HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2019 March 14.

Published in final edited form as:

J Immunol. 2010 August 01; 185(3): 1393–1403. doi:10.4049/jimmunol.0903528.

Implications for Gene Therapy-Limiting Expression of IL-2Rγ_c Delineate Differences in Signaling Thresholds Required for Lymphocyte Development and Maintenance

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Abstract

X-linked SCID patients are deficient in functional IL-2Rγ_c leading to the loss of IL-2/IL-4/IL-7/IL-9/IL-15/IL-21 signaling and a lack of NK and mature T cells. Patients treated with IL-2Rγ_c gene therapy have T cells develop; however, their NK cell numbers remain low, suggesting antiviral responses may be compromised. Similarly, IL-2R$\gamma_c^{-/-}$ mice reconstituted with IL-2Rγ_c developed few NK cells, and reconstituted T cells exhibited defective proliferative responses suggesting incomplete recovery of IL-2Rγ_c signaling. Given the shift toward self-inactivating long terminal repeats with weaker promoters to control the risk of leukemia, we assessed NK and T cell numbers and function in IL-2R$\gamma_c^{-/-}$ mice reconstituted with limiting amounts of IL-2Rγ_c. Reconstitution resulted in lower IL-2/−15–mediated STAT5 phosphorylation and proliferation in NK and T cells. However, TCR costimulation restored cytokine-driven T cell proliferation to wild-type levels. Vector modifications that improved IL-2Rγ_c levels increased cytokine-induced STAT5 phosphorylation in both populations and increased NK cell proliferation demonstrating that IL-2Rγ_c levels are limiting. In addition, although the half-lives of both NK and T cells expressing intermediate levels of IL-2Rγ_c are reduced compared with wild-type cells, the reduction in NK cell half-live is much more severe than in T cells. Collectively, these data indicate different IL-2Rγ_c signaling thresholds for lymphocyte development and proliferation making functional monitoring imperative during gene therapy. Further, our findings suggest that IL-2Rγ_c reconstituted T cells may persist more efficiently than NK cells due to compensation for suboptimal IL-2Rγ_c signaling by the TCR.

The IL-2 family of cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) signal through the IL-2Rγ_c (γ_c) chain that associates with JAK3. Following ligand/receptor engagement, JAK3 is phosphorylated, which in turn phosphorylates STATs, such as STAT5, resulting in their dimerization and translocation to the nucleus (1). The γ_c chain is an essential component of different heterodimeric or heterotrimeric receptor complexes for each of the above

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Disclosures

The authors have no financial conflicts of interest.

The online version of this article contains supplemental material.

cytokines. IL-2 uses a trimeric complex with γ_c (CD132), IL-2Rβ (CD122), and IL-2Rα (CD25), whereas IL-15 uses γ_c, IL-2Rβ–, and IL-15Rα–chains and IL-7 uses a dimeric complex consisting of γ_c and IL-7Rα (CD127) (2). Genetic depletion of individual cytokines or components of their receptor complexes in mice led to the discovery that IL-7 and IL-15 signaling are required for T and NK cell development, respectively (3, 4). Mice with genetic depletion of the γ_c chain have no T or B cells and only a very small number of NK cells and these mice are used as a model for X-linked SCID (SCID-X1) (5, 6).

Patients with mutations in the γ_c chain develop SCID-X1 (7). This is typically manifested by the absence of both T and NK cells but normal numbers of functionally deficient B cells. As γ_c signals through JAK3, patients with mutations in JAK3 also haveT^-B^+ NK^- SCID develop (8). Both forms of SCID are lethal and without treatment results in death within the first few months of life. In many cases, allogeneic stem cell transplantation is curative; however, compatible donors are not always available, which led to the initiation of γ_c gene therapy trials. Unfortunately, these trials have recently been stopped as some of the patients treated with gene therapy had leukemia develop. In some cases, this has been attributed to upregulation of the LIM domain only 2 oncogene due to insertional mutagenesis (9, 10). To overcome this adverse event, current research is aimed at creating vectors with self-inactivating long terminal repeats (LTRs) and internal promoters with less potent activity and transcriptional buffers. After cessation of the gene therapy trials, Woods et al. (11) demonstrated that reconstitution of the mouse model of SCID-X1 with γ_c resulted in T cell lymphomas in 33% of recipient mice, suggesting that γ_c is itself oncogenic. Another recent report has shown that high levels of γ_c in transformed B cell lines can result in self-sufficient growth, one of the acquired capabilities of the cancer phenotype (12). These two studies imply that the level of γ_c expression is important in determining whether oncogenesis will occur after gene therapy. Although gene therapy for γ_c-mediated SCID-X1 has generally been considered successful apart from the development of leukemia in some patients, careful examination of the literature brings to light other significant issues that should be addressed prior to the next round of gene therapy.

Gene therapy in both mice and humans for γ_c-mediated SCID-X1 results in reconstitution of both the T and NK cell compartments to varying degrees. However, the NK cell compartment appears to be particularly vulnerable after gene therapy. In the initial murine gene therapy studies, NK cells developed in only 42–88% of mice reconstituted with the γ_c chain, and the percentage of NK cells in the γ_c-reconstituted mice were significantly lower than in mice reconstituted with wild-type (WT) bone marrow (BM) (13–16). In addition, although most patients who were treated with γ_c gene therapy initially had NK cells develop, over time their NK cell numbers fell to very low or negligible levels, leaving these patients with the potential for only limited NK cell-mediated tumor surveillance and viral immunity (17, 18). Some patients treated with γ_c gene therapy express normal levels of γ_c; however, those patients already expressing a mutant version of γ_c may not express normal levels of WT γ_c because of the competition with the mutant version. Development of the T cell compartment appears to be more robust, as the reconstituted mice all displayed a normal level of T cell development. However, on closer examination, although the reconstituted T cells are somewhat responsive to the IL-2 family of cytokines, this response is significantly lower than in WT cells (14–16). As the gene therapy approach is currently under re-

evaluation, we believe it would be beneficial to further study development of the NK cell compartment and the T cell response to the IL-2 family of cytokines under conditions that may limit the propensity for development of leukemia.

A recent report has indicated the level of γ_c expression is important for signaling in the lymphoid compartment. Smyth et al. (19) showed that IL-7 signaling requires a higher level of γ_c expression than IL-2 or IL-15 signaling using in vitro reconstitution of a transformed B cell line (19). The development of gene therapy with vectors containing self-inactivating LTRs and internal promoters with less potent activity may result in the development of T and NK cells with lower levels of γ_c expression. In this study, we examine the development and signaling events involved in T and NK cells after in vivo reconstitution of γ_c null mice with low-level γ_c. We find that as the NK cell compartment develops, these cells appear to display normal functional activity; however, they display significantly reduced γ_c-mediated signaling and proliferation. The T cell compartment also develops, however, ex vivo γ_c-mediated signaling is significantly reduced and proliferation is ablated in γ_c-reconstituted cells compared with WT cells. In addition, we have shown in both the NK and T cell compartments that increasing γ_c expression correlates with increased signaling downstream of γ_c cytokines such as IL-2 and IL-15. These data suggest that low-level γ_c is sufficient for NK and T cell development, whereas a higher threshold is required for γ_c-mediated proliferation.

Materials and Methods

Mice

C57BL/6 and IL-2R$\gamma_c^{-/-}$ ($B^+T^+NK^-$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ ($B^-T^-NK^-$) mice were purchased from Taconic (Germantown, NY). IL-2R$\gamma_c^{-/-}$/LAT$^{-/-}$ ($B^+T^-NK^-$) mice were generated by crossing IL-2R$\gamma_c^{-/-}$ with LAT$^{-/-}$ mice (20). IL-15$^{-/-}$ ($B^+T^+NK^-$) mice were a kind gift from Dr. Thomas Waldman (National Institutes of Health, Bethesda, MD). All mice were used at 6–12 wk of age. The care of experimental mice was in accordance with National Institutes of Health guidelines.

Constructs and Abs

The IL-2Rγ_c IRES EGFP pMSCV (γ_c:I:EGFP) and the EGFP IRES IL-2Rγ_c pMSCV (EGFP:I:γ_c) constructs were described previously (21). Flow cytometry Abs (NK1.1 [PK136], CD122 [5H4], CD127 [A7R34], CD25 [PC61.5] CD49b [DX5], and CD107a [eBio1D4B]) were purchased from eBioscience (San Diego, CA), and (CD3 [145–2C11], CD132 [4G3], Ly49C/I [5E6], pSTAT5 pY694, and IFN-γ [XMG1.2]) were purchased from BD Biosciences (San Jose, CA). NK1.1 Alexa Fluor 647 was purchased from BioLegend (San Diego, CA). IL-15Rα Ab was purchased from R&D Systems (Mineapolis, MN). pSTAT3, STAT3, pErk, Erk, pAkt, and Akt Abs were purchased from Cell Signaling (Beverly, MA). JAK1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). N-terminal JAK3 Ab was a kind gift from Dr. John O'Shea (National Institutes of Health).

BM transduction and transplantation

BM transduction and transplantation protocol used has been described previously (21). Briefly, BM was harvested from 5-fluorouracil-treated C57BL/6, IL-2R$\gamma_c^{-/-}$, IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$, or IL-15$^{-/-}$ mice, retrovirally infected with empty vector pMSCV, EGFP:I:γ_c, or γ_c:I:EGFP (≈40–80% transduction efficiency) and then injected i.v. into the tail vein of sublethally irradiated (300 rads) IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$, IL-2R$\gamma_c^{-/-}$/LAT$^{-/-}$, or IL-15 $^{-/-}$ mice or sublethally irradiated (900 rads) C57BL/6 mice. After 8–12 wk post-BM transduction, the mice were euthanized and tissues were stained for FACS analysis (pSTAT5, IFN-γ, or CD107a), cells were adoptively transferred or NK and T cell populations were sorted and cell proliferation assay or Western blotting was performed as described below.

Cell preparation and flow cytometry

Splenocytes were mashed through 100-μm nylon cell strainers, and erythrocytes were lysed in ACK buffer. Livers were placed in filter bags and stomached for 2 min. The cells were washed in PBS and centrifuged through a Percoll gradient (40:80%) for 20 min. The mononuclear cell band was harvested. Splenocytes and liver cells were washed in PBS prior to staining. For surface staining, cells were preincubated with 2.4G2 to block Fc receptor binding, followed by incubation with various cell surface Abs. Cells were fixed with BD Cytofix and analyzed on BD FACSort or BD LSRII.

Intracellular phospho-STAT5 staining

The pSTAT5 staining protocol has been described previously (22). Briefly, splenocytes were stained for NK1.1 Alexa Flour 647 and CD3 Pacific Blue, stimulated with IL-2 (10,000 IU/ml), IL-7 (100 ng/ml), or IL-15 (100 ng/ml) for 20 min at 37°C, and then prepared for intracellular staining with Reagent A from the Caltag Fix and Perm cell permeabilization kit (Invitrogen, Carlsbad, CA). Cells were treated with a 30-min ice-cold methanol step between fixation and permeabilization of the cells to enhance intracellular staining. The methanol treatment step quenches fluorescence of PE and PerCP fluorochromes but not Alexa Fluor 647 or Pacific Blue. The cells were permeabilized in Caltag Reagent B and stained for PE-conjugated phospho-STAT5 (Tyr694) for 1 h at room temperature. NK1.1$^+$CD3$^-$ cells and NK1.1$^-$ CD3$^+$ cells were gated and analyzed for intracellular pSTAT5 staining on a BD LSRII.

Intracellular IFN-γ measurement

Fresh splenocytes resuspended in complete medium were plated onto wells (24-well plate) precoated with 1 μg/ml anti-NK1.1 (PK136), control IgG, or PBS. IL-12 (10 ng/ml) and IL-18 (100 ng/ml) or PMA (10 ng/ml) and ionomycin (1 μg/ml) were added to the PBS precoated wells. Brefeldin A (1 μg/ml) was then added to all wells. After 6 h of culture at 37°C, cells were harvested and Fc receptors were blocked with 2.4G2, followed by staining with PE-conjugated anti-DX5 and PerCPCy5.5-conjugated anti-CD3 or isotype controls. The cells were washed and fixed and permeabilized using BD Cytofix/Cytoperm solution. Fc receptor was blocked by incubation with 2.4G2 and stained with APC-conjugated anti–IFN-γ. DX5$^+$CD3$^-$ cells were gated and analyzed for intracellular IFN-γ staining.

Degranulation assay (CD107a)

Fresh splenocytes (1×10^6 cells) resuspended in complete medium were plated onto wells in a 24-well plate. Yac1 cells (2×10^5 cells) or PMA (10 ng/ml) and ionomycin (1 μg/ml) were added to the samples, followed by addition of PE-conjugated CD107a or isotype control. Cells were incubated at 3700B0030C for 1 h, followed by the addition of Golgi Stop (BD Biosciences) to all wells and further cultured for 4 h at 37°C. Cells were harvested and Fc receptors were blocked with 2.4G2, followed by staining with APC-conjugated anti-NK1.1 and PerCPCy5.5-conjugated anti-CD3 or isotype controls. The cells were washed and fixed in BD Cytofix solution. $NK1.1^+CD3^-$ cells were gated and analyzed for SSSCD107a staining.

Cell proliferation assay

Sorted NK ($NK1.1^+CD3^-$) and T cell ($NK1.1^-$ $CD3^+$) populations were seeded in triplicate in 96-well plates (5×10^4–1×10^5 cells/well) and cultured for 72 h in the presence or absence of IL-2 (1000 IU/ml or 10,000 IU/ml), IL-15 (10 ng/ml or 100 ng/ml), or IL-12 and IL-18 (10 ng/ml and 50 ng/ml). Cells were pulsed with 1 μCi/well of [^{3}H]thymidine (Perkin-Elmer, Waltham, MA) for the final 16–20 h. Cells were then harvested, and radioactivity was analyzed using a scintillation counter.

Western blots

Reconstituted $NK1.1^-CD3^+$ T cells were sorted using a FACS ARIA and cultured with ConA (2.5 mg/ml) for 36 h. Cells were acid washed, followed by serum-free medium washes and rested for 1 h at 37°C in serum-free medium. Cells were stimulated with and without IL-2 (10,000 IU/ml) for 5 min at 37°C. Cells were lysed with Triton X-100 lysis buffer (1% Triton X-100, 300 mM NaCl, 50 mM Tris pH7.4, 2 mM EDTA, 1 μg/ml aprotinin, 1 mg/ml leupeptin, 1 mM PMSF, and 2 mM sodium vanadate) and protein levels were normalized using a bicinchoninic acid protein assay. Four times reducing Nupage sample buffer was added to lysates and heated for 10 min at 70°C. Lysates were separated by SDS-PAGE (Nupage, Invitrogen), transferred to PVDF membrane (Millipore, Billerica, MA) and analyzed by Western blot.

CFSE labeling and adoptive transfer

Splenocytes were labeled with 5 μM CFSE (Molecular Probes, Invitrogen) for 10 min at 37°C, followed by the addition of RPMI 1640/10% FBS. Cells were washed three times and 3.5×10^6 CFSE-labeled splenocytes were injected i.v. into the tail vein of C57BL/6 mice.

Results

Expression of γ_c-dependent cytokine receptors in reconstituted NK cells

To examine the development and γ_c-mediated signaling of NK cells expressing low- level γ_c per cell, we reconstituted IL-2R$\gamma_c^{-/-}$ or IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ BM progenitors with a previously described retroviral construct, EGFP:IRES:IL-2Rγ_c pMSCV (EGFP:I:γ_c), expressing EGFP and low-level γ_c, ensuring that the resulting NK cells were $EGFP^+$ (21). IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ donors were used for some experiments to ensure adequate NK cell

numbers as IL-2R$\gamma_c^{-/-}$ mice develop a T cell lymphoproliferative disorder resulting in a lower percentage of NK cells during reconstitution. Analysis of γ_c levels on splenic and liver NK1.1$^+$CD3$^-$ NK cells in "γ_c transduced" EGFP:I:γ_c mice showed a lower level of γ_c expression than that found in mice reconstituted with WT BM (Fig. 1A). As IL-15 is required for NK cell development, we also examined the level of IL-15Rα in the reconstituted mice. IL-15 is found primarily in association with IL-15Rα on dendritic cells, which can activate NK cells via *trans* presentation. This makes it difficult to detect IL-15Rα; however, both EGFP:I:γ_c transduced and WT liver leukocytes displayed a similar level of detectable IL-15Rα expression (Fig. 1B). Further analysis revealed similar expression levels of IL-2Rβ, IL-2Rα, and IL-7Rα on EGFP:I:γ_c transduced, WT, and the small number of untransduced IL-2R$g_c^{-/-}$/Rag2$^{-/-}$ splenic and liver NK1.1$^+$CD3$^-$ NK cells (Fig. 1C). We have previously examined other phenotypic and maturation markers on the EGFP:I:γ_c NK cells and although most developmental markers are normal on these cells, CD27, CD11b, and Ly49D display slightly altered expression patterns suggesting a modest change in the maturation status of these cells (21). Therefore, low-level expression of γ_c appears to be sufficient for the development of NK cells and the level of γ_c expression does not affect expression of other members of the IL-2/−7/−15R dimeric or trimeric receptor complexes.

γ_c cytokine-mediated signaling of reconstituted NK cells

To determine whether signaling by the IL-2Rγ_c family of cytokines was normal in low level γ_c-transduced NK cells, we investigated the in vitro phosphorylation of STAT5. IL-2 and IL-15 stimulated minimal STAT5 phosphorylation in γ_c-transduced EGFP:I:γ_c NK cells compared with mock-transduced WT NK cells. IL-7 did not result in phosphorylation of STAT5 either in EGFP:I:γ_c or WT NK cells (Fig. 2A) likely due to the low expression of the IL-7Rα- chain (Fig. 1C). We further examined the downstream effects by determining the proliferation of NK cells after stimulation with IL-2 or IL-15. In agreement with the aforementioned reduction in pSTAT5 levels in γ_c-transduced NK cells, these cells also displayed a highly significant reduction in γ_c-mediated proliferation (11.9 ± 3.3% of WT response) (Fig. 2B). EGFP:I:γ_c-transduced NK cells also display a diminished response to IL-12/IL-18 (45.1 ± 4.4% of WT response) (Fig. 2C), however, this is much less severe than the defective response to γ_c cytokines. This suggests that although these cells may have a slightly less responsive phenotype overall possibly due to the low level of γ_c, their lack of response to γ_c cytokines is much more severe indicating a γ_c-specific defect. To determine whether the IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ environment played a role in the reduced signaling in EGFP:I:γ_c cells, EGFP:I:γ_c-transduced BM was transplanted into sublethally irradiated WT recipients. The EGFP:I: γ_c NK cells in WT recipients displayed similarly deficient γ_c-mediated signaling as EGFP: I:γ_c NK cells transplanted into sublethally irradiated IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ recipients indicating an intrinsic defect in the NK cells rather than their environment (Supplemental Fig. 1). These data suggest that although limiting levels of γ_c are sufficient for the development of NK cells, signaling in these cells is clearly defective, suggesting a higher threshold of γ_c signaling for NK cell proliferation than development.

Low-level IL-15 signaling is sufficient for NK cell development

As the introduction of low-level γ_c results in such reduced signaling in NK cells, we sought to determine whether IL-15 signaling was actually required for the development of NK cells

using this system or whether ectopic expression of IL-2Rγ_c overcomes the need for IL-15–mediated signaling. To that end, IL-15$^{-/-}$ mice were used to determine whether retroviral expression of low-level γ_c could negate the need for IL-15 signaling. NK cells developed to varying degrees in IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ reconstituted recipient mice when low-level γ_c was expressed in IL-2R$\gamma_c^{-/-}$, IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$, or IL-15$^{-/-}$ donor BM (Fig. 3A–C). A small number of NK1.1$^+$CD3$^-$ cells are present in the chimeric mice with γ_c-transduced IL-15$^{-/-}$ donor BM transferred to IL-15$^{-/-}$ recipients (Fig. 3A, 3B, Supplemental Table I); however, these cells do not display other characteristics of NK cells, such as expression of Ly49C/I (Fig. 3C), or low/no expression of DX5, IL-2Rβ, or other NK cells markers (data not shown). As these cells do not display typical NK cell markers, this suggests that the small percentage of NK1.1$^+$ cells observed in this study may represent the newly described γ_c-independent immature NK cells found in the BM of IL-15$^{-/-}$ and IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ mice (Fig. 3A–C) (6). This suggests that low levels of γ_c are sufficient for the IL-15–mediated signaling required for normal NK cell development and thus retroviral expression of γ_c does not bypass the need for IL-15 signaling.

γ_c-transduced NK cells are functional

As γ_c-mediated signaling of reconstituted NK cells is defective, we wanted to examine whether these cells displayed normal non-γ_c–mediated functional activity. Therefore, IFN-γ production was examined after NK1.1, IL-12/IL-18, or PMA/ionomycin stimulation. Previously, we have shown that EGFP:I:γ_c NK cells produce IFN-γ; however, the level of IFN-γ produced was not previously shown in comparison with WT cells (21). In this study, we show that expression of low-level γ_c appears to play no role in the production of IFN-γ as the EGFP:I:Sγ_c NK cells displayed similar IFN-γ production compared with WT cells (Fig. 4A). Similarly, EGFP:I:γ_c-transduced NK cell cytotoxic activity as assessed by CD107a expression was normal after coculture with Yac1 target cells or PMA/ionomycin (Fig. 4B). Levels of NKG2D, the receptor that recognizes Yac1 target cells, were comparable in both γ_c-transduced and WT NK cells (data not shown). These data indicate that although γ_c cytokine (IL-2 and IL-15)-mediated signaling is reduced in the presence of low levels of γ_c, non-γ_c–mediated functional ability of the reconstituted NK cells is normal.

Expression of γ_C-dependent cytokine receptors in reconstituted T cells

We next sought to determine how T cells respond to low levels of γ_c. Therefore, IL-2R$\gamma_c^{-/-}$ BM progenitors were transduced with the construct expressing EGFP and low levels of γ_c (EGFP:I:γ_c). Expression of γ_c in EGFP$^+$NK1.1$^-$CD3$^+$ T cells was significantly lower than in WT T cells (Fig. 5A). Analysis of IL-2Rβ, IL-2Rα, and IL-7Rα levels revealed comparable expression on EGFP:I:γ_c and WT NK1.1$^-$CD3$^+$ T cells (Fig. 5B).

Defective γ_c cytokine-mediated signaling of γ_c-reconstituted T cells

It has previously been shown that IL-2R$\gamma_c^{-/-}$ T cells transduced with γ_c display corrected signaling to some degree; however, reconstitution of γ_c-mediated signaling and proliferation remains incomplete when compared with WT γ_c-mediated signaling (16). Therefore, we examined the effect of low-level γ_c on transduced T cells. Phosphorylation of STAT5 after IL-2, IL-7, and IL-15 in EGFP:I:γ_c-transduced T cells was significantly reduced (Fig. 6A), although this reduction was not as severe as seen in EGFP:I:γ_c NK cells (Fig. 2A). We next

examined various aspects of IL-2–mediated signaling in reconstituted primary NK1.1^{-}CD3^{+} T cells cultured with ConA for 36 h, such as the phosphorylation of STAT3 and the protein levels of STAT3 and JAK1/3. The levels of the JAK/STAT proteins were normal in EGFP:I:γ_c T cells; however, phosphorylation of STAT3 in response to IL-2 was severely diminished in EGFP:I:γ_c T cells (Fig. 6B). IL-2 stimulation has previously been shown to result in the phosphorylation and activation of Erk (23) and Akt (24). The Akt pathway is essential for T cell proliferation and STAT5 activation is required for Akt activity during IL-2–stimulated T cell proliferation (24). Therefore, we examined activation of Erk and Akt after IL-2 stimulation of sorted primary reconstituted T cell populations. EGFP:I:γ_c T cells displayed greatly reduced phosphorylation of Akt and Erk compared with WT cells after IL-2 stimulation (Fig. 6C). Further downstream, proliferation of γ_c-transduced T cells was clearly absent, whereas WT T cells proliferated in response to IL-2 (Fig. 6D) or IL-15 (data not shown). However, activation of the TCR (ConA) in combination with IL-2 stimulation results in normal T cell proliferation (Fig. 6E).

We have previously ruled out environmental effects for the reduced signaling in NK cells (Supplemental Fig. 1), therefore, we decided to check whether aberrant expression of the EGFP:I:γ_c construct was the cause for reduced signaling in both the NK and T cell compartments. To this end, WT BM was transduced with the EGFP:I:γ_c construct; however, this does not affect the signaling, implying that aberrant expression of γ_c is not the cause of deficient signaling in the γ_c-transduced NK cells (data not shown) or γ_c-transduced T cells (Supplemental Fig. 2). These data suggest that γ_c signaling via the JAK/STAT and Akt pathways is required to reach a certain threshold to result in T cell proliferation. Therefore, similar to NK cells, significant levels of γ_c are required for normal T cell signaling and proliferation, whereas lower levels of γ_c are sufficient for development. However, activation of the TCR in combination with low-level γ_c signaling is sufficient for some T cell proliferation.

Increased γ_c levels result in increased γ_c-mediated signaling

We next decided to examine the possibility that the expression level of γ_c directly dictates the level of γ_c-mediated signaling. To this end, IL-2R$\gamma_c^{-/-}$ BM progenitors were transduced with the construct expressing EGFP and low levels of γ_c (EGFP:I:γ_c) or with a construct expressing higher levels of γ_c and low levels of EGFP (γ_c:I:EGFP). Expression of γ_c in EGFP:I:γ_c NK cells was low, increased in γ_c:I:EGFP NK cells, and further increased in WT NK cells (Fig. 7A). A similar pattern of γ_c expression was observed in T cells, although the increase between γ_c:I:EGFP and EGFP:I:γ_c T cells was not as dramatic as in the NK cells (Fig. 7B). IL-2–mediated induction of STAT5 phosphorylation was low in EGFP: I:γ_c NK cells, increased in γ_c:I:EGFP NK cells and further increased in WT NK cells, thereby, displaying increased STAT5 phosphorylation in accordance with the level of γ_c expression (Fig. 7C). The various T cell populations displayed a similar effect on STAT5 phosphorylation with increasing γ_c expression (Fig. 7D); however, the effect was not as pronounced in T cells as in NK cells. The level of proliferation of the NK cells (Fig. 7E) and the absolute number of NK cells in the reconstituted mice (Table I) also correlated with the level of γ_c expression and STAT5 phosphorylation. STAT5 phosphorylation and γ_c expression were only slightly increased in γ_c:I:EGFP T cells compared with EGFP: I:γ_c T

cells; however, this increase was not significant enough to result in increased proliferation (Fig. 7F). The absolute number of T cells in the reconstituted mice (Table I) correlated with the level of γ_c expression; however, the reduction in T cell number with reduced γ_c expression was not as severe as the reduction in NK cells. These data indicate that although limiting γ_c expression is sufficient for the development of the NK and T cell compartments, higher levels of γ_c expression are required for normal signaling and proliferation.

NK cells require more γ_c than T cells for normal maintenance

To determine whether the maintenance of NK or T cells was affected by the level of γ_c expression, we adoptively transferred CFSE-labeled WT or γ_c:I:EGFP chimeric IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ (IL-2R$\gamma_c^{-/-}$ and IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ BM transduced with intermediate levels of γ_c) splenocytes into WT recipients. We then assessed the time the NK and T cells persisted in these mice over a period of 2 wk. We found that both the WT and γ_c: I:EGFP-transduced NK and T cells were significantly reduced by day 7 after transfer. However, at day 7 the remaining γ_c:I:EGFP NK cells were only ≈1/20 of the remaining WT NK cells, whereas the remaining γ_c:I:EGFP T cells were ≈1/3 of the remaining WT T cells. These data again suggest a more severe defect in the maintenance of NK cells with reduced γ_c levels than in the equivalent T cells (Fig. 8).

Discussion

We have shown in this study that although low levels of γ_c are sufficient for the development of NK and T cells, γ_c-mediated signaling and proliferation are deficient in these cells. This correlates with previous ex vivo data showing that IL-2–mediated T cell proliferation is only partially restored after reconstitution with low levels of γ_c (14, 16). In this study, we further dissect the effect of expression of low levels of γ_c on the development and maintenance of NK and T cells.

Previously, the signaling of γ_c-reconstituted NK cells has not been closely examined. In this study, we show that NK cells develop, produce normal levels of IFN-γ, and display normal cytotoxicity with low levels of γ_c-mediated IL-15 signaling; however, IL-2/−15–mediated phosphorylation of STAT5 and proliferation are severely reduced. This suggests that the threshold for development of NK cells is lower than the threshold for proliferation/maintenance of these cells. IL-15 and IL-15Rα support NK cell homeostasis via *trans* presentation (25, 26) and mature NK cells transferred into IL-15$^{-/-}$ or IL-15R$\alpha^{-/-}$ mice do not survive long (27, 28). The $t_{1/2}$ of transferred NK cells is shortened from 7 d in WT mice to ~10 h in IL-15R$\alpha^{-/-}$ mice, suggesting that NK cells require a certain level of IL-15–mediated signaling every few hours to avoid programmed cell death (28). SCID-X1 patients treated with γ_c gene therapy initially have NK cells develop; however, they are unable to maintain normal numbers of NK cells (17, 18). Our data suggest that insufficient IL-15 signaling due to low levels of γ_c in mature NK cells in mice may explain their inability to maintain a normal mature NK cell pool. These data also suggest that the new gene therapy vectors under investigation with self-inactivating LTRs possibly expressing lower levels of γ_c may result in even lower NK cell numbers than the previous form of γ_c gene therapy and/or nonresponsive NK cells. This suggests that future gene therapy for SCID-X1 should

use the IL-2Rγ_c endogenous promoter in a retroviral vector with self-inactivating LTRs to ensure that the levels of IL-2Rγ_c will be very tightly controlled permitting a normal functional NK cell population while avoiding the development of leukemia.

The level of expression of γ_c appears to be extremely important for various reasons. We have shown in this study that too little γ_c can result in a seriously depleted NK cell compartment, whereas Amorosi et al. (12) showed that overexpression of γ_c in transformed B cell lines can result in self-sufficient growth, suggesting the possibility γ_c may have the ability to cause cancer at high levels of expression (12). Woods et al. (11) also suggested γ_c may be oncogenic as reconstitution of the SCID-X1 mouse model with γ_c resulted in T cell lymphomas in some mice. Our data combined with these two studies suggest γ_c expression levels must be carefully controlled during gene therapy. A significant proportion of the patients treated by gene therapy had leukemia develop (9, 10), and it is possible to speculate that these patients may have mounted a better antileukemic response if they had an intact NK cell compartment.

NK cells are important in the defense against viral infections, and data from patients lacking NK cells support this role. Patients lacking NK cells are susceptible to severe and/or recurrent infections with herpes viruses, including varicella zoser virus (29), HSV (30), EBV (31), and CMV (32). These patients had some of the above infections develop early in life, whereas other infections occurred during adolescence. These data suggest that patients lacking NK cells or with low levels of mature NK cells may be at risk for serious viral infections. Although it may be tempting to determine the ability of γ_c-transduced mice to mount an appropriate antiviral response, this would be extremely difficult to control due to the low levels of NK cells present in these mice compared with WT mice. Therefore, prior to the next round of gene therapy for SCID-X1 patients, the level of γ_c expression should be given serious consideration to improve NK cell homeostasis and, therefore, the patients' ability to ward off viral infections.

The T cell compartment develops normally in γ_c-reconstituted mice and in SCID-X1 patients who received treatment with γ_c gene therapy. However, IL-2/−7/−15 signaling is severely diminished in T cells expressing low levels of γ_c. IL-2 signaling results in the phosphorylation of JAK1/3 and STAT5/3, followed by dimerization and translocation of the STAT molecules to the nucleus (33). We have shown in this study that the levels of the JAK/STAT proteins are normal in g_c-reconstituted cells, but IL-2–induced phosphorylation of STAT3 and IL-2/−7/−15–induced phosphorylation of STAT5 are severely reduced. IL-2 stimulation of T cells also results in the activation of other pathways. JAK1/3 phosphorylates the IL-2R complex, thereby creating a docking site on IL-2Rβ for the adaptor protein Shc. The adapter protein Grb2 and guanine nucleotide exchange factor Son of Sevenless are then recruited to the IL-2R complex resulting in the activation of the Ras–Raf–MAPK pathway (34). IL-2 also stimulates activation of the PI3K–Akt pathway that is involved in antiapoptotic and proliferative activities of T cells in response to IL-2 (35). JAK1 has been shown to be important for the recruitment of PI3K p85 (36). In this study, we show that activation of Erk and Akt are severely reduced probably due to diminished proximal signaling from cells expressing low levels of γ_c. This then results in negligible levels of IL-2/−7/−15–induced T cell proliferation. These data show that like NK cells, although low-

level γ_c signaling is sufficient for the development of T cells, a higher threshold also exists for their proliferation or survival.

If IL-2Rγ_c signaling is defective in both T cells and NK cells, why then are T cells maintained in the periphery, whereas NK cells are not? One explanation may lie in the combined use of IL-2Rγ_c– and TCR-mediated signaling that results in the relatively long half-life (10–15 wk) of peripheral T cells. The T cell half-life is reduced to just 2–3 wk in the absence of either MHC class II ($CD4^+$ T cells) (37) or IL-7 (38) signaling. Consistent with these data, we find that T cells expressing low levels of IL-2Rγ_c proliferate poorly in response to cytokine alone; however, the proliferative response is comparable to WT T cells when cytokine is combined with TCR stimulation. In contrast to T cells, NK cell homeostasis has not been shown to involve constitutive ITAM-derived signals similar to the TCR and peripheral NK cell numbers are normal in mice lacking DAP12 or FcεRIγ signaling chains (39, 40). Instead, NK cells are critically dependent on IL-15 signaling and the absence of IL-15Rα reduces NK cell half-life to ~10 h (28). Our data show that although both NK and T cell maintenance are reduced with lower γ_c levels, the reduction is much more severe in NK cells when compared with WT cells. Therefore, reduced signaling from suboptimal IL-2Rγ_c expression in reconstituted cells appears to have a more profound impact on NK cell homeostasis than in T cells where the reduced IL-2Rγ_c signal is supported by tonic TCR engagement. Such a model is consistent with the relatively stable T cell compartment and transient NK cell reconstitution found during IL-2Rγ_c gene therapy in mice (14, 16) and SCID-X1 patients (17, 18).

Taken together, these data for the first time definitively suggest that careful consideration must be given to the level of γ_c expression for the next round of gene therapy. Previous data have suggested that overexpression of γ_c may be oncogenic and, in this study, we have shown that although low levels of γ_c expression will facilitate NK and T cell development, a higher threshold of γ_c signaling is required for efficient proliferation and maintenance of the NK and T cell populations. Both of these lymphoid populations are required for the efficient control of infectious diseases and tumor development and progression, therefore, development of future gene therapy strategies should aim to efficiently reconstitute both the NK and T cell populations and should include functional analysis of reconstituted cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank John Wine and Grace Williams for animal care for this project. We also thank Bill Bere for help with the preparation of some tissue samples and Kathleen Noer and Roberta Matthai for cell sorting; Dr. Howard Young, Dr. Massimo Gadina, Dr. John O'Shea, Dr. Thomas Waldman, and Dr. Cristina Bergamaschi for providing reagents and mice for this study; and Dr. Scott Durum, Dr. James Johnston, and Dr. Massimo Gadina for critically reading the manuscript.

This work was supported by the Intramural Research Program of the National Institutes of Health-National Cancer Institute.

Abbreviations used in this paper:

BM	bone marrow
γ_c	IL-2Rγ_c
LTR	long terminal repeat
MFI	mean fluorescence intensity
SCID-X1	X-linked SCID
Un	unstimulated
WT	wild-type

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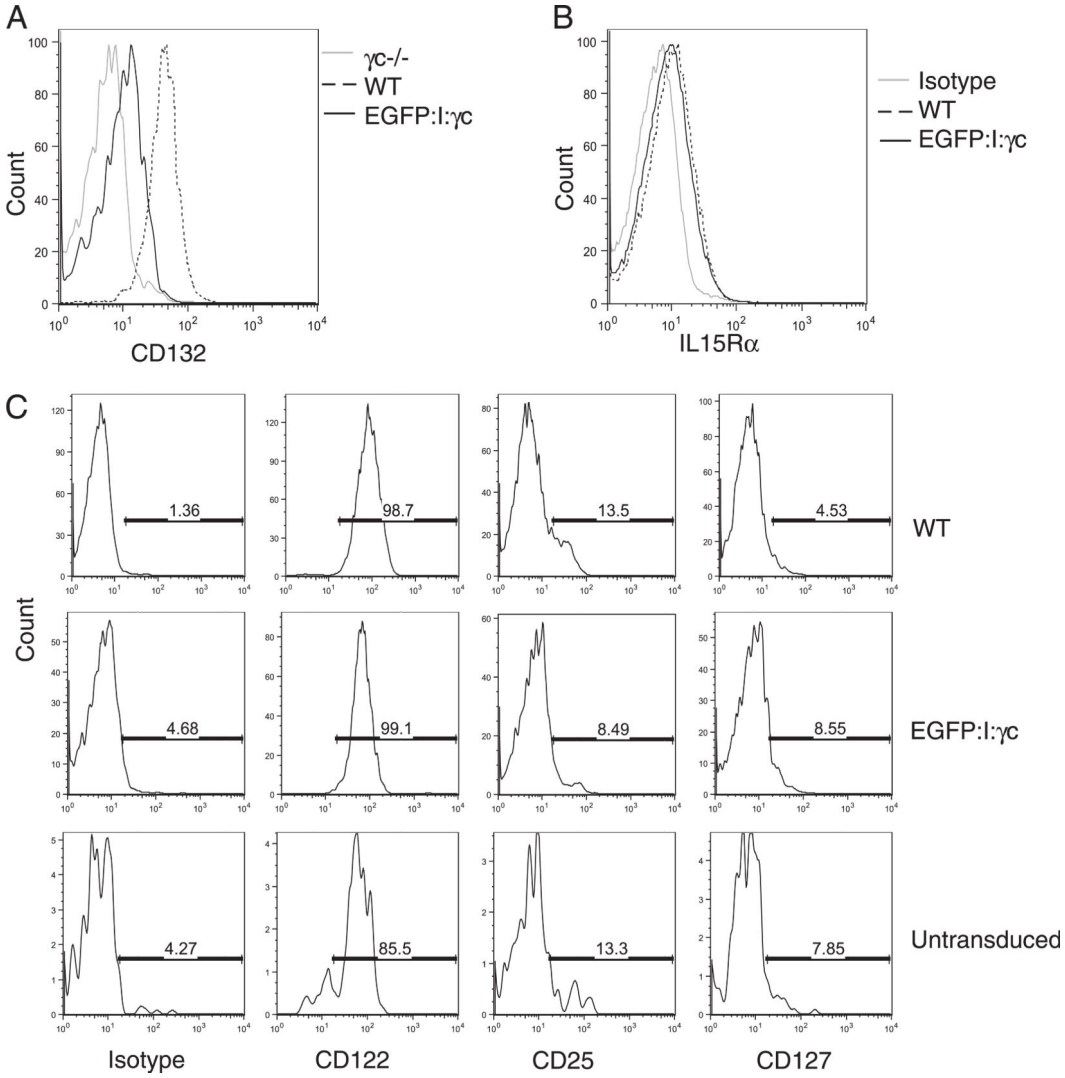

FIGURE 1.

Normal expression of γ_c and related receptor chains on NK cells from EGFP:I:γ_c chimeric mice compared with WT mice. Representative expression of (*A*) γ_c (CD132) on NK1.1$^+$CD3$^-$ cells from WT and EGFP:I:γ_c chimeric mice compared with IL-2R$\gamma_c^{-/-}$ CD3$^+$cells (*n* = 9–10). *B,* Representative expression of IL-15Rα on liver leukocytes from WT and EGFP:I:γ_c chimeric mice (*n* = 6). *C,* Representative expression of IL-2Rβ (CD122), IL-2Rα (CD25), and IL-7Rα (CD127) on NK1.1$^+$CD3$^-$ cells from WT, EGFP:I:γ_c chimeric mice and untransduced IL-2R$\gamma_c^{-/-}$/Rag$^{-/-}$ mice (*n* = 2–14).

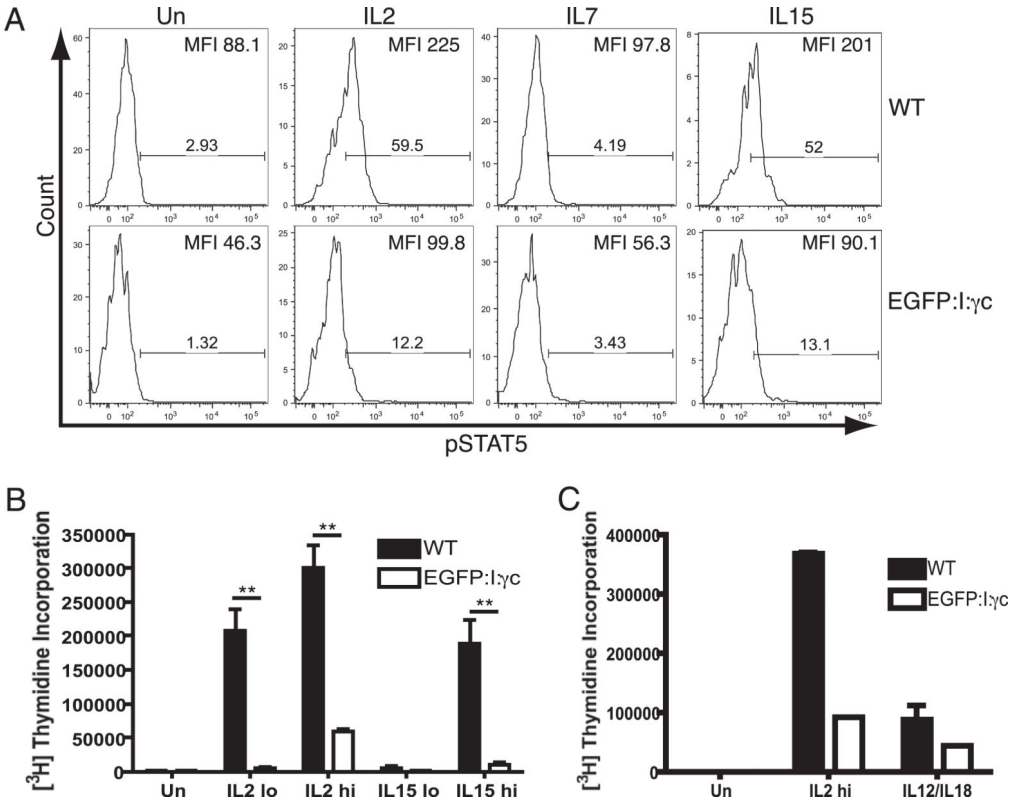

FIGURE 2.

Reduced STAT5 phosphorylation and proliferation in EGFP:I:γ_c NK cells on stimulation with IL-2Rγ_c cytokines. *A,* WT and EGFP:I:γ_c-transduced splenocytes were left unstimulated or stimulated with IL-2 (10,000 IU/ml), IL-7 (100 ng/ml), or IL-15 (100 ng/ml) for 20 min. pSTAT5 levels were detected by FACS analysis gated on NK1.1$^+$CD3$^-$ cells (n = 2–7). *B* and *C,* WT and EGFP:I:γ_c splenocytes were sorted to purify the NK1.1$^+$CD3$^-$ population. Purified NK cells were plated and left unstimulated or stimulated with IL-2 lo (1000 IU/ml), IL-2 hi (10,000 IU/ml), IL-15 lo (10 ng/ml), IL-15 hi (100 ng/ml), or IL-12/IL-18 (10 ng/ml IL-12 and 50 ng/ml IL-18) for 72 h. A total of 1 μCi [^{3}H]thymidine was added per well and proliferation was measured 18 h later. Graphs display the mean results ± SEM of two to six replicates. Statistical significance was calculated using the Mann-Whitney *U* test. *p <0.05; **p < 0.005. MFI, mean fluorescence intensity.

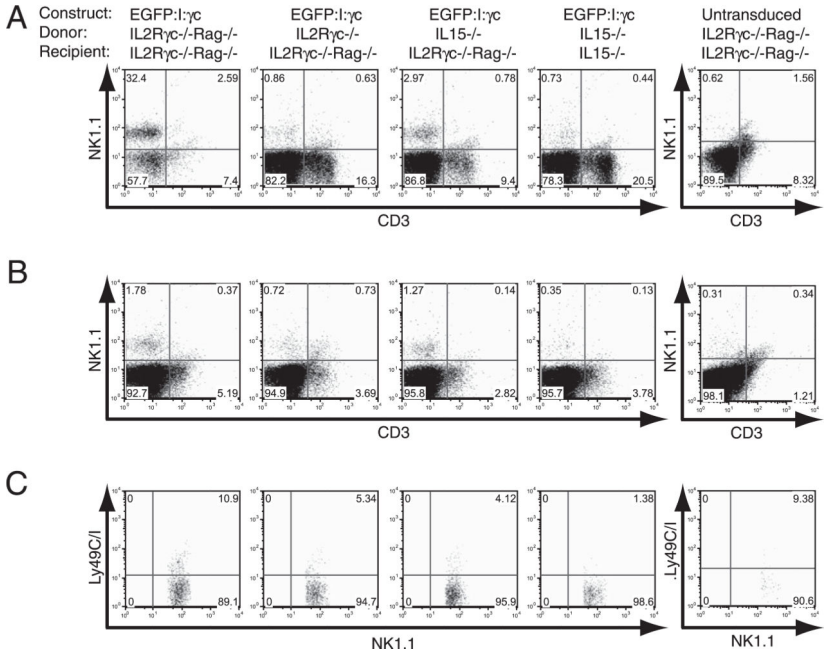

FIGURE 3.

Low-level γ_c facilitates sufficient IL-15 signaling for NK development. EGFP:I:γ_c-transduced IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$, IL-2R$\gamma_c^{-/-}$, IL-15$^{-/-}$, or untransduced IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ donor BM was transplanted into IL-2R$\gamma_c^{-/-}$/Rag2$^{-/-}$ or IL-15$^{-/-}$ recipient mice. Development of NK cells was determined by FACS analysis for the NK1.1$^+$ CD3$^-$ population in the (*A*) spleen (*n* = 2) and (*B*) BM (*n* = 2). *C*, Expression of Ly49C/I on the BM NK1.1$^+$CD3$^-$ population was determined by FACS analysis (*n* = 2).

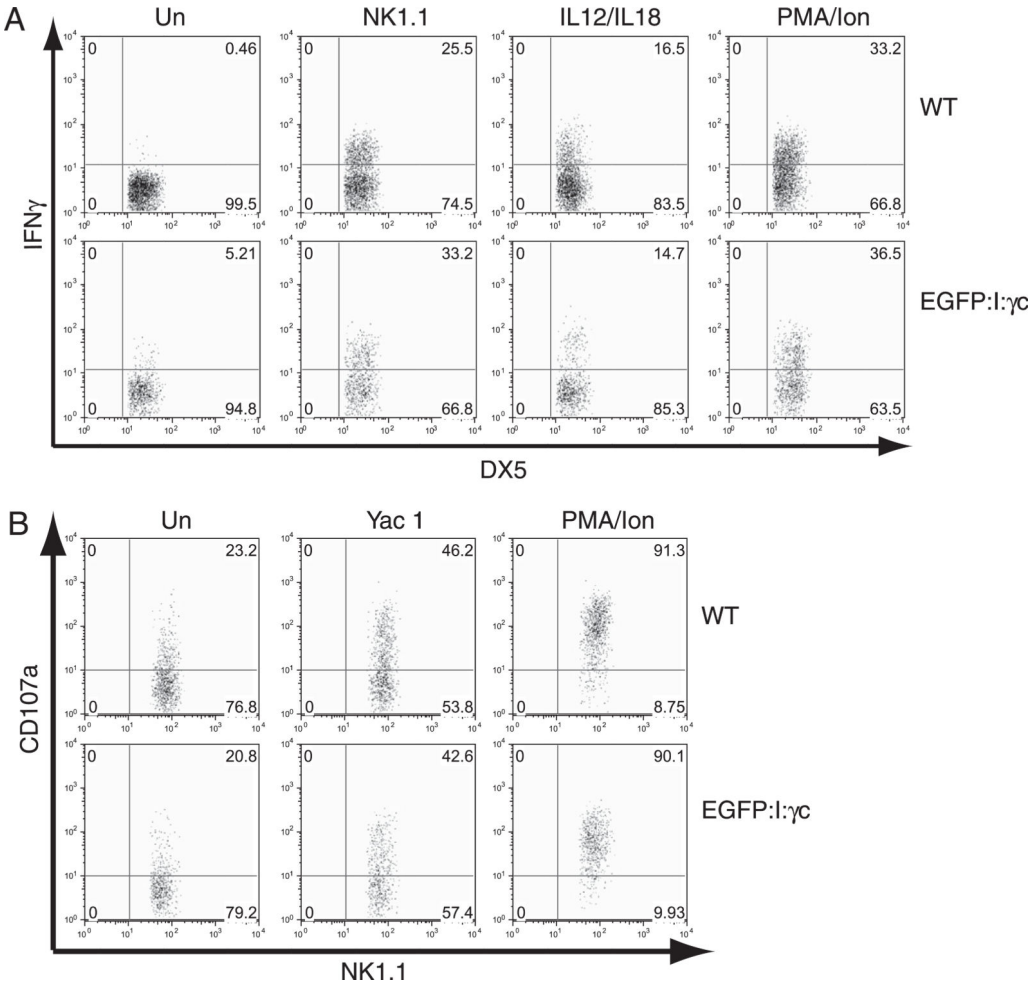

FIGURE 4.

EGFP:I:γ_c-transduced NK cells are functional. *A,* IFN-γ production was measured by intracellular staining on the $DX5^+CD3^-$ population after stimulation with mouse IgG (1 μg/ml), anti-NK1.1 (1 μg/ml), IL-12/IL-18 (100 ng/ml), or PMA/ionomycin ($n = 6–7$). *B*, NK cell degranulation was measured by FACS analysis of CD107a levels on the $NK1.1^+CD3^-$ population after coculture with Yac1 target cells or PMA/ionomycin ($n = 3$).

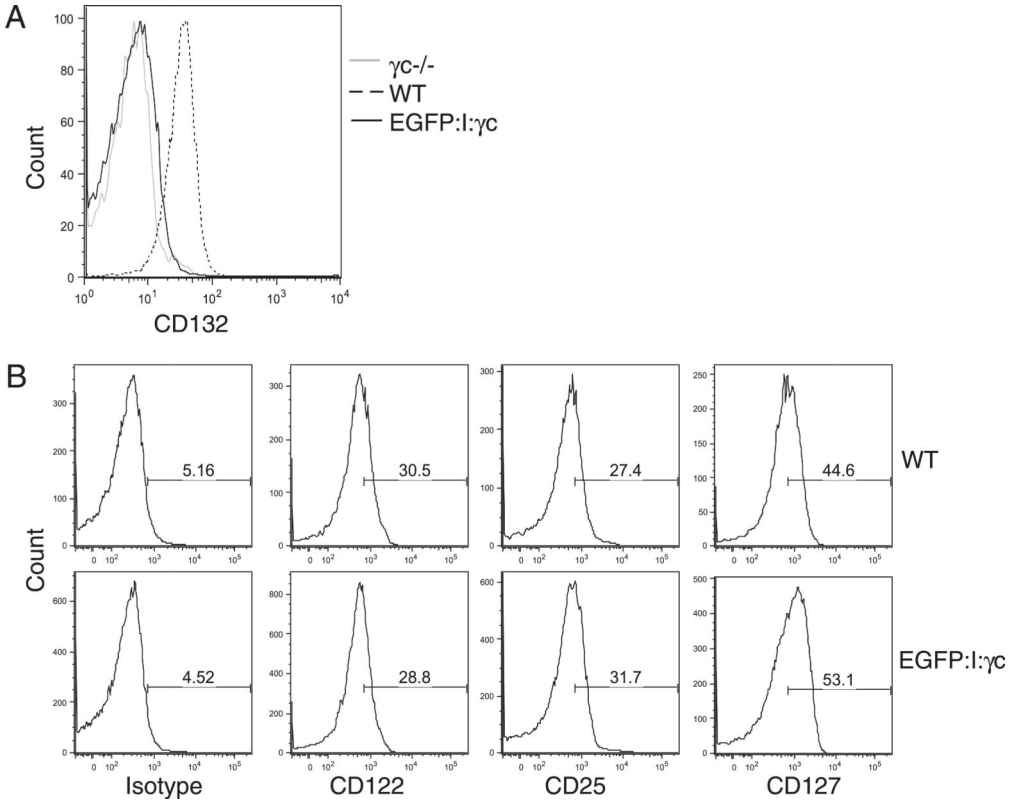

FIGURE 5.

Normal expression of γ_c and related receptor chains on T cells from EGFP:I:γ_c chimeric mice compared with WT mice. Representative expression of (*A*) γ_c (CD132) (*n* = 5–6), (*B*) IL-2Rβ (CD122), IL-2Rα (CD25), and IL-7Rα (CD127) on NK1.1$^-$CD3$^+$ T cells from WT and EGFP:I:γ_c chimeric mice compared with IL-2R$\gamma_c^{-/-}$ T cells or isotype control (*n* = 5–6).

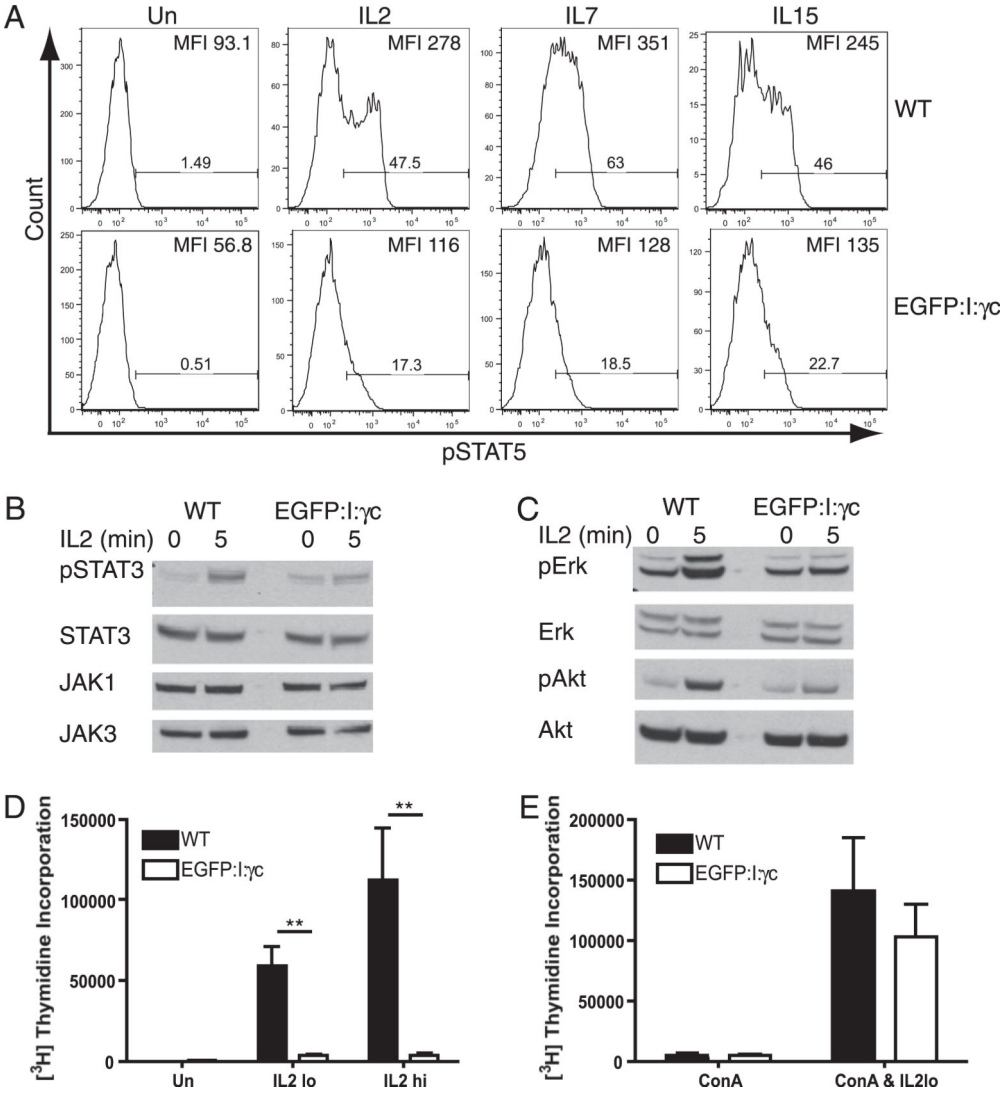

FIGURE 6.

Reduced activation and proliferation in EGFP:I:γ_c T cells on stimulation with IL-2Rγ_c cytokines. *A,* WT and EGFP:I:γ_c splenocytes were left unstimulated or stimulated with IL-2 (10,000 IU/ml), IL-7 (100 ng/ml), or IL-15 (100 ng/ml) for 20 min. pSTAT5 levels were detected by FACS analysis gated onNK1.1$^-$CD3$^+$ cells (n = 2–6). *B* and C, WT and EGFP:I:γ_c were sorted to purify the NK1.1$^-$CD3$^+$ population and cultured in 2.5 μg/ml ConA for 36 h. Whole cell lysates from unstimulated and IL2 stimulated T cells were immunoblotted with (*B*) pSTAT3, STAT3, JAK1, and JAK3 Abs (n = 2) or with (*C*) pErk, Erk, pAkt, and Akt Abs (n = 2). *D* and *E,* WT and EGFP:I:γ_c were sorted to purify the NK1.1$^-$CD3$^+$ population. Purified T cells were plated and left unstimulated or stimulated with IL-2 (1000 IU/ml or 10,000 IU/ml) or ConA (1 μg/ml) for 72 h. A total of 1 μCi [^{3}H]thymidine was added and proliferation was measured 18 h later. Graphs display the mean results ± SEM of four to six replicates from two independent experiments. Statistical significance was calculated using the Mann-Whitney *U* test. *p < 0.05; **p < 0.005. MFI, mean fluorescence intensity; Un, unstimulated.

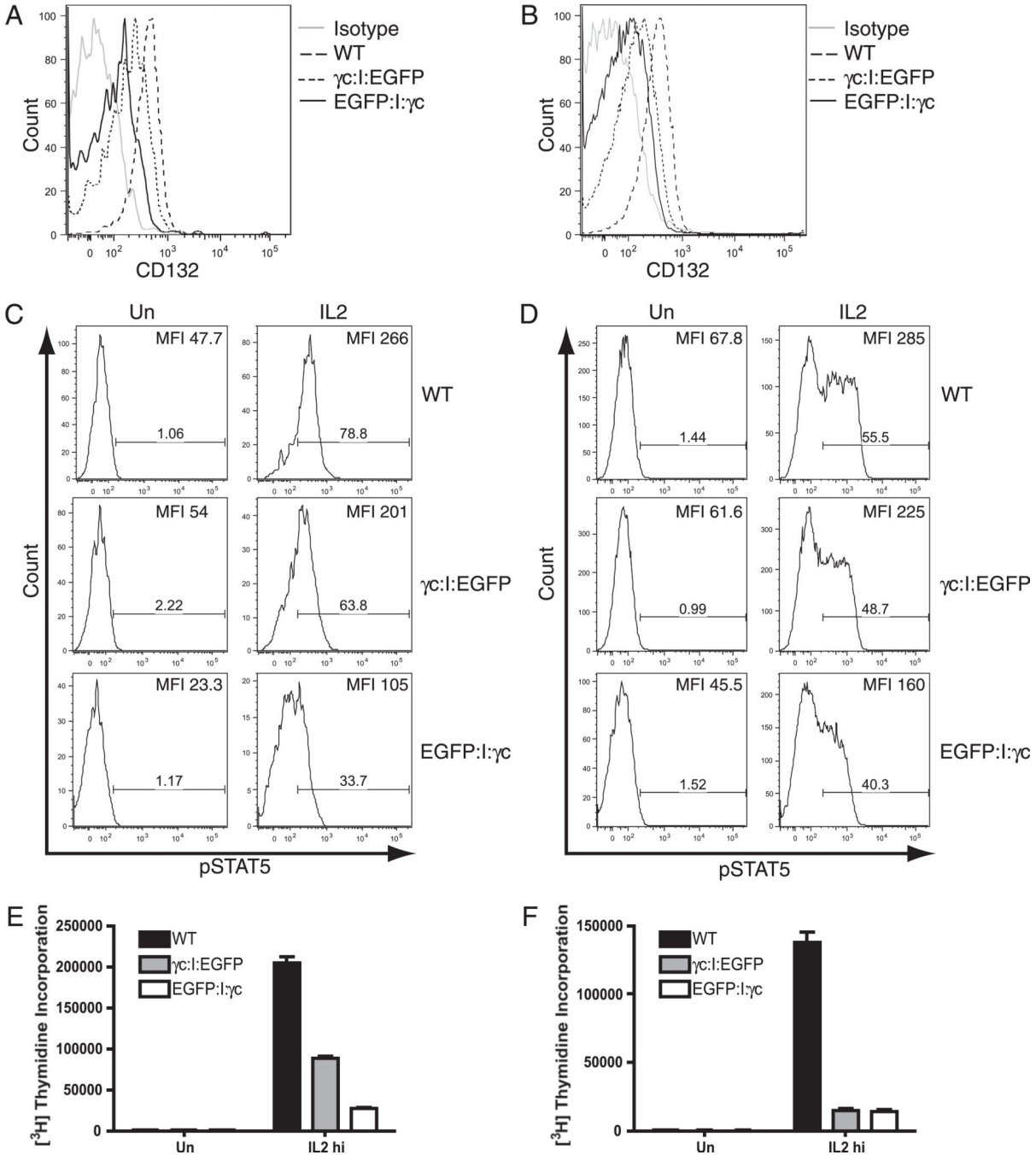

FIGURE 7.

γ_c expression level correlates with the level of STAT5 phosphorylation and proliferation. Representative expression of γ_c (CD132) on (*A*) NK1.1$^+$CD3$^-$ NK cells ($n = 3$) and (*B*) NK1.1$^-$CD3$^+$ T cells ($n = 3$) from WT, γ_c:I:EGFP and EGFP:I:γ_c chimeric mice. *C* and *D*, WT, γ_c:I:EGFP, and EGFP:I:γ_c splenocytes were left unstimulated (Un) or stimulated with IL-2 (10,000 IU/ml) for 20 min. pSTAT5 levels were detected by FACS analysis gated on (*C*) NK1.1$^+$CD3$^-$ NK cells ($n = 3$) or on (*D*) NK1.1$^-$CD3$^+$ T cells ($n = 3$). *E* and *F*, WT and EGFP:I:γ_c splenocytes were sorted to purify the NK1.1$^+$CD3$^-$ population (*E*) or the

NK1.1^{-}CD3^{+} population (*F*). Purified NK or T cells were plated and left unstimulated or stimulated with IL-2 hi (10,000 IU/ml) for 72 h. A total of 1 μCi [^{3}H]thymidine was added per well and proliferation was measured 18 h later. Graphs display the mean results ± SEM of three replicates. MFI, mean fluorescence intensity.

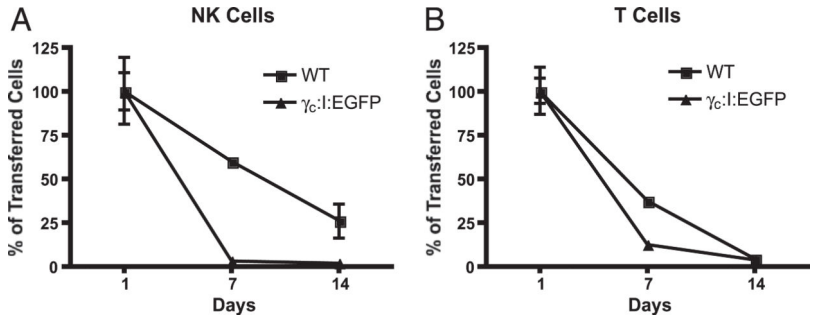

FIGURE 8.

Reduced γ_c expression results in a more severe defect in NK cell maintenance than T cell maintenance. CFSE-labeled WT or γ_c:I:EGFP spleno-cytes were adoptively transferred into WT mice. *A*, NK1.1$^+$CD3$^-$CFSE$^+$ (NK) or (B) NK1.1$^-$CD3$^+$ CFSE$^+$ (T) splenocytes were detected by FACS analysis at the indicated times. CFSE$^+$ NK and T cell numbers were normalized to the number of CFSE$^+$ NK or T cell numbers present on day 1 (100%). Graphs display the mean results ± SEM of three replicates.

Table I.

NK and T cell development correlates with γ_c expression level

Mice	NK Cell No. ($\times 10^6$)	T Cell No. ($\times 10^6$)
WT	2.14 ± 0.11	10.17 ± 0.57
γ_c:I:EGFP	0.77 ± 0.19	8.96 ± 0.59
EGFP:I:γ_c	0.34 ± 0.03	5.1 ± 1.33

Absolute splenocyte NK and T cell numbers were quantified ± SE from WT, γ_C:I: EGFP, and EGFP:I: γ_C-transduced IL-2R$\gamma_C^{-/-}$ mice