

IL-2–dependent adaptive control of NK cell homeostasis

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Activation and expansion of T and B lymphocytes and myeloid cells are controlled by Foxp3⁺ regulatory T cells (T reg cells), and their deficiency results in a fatal lympho- and myeloproliferative syndrome. A role for T reg cells in the homeostasis of innate lymphocyte lineages remained unknown. Here, we report that T reg cells restrained the expansion of immature CD127⁺ NK cells, which had the unique ability to up-regulate the IL2R α (CD25) in response to the proinflammatory cytokine IL-12. In addition, we observed the preferential accumulation of CD127⁺ NK cells in mice bearing progressing tumors or suffering from chronic viral infection. CD127⁺ NK cells expanded in an IL-2–dependent manner upon T reg cell depletion and were able to give rise to mature NK cells, indicating that the latter can develop through a CD25⁺ intermediate stage. Thus, T reg cells restrain the IL-2–dependent CD4⁺ T cell help for CD127⁺ immature NK cells. These findings highlight the adaptive control of innate lymphocyte homeostasis.

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Abbreviations used: DT, diphtheria toxin; LCMV, lymphocytic choriomeningitis virus.

Regulatory T cells (T reg cells), expressing the transcription factor Foxp3, exert a critical brake on the adaptive immune system as their acute ablation or developmental paucity leads to a fatal lymphoproliferative syndrome in mice and humans. Aside from limiting the activation and size of the peripheral effector T and B cell populations, T reg cells restrain the generation and activation of innate myeloid cells, for example, dendritic cells (Kim et al., 2007; Wing et al., 2008; Liu et al., 2009). Additional innate lineages include NK cells and a growing family of innate lymphoid cells, two major types of lymphocytes lacking Ig and TCR receptors. These lymphocytes function as important effectors of immune responses directed against pathogens and tumors; they participate in the positive and negative regulation of adaptive immune responses and contribute toward wound healing and tissue repair. In addition, these cells have been implicated in autoimmune and allergic inflammation (Spits and Di Santo, 2011; Monticelli et al., 2012). Innate lymphocytes share some important characteristics with T lymphocytes. For example, common gamma chain (γ c) receptor family cytokines are required for their generation and maintenance. NK cells use IL-15, whereas innate lymphoid cells and a subset of NK cells expressing IL-7R are dependent on IL-7. These and additional cytokines (IL-25, IL-33, and type-I IFNs) that govern the homeostasis and maturation of these

cells are produced by myeloid, endothelial, and stromal cells (Spits and Di Santo, 2011; Sun and Lanier, 2011; Monticelli et al., 2012; Vonarbourg and Diefenbach, 2012). It is unknown whether cells of the adaptive immune system influence the differentiation and numbers of innate lymphocytes and whether their homeostasis is controlled by T reg cells.

To address these questions, we used conditional ablation of T reg cells in *Foxp3^{DTTR}* mice and explored its impact on NK cells, a prototypic innate lymphocyte lineage. We found that a subset of immature splenic CD127⁺ NK cells preferentially expressed CD25 in response to IL-12. T reg cells limited the IL-2–dependent homeostasis of these cells, and CD127⁺ NK cells accumulated in tumor-bearing and chronically infected mice. Thus, our experiments revealed the IL-2–dependent adaptive help for the homeostasis of a subset of innate lymphocytes and its restraint mediated by T reg cells.

RESULTS

Expansion of CD127⁺ NK cells in the absence of T reg cells

Previous work demonstrated the expansion of NK cells upon diphtheria toxin (DT)–mediated

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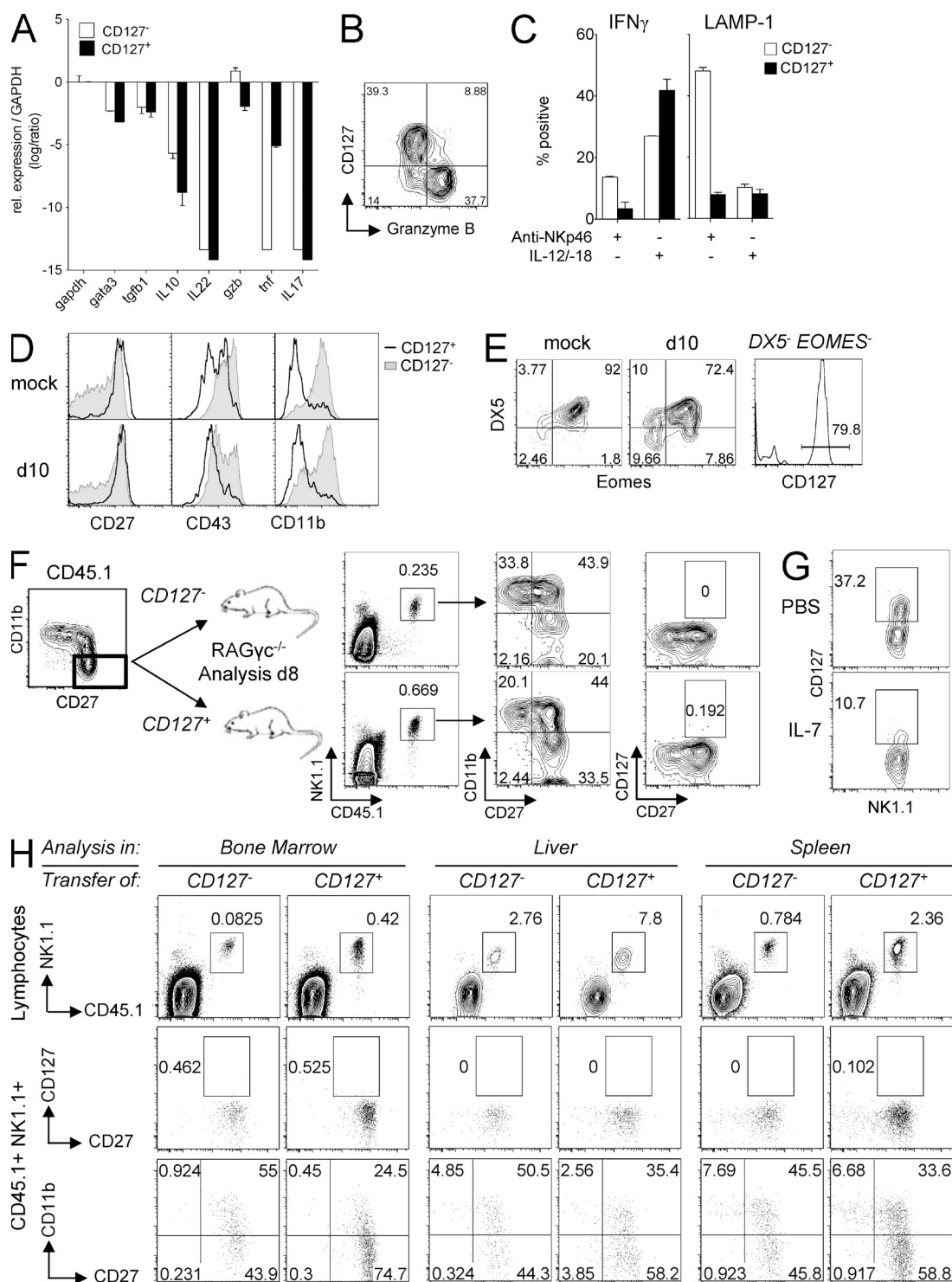


Figure 2. The CD127⁺ NK cell subset contains immature cells. (A–E) *Foxp3^{DTR}* mice were subjected to DT treatment to deplete T reg cells or mock treated, and splenic NK cells were isolated on day 10 of DT treatment. (A) Real-time PCR analysis of sort-purified CD27⁺CD11b⁺ splenic NK cells. (B) Intracellular staining for granzyme B expression in NK1.1⁺ CD3⁻ NK cells. (C) IFN- γ production and degranulation (LAMP-1⁺) of NK cell subsets (day 10 of DT treatment) stimulated as indicated. (D) Surface marker expression of CD127⁺ and CD127⁻ subsets of NK cells. (E) Intracellular staining for Eomes expression of NK1.1⁺ CD3⁻ NK cells. (F) CD27⁺CD11b⁻ CD127⁺ or CD127⁻ splenic NK cells were sort-purified from CD45.1⁺ *Foxp3^{-/-}* mice, and 2×10^4 cells were transferred i.v. into RAG γ C^{-/-} mice. The phenotype of transferred cells was analyzed in the spleen 8 d after transfer. (G) Phenotype of splenic NK cells from day 10 DT treatment mice after 4-h in vitro incubation with PBS or IL-7. (H) Experimental setup identical to F, but transferred cells were analyzed in indicated organs 30 d after transfer. All data are representative of two to three independent experiments. Error bars indicate SD.

CD127⁺ NK cells accumulate in tumor-bearing and chronically infected mice

To test whether the expansion of immature CD127⁺ NK cells is a unique feature of systemic autoimmunity observed in T reg cell-deficient mice or can be associated with other chronic inflammatory conditions, we analyzed mice with a pronounced tumor burden. CD127⁺ NK cells were enriched among tumor-infiltrating NK cells and in spleens of tumor-bearing B6 mice transplanted with B16 melanomas (Fig. 3 A). We also compared splenic NK cell subsets in acute and chronic viral infection. Although the frequency of CD127⁺ NK cells was reduced on day 6 of infection with lymphocytic choriomeningitis virus (LCMV) strain Armstrong (acute infection), it was increased on day 21 of infection with the chronically persisting LCMV clone 13 (Fig. 3 B). In both tumor-bearing and chronically infected mice, CD127⁺ NK cells shared the immature phenotype observed in mice subjected to T reg cell ablation (not depicted). These data suggest that CD127⁺ NK cells accumulate during chronic inflammatory conditions. Transplantation of B16 tumors and infection with LCMV clone 13 induced the relative expansion of CD127⁺ NK cells while the size of the splenic NK cell compartment was reduced in these mice (not depicted). In contrast, we observed a significant increase in absolute numbers of CD127⁺ NK cells in T reg cell-depleted mice, raising the possibility that T reg cells limited the expansion of CD127⁺ NK cells in tumor-bearing and LCMV-infected mice. We therefore decided to investigate the mechanisms controlling the homeostasis of CD127⁺ NK cells in the absence of T reg cells.

CD4⁺ T cells drive the expansion of CD127⁺ NK cells

Our recent observation that T reg cells controlled NK cell cytotoxicity by restricting T cell help (in this issue, see Gasteiger et al.) raised the question of whether the increase in numbers of CD127⁺ immature NK cells in *Foxp3^{DTR}* and *Foxp3^{KO}* mice was similarly dependent on activated T cells. Indeed, the

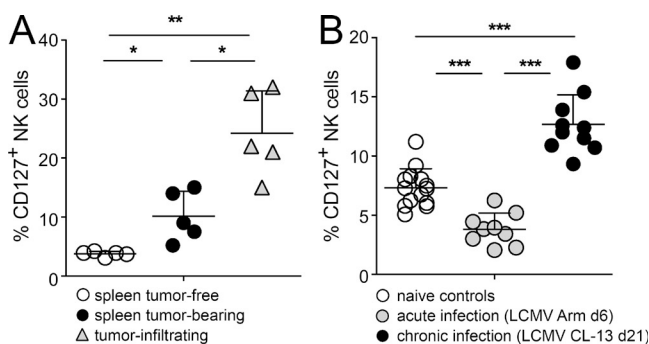


Figure 3. CD127⁺ NK cells accumulate in tumor-bearing and chronically infected mice. (A) Frequency of CD127⁺ cells within splenic or tumor-infiltrating NK1.1⁺ CD3⁻ NK cell subsets from B6 mice on day 16 after intradermal B16 melanoma implantation. (B) Frequency of CD127⁺ cells within splenic NK1.1⁺ CD3⁻ NK cell subsets in acutely (day 6, LCMV Armstrong) or chronically (day 21, LCMV clone 13) infected B6 mice. The data are representative of at least two independent experiments. Error bars indicate SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

transfer of effector T cells from *Foxp3^{KO}* mice into lymphopenic (RAG or TCRβδ deficient) recipients was sufficient to trigger the expansion of host CD127⁺ immature NK cells in the absence of T reg cells (Fig. 4 A). Furthermore, transfer of CD4⁺ but not CD8⁺ T cells alone resulted in increased CD127⁺ NK cell numbers (Fig. 4 B), and the antibody-mediated depletion of CD4⁺ T cells prevented the expansion of NK cells and dramatically reduced the number of CD127⁺ NK cells in T reg cell-depleted *Foxp3^{DTR}* mice (Fig. 4 C).

CD127⁺ NK cells preferentially up-regulate CD25 in response to IL-12

Interestingly, we found that a fraction of CD127⁺ NK cells were the only NK cells in unchallenged mice to express CD25, the high-affinity subunit of the IL-2 receptor. The frequency of CD25⁺ cells was markedly increased in DT-treated *Foxp3^{DTR}* as well as *Foxp3^{KO}* mice (Fig. 5 A). Intriguingly, the majority

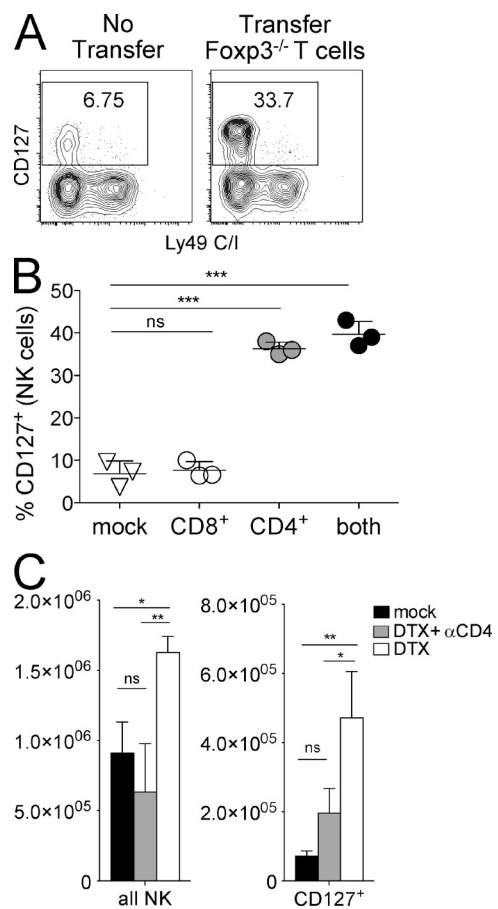


Figure 4. CD4⁺ T cells drive the expansion of CD127⁺ NK cells. (A and B) CD127 expression by endogenous NK cells from TCRβδ-deficient mice 30 d after transfer of CD4⁺, CD8⁺, or both subsets of CD45.1⁺ *Foxp3^{-/-}* T cells. (C) Absolute numbers of total NK1.1⁺ CD3⁻ splenic NK cells (left) or the CD127⁺ NK subset (right) on day 10 of DT treatment (DTX). One group of mice additionally received CD4-depleting antibody 12 h before the first DT administration. The data are representative of two to three independent experiments. Error bars indicate SD. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.

of CD127⁺ CD25⁺ appeared to be DX5^{lo} Eomes⁻ (not depicted). These observations suggested that CD25-dependent IL-2 signaling conferred a competitive advantage to immature CD127⁺ NK cells over their CD127⁻ counterparts in the absence of T reg cell-mediated suppression and in tumor-bearing and chronically infected mice (Fig. 5, B and C). Therefore, we tested which signals were able to induce CD25 expression preferentially in CD127⁺ NK cells. In accordance with a recent study (Lee et al., 2012), IL-18 alone and in combination with IL-12 induced high amounts of CD25 on all NK cells (not depicted). Because IL-12 is increased upon T reg cell depletion (Chinen et al., 2010), we tested its ability to drive the preferential expression of CD25 by CD127⁺ NK cells. Titration of IL-12 revealed that CD127⁺ NK cells had a unique ability to express CD25 upon stimulation with a low dose of IL-12 (Fig. 5 D). Importantly, TGF- β , which inhibits mature NK cells (Marcoe et al., 2012), but not IL-10 prevented IL-12-induced CD25 up-regulation by CD127⁻ cells, whereas CD127⁺ NK cells were resistant to TGF- β -mediated blockade of CD25 expression. Consistent with the observed IL-12-driven in vitro induction of CD25, the acute treatment of mice with IL-12 also induced selective up-regulation of CD25 on CD127⁺ NK cells (Fig. 5 E). This selective effect of IL-12 can be explained at least in part by increased levels of IL-12R β 1 on CD127⁺ NK cells (Fig. 5 F).

T reg cells control the IL-2-dependent homeostasis of immature CD127⁺ CD25⁺ NK cells

The aforementioned observations raised the possibility that the preferential expression of CD25 by CD127⁺ immature NK cells enabled the expansion of this subset fueled by increased availability of IL-2 upon the removal of T reg cells. T reg cells are known to restrict the availability of IL-2 by direct competition and consumption and by restraining the activation of IL-2-producing T cells (Pandiyani et al., 2007; Yamaguchi et al., 2011; Gasteiger and Kastenmuller, 2012). Importantly, targeting of IL-2 (Boyman et al., 2006) to CD25 in the form of agonistic immune complexes of IL-2 bound to JES6-1 antibody, which prevents IL-2 binding to CD122 alone, led to preferential expansion of CD127⁺ NK cells in T cell-deficient mice. In contrast, IL-2 bound to S4B6 antibody, which blocks binding to CD25 but allows binding to CD122, led to expansion of all NK cells (Fig. 6 A). Furthermore, blockade of IL-2 prevented the expansion of CD127⁺ NK cells in T reg cell-depleted mice (Fig. 6 B). In analogy to its function in peripheral T cells, CD25 expression seemed to confer competitive fitness but was dispensable for CD127⁺ NK cell development because CD25-deficient *Il2ra*^{-/-} mice had normal numbers of CD127⁺ NK cells (not depicted).

To directly test whether cell-intrinsic CD25 signaling accounts for the ability of CD127⁺ NK cells to expand in the absence of T reg cells, we generated mixed chimeras by transferring BM from CD45.2⁺ *Il2ra*^{-/-} mice mixed with BM from CD25-sufficient CD45.1⁺ *Foxp3*^{DTR} mice into RAG- γ c double-deficient recipients lacking endogenous NK cells (CD45.2⁺ *Il2ra*^{-/-} + CD45.1⁺ *Foxp3*^{DTR} \rightarrow RAG- γ c; Fig. 6 C).

Because high-affinity IL-2R signaling facilitates differentiation of T reg cells and is required for their homeostasis, virtually all T reg cells in these chimeric mice were derived from CD25-sufficient *Foxp3*^{DTR} precursors and, therefore, expressed DT

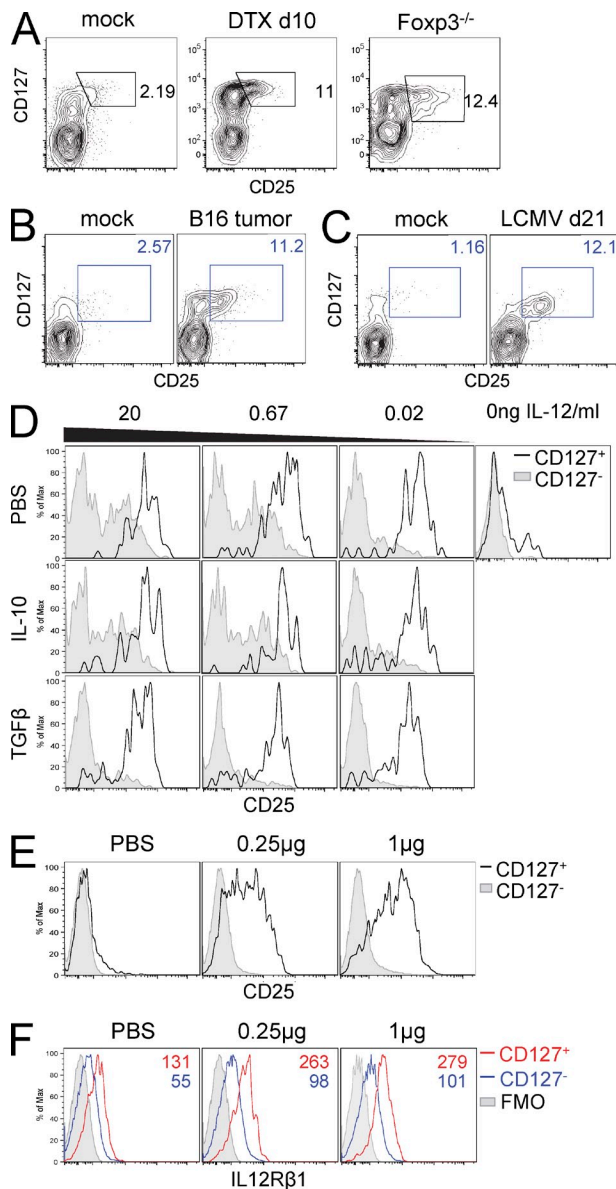


Figure 5. CD127⁺ NK cells preferentially induce CD25 expression in response to IL-12. (A–C) Cell surface expression of CD127 and CD25 on NK cells from mock treated, day 10 DT treatment (DTX) *Foxp3*^{DTR} mice, or 3-wk-old *Foxp3*^{-/-} mice (A) and tumor-bearing mice on day 16 of s.c. B16 melanoma implantation (B) or on day 21 of chronic viral infection with LCMV clone 13 (C). (D) CD25 expression of NK cells stimulated for 18 h in vitro with the indicated concentrations of IL-12 and PBS control, IL-10, or TGF- β . (E and F) Expression of CD25 (E) and IL12R β 1 (F) on splenic NK cells from mice that received (i.v.) the indicated doses of IL-12 16 h before analysis. Numbers in histograms show background-subtracted mean fluorescence intensity. FMO, full minus one control. The data are representative of two to three independent experiments.

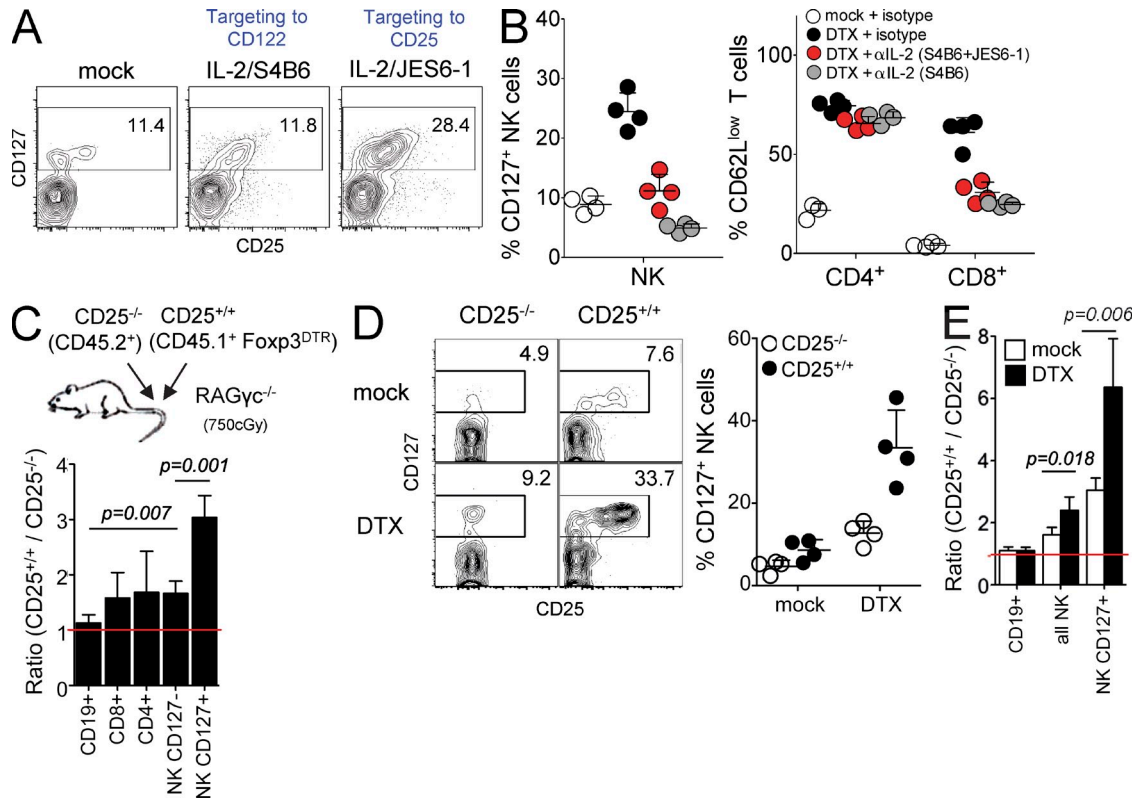


Figure 6. T reg cells control the IL-2-dependent homeostasis of immature CD127⁺ CD25⁺ NK cells. (A) CD127 expression by endogenous NK cells from TCR $\beta\delta$ -deficient mice on day 7 of treatment with the indicated IL-2-anti-IL-2 complexes. (B) Foxp3^{DTR} mice were subjected to DT treatment (DTX) or mock treated and additionally received IL-2 blocking antibodies JES6-1A and/or S4B6-1 or isotype control IgG, as indicated. Splenocytes were analyzed on day 10 of DTX. (C–E) Mixed chimeras were generated by cotransfer of BM from CD45.2⁺ *Il2ra*^{-/-} mice (CD25^{-/-}) and CD25-sufficient CD45.1⁺ Foxp3^{DTR} mice (CD25^{+/+}) into irradiated RAG- γ c^{-/-} mice. (C) Ratios of CD25^{+/+} to CD25^{-/-} among splenic lymphocytes 10 wk after BM transfer. (D and E) Frequency of CD127⁺ cells among CD25^{+/+} and CD25^{-/-} NK cells (D) and ratios of CD25^{+/+} to CD25^{-/-} among NK subsets on day 10 of T reg cell depletion (E). The data are shown as mean \pm SD and are representative of two to three independent experiments.

receptor (DTR; not depicted). Equal ratios of CD25-deficient and -sufficient CD19⁺ B cells observed 10 wk after BM transplantation suggested equal engraftment of CD25-sufficient and -deficient hematopoietic precursor cells (Fig. 6 C). Similar to peripheral T cells, mature CD127⁻ NK cells demonstrated a consistent, albeit moderate, bias in favor of CD25-sufficient NK cells, which is in agreement with our observation that some CD127⁺ CD25⁺ NK cells give rise to mature NK cells upon transfer into NK cell-deficient mice (Figs. 6 C and 2 F). In contrast, the CD127⁺ NK cell subset exhibited a pronounced bias toward CD25-sufficient over CD25-deficient cells even in the presence of T reg cells in the mixed chimeras, suggesting that IL-2 receptor signaling confers superior fitness to immature NK cells (Fig. 6 C). Depletion of T reg cells in mixed chimeras induced activation and expansion of T cells similar to that observed in Foxp3^{DTR} mice (not depicted). In addition, CD25-sufficient but not CD25-deficient CD127⁺ NK cells underwent additional expansion resulting in a further amplified skewing toward the former (Fig. 6, D and E). T reg cell depletion caused comparable skewing toward CD25-sufficient cells among CD4⁺ and CD8⁺ T cell subsets, known to respond to IL-2 in a CD25-dependent manner,

but not among other major hematopoietic cell populations (not depicted). Thus, these results suggest that the CD127⁺ NK cell subset contains immature NK cells and precursors of mature NK cells capable of expressing CD25 and responding to IL-2. T reg cells control the homeostasis of these immature NK cells by restricting CD4⁺ T cell-dependent IL-2 availability.

DISCUSSION

In addition to driving the expansion and differentiation of effector T cells, IL-2 is a key cytokine for the generation, maintenance, and suppressor function of T reg cells. Increased amounts of IL-2 produced by activated T cells lead to a sharp rise in numbers of T reg cells and their suppressor activity (Furtado et al., 2002; O’Gorman et al., 2009). Therefore, one important function of IL-2 is to establish a negative regulatory feedback loop for developing immune responses and the expansion of immune effector cells (Malek and Castro, 2010). Our findings suggest that IL-2 can additionally serve as a link between the homeostatic control of innate lymphocytes and adaptive immune responses, i.e., the activation of CD4⁺

T cells. Our work implicates T reg cell–mediated restraint of IL-2 availability as an important control mechanism of the homeostasis of innate lymphocytes.

Analyses of NK cell subsets in the presence or absence of T reg cells and in mixed BM chimeras suggested that NK cell differentiation and homeostatic fitness is facilitated by the expression of CD25 by immature CD127⁺ NK cells. We found that CD127⁺ NK cells have the propensity to respond to low levels of IL-12 stimulation with the up-regulation of CD25. This was at least in part caused by increased IL12Rβ1 expression. However, the observed failure of CD127⁻ NK cells to induce CD25 expression *in vivo* in response to seemingly high amounts of IL-12 (0.25–1 µg/mouse) suggests that additional signals might affect this response. In this context, it is of interest that CD127⁺ NK cells were resistant to the TGF-β–mediated inhibition of IL-12–driven CD25 expression, which we observed in their CD127⁻ counterparts. Thus, unlike mature cells, CD127⁺ immature NK cells likely undergo IL-2–dependent expansion even in the presence of TGF-β. This observation might explain why TGF-β inhibits predominantly mature NK cells (Marcoe et al., 2012). Collectively, our results point to a remarkable parallel between CD127⁺ NK cell maturation and the differentiation of CD8⁺ T cells in regards to the influence of IL-2 and CD25 signaling on the transition from less differentiated CD127⁺ KLRG-1⁻ cells to CD127⁻ KLRG-1⁺ terminally differentiated cells, which might be more susceptible to TGF-β–mediated suppression (Sanjabi et al., 2009; Kalia et al., 2010). For NK cells, this concept might be expandable to additional immunosuppressive mechanisms. A recent study suggested that calorically restricted mice, which have increased levels of corticosteroids, display increased frequencies of CD127⁺ NK cells as a consequence of a reduction of mature NK cells (Clinthorne et al., 2013).

Interestingly, CD127⁺ NK cells controlled by T reg cells resemble in part human CD56^{hi} NK cells in that both constitute a small fraction of Ly49^{lo}/KIR^{lo}, CD94^{hi}, NKG2A^{hi} cells that are enriched in secondary lymphoid organs and produce high amounts of cytokines in response to IL-12 and IL-18 (Fehniger et al., 2003). Human CD56^{hi} NK cells, which have been considered immature (Nagler et al., 1989), also express CD25 (Caligiuri et al., 1990). Furthermore, prolonged low-dose IL-2 treatment of cancer patients preferentially expanded this NK cell subset (Caligiuri et al., 1993). This observation raises the possibility that similar to CD127⁺ murine NK cells, human CD56^{hi} NK cells expand upon CD25 signaling. Intriguingly, these cells preferentially accumulate at sites of chronic inflammation in patients suffering from tuberculosis, sarcoidosis, rheumatoid arthritis, and cancer (Dalbeth and Callan, 2002; Bauernhofer et al., 2003; Katchar et al., 2005; Schierloh et al., 2005). The observed accumulation of CD127⁺ NK cells during systemic autoimmunity and in tumor-bearing and chronically infected mice warrants further investigation of origins, differentiation potential, and functions of these cells and their regulation by T reg cells in inflammatory diseases and cancer.

MATERIALS AND METHODS

Animals. *Foxp3^{DTR}* and *Foxp3^{KO}* mice have been previously described (Fontenot et al., 2003; Kim et al., 2007). C57BL/6J, TCRβ8^{KO}, and *Il2ra^{-/-}* mice were purchased from The Jackson Laboratory. RAGγc^{KO} and *Foxn1^{nu}* (nude) mice were purchased from Taconic. All of the mice were bred and housed in the specific pathogen–free animal facility at the Memorial Sloan-Kettering Cancer Center and used in accordance with institutional guidelines.

Cell depletions, IL-2 blockade, and treatment with IL-2–anti-IL-2 complexes. To deplete T reg cells, *Foxp3^{DTR}* mice were injected i.p. with 50 µg/kg DT (Sigma Aldrich) every other day (q2d). Chimeras received 15 µg/kg DT q2d. NK cells and CD4⁺ and CD8⁺ T cells were depleted by i.p. injections of 300 µg PK136, 400 µg GK1.5, and 2.43 antibody, respectively (BioXcell). To allow for complex formation, 1 µg of recombinant murine IL-2 (PeproTech) was incubated for 10 min at room temperature with 5 µg anti-IL-2 antibody (JES6-1A12 or S4B6-1; BioXcell) before i.p. injection q2d. To block IL-2 *in vivo*, mice received 200 µg of the indicated antibodies q2d i.p.

Tumor transplantation and viral infection. Mice were injected intradermally with 10⁶ B16/BL6 tumor cells on both flanks, and tumors were allowed to grow until they reached 1.5 cm in diameter. Tumors were dissociated with Liberase TL (Roche) according to the manufacturer's protocol; lymphocytes were enriched on a Ficoll gradient and stained for flow cytometric analysis using an LSRII flow cytometer (BD). Mice were infected i.p. with 2 × 10⁵ LCMV Armstrong or i.v. with 2 × 10⁶ LCMV clone 13 and analyzed on day 6 or 21 of infection, respectively.

Adoptive transfer. NK cells or T cells were negatively enriched after labeling with biotinylated antibodies against CD4, CD8, CD19, and Ter-119 and anti-biotin MACS–beads using LD columns (Miltenyi Biotec), goat anti-rat IgG beads (QIAGEN), or using the Dynabeads FlowComp mouse CD4 or T cell kits (Invitrogen), stained with surface antibodies, and then sorted on a FACSAria II cell sorter (BD), washed, and transferred by i.v. injection. Purity of sorted cell populations was typically >98%.

Analysis of phenotype, cytokine production, and degranulation. Cell suspensions of spleens were stained with fluorophore–conjugated antibodies purchased from eBioscience, BioLegend, or BD. Intracellular staining of Ki-67, Foxp3, and Eomes was performed using the Foxp3 mouse T reg cell staining kit (eBioscience). IL12Rβ1 was detected using biotinylated primary anti-IL12Rβ1 and secondary staining with Streptavidin-PE (Molecular Probes). The FMO (full minus one) control lacked anti-IL12Rβ1. CD127 down-regulation was tested in the presence of 10 ng/ml IL-7. To analyze cytokine production and degranulation, 10⁶ splenocytes/well were incubated on MaxiSorp 96–well plates (Thermo Fisher Scientific) coated with 10 µg/ml of agonist antibodies in the presence of 1 µg/ml Brefeldin A (Sigma-Aldrich) and 2.5 µg/ml CD107a antibody. Alternatively, cells were stimulated as indicated with 20 ng/ml IL-12, 10 ng/ml IL-18 (R&D Systems), or 50 ng/ml PMA and 500 ng/ml ionomycin. Intracellular cytokine staining was performed using the Perm/Wash kit (BD). For live/dead discrimination, propidium iodide or LIVE/DEAD fixable yellow dye (Molecular Probes) was used. Cells were analyzed on an LSRII cytometer and analyzed with FlowJo software (Tree Star).

Generation of mixed BM chimeras. NK cell–deficient RAGγc^{KO} mice were irradiated (750 cGy) and rested overnight. The next morning, BM from femurs and tibiae of CD45.2⁺ *Il2ra^{-/-}* and CD45.1⁺ *Foxp3^{DTR}* mice was prepared and depleted of CD90⁺ and NK1.1⁺ cells, and 2.5 × 10⁶ cells of each were i.v. injected into recipients.

Quantitative PCR. Total RNA was extracted with TRIzol reagent (Invitrogen) from FACS–purified NK cells. cDNA was synthesized with SuperscriptIII Reverse Transcription (Invitrogen), followed by real-time PCR analysis (SYBR green; Applied Biosystems).

Statistical analysis. All statistical analyses were performed using Prism5 software (GraphPad Software). Results are expressed as means \pm SD. Differences between individual groups were analyzed for statistical significance using the unpaired Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

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The authors declare no competing financial interests.

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