokine when compared with the NKp46<sup>-</sup> NKT cells (81.0%  $\pm$  1.4% vs. 46.5%  $\pm$  4.7%, respectively;  $P < 0.05$ ,  $n = 3$ ). (C) Representative figure and summary data show that when activated by LPS, NKp46<sup>+</sup> NKT cells produce more IL-10 than NKp46<sup>-</sup> NKT cells (78.5%  $\pm$  6.3% vs. 38.1  $\pm$  3.6%, respectively;  $P < 0.05$ ,  $n = 3$ ). Cells were first gated on NK1.1<sup>+</sup>CD3<sup>+</sup>, followed by an analysis of intracellular staining of (A) anti-IFN- $\gamma$  mAb, (B) anti-TNF- $\alpha$  mAb, or (C) anti-IL-10 mAb or (A–C) their isotype nonreactive control mAb for NKp46<sup>+</sup> and NKp46<sup>-</sup> subsets. NKp46<sup>+</sup> NKT cells (black solid line; dark gray filled), NKp46<sup>-</sup> NKT cells (black dashed line; light gray filled), and the isotype control staining (light gray solid line; unfilled) are include for comparison (A–C, left). Numbers in histograms indicate percentages of cells within the subset that are staining positive for intracellular cytokine. The bar graphs represent mean  $\pm$  SD.

rated over 2-fold more BrdU in IL-15tg mice than in littermate WT mice (Figure 5C; the summary of the 4 pairs of experiments is not shown;  $P < 0.05$ ,  $n = 4$ ).

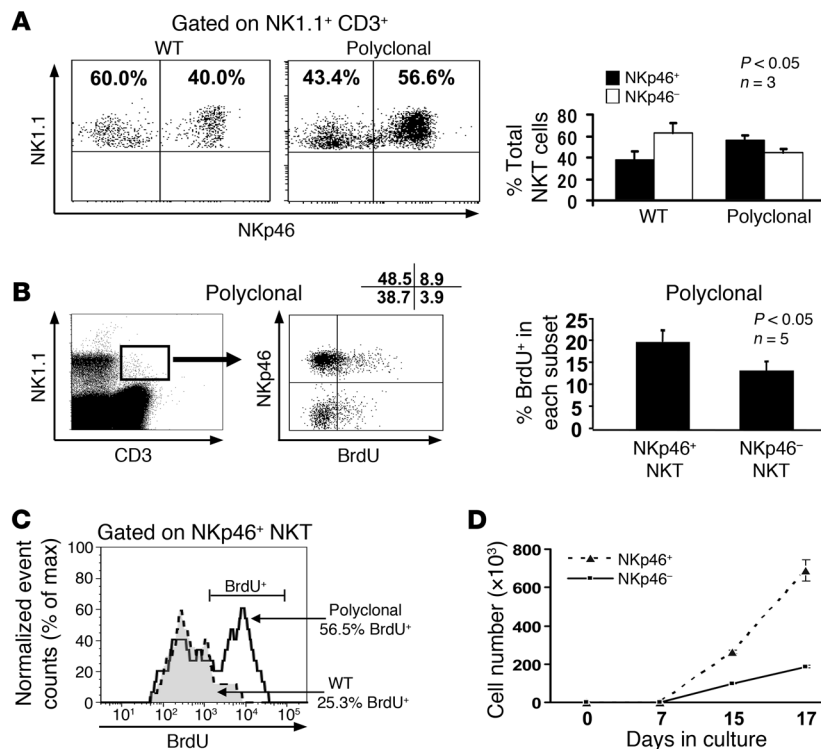
To directly prove that NKp46<sup>+</sup> NKT cells have a greater responsiveness to IL-15 than their NKp46<sup>-</sup> counterparts, we pooled spleen cells from 7–10 mice, sorted the 2 subsets using FACS, and treated the sorted cells ( $\geq 95\%$  purity) with IL-15 in culture. We counted cells at several time points and found that NKp46<sup>+</sup> NKT cells had significantly greater proliferation in IL-15 when compared with NKp46<sup>-</sup> NKT cells in IL-15, starting on day 15 (Figure 5D;  $P < 0.05$ ,  $n = 5$ ). These results were consistent with the data from Figure 2, showing that NKp46<sup>+</sup> cells had higher IL-2/15R $\beta$  chain (CD122) expression than NKp46<sup>-</sup> NKT cells. Collectively, our data show that NKp46<sup>+</sup> NKT cells have higher responsiveness to IL-15 than NKp46<sup>-</sup> NKT cells in vivo and in vitro and undergo greater proliferation with overexpression of IL-15, compared with normal expression of IL-15.

We assessed the TCR V $\beta$  repertoire of NKp46<sup>+</sup> NKT splenocytes from WT FvB/NJ, polyclonal IL-15tg, and IL-15tg T-LGL leukemia

mice by flow cytometry to look for any skewing of the TCR repertoire in this subset during expansion in the presence of excessive endogenous IL-15. Early skewing of the TCR repertoire was observed in NKp46<sup>+</sup> NKT cells in polyclonal IL-15tg mice, with small but marked expansions when compared with those in WT mice (e.g., expansions of the V $\beta$ 6 and V $\beta$ 2 subpopulations in the polyclonal IL-15tg mouse presented in Supplemental Figure 1A). With the appearance of T-LGL leukemia, there was abundant evidence of clonal expansion in NKp46<sup>+</sup> NKT cells (Supplemental Figure 1A) but not in NKp46<sup>-</sup> NKT cells (data not shown). The clonality of NKp46<sup>+</sup> NKT leukemia was confirmed by PCR analysis, using normal T cells as a control (Supplemental Figure 1B).

*Evidence for T-LGL leukemia originating from a minute NKp46<sup>+</sup> population in mice.* Our data thus far show that virtually all IL-15tg leukemia cells from T-LGL mice ( $n = 10$ ) expressed NKp46, suggesting that they could originate from the minute population of NKp46<sup>+</sup> NKT cells that selectively expand in the polyclonal IL-15tg mice. Several observations detailed in this report favor this possibility. First, CD122 expression was constitutively higher on WT CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> cells when compared with that

on their NKp46<sup>-</sup> counterparts (Figure 2). Second, the former was constitutively more activated and was more responsive to IL-15 in vitro and in vivo (Figures 4 and 5). Nonetheless, it is possible that NKp46 expression could be induced on the NKp46<sup>-</sup> NKT cells in vivo. To determine whether CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>-</sup> NKT cells can become CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> NKT cells in vivo, we undertook an adoptive transfer experiment. Sorted CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>-</sup> NKT cells (at least 99% purity) from CD45.2 IL-15tg mice with polyclonal lymphocyte expansion were adoptively transferred into congenic CD45.1 WT mice. Donor IL-15tg CD45.2<sup>+</sup>CD3<sup>+</sup>NK1.1<sup>+</sup> NKT cells were found engrafted in recipient spleen, bone marrow, and blood 9 months after initial adoptive transfer, all of which had the NKp46<sup>-</sup> phenotype as noted by flow cytometric analysis (Figure 6). These data, together with our previous data showing differential dependence of the NKp46<sup>+</sup> and NKp46<sup>-</sup> subsets on CD1d, imply that the two subsets may have different cell origins. Moreover, all 10 cases of T-LGL leukemia tested were NKp46<sup>+</sup>, without any evidence of intermediate NKp46 staining or CD1d expression. Collectively, these data suggest that IL-15-induced T-LGL leuke-

**Figure 5**

Mouse NKp46<sup>+</sup> NKT cells have a higher IL-15 responsiveness compared with that of NKp46<sup>-</sup> NKT cells. **(A)** Representative figure and summary data showing that in resting WT mice the percentage of CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>-</sup> cells is significantly greater than that of CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> cells (62.8% ± 8.9% vs. 37.2% ± 8.9%;  $P < 0.05$ ,  $n = 3$ ). However, after chronic exposure to higher levels of endogenous IL-15 in polyclonal IL-15tg mice, CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> cells become the predominant population, compared with CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>-</sup> cells (55.8% ± 4.3% vs. 44.2% ± 4.3%;  $P < 0.05$ ,  $n = 3$ ). Percentages of cells in the respective quadrants are indicated. **(B)** Representative figure and summary BrdU data showing that a significantly greater fraction of CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> NKT cells incorporate BrdU than CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>-</sup> NKT cells in vivo in polyclonal IL-15tg mice (19.7% ± 2.6% vs. 13.2% ± 1.8%;  $P < 0.05$ ,  $n = 5$ ). Numbers above the dot plot on the right indicate the percentages of cells for each quadrant. **(C)** Representative figure of in vivo BrdU data showing that a significantly greater fraction of CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> NKT cells from IL-15tg mice incorporate BrdU than CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> NKT cells from littermate WT mice ( $n = 4$ ). Percentages indicate the fraction of CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> NKT cells positive for BrdU incorporation. **(D)**  $1 \times 10^3$  FACS-purified NKp46<sup>+</sup> and NKp46<sup>-</sup> mouse splenic NKT cells were cultured in the presence of IL-15 and then counted, as detailed in the Methods section. Compared with NKp46<sup>-</sup> NKT cells, NKp46<sup>+</sup> splenic NK cells showed a greater IL-15 responsiveness at day 15 and day 17 ( $P < 0.05$ ,  $n = 5$ ). **(A, B, and D)** Error bars indicate SD.

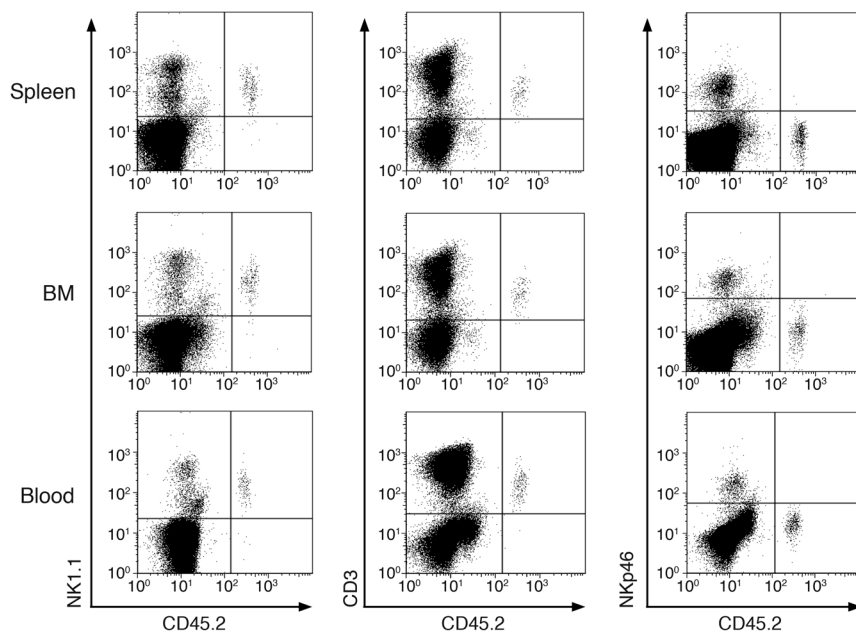
mia is most likely derived from the malignant transformation of the minute population of WT NKp46<sup>+</sup> NKT cells.

**Immunoreceptor tyrosine-based activation motif signaling in NKp46<sup>+</sup> NKT cells.** The data above indicate that NKp46<sup>+</sup> NKT cells have phenotypic and functional evidence of enhanced activation compared with their NKp46<sup>-</sup> NKT counterparts and that overexpression of IL-15 in vivo contributes to the leukemic transformation of NKp46<sup>+</sup> NKT-like cells. We looked for confirmation of enhanced activation by examining cell signaling molecules. Previous studies showed that NKp46 interacts with immunoreceptor tyrosine-based activation motif-containing (ITAM-containing) CD3 $\zeta$  (19, 20), which can recruit tyrosine kinases and induce cell activation and proliferation (27). In vitro, we found that activation with IL-15

enhanced some tyrosine phosphorylation in freshly isolated NKp46<sup>+</sup> NKT cells (Figure 7A). The same induction was not seen in NKp46<sup>-</sup> NKT cells exposed to IL-15. We also found that cross-linking NKp46 induced the phosphorylation of Lck and Zap70, both of which are downstream signaling components of CD3 $\zeta$  (Figure 7B and ref. 27). Consistent with the data from mice, we found that NKp46 and CD3 $\zeta$  colocalized with each other in human NK cells and in human LGL leukemia (Supplemental Figure 2).

To further investigate the putative susceptibility of WT NKp46<sup>+</sup> NKT cells to leukemic transformation by overexpression of IL-15 and their activation phenotype, we performed a microarray analysis on the NKp46<sup>+</sup> and NKp46<sup>-</sup> resting WT populations. The resting WT NKp46<sup>+</sup> population has a unique gene expression pattern compared with that of the NKp46<sup>-</sup> resting WT populations (e.g., lower expression of inhibitory or antiproliferative genes *Ctla4*, *Foxp3*, and *Cd81*; Figure 7C and Supplemental Table 2). Expression of *Cd4* gene ranked first among genes downregulated in the NKp46<sup>+</sup> NKT population (Supplemental Table 2), consistent with our flow data showing that NKp46<sup>+</sup> NKT cells were CD4<sup>-</sup> (Figure 3A). More interestingly, *DAPI2* ranked next to *NKp46* on the top of the list, displaying an ~18-fold higher gene expression compared with that of NKp46<sup>-</sup> NKT cells (Figure 7C and Supplemental Table 2). The expression of *DAPI2* was confirmed by a real-time analysis (Figure 7D). It has been previously reported that DAPI2 interacts with activating molecules Ly49D, Ly49H, NKG2C, NKp44, Zap70, and spleen tyrosine kinase (Syk) (28–32). These interactions may in part contribute to an activation phenotype and subsequent leukemic transformation of the NKp46<sup>+</sup> NKT cell subset. In support of this, our microarray data also indicate that NKp46<sup>+</sup> NKT cells had 6.3-fold higher Syk expression than NKp46<sup>-</sup> NKT cells ( $P = 0.00028$ , not shown in Supplemental Table 2 because the  $P$  value is slightly over 0.00025, the cut-off  $P$  value used in the study).

**Treatment of IL-15tg mice by TM- $\beta$ 1 mAb.** In order to directly implicate the interplay between IL-15 and IL-15R $\beta$  in the leukemic transformation of NKp46<sup>+</sup> NKT cells (which have higher CD122 surface expression than NKp46<sup>-</sup> NKT cells [Figure 2]), we delivered weekly anti-CD122 mAb (TM- $\beta$ 1) intraperitoneal injections to 4- to 5-week-old IL-15tg mice. At nearly 5 months after the initial treatment, 100% of the 10 IL-15tg mice in the TM- $\beta$ 1-treated group were alive and well, while 6 out of the 10 IL-15tg mice receiving the placebo died, 4 of which could be autopsied and were found to have fulminant T-LGL leukemia (Figure 8A,  $P = 0.004$ , and data not shown). For a certain period, the TM- $\beta$ 1 treatment also prevented the alopecia that occurs in IL-15tg mice (Figure 8B). The efficacy of the TM- $\beta$ 1 treatment was coincident with the nearly complete depletion of NKp46<sup>+</sup>CD122<sup>+</sup> cells in these mice (Figure 8, C and D). Documentation of fulminant leukemia in the placebo-treated group, com-



**Figure 6**  
NKp46<sup>-</sup> NKT cells adoptively transferred from IL-15tg polyclonal mice fail to acquire NKp46 surface expression in WT recipient mice. NKp46<sup>-</sup> NKT (NK1.1<sup>+</sup>CD3<sup>+</sup>) cells were purified from C57BL/6 IL-15tg (CD45.2) whole spleen cells of mice with polyclonal NK and NKT cell expansion by FACS to at least 99% purity. Purified NKp46<sup>-</sup> NKT cells were adoptively transferred into irradiated CD45.1 mice by tail vein injection. The recipient mice were sacrificed 9 months after the injection, and their spleen, bone marrow, and blood cells were isolated for analyzing coexpression of CD3, NK1.1, or NKp46 and CD45.2. Data demonstrated that IL-15tg NKp46<sup>-</sup> NKT donor cells do not acquire NKp46 surface expression in vivo. Representative plots from 1 out of 3 independent experiments are shown.

pared with the absence of leukemia in the TM-β1-treated group, is shown in Figure 9, A-D.

**Identification and characterization of NKp46<sup>+</sup> NKT cells in normal human blood.** To assess the NKp46<sup>+</sup> expression on human NKT cells, we analyzed cell surface markers from human PBMCs isolated from peripheral blood leukopacks of 3 healthy donors. NKT cells were identified as CD56<sup>+</sup>CD3<sup>+</sup>, and NKp46 was expressed on a minute fraction (1.3% ± 0.7%) of the NKT lymphocytes (Figure 10A, a representative donor). Like the mouse, when compared with the NKp46<sup>-</sup> subset, the human NKp46<sup>+</sup> NKT subset also showed significantly higher expression of CD94, NKG2A, and CD62L and also higher expression for CD161 and killer cell immunoglobulin-like receptors (KIRs) NK maturation markers (Figure 10B;  $P < 0.05$ ,  $n = 3$  for each marker). Our characterization also indicated that like the mouse, the NKp46<sup>+</sup> NK subset has significantly higher expression of IL-15Rβ, suggesting that it has a stronger responsiveness to IL-15 than its NKp46<sup>-</sup> counterpart as well. The latter observation was confirmed by an in vitro proliferation assay of sorted NKp46<sup>+</sup> and NKp46<sup>-</sup> NKT (CD56<sup>+</sup>CD3<sup>+</sup>) subsets, showing that only the NKp46<sup>+</sup> subset cells survived and proliferated in the presence of IL-15 and cross-linking of NKp46 receptor (Figure 10C). The ex vivo 5-ethynyl-2'-deoxyuridine (EdU) assay of cells freshly isolated from peripheral blood showed a significantly greater fraction of resting NKp46<sup>+</sup> NKT cells expressing EdU ( $P < 0.05$ ,  $n = 3$ ), suggesting a greater number of NKp46<sup>+</sup> NKT cells proliferating in vivo compared with that in the NKp46<sup>-</sup> NKT cell subset (Figure 10D). We also assessed the IFN-γ secretion of human NKp46<sup>+</sup> and NKp46<sup>-</sup> NKT cells, and consistent with mouse NKp46<sup>+</sup> NKT cells, human NKp46<sup>+</sup> NKT cells produce significantly more IFN-γ than NKp46<sup>-</sup> NKT cells (21.1% ± 4.8% vs. 10.7% ± 3.8%;  $P < 0.05$ ,  $n = 3$ ) (Figure 10E).

**Expression of NKp46 on human T-LGL leukemia.** T-LGL leukemia cells express both T cell-associated (e.g., CD3 and CD8) and NK cell-associated (e.g., CD56 or CD57) markers on their surface (33). To assess NKp46 expression on human T-LGL leukemia cells as was done in the mouse, we analyzed *NKp46* mRNA expression in CD3<sup>+</sup>CD57<sup>+</sup> T-LGL leukemia cells using microarray analysis.

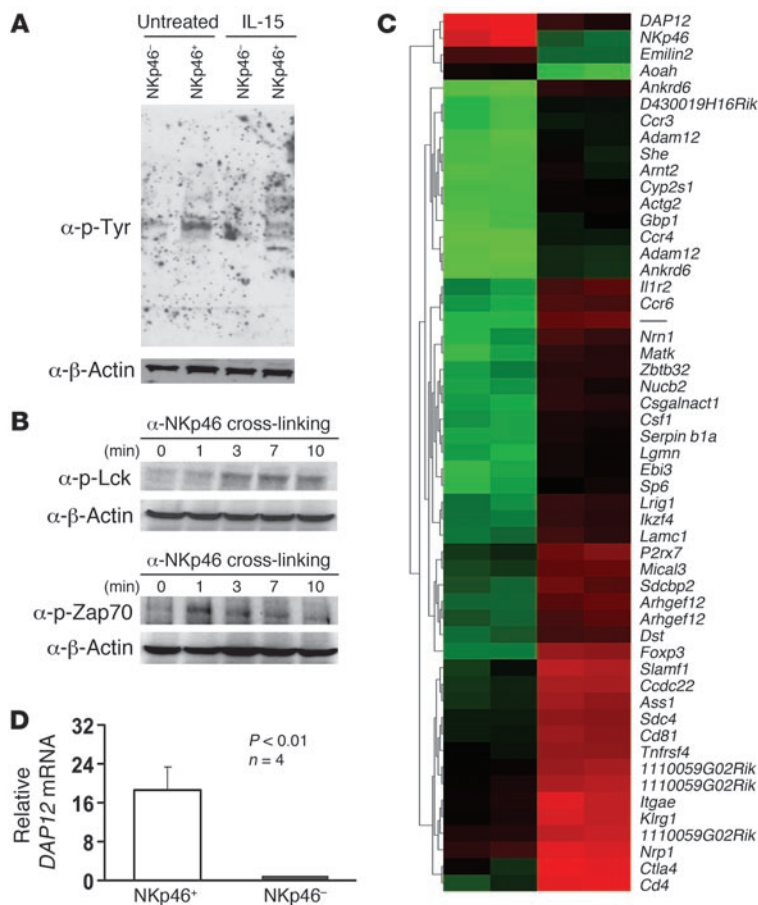
NKp46 expression was significantly upregulated in T-LGL leukemia cases compared with that in both normal PBMCs and CD8<sup>+</sup> T cells (Figure 11A;  $P < 0.001$ ,  $n = 34$ ), which is consistent with the higher protein expression on the T-LGL leukemic cell surface determined by flow cytometry (Figure 11B, right). Data similar to that found in CD3<sup>+</sup>CD57<sup>+</sup> T-LGL were also revealed in CD3<sup>+</sup>CD56<sup>+</sup> T-LGL (data not shown).

We noticed that the surface density expression of NKp46 on NKT cells from both healthy donors and T-LGL leukemia patients was relatively low compared with that of normal NKp46<sup>+</sup>CD3<sup>-</sup> human NK cells (Figure 11B) and the T-LGL leukemia cells of IL-15tg mice (Figure 1B, right). We therefore assessed the NKp46<sup>-</sup>CD3<sup>+</sup> T-LGL leukemia cells, NKp46<sup>+</sup>CD3<sup>+</sup> T-LGL leukemia cells, and normal NKp46<sup>-</sup>CD3<sup>-</sup> NK cells from a T-LGL leukemia patient for expression of *NKp46* mRNA and protein. After FACS sorting, RT-PCR (Figure 11C, top), and Western blotting (Figure 11C, bottom) demonstrated that sorted T-LGL leukemia cells lacking flow cytometric evidence of surface NKp46 expression (NKp46<sup>-</sup>CD3<sup>+</sup>) expressed *NKp46* at the mRNA and protein levels but in an amount that was less than that of sorted T-LGL leukemia cells with surface NKp46 expression (NKp46<sup>+</sup>CD3<sup>+</sup>) and less than that of normal NK cells (NKp46<sup>-</sup>CD3<sup>-</sup>). We also assessed both normal NK cells and T-LGL leukemia cells for intracellular NKp46 protein expression by immunofluorescent staining. We noted that some NKp46 expression located in the cytoplasm and coexisted in the same region of CD3ζ expression, not only in normal NK cells (Supplemental Figure 2A) but also in T-LGL leukemia cells (Supplemental Figure 2B). This is consistent with previous studies showing that the 2 molecules interact with each other in NK cells (19, 20). Finally, we found that T-LGL leukemia cells have upregulation of IL-15, IL-15Rα, and IL-15Rγ expression, as compared with that of normal CD8<sup>+</sup> T cells (Supplemental Figure 3), in addition to the expression of IL-15Rβ (data not shown).

## Discussion

Previous studies proposed that NKp46 is a specific marker for NK cells across species (24, 25). However, CD3<sup>+</sup>NKp46<sup>+</sup> expression





**Figure 7**

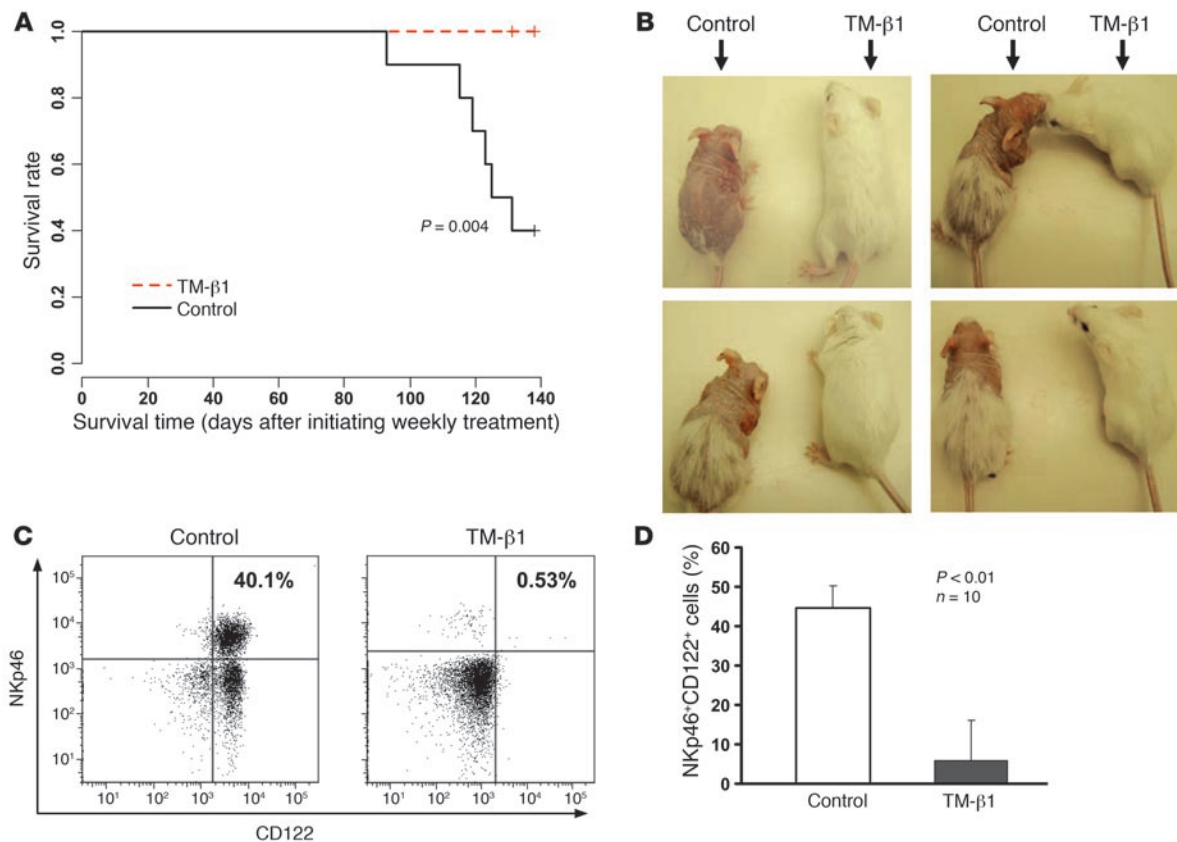
Examination of ITAM signaling in NKp46<sup>+</sup> NKT cells. **(A)** Fresh NKp46<sup>+</sup> and NKp46<sup>-</sup> NKT cells were purified from pooled splenic cells of WT FvB/NJ mice by column selection, followed by FACS sorting. Cells were exposed to medium alone or IL-15 and then subjected to Western blotting using the mixture of pan-phospho-tyrosine mAbs PY20 and 4G10. The same membranes were reprobbed with an anti-actin mAb to ensure equal loading of protein. **(B)** NKp46<sup>+</sup> cells were starved in serum-free medium, following expansion by IL-2 or IL-15. These starved cells were incubated with anti-NKp46 mAb for 30 minutes on ice, followed by cross-linking with a secondary mAb at 37°C for the time points indicated. Protein lysates from these cells were analyzed by Western blotting using anti-phospho-Lck or anti-phospho-Zap70 mAb. The same membranes were reprobbed with an anti-actin mAb to ensure equal loading of protein. Results show that cross-linking of NKp46 receptor induced Lck and Zap70 tyrosine phosphorylation. **(C)** Gene profile analysis of resting WT NKp46<sup>+</sup> (left 2 columns) and resting WT NKp46<sup>-</sup> (right 2 columns) NKT cell subsets identified that *DAP12* is dramatically upregulated in the NKp46<sup>+</sup> NKT subset, with each subset displaying a unique gene expression pattern (see also Supplemental Table 2). Green and red denote low and high gene expression levels, respectively. **(D)**. Confirmation of *DAP12* expression by real-time RT-PCR analysis. The data are presented as mean ± SEM.

has also been reported in WT mice, in patients with celiac disease, and in cultured human umbilical cord blood CD8<sup>+</sup> T cells (24, 34, 35), but to our knowledge, they have not yet been identified and characterized in healthy humans. In this report, we demonstrated that a minute population of NKp46<sup>+</sup> NKT cells exists in normal humans as well as in several species of WT mice. Both human and mouse NKp46<sup>+</sup> NKT cells showed a more activated phenotype compared with that of NKp46<sup>-</sup> NKT cells, and NKp46<sup>+</sup> NKT cells from both species demonstrated a significantly greater intrinsic proliferation and/or significantly greater proliferative response to IL-15 when compared with that of NKp46<sup>-</sup> NKT cells. We observed that virtually 100% of murine IL-15tg T-LGL leukemia cells examined did express NKp46, in contrast to the minute fraction in WT mice, and that IL-15-expressing NKp46<sup>-</sup> cells did not upregulate NKp46 on their cell surface, despite months of tracking after adoptive transfer to congenic WT mice. Finally, interruption of IL-15 signaling via the regular administration of the TM-β1 mAb completely prevented NK or T-LGL leukemic transformation in IL-15tg mice. Thus, we believe that our data support the notion that NKp46<sup>+</sup> NKT cells likely represent the fraction of cells that undergoes malignant transformation to T-LGL leukemia after chronic exposure to overexpression of IL-15 in vivo. Notably, a majority of human T-LGL leukemia samples expressed higher levels of mRNA of *NKP46* and *IL15* as well as *IL15RA* and *IL2RG* when compared with their nonmalignant CD8<sup>+</sup> counterparts. Together, the observational and experimental mouse and human data noted above suggest that at least some human T-LGL leukemia cells are

likely transformed from the minute NKp46<sup>+</sup> NKT population in the presence of excessive IL-15 expression.

The phenotypic analysis performed in this study for both mice and humans defines the NKp46<sup>+</sup> subset of NKT cells as more closely resembling NK cells and more activated when compared with the NKp46<sup>-</sup> NKT cells. Consistent with this, NKp46<sup>+</sup> NKT cells secrete more IFN-γ, TNF-α, and IL-10 cytokines. We recently showed that mouse CD94<sup>hi</sup> NK cells have higher IFN-γ production than CD94<sup>lo</sup> NK cells (36) as do human CD94<sup>hi</sup> NK cells (37). Consistent with these earlier findings, here we show that both mouse and human NKp46<sup>+</sup> NKT cells have significantly higher CD94 expression than NKp46<sup>-</sup> NKT cells. Likewise, we noted that compared with NKp46<sup>-</sup> NKT cells, NKp46<sup>+</sup> NKT cells have higher expression of the activating molecule DAP12, which interacts with CD94/NKG2C (29). Nonetheless, we cannot yet imply that the NKp46<sup>-</sup> NKT cell subset undergoes further differentiation to the NKp46<sup>+</sup> subset in either mice or humans. Indeed, we tracked NKp46<sup>-</sup> cells for 9 months in vivo and did not see any acquisition of NKp46 expression. Consistent with this, we found that the 2 subsets had differential dependence on CD1d, allowing us to classify NKp46<sup>+</sup> NKT cells as non-CD1d-restricted NKT-like cells. However, the developmental relationship between NKp46<sup>-</sup> and NKp46<sup>+</sup> NKT cells is not very clear yet and will require further study.

A number of studies here show that the WT NKp46<sup>+</sup> NKT-like cells were more activated than their NKp46<sup>-</sup> counterpart in both species, especially given the fact that NKp46<sup>-</sup> NKT-like cells have a higher proliferative capacity than NKp46<sup>-</sup> NKT cells in vivo and



**Figure 8**

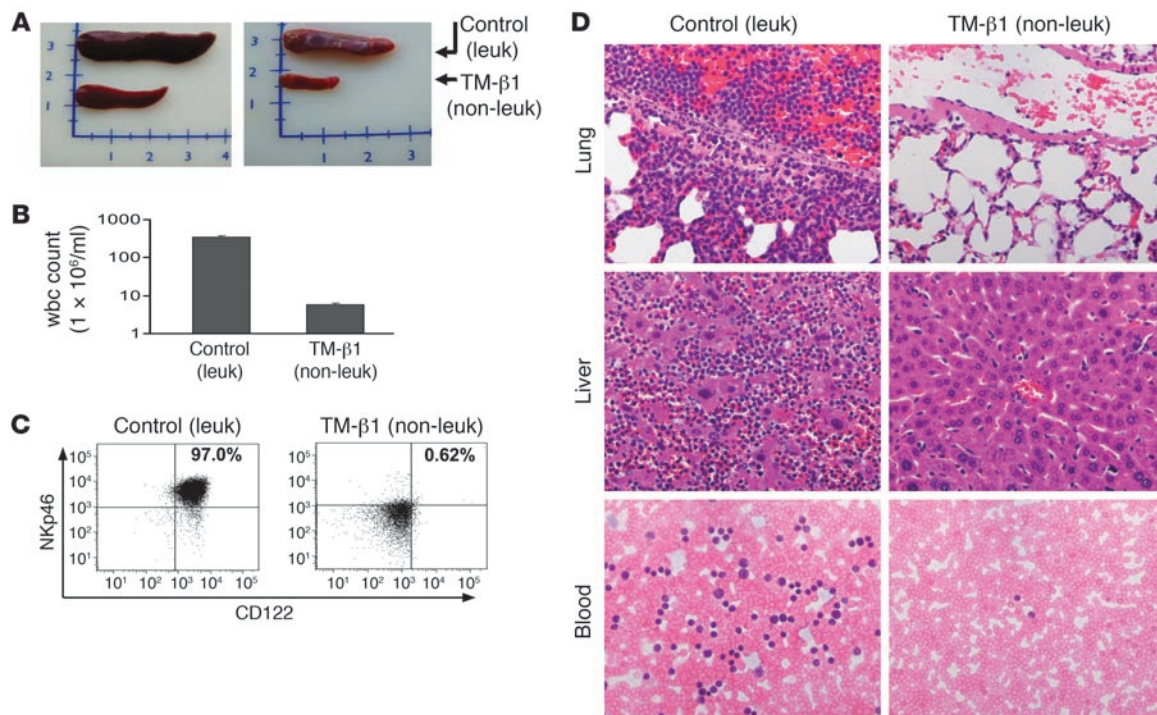
TM-β1 mAb treatment enhances survival of IL-15tg mice and depletes NKp46<sup>+</sup>CD122<sup>+</sup> cells. **(A)** The Kaplan-Meier curve shows that 4- to 5-week-old IL-15tg mice given TM-β1 mAb weekly, at a dose of 200 μg per mouse, exhibited prolonged survival compared with control mice treated with PBS. **(B)** TM-β1 mAb treatment prevented early onset of alopecia that occurs in the IL-15tg mice. Four out of ten littermate pairs of WT and IL-15tg mice are shown. **(C)** Flow cytometric analysis indicated the nearly complete depletion of NKp46<sup>+</sup>CD122<sup>+</sup> cells in the TM-β1-treated mice but not in the control-treated mice. Percentages of NKp46<sup>+</sup>CD122<sup>+</sup> cells in the respective quadrants are indicated. **(D)** Summary data of **C** from 10 pairs of littermate mice. The bar graph indicates mean ± SD.

in vitro. Consistent with these findings, our data showed that the minute NKp46<sup>+</sup> NKT-like cell subset expressed higher IL-15Rβ in both mice and humans than the larger NKp46<sup>-</sup> NKT subset. In fact, together with our previous studies (14), we show that IL-15 can transform NK and NKp46<sup>+</sup> NKT cells into leukemia in our IL-15tg mice, but IL-15 cannot do so for other types of cells (e.g., B, classical T, or monocytic cells). Therefore, presumably, the abnormally high expression of IL-15, combined with the relatively enhanced responsiveness to this cytokine, leads the NKp46<sup>+</sup> cell to malignant transformation. We previously reported that the CD3<sup>+</sup> T-LGL leukemias from the different IL-15tg mice are all clonal, but the TCR Vβ rearrangement in each case can vary (14). This variation in clonality likely results from a “field effect” of prolonged and excessive exposure to IL-15 on whole populations of susceptible NKp46<sup>+</sup> NK or NKT cells. We believe the clone that emerges depends on which ultimately has the strongest proliferative signal. The specific clonal expansion likely starts at the polyclonal stage, since in this study we show that TCR skewing has already begun in polyclonal IL-15tg mice.

Since the in vivo overexpression of IL-15 as a single gene product can seemingly drive IL-15-responsive cells into cancer cells, and since disruption of the signaling between IL-15 and IL-15Rβ

prevents leukemia in our mouse model, our accumulated studies would suggest that IL-15 acts as an oncogene. This function is in addition to its known roles in NK cell development, T cell homeostasis, and proinflammation (38). Surprisingly, despite the fact that both IL-15 and IL-2 share the IL-2/15Rβ cell surface receptors that transmit their signals following ligand binding, IL-2 does not appear to share this oncogenic property with IL-15 (39, 40). This notion of IL-15 as an oncogene is also supported by our earlier studies showing that overexpression of IL-15 can cause nonrandom gene silencing of some putative tumor suppressor genes by abnormal methylation at the genome level along with nonrandom cytogenetic changes (i.e., trisomy 15 and/or 17) (41, 42). A recent elegant study from the Young laboratory indicates that IL-15 reduces expression of the proapoptotic gene *Bid* in NK and T-LGL leukemias and is therefore also in line with this notion (43). Moreover, consistent with our hypothesis, a recent computer modeling study from Zhang and colleagues suggests that the persistence of IL-15 and PDGF is sufficient to reproduce all known deregulations in T-LGL leukemia (13).

It has previously been reported that loss of *p53* expression in cancer results in deregulation of both IL-15 and IL-15Rα (44), suggesting that deregulation of IL-15 signaling as determined by loss

**Figure 9**

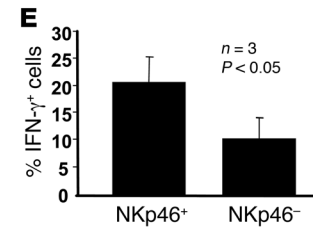
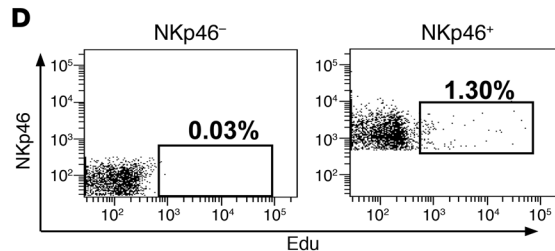
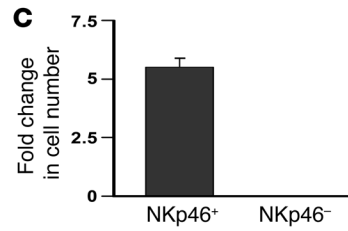
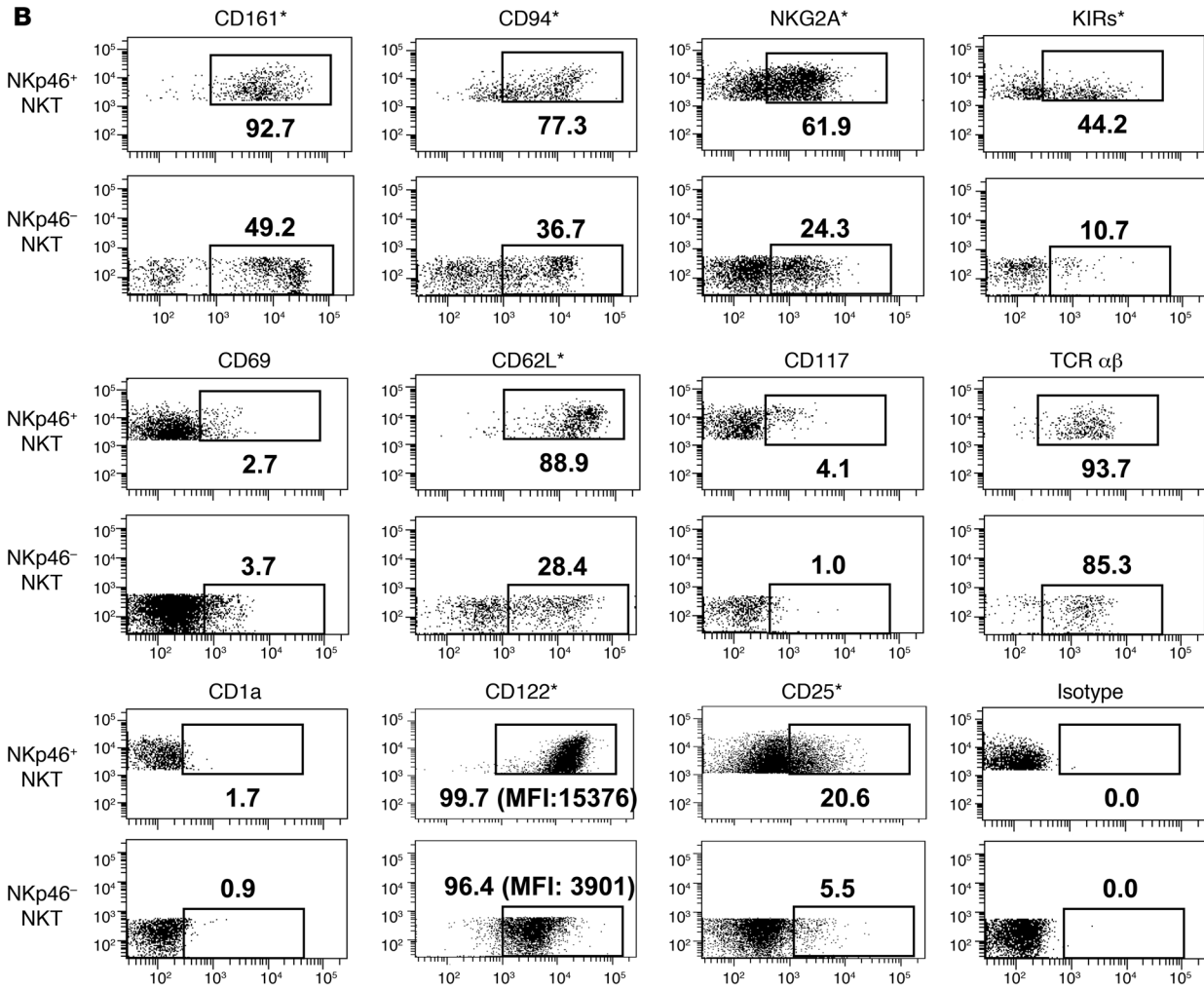
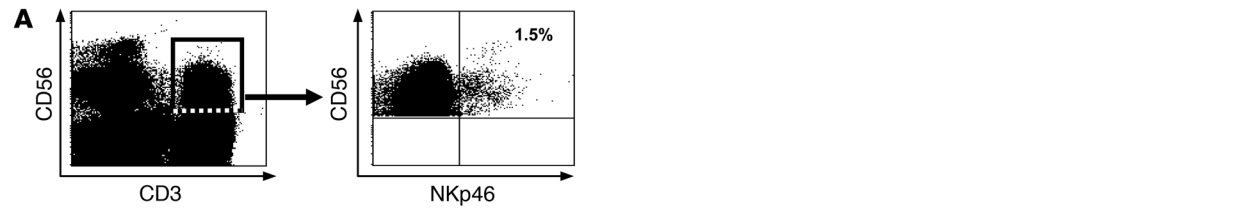
TM- $\beta$ 1 mAb treatment prevents leukemia in mice treated from the age of 4 to 5 weeks. **(A)** Size comparison of spleens from leukemia mice (leuk mice) in the control group and from non-leukemia mice (non-leuk mice) from the TM- $\beta$ 1-treated group. **(B)** Cell count comparison of white blood cells of leukemia mice in the control group and of non-leukemia mice from the TM- $\beta$ 1-treated group. The bar graph indicates mean  $\pm$  SD. **(C)** A flow cytometric analysis of splenic cells from leukemia mice in the control group and non-leukemia mice from the TM- $\beta$ 1-treated group. Percentages of NKp46<sup>+</sup>CD122<sup>+</sup> cells in the respective quadrants are indicated. **(D)** H&E tissue staining of lungs, livers, and blood from leukemia mice in the control group and from non-leukemia mice in the TM- $\beta$ 1-treated group. Original magnification,  $\times$ 200.

of p53 may contribute to oncogenesis. Likewise, the STAT genes, such as *STAT3* and *STAT5*, which are a part of IL-15 signaling, have been described as oncogenes, and their enhanced activation in states of inflammation can contribute to an increase in cell growth and/or tumorigenesis (45, 46). Similarly, the overexpression of IL-15 itself may induce leukemic transformation by a combination of aberrant gene methylation and consequent silencing of tumor suppressor genes, along with enhanced cell proliferation and/or resultant increase in resistance to apoptosis (42, 47). In this study, we showed that IL-15 can induce tyrosine phosphorylation in NKp46<sup>+</sup> NKT cells but not in NKp46<sup>-</sup> NKT cells. The cumulative effect of these changes caused by IL-15 deregulation could result in uncontrolled proliferation, genomic instability, and consequent malignant transformation of a susceptible population, as is seen in our NKp46<sup>+</sup> NKT cell population (48).

The IL-15-driven oncogenesis may also, at least partially, be mediated by NKp46. In literature, it has been shown that NKp46 associates with cytoplasmic CD3 $\zeta$  in NK cells (19, 49). Consistent with this, we found that in both normal NK cells and T-LGL leukemia cells, NKp46 appears to be mainly expressed on the cell surface and/or cytoplasm and that cytoplasmic CD3 $\zeta$  expression coexists in the same region of a cell (Supplemental Figure 2). We speculate that this association may trigger a series of inhibitory and activating signaling networks, which, in the presence of excessive IL-15, converge to result in oncogenesis through interfering with normal cell growth and/or cell survival/apoptosis. In fact,

as mentioned earlier, we found that IL-15 stimulation induced tyrosine phosphorylation in NKp46<sup>+</sup> NKT cells, and cross-linking of NKp46 enhanced phosphorylation of both Zap70 and Lck. Interestingly, our unpublished data demonstrated that IL-15 can further upregulate NKp46 expression in NKp46<sup>+</sup> NK cells, and this may also be true for NKp46<sup>+</sup> NKT cells that we characterized in this study. According to this notion, the cells that have both NKp46 and cytoplasmic CD3 $\zeta$  expression will have higher surface expression of IL-15 receptor components and are more likely to undergo malignant transformation in the presence of excessive IL-15. Thus, NK cells (all of which express NKp46 and cytoplasmic CD3 $\zeta$ ) and NKp46<sup>+</sup> non-CD1d-restricted NKT-like cells would be susceptible to malignant transformation under such conditions. Indeed, as mentioned earlier, our mice overexpressing IL-15 only have NKp46<sup>+</sup> NK and NKp46<sup>+</sup> NKT (T-LGL) leukemia not NKp46<sup>-</sup> NK LGL leukemia, NKp46<sup>-</sup> T-LGL leukemia, or T, B, or monocytic leukemia (14). Therefore, our data show that NKp46 expression can mark T-LGL leukemia in both mice and humans. Since unique and specific markers to identify LGL leukemia are lacking, NKp46 adds a valuable marker to the current potential marker pool for this disease.

Consistent with this report is the finding that many if not all human cell lines that are derived from NK or T-LGL leukemias are in fact dependent on exogenous IL-15 or IL-2 stimulation, both of which work through the IL-2/15R $\beta$  signaling components (50–52). This would suggest that other cells, such as DCs or stromal cells,



**Figure 10**

Characterization of NKp46<sup>+</sup> NKT cells in human PBMCs. **(A)** Representative plot shows that human NKp46<sup>+</sup> NKT cells (CD56<sup>+</sup>CD3<sup>+</sup>) in peripheral blood of healthy donors represented approximately 1.5% of NKT cells and approximately 0.2% of human lymphocytes (data not shown). **(B)** Assessment of NKp46<sup>+</sup> and NKp46<sup>-</sup> subsets for surface marker expression. The cell number of the NKp46<sup>-</sup> NKT subset was roughly equivalent to the number of the minute population of the NKp46<sup>+</sup> NKT subset. The numbers above or below each box in each plot are the percentages averaged from at least 3 donors and representing the mean percent positive cells for the surface marker, compared with its isotype control. Mean fluorescence intensity of CD122 is also included and is represented by the numbers in the parenthesis. \**P* < 0.05. **(C)** Costimulation of human NKT cells with IL-15 and NKp46 cross-linking shows that FACS-sorted NKp46<sup>+</sup> human NKT cells can survive and proliferate for 15 days after costimulation but FACS-sorted NKp46<sup>-</sup> NKT cells cannot. **(D)** EdU incorporation assay shows that NKp46<sup>+</sup> NKT (CD56<sup>+</sup>CD3<sup>+</sup>) cells incorporate higher EdU than NKp46<sup>-</sup> NKT cells. The assay was conducted on PBMCs freshly isolated from peripheral blood of healthy donors. The percentages in the dot plots indicated EdU<sup>+</sup> cells for each subset. **(E)** IFN- $\gamma$  intracellular staining indicates that human NKp46<sup>+</sup> NKT (CD56<sup>+</sup>CD3<sup>+</sup>) cells produce more IFN- $\gamma$  than NKp46<sup>-</sup> NKT cells (*P* < 0.05, *n* = 3). **(A, B, and D)** Representative plots from 1 out of at least 3 independent experiments are shown. **(C and E)** Error bars indicate SD.

may have deregulated *IL15* gene expression, which leads to malignant conversion of the NKp46<sup>+</sup> LGL *in vivo*. Our data strongly suggest that IL-15-induced NKp46<sup>+</sup> T-LGL leukemia develops from functionally more activated cells expressing a variety of NK cell maturation markers. This could suggest that a leukemia stem cell may exist within a mature cell population, a notion that has recently been suggested in other studies (53).

Over the past decade, certain mAbs have been shown to provide an effective approach to treat hematological malignancies, and the anti-CD122 mAb (against IL-2/15R $\beta$ ) is one of the mAbs being studied in clinic for the treatment of leukemia (54). In a phase I trial, 12 T-LGL leukemia patients received 4 doses of murine M $\mu$ k- $\beta$ 1 mAb against human IL-15R $\beta$  at 4-day intervals. Downregulation of CD122 was demonstrated in some patients, but no responses were seen, which might be due to the short time period (10–12 days) of administration and questionable effectiveness of this murine mAb compared with humanized mAb (55). Because of high expression of IL-15R $\beta$  in both normal NKp46<sup>+</sup> NKT cells and their LGL leukemia counterparts in IL-15tg mice, we administered weekly doses of 200  $\mu$ g TM- $\beta$ 1 mAb to IL-15tg mice, starting at the age of 4–5 weeks for a longer period of time (nearly 5 months) than that reported in the human trial. Results showed that the treatment was 100% effective in preventing the development of T-LGL leukemia, compared with mice receiving the placebo who had a 40% incidence of leukemia. The same treatment in older leukemic mice (13- to 14-week old) showed little or no effectiveness (data not shown). Thus, the dose and schedule of mAb administration compared with the number of malignant cells, the extent of the IL-15R $\beta$  expression, and/or their lack of dependence on IL-15 over time could all be factors for the failure of this mAb therapy in these older mice.

In summary, using NKp46 surface density expression on T cells, we have characterized a minute NKp46<sup>+</sup> NKT-like population in mice and identified and characterized a similar population in healthy human donors that appears more susceptible to malig-

nant transformation in the presence of excessive IL-15, compared with the NKp46<sup>-</sup> T cell population. The work here also suggests that NKp46 can be a marker for these malignant cells and their precursors and, as such, should be considered a tumor-associated antigen. In humans, there is no curative therapy for LGL leukemia (56). Targeting of IL-15R $\beta$  can be a promising approach, and NKp46 expression might also prove to be a useful target in states of minimal residual disease for NK and T-LGL leukemia.

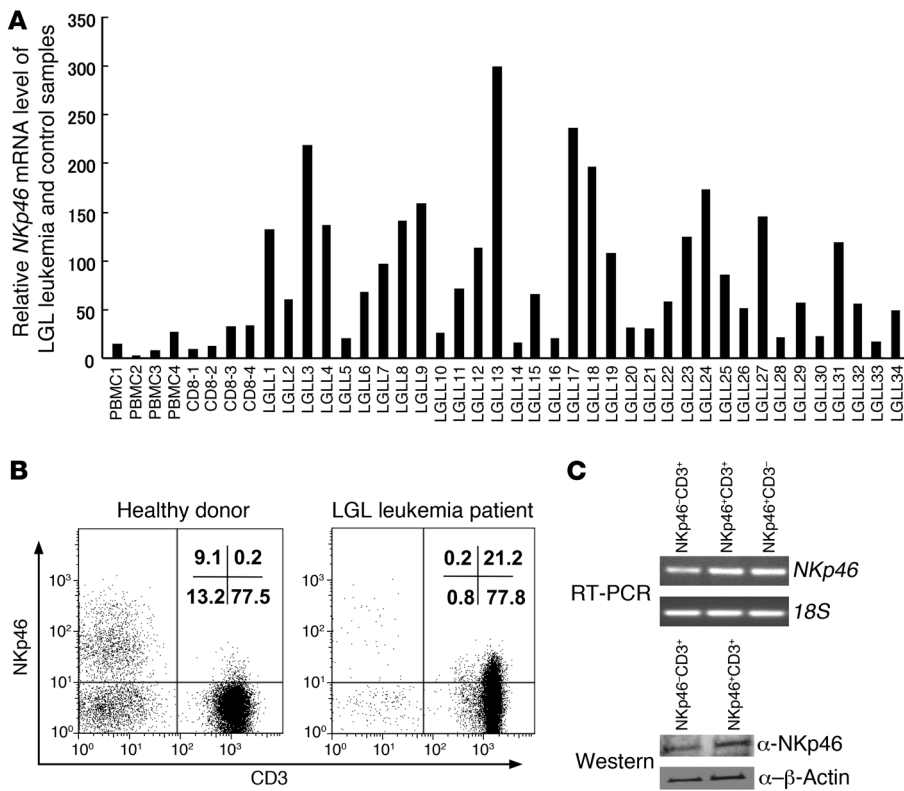
**Methods**

**Mice.** IL-15tg FvB/NJ background and C57BL/6 background mice were engineered as described and maintained at The Ohio State University (14). All studies were reviewed and approved by The Ohio State University Institutional Laboratory Animal Care and Use Committee.

**Human PBMC preparation.** Human PBMCs and CD8<sup>+</sup> T cells were isolated from peripheral blood leukopacks of healthy donors (American Red Cross) by Ficoll-Hypaque density gradient centrifugation. LGL leukemia cells were obtained from 34 T-LGL leukemia patients. All patients met the clinical criteria of T-LGL leukemia, with increased LGL counts and clonal T cell antigen receptor gene rearrangement. Informed consent was obtained from all patients. All experiments involving human blood were in accordance with a human subjects protocol approved by the Institutional Review Boards of The Ohio State University or of Milton S. Hershey Medical Center (Hershey, Pennsylvania, USA).

**Flow cytometric analysis.** The following mAbs reactive with murine cells were purchased from BD Biosciences: CD3 (145-2C11), CD5 (53-7.3), CD4 (RM4-5), CD8a (53-6.7), CD27 (LG.3A10), CD43 (S7), CD44 (IM7), CD45.1 (A20), CD45.2 (clone 104), CD69 (H1.2F3), CD122 (TM- $\beta$ 1), CD62L (MEL-14), D $\alpha$ 5 (HM $\alpha$ 2), CD107a (1D4B), NK1.1 (PK136), Ly49G2 (4D11), Ly49D (4E5), Ly6A/E (Sca-1) (D7), NKG2 A/C/E (20d5), CD11b (M1/70), CD117 (2B8), TCR $\alpha\beta$  (H57-597), TCR $\gamma\delta$  (GL3), IL-10 (JESS-16E3), TNF (MP6-XT22), and IFN- $\gamma$  (XMG1.2); these were used as direct conjugates to FITC, PE, PerCP, PE Cy7, or APC. CD94 (18d3) mAbs reactive with murine cells were purchased from eBioscience and used as direct conjugates to FITC or PE. The mouse CD1d tetramer loaded with the  $\alpha$ -C-galactosylceramine analogue PBS-57 and conjugated with APC and the unloaded control are gifts provided by NIH Tetramer Core Facility. Anti-human CD3-FITC (SK7), CD3-V450 (UCHT1), CD8-PerCP-Cy5.5 (SK1), CD57-FITC (NK-1), IFN- $\gamma$ -APC (4S. B3), NKp46-APC (9E2/nkp46), CD69-PE (FN50), CD62L-PE (DREG-56), CD94-PE (HP-3D9), CD117-PE (104D2), CD25-PE (M-A251), CD122-PE (Mik- $\beta$ 3), CD-132-PE (AG184), CD161-PE (HP-3G10), CD1a-PE (HI149), and TCR  $\alpha\beta$ -PE (T10B9.1A-31) were purchased from BD Biosciences. Anti-human CD158a-PE (HP-3E4; BD), CD158b-PE (CH-L; BD), KIR-NKAT2-PE (DX27; BD), KIR3DL1-PE (177407; R&D Systems), and KIR2DL3-PE (180701; R&D Systems) were mixed to examine KIRs expression. PE conjugated anti-human NKp46 (BAB281) and APC- and PE Cy7-conjugated anti-human CD56 (N901) were purchased from Beckman Coulter. Human LGL leukemia cells and normal PBMCs were stained with anti-human CD3 (BD Biosciences), CD57 (BD Biosciences), CD56 (BD Biosciences), CD8 (BD Biosciences), and NKp46 mAbs (Beckman Coulter). Cell preparation and analysis were performed as previously described (14, 36, 37).

**Determination of cytokine production.** For IFN- $\gamma$  analysis, whole spleen cells from WT FvB/NJ or C57BL/6 mice and human PBMCs were prepared and either rested or costimulated with recombinant mouse IL-18 (100 ng/ml) plus recombinant mouse IL-12 (10 ng/ml) and recombinant human IL-18 (100 ng/ml) plus recombinant human IL-12 (20 ng/ml), respectively (R&D Systems). At approximately 20 hours after stimulation, Golgi-plug (BD Biosciences) was added to the cells that were subsequently incubated for an additional 4 hours and stained for surface antigens (NK1.1 or CD56, CD3, and NKp46) and intracellular IFN- $\gamma$ . TNF- $\alpha$  and IL-10 production were assayed



**Figure 11**

Expression of NKp46 on human T-LGL leukemia cells. **(A)** Microarray analysis comparing NKp46 expression in PBMCs and isolated CD8<sup>+</sup> T cells from healthy donors to that in T-LGL leukemia patient samples (majority of cases are CD8<sup>+</sup>) indicates that NKp46 expression is upregulated in the majority of T-LGL leukemia cases ( $P < 0.001$ ,  $n = 34$ ). **(B)** A representative plot assessing a disproportionately greater fraction of CD3<sup>+</sup>NKp46<sup>+</sup> cells in the blood of 1 out of 5 T-LGL leukemia patients. The numbers in the dot plots indicate the percentages of cells in the respective quadrants. **(C)** RT-PCR and Western blotting demonstrated that sorted T-LGL leukemia cells lacking surface NKp46 expression by flow cytometry (NKp46<sup>-</sup>CD3<sup>-</sup>) express *NKp46* at the mRNA and protein levels in an amount that is less than that of sorted NKp46<sup>+</sup>CD3<sup>+</sup> T-LGL leukemia cells and normal NKp46<sup>+</sup>CD3<sup>-</sup> NK cells from the same patient. Due to low cell numbers, Western blotting for NKp46<sup>-</sup>CD3<sup>-</sup> NK cells of patients was not performed.

similarly, except that splenic cells from WT or IL-15tg FvB/NJ mice were cultured in the presence or absence of IL-2 (300 U/ml) or LPS (1 μg/ml).

**In vivo BrdU incorporation study.** To compare BrdU incorporation of NKp46<sup>+</sup> NKT cells and NKp46<sup>-</sup> NKT cells from the same mouse, 5 WT FvB/NJ mice and 5 polyclonal IL-15tg mice (nonlittermates) were given BrdU drinking water, changed daily at a concentration of 0.8 mg/ml for 6 consecutive days prior to sacrifice. After sacrifice, isolated spleen cells were stained for BrdU incorporation with the BD Pharmingen BrdU FITC Flow Kit according to the manufacturer's instruction. The experiment was repeated on 4 littermate pairs of WT and IL-15tg mice to compare BrdU incorporation of NKp46<sup>+</sup> NKT cells between WT mice and IL-15tg mice.

**Proliferation assay of mouse and human NKp46<sup>+</sup> and NKp46<sup>-</sup> NKT cells.** FACS-sorted NKp46<sup>+</sup> and NKp46<sup>-</sup> splenic NKT cells of WT FvB/NJ mouse or of human peripheral blood ( $1 \times 10^3$ ) were plated in triplicate in 96-well plates in 200 μl RPMI 1640 with 20% FBS in the presence of recombinant human IL-15 (Miltenyi Biotec; 100 ng/ml) or high-dose human IL-2 (900 U/ml) for assessment of in vitro cell growth. For mouse cells, 2-ME (50 μM) was also added to the culture. For human cells, some wells were costimulated with IL-15 and NKp46. For this purpose, anti-mouse IgG secondary mAb (Fab specific; Sigma-Aldrich) in PBS (10 μg/ml) was coated onto flat-bottom 96-well plates overnight. On the second day, cells were sorted and incubated with mouse anti-human NKp46 mAb (195314; R&D System) for 30 minutes on ice, followed by centrifugation and resuspension in the medium with IL-15. Finally, cells were cultured on the coated 96-well plates after the removal of PBS. Viable cells were enumerated at indicated points using a trypan blue (Invitrogen) exclusion assay.

For EdU assay, 10 million freshly isolated human PBMCs from peripheral blood leukopacks of 5 healthy donors were cultured with EdU for 3–4 hours in medium and were assayed for EdU incorporation by a Click-iT EdU Kit (Invitrogen) using a cytometric analysis, according to manufacturer's instructions. CD56<sup>+</sup>CD3<sup>+</sup> gate was set for total NKT cells that

were then divided between NKp46<sup>+</sup> and NKp46<sup>-</sup>. EdU incorporation of these 2 subsets was compared. Controlled samples were assayed in the same way without the addition of EdU and showed no any EdU<sup>+</sup> events in the flow cytometric analysis.

**Adoptive transplantation.** Six- to 8-week-old recipient WT C57BL/6 mice (CD45.1) were irradiated with a sublethal dose of 6 Gy from gamma irradiator. Splenic NKp46<sup>-</sup> NKT (NK1.1<sup>+</sup>CD3<sup>+</sup>) cells of C57BL/6 IL-15tg (CD45.2) mice with polyclonal NK and NKT cell expansion were sorted to at least 99% purity and transplanted into the irradiated CD45.1 mice by tail vein injection. To assess donor cell development, blood was drawn from the recipient tail veins, and CD45.2-positive cells were analyzed for CD3, NK1.1, and NKp46 expression once every 2 weeks in the first month after adoptive transfer. Total spleen, bone marrow, and blood cells were isolated from sacrificed recipient mice 9 months after the initial adoptive transfer to repeat the flow analysis.

**Western blotting and RT-PCR.** To assess induction of total tyrosine phosphorylation by IL-15 in fresh WT NKp46<sup>+</sup> and NKp46<sup>-</sup> NKT cells, NK1.1<sup>+</sup>/DX5<sup>+</sup> cells were first enriched by column selection (Miltenyi Biotec), after NK1.1-PE, DX5-PE, and PE-bead (Miltenyi Biotec) staining of pooled splenic cells of 7 to 15 WT FvB/NJ mice. The enriched cells were then FACS sorted for NKp46<sup>+</sup> NKT cells and NKp46<sup>-</sup>NKT cells, resulting in the collection of 100,000 to approximately 250,000 cells for the NKp46<sup>+</sup> NKT fraction. An equal number of the cells were either directly lysed by laemmli sample buffer (Bio-Rad) or lysed after stimulation with IL-15 for 5 minutes at 37°C. Lysates were subjected to Western blotting using the mixture of PY-20 (Calbiochem) and 4G10 (Millipore) anti-phospho-tyrosine antibodies.

To assay whether Lck and Zap70 phosphorylation can be induced by NKp46 stimulation, NKp46<sup>+</sup> cells were first expanded with IL-2 or IL-15 for about a week, starved overnight, and then were incubated with an anti-mouse NKp46 mAb (M-20; Santa Cruz Biotechnology Inc.) for 30 minutes



on ice, followed by cross-linking with a secondary mAb at 37°C for 0, 1, 3, 7, or 10 minutes. Protein lysates of these cells were analyzed by Western blotting with phospho-Lck or phospho-Zap70 mAb (Cell Signaling Technology). The same membranes were reprobbed with Actin mAb (Santa Cruz Biotechnology Inc.) to ensure equal loading of protein.

To determine the NKp46 expression level of NKp46<sup>+</sup> and NKp46<sup>-</sup> NKT subsets of T-LGL leukemia, the 2 subsets were sorted from CD3<sup>+</sup> cells of CD3<sup>+</sup>CD56<sup>+</sup> T-LGL patients. A portion of sorted subset cells were used to extract RNA for cDNA syntheses for RT-PCR analysis, and the rest portion was used for lysis for Western blotting analysis. Both assays were applied to compare NK46 expression in the 2 T-LGL leukemia subsets. The forward PCR primer was 5'-GGCTGTGTCTGAGTCAGAG-3', and the reverse primer was 5'-GAGTTCATGTCCGGGATGTAG-3'. NKp46 (M-20) mAb used for Western blotting was purchased from Santa Cruz Biotechnology.

The *DAPI2* mRNA level was determined by real-time RT-PCR analysis of cDNA synthesized from RNA extracted from FACS-purified NKp46<sup>+</sup> NKT cells and NKp46<sup>-</sup> NKT cells. SYBR Green PCR Master Mix was purchased from Applied Biosystems, and primers were forward, 5'-CGTACAG-CCCCAGAGTGAC-3' and reverse, CACCAAGTCACCCAGAACAA-3'.

**Microarray analyses.** To compare the global gene expression profile of NKp46<sup>+</sup> NKT cells and NKp46<sup>-</sup> NKT cells, the 2 subset cells were sorted from fresh pooled splenic cells of 7 to 10 WT FvB/NJ mice. Total RNA was isolated by TRIzol (Invitrogen) and purified with the RNeasy Mini Kit (Qiagen). cDNA was synthesized and amplified with the Ovation RNA Amplification System V2 Kit (NuGEN) and fragmented by the Encore Biotin Module V2 Kit (NuGEN), followed by hybridizing with Mouse Genome 430 arrays (Affymetrix). Signal intensities were quantified by Affymetrix software. Background correction and normalization were performed to correct technical bias, and gene expression levels were summarized over probe sets using RMA method (57). A filtering method was applied to filter out low or no expression genes, with log<sub>2</sub> expression levels below 5 for over 80% samples. For the remaining genes, linear models were performed to detect differentially expressed genes between NKp46<sup>+</sup> NKT and NKp46<sup>-</sup> NKT cells. In order to improve the estimates of variability and statistical tests for differential expression, variance shrinkage methods were used (58). The significance level was adjusted by controlling the mean number of false positives at 5 out of all probe sets (~20,000), i.e., 0.00025. A gene tree by hierarchical clustering method was generated by TMEV software for genes showing significant expression difference of more than 10 folds between the 2 cell subsets.

Expression of NKp46 in normal donor and T-LGL leukemia patient samples was analyzed by microarray profiling. In this microarray analysis, total RNA from human PBMCs and CD8<sup>+</sup> T cells obtained from buffy coats of normal donors and T-LGL leukemia samples was isolated with the RNeasy Mini Kit (Qiagen). cDNA was synthesized with random hexamers (Invitrogen). T-LGL leukemia samples and the PBMC and CD8<sup>+</sup> cell controls were subjected to microarray analysis as previously described (59), and data were deposited in the NCBI database (accession number GSE10631; <http://www.ncbi.nlm.nih.gov/geo>). Buffy coats for this experiment were

obtained from Hershey Medical Center Blood Bank, according to a human subjects protocol approved by Milton S. Hershey Medical Center.

**Treatment of IL-15tg mice by CD122 mAb TM-β1.** Twenty 4- to 5-week-old littermate IL-15tg mice were randomly separated into 2 groups. One group of mice was intraperitoneally injected with purified CD122 mAb TM-β1, and the other group was intraperitoneally injected with PBS. A dose of 200 μg mAb per mouse (as previously reported in ref. 60) was administered to the treatment group once per week for 138 days or until the mice were moribund. Five weeks after the initial treatment and immediately before the weekly dose, blood cells were collected for flow cytometric analysis to determine the efficacy of NKp46<sup>+</sup>CD122<sup>+</sup> cell depletion. Seven weeks after the initial treatment, pictures of live mice were taken. Mice were monitored daily, and once the mice were sacrificed, the spleen size was measured, white blood cells were counted, splenic cells were subjected to a flow cytometric analysis, and lung, liver, and blood tissues were prepared for H&E staining to determine the cause of death.

**TCR Vβ repertoire analysis.** Splenic cells from WT, IL-15tg polyclonal, and IL-15tg T-LGL leukemic FvB/NJ mice were prepared. The TCR Vβ screening panel was purchased from BD Biosciences, and analysis was performed as previously described (14). NKp46<sup>+</sup>NK1.1<sup>+</sup>CD3<sup>+</sup> leukemia cells of IL-15tg mice and CD3<sup>+</sup>CD19<sup>+</sup>NK1.1<sup>-</sup> T cells (as a control) of WT mice were purified by FACS (≥99% purity). RNA was isolated from these purified cells with the RNeasy Mini Kit (Qiagen), and cDNA was synthesized with random hexamers (Invitrogen) from the RNA. The cDNA was used for PCR amplification of 24 TCR Vβs using spectratyping primers as previously reported (61).

**Statistics.** An unpaired *t* test was used to compare 2 independent groups. A paired *t* test was applied to the comparison between 2 correlated groups, with observations from the same donor cells. Logit transformation was performed on the proportions of NKp46 cells versus NKp46<sup>-</sup> cells. Arcsine square root transformation was performed on the data set of extremely small proportions (close to 0), in the ex vivo EdU assay of human NKp46<sup>-</sup> and NKp46<sup>+</sup> NKT cells. All tests were 2 tailed. The Holm's procedure was used to correct for multiple comparisons. *P* < 0.05 was considered significant for single comparisons and for multiple comparisons after adjustment.

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Address correspondence to: Michael A. Caligiuri or Jianhua Yu, The Ohio State University Comprehensive Cancer Center, 521B James Cancer Hospital, 300 W. 10th Ave., Columbus, Ohio 43210, USA. Phone: 614.293.7521; Fax: 614.293.3132; E-mail: Michael.caligiuri@osumc.edu (M. Caligiuri); Jianhua.yu@osumc.edu (J. Yu).

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