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*J Immunol* 2010; 184:2769-2775; Prepublished online 8 February 2010; doi: 10.4049/jimmunol.0900685
http://www.jimmunol.org/content/184/6/2769

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2010/02/05/jimmunol.0900685.DC1

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In Vivo Role of Flt3 Ligand and Dendritic Cells in NK Cell Homeostasis

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IL-15 is required for NK cell development and homeostasis in vivo. Because IL-15 is presented in trans via its high-affinity IL-15Rα–chain to cells expressing the IL-15Rβγ complex, we postulated that certain IL-15–bearing cells must be required for NK cell homeostasis. Using IL-15WT/WT and IL-15−/− mice, bone marrow chimeras with normal cellularity, and a selective depletion of CD11chi dendritic cells (DCs), we demonstrate that ablation of the resting CD11chi DC population results in a highly significant decrease in the absolute number of mature NK cells. In contrast, administration of Flt3 ligand increases the CD11chi DC population, which, when expressing IL-15, significantly expands mature NK cells via enhanced survival and proliferation. In summary, a CD11cDC population expressing IL-15 is required to maintain NK cell homeostasis under conditions of normal cellularity and also is required to mediate Flt3 ligand-induced NK cell expansion in vivo. The Journal of Immunology, 2010, 184: 2769–2775.

Interleukin-15 is the only cytokine that has been shown to directly support NK cell development in vivo. In mice and in humans, NK cell precursors have been described to respond to IL-15 and differentiate into mature NK cells (1). Overexpression of endogenous IL-15 leads to expansion and activation of NK cells (2), whereas disruption of the IL-15 gene (3) or the high-affinity IL-15Rα–chain (4) is sufficient to abrogate NK cell development and impair survival of mature NK cells. Altogether, these observations indicate that IL-15 signaling is critical for NK cell homeostasis and necessary for both differentiation and survival of mature NK cells in vivo (5).

IL-15 undergoes inefficient translation and modest secretion. It is presented to the IL-15Rβγ heterodimer in trans via its high-affinity IL-15Rα–chain that cycles to the surfaces of APCs and some stromal cells (6, 7). Although there is ample evidence that IL-15 produced by activated APCs is critically involved in NK cell activation, little is known about the biological relevance of IL-15 produced by nonactivated APCs with respect to NK cell homeostasis. Because mature NK cell numbers are diminished greatly in the absence of endogenous IL-15 and effective in trans presentation of the cytokine necessitates coexpression of IL-15 and IL-15Rα by the same non-NK cell (8, 9), we postulated that under conditions of normal cellularity NK cell homeostasis might depend on an IL-15Rα− cell type(s) that must present IL-15. Flt3 ligand (FL) is also an essential growth factor for dendritic cell (DC) and NK cell homeostasis in vivo (10), yet mature NK cells neither express the receptor for FL (Flt3) nor respond to FL in vitro (11). Exogenous administration of FL results in potent antitumor activity, likely mediated via NK cells (12), yet the mechanism of this FL-mediated immune modulation is unknown.

In this study, we provide in vivo evidence that under conditions of normal cellularity the IL-15–bearing CD11c+ DCs are critical for maintenance of NK cell homeostasis and that exogenously administered FL expands this population, which in turn alters mature NK cell homeostasis via enhanced survival and proliferation.

Materials and Methods

Animals

Female C57BL/6 (Ly5.1 and Ly5.2) and C57BL/6 CD11c−diphtheria toxin receptor (DTR) transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The CD11c−DTR mice express DTR under the control of the CD11c promoter as described by Jung et al. (13). C57BL/6 IL-15−/− mice were generously provided by Dr. Jacques Peschon (Amgen, Seattle, WA). All of the mice were housed in microisolator cages within a pathogen-free animal facility. All of the animal research conducted in this study was approved by the Institutional Laboratory Animal Care and Use Committees at The Ohio State University or at the National Institutes of Health.

Drug administration

Human recombinant FL was kindly provided by Amgen (Thousand Oaks, CA). Mice were treated with one daily i.p. injection of FL (10 μg) or vehicle (PBS) for 14 or 28 consecutive days.

Isolation of spleen, bone marrow, and blood cells

Mice were anesthetized with 0.3 ml of a mixture of 4.5 mg/ml ketamine and 1.1 mg/ml xylazine, followed by retro-orbital bleeding. Mice then were sacrificed by cervical dislocation while under anesthesia. Spleens were excised, weighed, and disrupted into single-cell suspensions. Bone marrow...
B(1)M cells were obtained from one femur and tibia. Cells from each tissue were treated with RBC lysis buffer, washed, and resuspended in RPMI 1640 containing 10% FCS.

Flow cytometric analysis
Expression of cell surface Ags was evaluated by direct immunofluorescence (14), except IL-15Rα, which required indirect immunofluorescence. Approximately 5 × 106 cells per sample were incubated with mAb for 30 min, washed with PBS containing 2% FCS, and resuspended in 1% formalin. The following mAbs were used from BD PharMingen (San Diego, CA): CD3-FITC, CD4-PerCP, CD8-PE, CD11b-FITC, CD11c-PE, CD19-allophycocyanin, CD27-PE, CD45.1-PE, DX5-allophycocyanin, NK1.1-PE, and Ly49D-FITC. CD45.1-allophycocyanin was purchased from eBioscience (San Diego, CA). IL-15Rα surface expression was determined by staining using a biotinylated anti-mouse Ab against IL-15Rα purchased from R&D Systems (Minneapolis, MN), followed by a secondary staining with streptavidin from BD PharMingen. Apoptosis was evaluated using annexin V-FTTC (BD PharMingen) and 7-aminoactinomycin D ( Molecular Probes, Eugene, OR). Immunofluorescence reactivity was determined on 105 cells per sample by automated multiparameter flow cytometry on a FACS Calibur analyzer (Becton Dickinson, Mountain View, CA). For CFSE analysis, between 1 × 106 and 106 events were acquired. CellQuest (BD Biosciences, San Jose, CA) or FlowJo (Tree Star, Ashland, OR) software was used for data analysis.

Generation of BM chimeras
BM cells were obtained from the tibias and femurs of Ly5.2 C57BL/6 WT-15/−− and IL-15WT/WT donor mice. Ly5.1 recipient mice were irradiated with 1300 rad (2 × 650 rad) from a 137 Cs source and injected i.v. with 106 BM cells. Five weeks later, BM chimeric mice were treated daily with 105 g/FL or PBS for a period of 28 d. Mice then were sacrificed for analysis.

Detection of IL-15 and IL-15Rα mRNA
The relative quantification of IL-15 and IL-15Rα transcripts was performed using real-time PCR (2). The forward and reverse primer sequences for IL-15 were 5′-TCTATTAGGCAACCTTACACTGACA-3′ and 5′-GCAATTCCAGGAGAAGCATTG-3′, respectively, and the fluorogenic probe was 6FAM-CTTTCATCCAGTTGCAAAGTTACTGCATG-TAMRA. The forward and reverse primer sequences for IL-15Rα were 5′-AGTGACCAAAGGTTGACCTA-3′ and 5′-TGGGAGAGAAA-GCTTCTGGCT-3′, respectively, and the fluorogenic probe was 6FAM-AGAAGCCTCTCCTCTGCCTGCAAAT-TAMRA. The total reaction volume was 25 μl and consisted of 2.5 μl sample cDNA, 2.5 μl mixture of 10× forward and reverse primers (9.0 μM each) and 10× fluorogenic probe (1.0 μM), 2.5 μl 10× mixture of primers and fluorogenic probe for 18S rRNA, 5.0 μl water, and 12.5 μl 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Real-time PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems). The real-time PCR sample was processed first to activate uracil-DNA glycosylase and AmpliTaq at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Quantitative real-time PCR reactions were performed and analyzed by the ΔCT method after normalizing to internal 18S rRNA control reactions as described (15).

Adoptive transfer of CFSE-labeled NK cells
Mouse NK cells were enriched freshly from spleens of female C57BL/6 CD45.1 mice by positive bead selection (>95% purity by flow cytometry). Spleen cells were labeled using PE-conjugated rat anti-mouse mAb directed against NK1.1 Ag followed by anti-PE microbeads (Miltenyi Biotec, Auburn, CA). Approximately 0.5 × 106 NK1.1+CD45.1+ cells were labeled with 5 μM CFSE (Molecular Probes), washed, and then injected i.v. into wild-type (WT) CD45.2+ mice. In some experiments, recipients received daily i.p. injections with either FL or PBS.

In vivo depletion of CD11c+ DCs
BM chimeric mice were created using Ly5.1 C57BL/6 CD11c-DTR donor BM transplanted into Ly5.2 C57BL/6 recipients following irradiation with 1300 rad. Five weeks following BM transplantation, 5 × 106 NK1.1+CD45.1+Ly5.2+ splenocytes were enriched and administered i.v. to the BM chimeric mice. BM chimeric mice were treated i.p. with PBS or diphtheria toxin (DT) (4 μg/kg body weight) four times (day 0, +3, +6, and +9) to ablate CD11c+ DCs. Congenic NK cells were analyzed at day +10. In experiments where chimeric mice were treated daily with 10 μg/FL or PBS for 14 d, CD11c+ DCs were ablated in vivo by four i.p. injections of 4 ng/g body weight DT given on days 0, +4, +8, and +12 of FL treatment (13). At the end of the treatment, the chimeric mice were sacrificed for analysis.

Statistical analysis
All of the comparisons between treatment groups were performed using the unpaired Student t test, with <0.05 designated as significant. In univariate analysis, we used the Spearman rank correlation to test whether there was a significant correlation between the proportion of NK cells and the different cell subset, such as DCs, monocytes, or B lymphocytes.

Results
IL-15 produced by hematopoietic cells is necessary for mature NK cell homeostasis
We and others have demonstrated that IL-15 is necessary for mature NK cell survival (5, 16). To determine whether the cellular source of IL-15 is derived from radiosensitive stromal cells or radiosensitive hematopoietic cells (HCS), we created BM chimeras using donor IL-15−/− or IL-15WT/WT BM cells for engraftment. Five weeks after myeloablation and BM transplantation, chimeric mice showed normal blood counts (data not shown). Following engraftment, highly enriched congenic (CD45.1+) mature NK1.1+ NK cells from WT mice were transferred into the chimeric mice, 10 d after adoptive transfer the mice were sacrificed, and the splenic NK cells were enumerated. In IL-15−/− → IL-15WT/WT BM chimeras, the total number of splenic NK1.1+CD45.1+ cells averaged 2.4 × 105 ± 3527, whereas the total number of splenic NK1.1+CD45.1+ cells from IL-15WT/WT → IL-15+/− chimeras averaged 6.1 × 105 ± 7244 (p < 0.005). The latter value was not significantly different from the number of splenic NK1.1+CD45.1+ cells recovered from IL-15WT/WT → IL-15WT/WT control mice (Fig. 1A). These results demonstrate that under normal conditions HC-derived IL-15 is necessary for optimal mature NK cell survival in vivo. We next sought to investigate the cell type(s) responsible for this effect.

In vivo depletion of CD11c+ DCs alters the normal homeostasis of mature NK cells
Previous work combined with the results above prompted us to investigate the potential role of CD11c+ cells in regulating mature NK cell survival (9, 17). Real-time PCR performed on enriched CD11c+ DCs obtained from WT animals confirmed the presence of IL-15 mRNA (data not shown). To investigate whether CD11c+ DCs are critical for mature NK cell survival in vivo, we created BM chimeras using CD11c-DTR donor BM cells transplanted into lethally irradiated C57BL/6 WT recipient mice (CD11c-DTR → C57BL/6 WT/WT) (Fig. 1B). Chimeric mice recovered with normal cellularity. Repeated administration of DT to these mice efficiently depleted CD11c+ DCs without systemic toxicity (Fig. 1C, 1D). To eliminate any direct toxic effect of DT administration on NK cells derived from CD11c-DTR transgenic BM progenitors, we adoptively transferred congenic NK1.1+ cells obtained from spleens of CD45.1+ WT mice and assessed the impact of CD11c+ DC ablation on nontransgenic mature NK cells. Following infusion of 0.5 × 106 NK1.1+CD45.1+ NK cells into chimeric mice, the mice received DT treatment on days 0, +3, +6, and +9 and then were sacrificed on day +10 to enumerate splenic NK1.1+CD45.1+ NK cells (Fig. 1B). The number of splenic NK1.1+CD45.1+ NK cells recovered from PBS-treated animals (n = 5) averaged 5.2 ± 0.01 × 105. In contrast, 3.9 ± 0.1 × 105 splenic NK1.1+CD45.1+ cells were recovered from DT-treated, CD11c+ -depleted animals (n = 5; p < 0.0001) (Fig. 1E). Therefore, we conclude that under resting conditions of normal cellularity CD11c+ DCs are integral to the peripheral niche that regulates mature NK cell numbers in vivo. Considering that HC-derived IL-15 and CD11c+ DCs are involved critically in mature NK cell homeostasis, we next asked whether expansion of the CD11c+ DC pool was sufficient to alter mature NK cell homeostasis.
FIGURE 1. IL-15 produced by HCs and CD11c<sup>hi</sup> DCs regulates the homeostasis of mature NK cells. A, Reciprocal BM chimeras were generated using CD45.2<sup>+</sup>IL-15<sup>−/−</sup> and CD45.2<sup>+</sup>IL-15<sup>WT/WT</sup> B6 mice as a source of donor BM and as host recipients. Five weeks after transplantation, highly enriched mature WT NK1.1<sup>+</sup>CD45.1<sup>+</sup> cells were adoptively transferred into the CD45.2<sup>+</sup> chimeric mice. Ten days later, mice were sacrificed and analyzed for splenic NK1.1<sup>+</sup>CD45.1<sup>+</sup> cells. Significantly more NK1.1<sup>+</sup>CD45.1<sup>+</sup> cells were recovered per spleen from IL-15<sup>WT/WT</sup> → IL-15<sup>−/−</sup> chimeras compared with IL-15<sup>−/−</sup> → IL-15<sup>WT/WT</sup> chimeras. B, Schematic representation of the experimental design where enriched NK1.1<sup>+</sup>CD45.1<sup>+</sup> cells were administered to congenic CD45.2<sup>+</sup>CD11c-DTR transgenic → CD45.2<sup>+</sup> WT BM chimeras. C, Representative flow cytometric analysis of splenic CD11c<sup>+</sup> cells in PBS- and DT-treated animals. D, Graphical summary of percentage ± SE CD11c<sup>+</sup> DCs for PBS- and DT-treated BM chimeras. E, Absolute number of adoptively transferred NK1.1<sup>+</sup>CD45.1<sup>+</sup> cells after CD11c<sup>hi</sup> DC depletion (five mice per group).

**FL administration expands NK cells in a DC-dependent manner**

FL-deficient mice are depleted severely of CD11c<sup>hi</sup> DCs, whereas CD11c<sup>hi</sup> DCs expand in response to in vivo FL administration (18, 19). As noted above, mature NK cells neither express Flt3R nor respond to this cytokine in vitro (11, 20). Nonetheless, we observed that a 14 d course of FL administration to WT mice resulted in a 12-fold expansion of NK cells in blood PBS (69.9 ± 67.4 cells per microliter versus FL 890.6 ± 154.5 cells per microliter [p < 0.0001]) and a 4-fold expansion of NK cells in the spleen (PBS 4.9 ± 0.6 × 10<sup>6</sup> cells per microliter versus FL 19.2 ± 2.3 × 10<sup>6</sup> [p = 0.0007]) (Fig. 2A, 2B). The significant changes in the absolute number of NK cells in blood and spleen in WT mice paralleled those seen for the percentage of NK cells. In stark contrast IL-15<sup>−/−</sup> mice showed no changes in the percentage of NK cells following treatment with FL (Fig. 2C–E).

With the evidence above that depletion of CD11c<sup>hi</sup> DCs reduces the absolute number of mature NK cells under conditions of normal cellularity in vivo, we hypothesized that the increase of NK cell numbers in response to FL occurs indirectly via the expansion of CD11c<sup>hi</sup> DCs that express IL-15. Consistent with this hypothesis, studies utilizing IL-15<sup>−/−</sup> → IL-15<sup>WT/WT</sup> and IL-15<sup>WT/WT</sup> → IL-15<sup>−/−</sup> chimeric mice demonstrated that the endogenous IL-15 necessary for FL-mediated NK cell expansion comes from within the HC compartment and not the stroma (Fig. 3A, 3B). Furthermore, by examining the temporal relationship between the expansions of the CD11c<sup>+</sup> and NK1.1<sup>+</sup> cell populations during FL administration, we noted that expansion of CD11c<sup>+</sup> cells was followed sequentially by expansion of NK1.1<sup>+</sup> cells, supporting the hypothesis that the CD11c<sup>hi</sup> DC population expanded by FL therapy could be a source of endogenous IL-15 required for expanding mature NK cells (Supplemental Fig. 1A, 1B).

**In vivo ablation of CD11c<sup>hi</sup> DCs reduces FL-mediated NK cell expansion**

To prove that the FL-mediated expansion of NK cells was dependent on the expansion of the CD11c<sup>hi</sup> DC population, we again used our CD11c-DTR CD45.2<sup>+</sup> transgenic BM progenitors → C57BL/6<sup>WT/WT</sup> BM chimeric mice, this time treating with both DT and FL (Fig. 3C). We again adoptively transferred NK1.1<sup>+</sup> CD45.1<sup>+</sup> congenic mature NK cells obtained from CD45.1<sup>+</sup> WT mice into BM chimeras to assess the impact of CD11c<sup>hi</sup> DC ablation during FL administration on the homeostasis of nontransgenic NK cells following treatment with FL (Fig. 2A, 2B).
adoptionally transferred mature NK cells. Concurrent administration of DT and FL significantly inhibited the expansion of NK1.1+CD45.1+ congenic mature NK cells compared with administration of PBS and FL (PBS + FL 24 ± 1.0 × 10^6 cells per milliliter versus DT + FL 17 ± 1.7 × 10^6 cells per milliliter [p < 0.01]) (Fig. 3D). Flow cytometric analysis revealed a highly significant (p < 0.0001) yet incomplete depletion of the CD11c+ DC population when compared with the control group (Fig. 3E, 3F). The incomplete depletion of CD11c+ DC subsets could be due to the continuous treatment of FL. Regardless, the data demonstrate that CD11c+ DCs contribute substantially to the FL-mediated expansion of NK cells in vivo. Therefore, the primary factor regulating mature NK cell expansion in the setting of FL administration is related to the accessibility to CD11c+ DCs that produce IL-15.

Because IL-15 and IL-15Ra are critical to NK cell homeostasis and are expressed on both CD11b+CD11c+ and CD11b+CD11c- populations, we asked whether FL administration increased the expression of either or both of these molecules. We first assessed IL-15 and IL-15Ra mRNA in unsorted blood and splenocytes of mice treated with either FL or PBS, showing the anticipated large increases in these transcripts following FL treatment (Fig. 4A). The CD11b+CD11c+ and CD11b+CD11c- populations were the two splenic populations largely responsible for the increase in IL-15 and IL-15Ra mRNA in the FL-treated mice (Fig. 4B). However, when IL-15 and IL-15Ra mRNA were quantified in equal numbers of CD11b+CD11c+ populations obtained from PBS- and FL-treated mice, there was no significant difference in IL-15 or IL-15Ra mRNA on a per-cell basis (Fig. 4C, 4D). A cytometric analysis confirmed that the FL treatment does not increase surface expression of IL-15Ra protein on a single-cell basis (Fig. 4E). Therefore, we conclude that FL alone does not activate DCs to produce more IL-15 or IL-15Ra and that the FL-mediated increase in IL-15 and IL-15Ra levels results from a selective expansion of APCs already producing these factors.

**FL-induced NK cell expansion occurs via enhanced survival and increased proliferation of mature NK cells**

One potential explanation for NK cell expansion by FL is via increased survival of mature NK cells. To examine whether FL-mediated expansion of CD11c+ cells could increase mature NK cell survival, mice were treated daily with FL for 14 d and then sacrificed 1, 3, or 5 d after discontinuation of FL therapy to assess for apoptosis by annexin V staining. Although the proportion of apoptotic annexin V+ NK1.1+ cells was unchanged from day +1 to day +3 following cessation of FL treatment, by day +5 the proportion of apoptotic annexin V+ NK1.1+ cells had increased 3-fold compared with day +3 (24.6 versus 8.1%; p = 0.002) (Fig. 5A). This increase in NK cell apoptosis was correlated to an increase in DC apoptosis (Supplemental Fig. 3) and a decrease in absolute numbers of splenic NK1.1+ cells, such that by day +5 after cessation of FL treatment NK cell numbers had returned to normal levels (Fig. 5B and inset).

To determine whether FL-mediated NK cell expansion is also due to increased NK cell proliferation, we isolated 0.5 × 10^6 congenic NK1.1+CD45.1+ cells, labeled them with CFSE, and transferred them into congenic CD45.2+ WT recipients treated daily with FL or PBS for 14 d. Notably, a significantly greater number of adoptively transferred mature NK1.1+CD45.1+ cells were recovered from the spleens of FL-treated mice compared with PBS-treated mice (FL 17 ± 4 × 10^6 cells per microliter versus PBS 5 ± 0.7 × 10^6 cells per microliter [p < 0.01]) (Fig. 5C). Further, adoptively transferred mature NK cells recovered from FL-treated mice demonstrated significantly enhanced proliferation compared with PBS-treated mice (percentages of congenic NK cells with diluted CFSE: FL 68.0 ± 5.7% versus PBS 28.8 ± 4.8% [p < 0.001]) (Fig. 5D, 5E). We conclude that the FL-expanded CD11c+ IL-15-bearing DCs increase NK cells via enhanced survival and proliferation of the mature NK pool.

**Discussion**

In this report, we have described a novel role for CD11c+ DCs in the regulation of mature NK cell homeostasis in vivo under conditions of normal cellularity. Our data demonstrate that HCs play an important role in maintaining NK cells in the periphery through production of IL-15 and that CD11c+ DCs represent essential regulators of the mature NK cell population (3, 5). The elimination of CD11c+ DCs from mice with normal cellularity resulted in a dramatic decrease in mature NK cells. In contrast, expansion of the CD11c+ DCs by exogenous administration of FL increased NK cell numbers via augmented survival and proliferation of mature NK cells. Notably, the partial elimination of CD11c+ cells...
during the course of FL treatment significantly reduced the potency of FL to expand mature NK cells. Taken together, our data provide compelling evidence that CD11c<sup>hi</sup> DCs are an integral part of the peripheral niche that controls mature NK cell homeostasis in the absence of lymphopenia or immune activation.

The observation that the number of NK cells is kept at relatively constant levels in humans and mice signifies that these cells are normally under tight homeostatic control. We do know that IL-15 is necessary for NK cell differentiation from NK cell precursors (3) and necessary for mature NK cell survival in the periphery (5, 16, 21). Our data and that of others (22) indicate that NK cell development depends on stromal and HC-derived IL-15 and that expression of IL-15<sub>ra</sub> by HCs is required for mature NK cell survival (7, 23). In this study, we provide conclusive evidence that HCs producing IL-15 are the primary regulators of mature NK cell survival in the periphery. Stromada-derived IL-15 has only a modest if any effect on mature NK cell survival, because the recovery of adoptively transplanted mature congenic NK cells was essentially the same whether IL-15 production was confined to HCs alone or produced by both HCs and the stroma. The inability of mature NK cells to survive in the presence of stroma-derived IL-15 alone could be related to their trafficking patterns, the mechanism of IL-15 delivery by stromal cells, or the absence of other necessary growth signals normally provided by HCs. Indeed, recent evidence indicates that stroma-derived IL-15 is not readily available within the BM microenvironment and that interactions between lymphotxin-α and lymphotxin-βR expressed by NK precursors and stroma cells, respectively, are critical in this process (24, 25). Because resting mature NK cells do not express lymphotxin α (26), this may prevent these cells from using stroma-derived IL-15. The observation that NK cell numbers are diminished greatly in mice lacking either stroma- or HC-derived IL-15 suggests that NK precursors and mature NK cells might occupy distinct niches that minimally overlap with each other.

Several reports have demonstrated previously that APCs produce IL-15 and IL-15<sub>ra</sub> (27–30), yet the potential role of APCs in the setting of mature NK cell homeostasis has not been, to our knowledge, directly addressed in vivo. Previous studies concluded that IL-15 produced by activated CD11c<sup>hi</sup> cells was important for NK cell activation (9, 17, 31–33) and, under lymphopenic conditions induced by γ irradiation, critical for NK cell proliferation (34). Given that resting APCs are poor producers of IL-15 (9), one might assume that IL-15 produced by resting CD11c<sup>hi</sup> cells may not be important for NK cell homeostasis under noninflammatory and normal cellular conditions. The studies presented in this paper show clearly that CD11c<sup>hi</sup> DCs are a critical component of the peripheral NK cell niche. We further demonstrate that both CD11b<sup>−</sup>CD11c<sup>−</sup> and CD11b<sup>−</sup>CD11c<sup>+</sup> cells constitute an important reservoir of IL-15 and IL-15<sub>ra</sub> and that the ablation of the resting CD11c<sup>hi</sup> DCs decreases mature resting NK cell numbers. We therefore suggest that at a minimum IL-15-bearing CD11c<sup>hi</sup> DCs are themselves responsible for producing and delivering IL-15 to mature resting NK cells and regulating their homeostasis under normal physiologic conditions. Given that the total CD11c<sup>hi</sup> population includes both myeloid- and lymphoid-derived DCs, it remains unclear whether both or one specific cell type controls NK cell homeostasis in vivo. Interestingly, however, multiple clinical studies have demonstrated that lymphopenic insults are followed by relatively rapid regeneration of myeloid DCs and NK cells, whereas the normalization of lymphoid DC numbers generally occurs later (35, 36). Furthermore, although there is ample evidence that myeloid DCs produce and present IL-15 in trans, strong evidence that lymphoid DCs, specifically plasmacytoid DCs, could express IL-15<sub>ra</sub> and present IL-15 in trans to mature NK cells has been lacking. Therefore, the data presented in this study are more consistent with a model wherein myeloid DCs are serving a role as regulators of mature NK cell homeostasis in vivo. We observed that exogenous FL administration can efficiently drive mature NK cell survival and proliferation and that ablation of CD11c<sup>hi</sup> DCs greatly reduces this effect, suggesting that these DCs play a critical role in NK cell expansion during FL therapy. Surprisingly, we were unable to provide evidence that FL could increase NK cell differentiation from BM progenitor cells. Whereas FL efficiently stimulates NK precursor formation and accumulation in an IL-15-independent manner, it does not appear to affect the distribution of immature BM NK cells expressing CD11b, CD27, or both...
NK1.1+CD45.1+ cells recovered from the mature congenic NK cell proliferation.

Summary (mean ± SE) of data as shown in A–E shows the absolute number of NK cells enumerated in PBS-treated mice.

C–E. Effect of FL treatment on mature congenic NK cell proliferation. C, Summary (mean ± SE) of number of viable splenic NK+CD3−CD11b+ cells, which may have contributed an alternative source of viable splenic NK+CD3− cells enumerated over time following discontinuation of FL administration. Inset shows the absolute number of NK cells enumerated in PBS-treated mice.

 Although we have clear evidence that CD11c<sup>hi</sup> DCs expand readily in FL-treated animals and present IL-15 to mature NK cells, it is important to note that we did not exclude other IL-15<sup>+</sup> cells producing IL-15 (e.g., DT-resistant CD11c<sup>low</sup> or CD11b<sup>+</sup> cells), which may have contributed an alternative source of HC-derived IL-15 to partly support NK cell expansion. Evidence of additional IL-15–producing HCs is provided by our observation that BM chimeras in which CD11c<sup>hi</sup> DCs had been ablated display stronger NK cell recovery compared with chimeric mice reconstituted with IL-15−/− HCs (Fig. 1A–E). Thus, other IL-15–producing HC-derived cell types in addition to the CD11c<sup>hi</sup> DC population may sustain mature NK cell homeostasis in vivo. This may explain how M-CSF, which primarily modulates monocytes and macrophages, could increase the number of NK cells in vivo (37). Nonetheless, it is clear that these other supporting HC-derived cells alone cannot sustain normal NK cell numbers that are observed when CD11c<sup>hi</sup> DCs are present. Under normal circumstances, the vast majority of mature NK cells do not undergo homeostatic proliferation, presumably because the NK pool is at equilibrium within its niche, including DCs. Homeostatic proliferation can be seen following adoptive transfer of mature NK cells into a mouse that has radiation-induced lymphopenia or is genetically deficient of NK cells (e.g., Rag<sup>−/−</sup> γ<sup>−/−</sup> mice), and it requires both IL-15 and DCs (21, 34, 38). In this study, we show that in vivo alteration of the NK cell niche via FL-mediated expansion of the CD11c<sup>hi</sup> DC population is another way to induce proliferation and expansion of mature NK cells. Administration of FL to humans is very safe and nontoxic relative to the administration of other lymphocytotropic factors, such as IL-2 (39, 40). Exogenous FL administration may provide the best way to selectively expand and activate NK cells when considering this therapy that benefits leukemia patients (41).

Although this study explored the role of CD11c<sup>hi</sup> DC-derived IL-15 on NK cell homeostasis, the same signal also can activate mature NK cells. Previously, it was demonstrated that NK cell priming occurs primarily in the lymph node and requires IL-15 production by activated DCs (9, 17). Because activated DCs produce more IL-15 and IL-15Ra per cell (9), it is likely that the quantity of IL-15 signaling on mature NK cells could differ considerably from that delivered by resting DCs. In this study, we have demonstrated that IL-15 produced by resting CD11c<sup>hi</sup> DCs is essential for mature NK cell survival, and this appears to occur without DC activation using, for example, whole-body irradiation, LPS, or polyinosinic-polycytidylic acid. Furthermore, although FL supports mature NK cell survival and proliferation, it has been shown that DCs expanded by FL express low levels of costimulatory molecules (42). Finally, we demonstrate that in vivo administration of FL does not increase IL-15 or IL-15Ra gene expression in CD11c<sup>hi</sup> DCs on a per-cell basis. Together, the data presented in this work are consistent with the notion that resting DCs can present IL-15 in trans, such as to support mature NK cell homeostasis under conditions of normal NK cellularity. Whether NK cells also are activated by the interaction with DCs in vivo may depend on quantitative differences in IL-15 signal received from resting versus activated DCs, a qualitative difference in the nature of the other costimulatory signals presented by the DCs, or both.

In summary, we demonstrate that CD11c<sup>hi</sup> DCs expressing IL-15 are required for mature NK cell survival in vivo under conditions of normal cellularity. Because such cells constitutively

![Figure 5](http://www.jimmunol.org/Downloaded.png)

**FIGURE 5.** FL-mediated NK cell expansion results from increased survival and proliferation of the mature NK cell pool. WT mice were treated with FL for 14 d, and NK cells were analyzed for annexin V expression. A, Summary (mean ± SE) of the proportion of splenic NK1.1+CD3<sup>−</sup> cells undergoing apoptosis over time following discontinuation of FL administration. B, Summary (mean ± SE) of absolute number of viable splenic NK1.1+CD3<sup>−</sup> cells enumerated over time following discontinuation of FL administration. Inset shows the absolute number of NK cells enumerated in PBS-treated mice.
express IL-15 and IL-15Ra and present IL-15 in trans to mature NK cells (9), our work suggests that DC and NK cell homeostasis are tightly linked and offers an explanation as to why mice lacking FL have deficiencies in both DCs and NK cells.

Acknowledgments
We thank Donna Bucci, Dr. Rebecca Bruner Klisovic, and Dr. Sameek Roychowdhury for technical and scientific assistance.

Disclosures
The authors have no financial conflicts of interest.

References
The *in vivo* role of flt3 ligand and dendritic cells in natural killer cell homeostasis

Guimond et al.

Supplemental data

Figure S1. The expansion of CD11b⁺CD11c⁺ DC precedes NK cell expansion during FL treatment.
(A) Summary (mean ± SE) of CD11b⁺CD11c⁺ splenic DC and NK1.1⁺CD3⁻ splenic NK cell counts in FL-treated mice at days 0, +4, +7 and +11. CD11b⁺CD11c⁺ DC undergo significant (4- to 5-fold) expansion between day 0 and +4 while NK cell expansion begins between on day +4 and +7. Each point represents mean ± SE. (B) Regression analysis between splenic CD11b⁺CD11c⁺ DC and splenic NK1.1⁺CD3⁻ NK cells (R²=0.92), after 14 days of FL treatment. Fourteen mice were analyzed in this experiment.
Figure S2. FL administration expands NK precursors but does not promote NK cell differentiation. (A) Limiting dilution assays (LDA) (11) were used to quantify BM NK precursors (NKp) after 14 days of exogenous PBS or FL administration. FL treatment significantly expanded the NKp numbers in BM when compared to PBS in both IL-15^{wt/wt} and IL-15^{-/-} mice, yet there was no significant difference in NKp frequency between IL-15^{wt/wt} mice treated with FL and IL-15^{-/-} mice treated with FL. (B) Histogram illustrating CD27 versus CD11b expression on gated NK1.1{+} NK cells in the BM. The lower right square captures the immature NK1.1^{+}CD11b^{-}CD27^{lo} NK cells, while the upper rectangle captures the mature NK1.1^{+}CD11b^{+}CD27^{hi} NK cells. (C and D) Summary (mean ± SE) of percent NK1.1^{+} subsets in the BM of PBS- and FL-treated mice (>4/group). FL administration did not alter the relative proportions of immature NK1.1^{+}CD11b^{-}CD27^{lo} and mature NK1.1^{+}CD11b^{+}CD27^{hi} NK subsets in BM.
Figure S3. WT mice were treated with FL for 14 days and CD11b⁺CD11c⁺ cells were analyzed for Annexin V expression. Summary (mean ± SE) of the proportion of splenic CD11b⁺CD11c⁺ cells undergoing apoptosis over time following discontinuation of FL administration. Inset showed the proportion of Annexin V expression in PBS-treated mice.