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Signal Via Lymphotoxin-βR on Bone Marrow Stromal Cells Is Required for an Early Checkpoint of NK Cell Development[1]

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NK cells play an important role in the immune system but the cellular and molecular requirements for their early development are poorly understood. Lymphotoxin-α (LTα)$^{-/-}$ and LTβR$^{-/-}$ mice show a severe systemic reduction of NK cells, which provides an excellent model to study NK cell development. In this study, we show that the bone marrow (BM) or fetal liver cells from LT$\alpha^{-/-}$ or LTβR$^{-/-}$ mice efficiently develop into mature NK cells in the presence of stromal cells from wild-type mice but not from LT$\alpha^{-/-}$ or LTβR$^{-/-}$ mice. Direct activation of LTβR-expressing BM stromal cells is shown to promote to early NK cell development in vitro. Furthermore, the blockade of the interaction between LT and LTβR in adult wild-type mice by administration of LTβR-Ig impairs the development of NK cells in vivo. Together, these results indicate that the signal via LTβR on BM stromal cells by membrane LT is an important pathway for early NK cell development. ***The Journal of Immunology,*** **2001, 166: 1684–1689.**

Natural killer cells represent a distinct population of lymphocytes. They exhibit spontaneous non-MHC-restricted cytotoxic activity against virally infected cells or tumor cells in vitro and mediate resistance to viral infections and cancer development in vivo (1, 2). NK cells express various inhibitory and activating molecules on NK cells (2, 3). Despite the increasing knowledge of NK cell function and recognition, their differentiation into mature NK cells from hemopoietic stem cells is not well defined (4–6).

Early experiments on mice treated with radioisotope suggested that bone marrow (BM)[3] was the essential site for NK cell development (7). Several cytokines are required for a coordinated development and differentiation of NK cells. Several cytokines such as Flt3-L and c-*kit* promote the differentiation of NK progenitor cells in vitro from CD34$^+$ primitive hemopoietic progenitor cells. IL-15 is then required for further differentiation and proliferation of NK cell lineages (4–6, 8). Furthermore, IL-15 was found to be essential for NK cell development in vivo (9, 10). Other studies have suggested that stromal cells may play an important role in the development of NK cells in vivo and in vitro (11, 12). In the absence of stromal cells, a mixture of soluble cytokines acts on progenitors to generate IL-15 responsive cells. The addition of IL-15 leads to their differentiation into NK1.1$^+$ cells (5). Interestingly, the cells generated in the absence of stromal cells failed to express the various Ly49 receptors that are normally expressed on wild-type (wt) splenic NK cells or NK cells that developed in the presence of stromal cells (5). These data suggest that stromal cells play an important role in NK cell development. However, neither the precise physiological role of stromal cells nor the role of cytokines in NK cell development has been well defined.

Lymphotoxin (LT), a cytokine of the TNF family, is necessary for the presence of NK cells in the spleen (13–15). We have reported that LT$\alpha^{-/-}$ mice had fewer NK cells in the spleen. The action of LT is independent of IL-15 (13). Other groups also independently demonstrated a compromised NK cell-mediated antitumor function in the spleen of LT$\alpha^{-/-}$ mice (14, 15). In addition, impaired NK cell recruitment had been demonstrated in these studies.

LT can form either soluble LTα3 homotrimers that bind to TNFRs on both hemopoietic cells and stromal cells or membrane LT$\alpha_1\beta_2$ heterotrimers bind to LTβRs on stromal cells (16, 17). We found that TNFR-I$^{-/-}$ mice showed no reduction of NK cells in the spleen (13). These findings raise the possibility that potential cell-cell contact between membrane LT-expressing lymphocytes and their surrounding stromal cells is required for NK cell development. Interestingly, LT also regulates the expression of several lymphoid tissue chemokines that direct the migration or localization of various immune cells to the spleen (18, 19). Therefore, the reduction of NK cells in the spleens of LT$\alpha^{-/-}$ mice may be attributed to the following mechanisms: 1) a reduced migration of NK cells into lymphoid tissues due to improper production of lymphoid tissue chemokines; 2) an intrinsic defect in NK cell progenitors that fail to respond to cytokines; and 3) a defective microenvironment, such as the lack of BM cytokine(s) or stromal cells needed for NK cell development.

To dissect LT-mediated NK cell development, we have used reciprocal BM reconstitution and reciprocal BM culture using early hemopoietic progenitors and stromal cells from wt and LT or LTβR-deficient mice. The results indicated that impaired NK cell development in BM is attributed to a defect in stromal cells. Our study has revealed that the close interaction between NK cell lineage and LT-activated stromal cells is essential for NK cell development at an early checkpoint.

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[3] Abbreviations used in this paper: BM, bone marrow; wt, wild type; LT, lymphotoxin; ProGP, progenipoietin; FL, fetal liver; BMT, BM transplantation; FDC, follicular dendritic cell; IRF-1, IFN-regulatory factor-1.

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Materials and Methods

Mice, Abs, and soluble receptor fusion protein

LTβR$^{-/-}$ mice and LT$\alpha^{-/-}$ mice were backcrossed for five and seven generations, respectively, to C57BL/6 mice and were maintained under specific pathogen-free conditions. Animal care and use were in accordance with institutional guidelines. Murine LTβR-human IgG1 Fc, human IgG, anti- LTβR Ab (an agonist to the receptor; kindly provided by Jeff Browning, Biogen, Cambridge, MA), and anti-LTβ mAb were previously described (19–21). Progenipoietin (ProGP-1) that activates Flt-3 and G-CSFRs was provided by Searle (Chesterfield, MO).

Analysis of NK cell development

Anti-CD3ϵ, anti-NK1.1 (PK136), anti-pan NK (DX5), anti-Ly49C/I, and anti-B220 Abs conjugated with FITC or PE were all obtained from PharMingen (San Diego, CA). Cells from spleens, lungs, blood, livers, and BM were collected, stained, and analyzed by using two-color flow cytometry on a FACScan with CellQuest software (Becton Dickinson, San Jose, CA).

Reciprocal transfer of BM

BM cells were harvested and recipients were prepared as described previously (22). Briefly, BM cells (2×10^6) from C57BL/6 or LTβR$^{-/-}$ mice (5–6 wk) were i.v. transferred into heavily irradiated recipient 5- to 6-wk-old mice (1050 rad). Six to 8 wk later, splenocytes were collected. The numbers of NK cells were monitored by flow cytometry as described above.

NK cytotoxicity assay

Fresh splenocytes from 6- to 10-wk-old mice were recovered by using a Lympholyte-M gradient (Cedarlane Laboratories, Hornby, Ontario, Canada) and were used in a standard ^{51}Cr release assay against YAC-1 cells for determining NK activity in vitro using several E:T ratios (23). Spontaneous ^{51}Cr release was $<$15% of maximum release.

Generation of stromal cells for NK cell development

A stromal cell line (OP9) that supports NK cell development was purchased from Riken Cell Bank (Ibaraki, Japan) (24). To prepare fresh BM stromal cells, BM cell preparations were cultured with RPMI 1640 culture medium containing 10% FCS in the absence of IL-15 for 2–3 wk. Under these conditions, stromal cells expand but few hemopoietic cells can survive. In some experiments, mitomycin C (50 μg/ml) was added into the culture for 30 min to prevent the potential growth of any surviving cells. Mitomycin C in the culture was washed out three times with RPMI 1640. Fetal liver (FL) cells from gestational days 11–13 were added to such culture and the effect of these stromal cells on NK cell development was evaluated. Mature NK cells were enumerated by flow cytometry for expression of NK1.1, DX5, or Ly49 C/I and by their cytotoxic activity to YAC-1 cells as targets.

Results and Discussion

Reduced numbers of NK and NK/T cells in both lymphoid and nonlymphoid tissues of LT$\alpha^{-/-}$ mice and LTβR$^{-/-}$ mice

To investigate whether the reduced number of NK cells is attributed to the lack of LT-mediated chemokine expression in lymphoid tissue, the percentages of NK cells and NK/T cells in the spleen, blood, and lungs of LT$\alpha^{-/-}$ mice were determined by flow cytometric analysis. Both NK1.1 and DX5 were used as NK markers because NK1.1 is a NK marker for C57BL/6 mice, whereas DX5 is a NK marker for most strains of mice. The percentages of NK cells and NK/T cells in LT$\alpha^{-/-}$ mice were greatly reduced in both lymphoid and nonlymphoid tissues (Fig. 1*A*). Therefore, the reduced number of NK cells is not the result of a migratory defect but rather a defect at the developmental stage.

To test whether interaction of membrane LT with LTβR contributes to the development of NK cells, we compared the percentage of NK cells and NK T cells in different tissues from LTβR$^{-/-}$ mice with those of wt mice by flow cytometry. As with LT$\alpha^{-/-}$ mice, LTβR$^{-/-}$ mice also exhibited a reduction in NK cells in both lymphoid and nonlymphoid tissues (Fig. 1*B*) and had an impaired NK cell activity against NK-sensitive target cells, YAC-1 (data not shown). Therefore, it seems that the interaction between

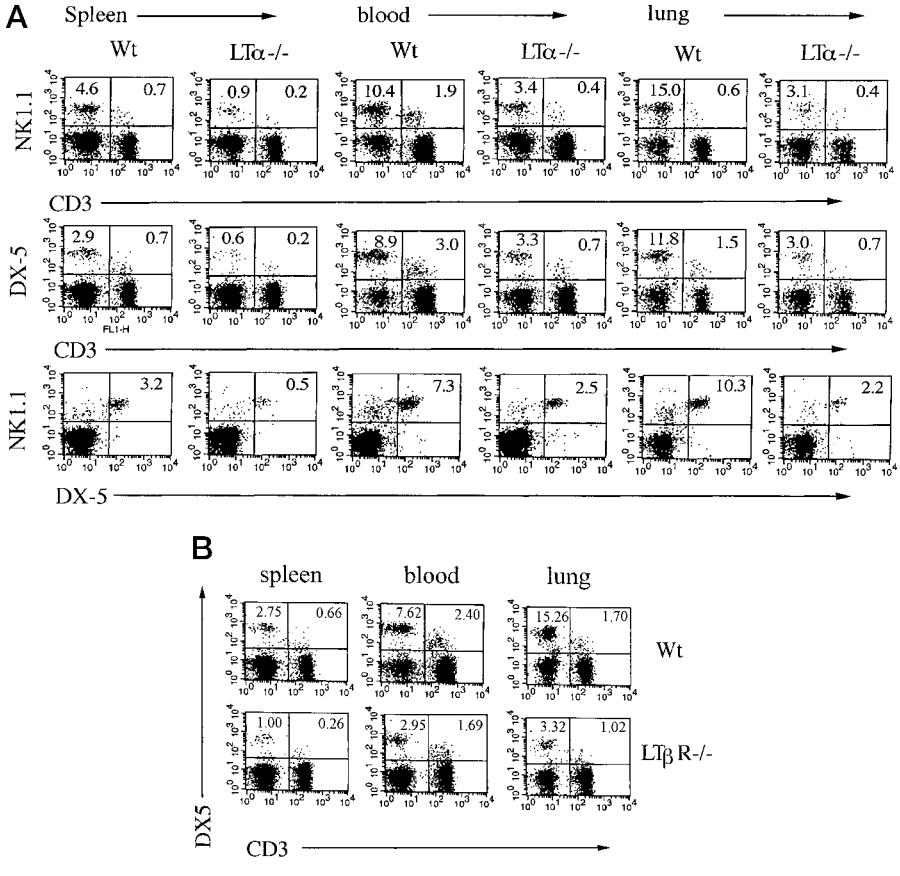

FIGURE 1. Reduced number of NK cells in lymphoid and nonlymphoid tissues in C57BL/6 LT$\alpha^{-/-}$ mice and LTβR$^{-/-}$ mice. *A*, Cells from spleen, blood, and lung of 6- to 8-wk-old wt and LT$\alpha^{-/-}$ mice were collected and stained for the NK cell markers PK136 (for NK1.1), DX5 (for pan NK), and anti-CD3. *B*, Cells from spleen, blood, and lung of 6- to 8-wk-old wt and LTβR$^{-/-}$ mice were collected and stained for the NK cell markers DX5 (for pan NK) and anti-CD3. We have not backcrossed LTβR$^{-/-}$ mice into B6 mice that express NK1.1 because the gene for LTβR is physically linked to NK1.1 loci. Four to six experiments were performed; data from one representative experiment are shown. Similar reduction can be detected in the liver of LT$\alpha^{-/-}$ mice (data not shown).

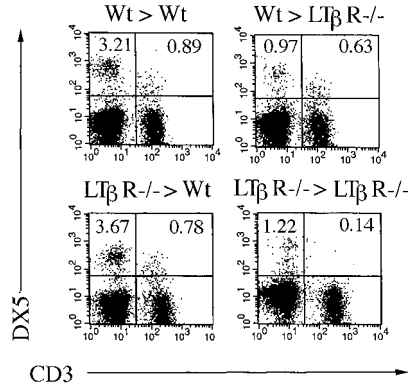

FIGURE 2. LTβR on stromal cells but not hemopoietic cells is required for the development of NK cells in vivo. Reciprocal BM reconstitution of wt and LTβR$^{-/-}$ mice was performed. The splenocytes were collected 5–6 wk after BM transfer. The percentage of splenic NK cells was determined by flow cytometry analysis. NK cell progenitors from LTβR$^{-/-}$ mice can develop normally in irradiated wt mice, whereas wt NK cell progenitors fail to grow inside LTβR$^{-/-}$ mice. Data from one of four experiments are shown.

membrane LT and LTβR is essential for normal NK cell development.

No intrinsic developmental defects of NK cell progenitors from LTβR$^{-/-}$ mice

BM is a major site for NK cell development. A reciprocal BM transfer between wt and LTβR$^{-/-}$ mice was used to dissect the roles of donor LT-expressing hemopoietic cells and LTβR-expressing recipient stromal cells in LT-mediated NK cell development. To study whether there was an intrinsic developmental defect of the NK cell lineage in LTβR$^{-/-}$ mice, BM cells from either wt or LTβR$^{-/-}$ mice were i.v. transferred into lethally irradiated adult wt recipients. Six weeks later, the splenocytes were collected and percentages of splenic NK cells were determined by flow cytometry analysis. There was no difference in the percentages of splenic NK cells in mice reconstituted with LTβR$^{-/-}$ BM or wt BM cells (Fig. 2). The NK lineage from LTβR$^{-/-}$ mice is able to respond to cytokines and LT-deficient NK cells can migrate to the spleen as occurs in wt mice. These data indicate that there are comparable numbers of NK cell progenitors in LTβR$^{-/-}$ mice and that these hemopoietic precursors from LTβR-deficient mice can develop normally into mature NK cells in a wt microenvironment. Therefore, the data suggest that the defective NK cell development in LTβR$^{-/-}$ mice is probably associated with a defective BM microenvironment.

Failure of LTβR$^{-/-}$ microenvironment to support NK cell progenitors development in vivo

To directly test whether a LTβR-deficient microenvironment could support wt NK cell progenitors developing into mature NK cells, we transferred BM cells from wt C57BL/6 mice into lethally irradiated wt C57BL/6 and LTβR$^{-/-}$ recipients. Six to 8 wk after reconstitution, the percentage of splenic NK cells was restored in wt recipients. In contrast, the percentage of recovered NK cells remained very low in the LTβR$^{-/-}$ recipient (Fig. 2), whereas the number of splenic B and T cells were completely restored in LTβR$^{-/-}$ recipients (data not shown). These data suggest that a LTβR-dependent signal through radioresistant cells (such as stromal cells) is essential for NK cell development.

Continuous signaling via LTβR is required for the maintenance of NK cell homeostasis

We previously reported that some LT-mediated lymphoid structures were developmentally fixed and were not restored in adult mice after BM reconstitution, whereas others were dynamically maintained by the action of LT through life (16, 25). To study whether LTβR signaling is required for the maintenance of NK cell homeostasis in adult mice, adult C57BL/6 mice were treated with LTβR-Ig once a week for 4 wk to block interaction between membrane LT and its receptor. The percentage of NK cells gradually declined in both lymphoid tissues and nonlymphoid tissues after such prolonged treatment (Fig. 3). Therefore, LT-mediated microenvironment supporting NK cell development is dynamically maintained by the persistent action of membrane LT through life.

LT$\alpha^{-/-}$ mice have a defect in the early stage of NK cell development in BM

Although IL-15 plays an important role in the development of NK cells, IL-15 expression and the response of LT-deficient BM cells to IL-15 is sustained in LT$\alpha^{-/-}$ mice (13). These results raise a 2possibility that the action of membrane LT on NK cell development is independent of or upstream of the IL-15 pathway. Once NK cell progenitors pass the early checkpoint that depends on stromal cells, IL-15 alone is sufficient to drive these cells becoming NK1.1$^+$ cells. We observed that NK1.1$^+$ cells from BM preparation rapidly died in 1 wk in the absence of IL-15 (data not shown). Therefore, we set up a culture system to study the interaction between early NK cell progenitors and stromal cells. BM cells from wt mice and LT$\alpha^{-/-}$ mice were cultured in the absence of IL-15 for 14 days to eliminate IL-15-dependent NK cells (more mature NK cells). IL-15 was then added into the culture to drive the proliferation and differentiation of stromal dependent NK progenitors into NK1.1$^+$ cells. Correspondingly, NK cell progenitors from wt BM generated 2- to 4-fold more NK1.1$^+$ cells than NK cell progenitors from LT-deficient BM after 10 days in such condition (Fig. 4). Therefore, the data indicate that there is a defect at the early stage of NK cell development (before the IL-15-dependent stage) in BM of LT$\alpha^{-/-}$ mice.

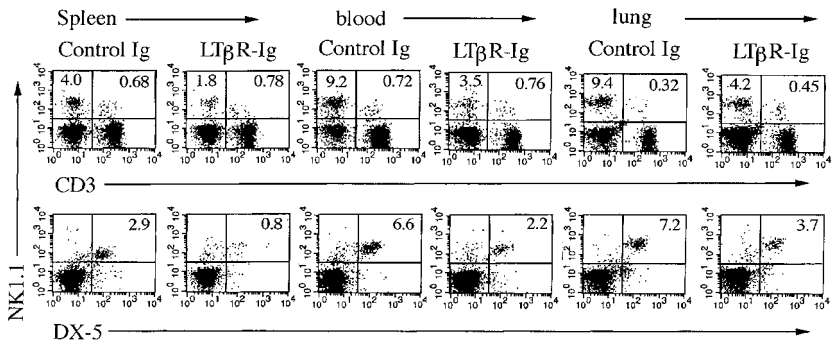

FIGURE 3. Constant signaling via LTβR is required for the maintenance of NK cells in adult mice. Adult wt mice (4–5 wk old) were treated with 100 μg of LTβR-Ig once a week for 4 wk. The percentage of NK cells was determined by flow cytometry analysis 1 wk after last treatment. Blocking of membrane LT by LTβR-Ig in adult mice prevents NK cell development.

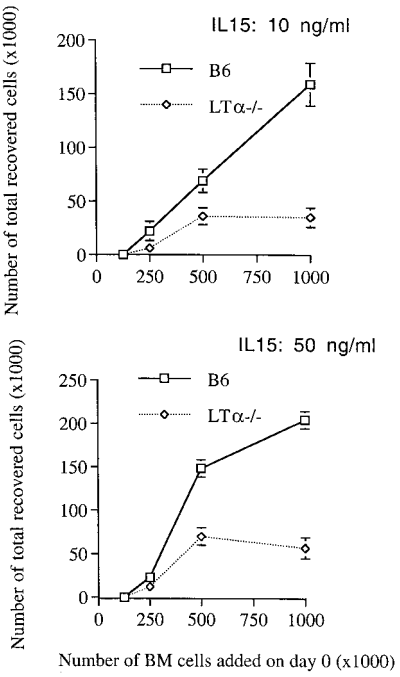

FIGURE 4. The early NK cell development is impaired in BM of LT$\alpha^{-/-}$ mice. Freshly isolated BM cells from wt and LT$\alpha^{-/-}$ mice were cultured in vitro for 10 days without exogenous cytokines to remove IL-15-dependent NK1.1$^+$ cells. IL-15 (10 ng or 50 ng/ml) was then added for another 10 days. Fewer NK1.1$^+$ cells in LT$\alpha^{-/-}$ mice were generated in this culture system. Data from one of four experiments are shown.

Normal stromal cells efficiently support LT-deficient NK cell progenitors

Because BM cells contain both stromal cells and NK cells at various developmental stages, the reduced production of NK cells in LT$\alpha^{-/-}$ mice could be the result of a defect in early NK cell progenitors, a selective survival defect in LT$\alpha^{-/-}$ BM cells or stromal cells in BM. We have already suggested based upon the BM transplantation (BMT) experiment that the progenitors are relatively normal but the stromal cells may be defective in LTβR$^{-/-}$ mice. To further study the role of LT-mediated stromal cells in NK cell development, we performed the following experiments. FL cells before 15 days of gestation contain early NK cell progenitors but no NK1.1$^+$ cells and show no NK activity (12). In addition, we found that FL cells did not respond to IL-15 alone at all unless additional stromal cells were also provided in the culture. Therefore, these stroma-dependent FL cells provide a useful source of NK progenitors to study the role of stromal cells. FL cells from wt and LT$\alpha^{-/-}$ mice were cultured with equal numbers of stromal cells, OP9 cell line. Along with cytokines, this cell line is able to support the development and differentiation of early NK cell progenitors (24). The OP9 cell line functions as stable stromal cells that support the differentiation of NK precursors. The growth of the cell line is not dependent on the action of LT. The number of NK cells growing from FL cells of LT$\alpha^{-/-}$ mice in the presence of IL-15 and the stromal cell line is similar to that of wt cells (Fig. 5*A*). It suggests that LT-deficient NK cell progenitors can develop into NK cells once stable stromal cells are provided.

Inability of stromal cells from LT$\alpha^{-/-}$ mice or LTβR$^{-/-}$ mice to efficiently support early NK cell progenitors

To directly test whether stromal cells from LT-deficient mice were able to efficiently support early NK cell development, we generated a primary stromal cell line in vitro from BM preparations of

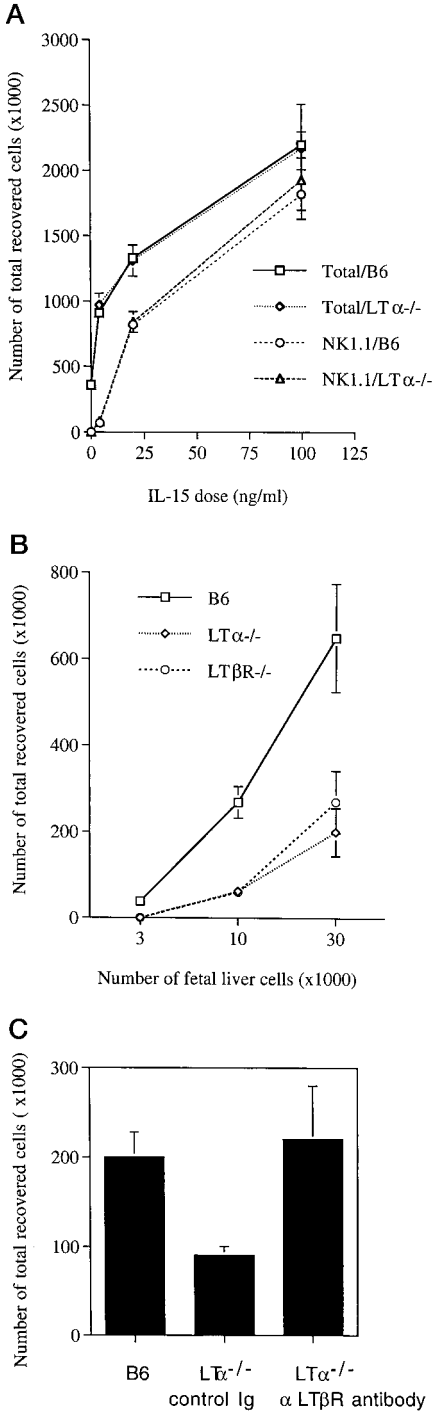

FIGURE 5. Impaired development of stromal cells but not NK cell progenitors in LT$\alpha^{-/-}$ mice. *A*, FL cells (2.5×10^4/ml) from wt and LT$\alpha^{-/-}$ mice (11–12 days of gestation) were cultured with (2.5×10^4/ml) stromal cell line, OP9, in the presence of 50 ng/ml IL-15. Fourteen days later, the number of cells in the culture was counted. FL cells from wt and LT$\alpha^{-/-}$ mice respond similarly in the presence of IL-15 and stromal cell lines. *B*, BM stromal cells from wt, LT$\alpha^{-/-}$ mice, and LTβR$^{-/-}$ mice were generated after 2 wk of culturing a BM cell preparation in the absence of IL-15 and then treating with mitomycin C. FL cells (2.5×10^4/ml) from wt mice were added in the culture along with 50 ng/ml IL-15. Fourteen days later, the number of cultured cells was counted. Stromal cells from LT$\alpha^{-/-}$ mice and LTβR$^{-/-}$ mice failed to efficiently generate NK cells. Data are shown from one of three experiments. *C*, Activation of LTβR-expressing stromal cells promotes NK cell development in vitro. BM cells from LT$\alpha^{-/-}$ mice were incubated with 10 μg/ml AH-F6 for 7 days and then 50 ng/ml IL-15 was added. Ten days later, the number of cells was counted. Addition of anti-LTβR Ab stimulated stromal cells that enhanced the growth of NK cells.

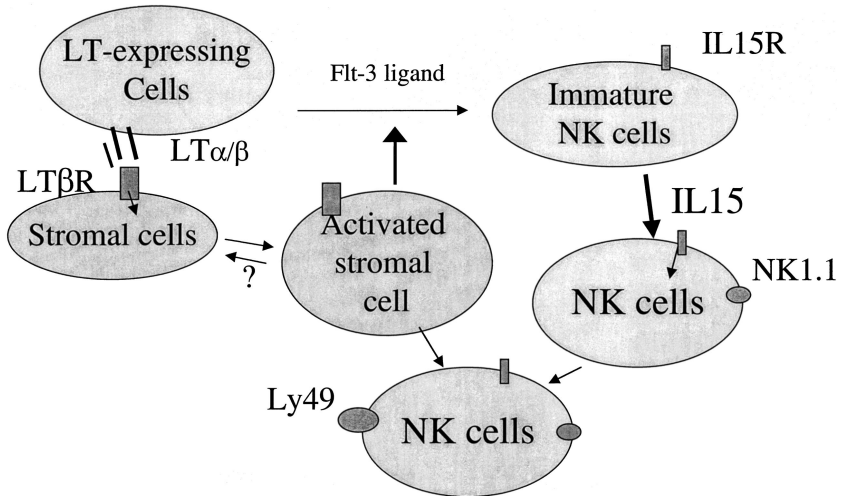

FIGURE 6. Proposed model of murine NK cell differentiation. The interaction between membrane LT and stromal cells is depicted. In the early step, LT-expressing cells, including NK progenitor cells, activate stromal cells via cell-cell contact. Activation of stromal cells via LTβR then promotes the differentiation of NK progenitors to IL-15R responding cells by both cytokines (such as Flt-3 ligand) and cell-cell contact mechanisms. IL-15 is then sufficient to drive these IL-15-responsive NK precursors into NK1.1 NK cells without the participation of stromal cells. The stromal cells are required for the expression of Ly49 on mature NK cells.

wt, LT$\alpha^{-/-}$, and LTβR$^{-/-}$ mice. BM cells were cultured for 2–3 wk without cytokines to allow stromal cells to expand. The cultured cells were then treated with mitomycin C to inhibit their further proliferation. Equal numbers of FL cells from wt mice (gestational days 11–13) were cocultured with these stromal cells as a source of early NK cell progenitors. In the presence of IL-15, NK cells were gradually generated during a 14-day culture. Interestingly, 2- to 4-fold fewer NK1.1$^+$ cells were generated in the presence of stromal cells prepared from LT$\alpha^{-/-}$ mice or LTβR$^{-/-}$ mice than in the presence of those from wt mice (Fig. 5*B*). In addition, our preliminary data showed that the number of NK cells remains lower in LT$\alpha^{-/-}$ mice treated with a large dose of ProGP that activates Flt-3 systemically increasing the number of NK cell than that in treated wt mice (data not shown). The data suggest that reduced NK cell development in LT$\alpha^{-/-}$ and LTβR$^{-/-}$ mice is attributed to the inability of their stromal cells to support NK progenitors developing into NK1.1$^+$ NK cells.

Activation of LTβR on stromal cells from LTα$^{-/-}$ mice promotes NK cell development in vitro

To investigate whether signaling via LTβR on stromal cells could restore their ability to support NK cell development, an anti-LTβR Ab (AH-F6) was used to stimulate the stromal cells from LT$\alpha^{-/-}$ mice. This Ab has been shown to be an agonist for LTβR (21). Seven days after BM cells from wt mice and LT$\alpha^{-/-}$ mice were cultured in the presence or absence of this Ab, IL-15 was added into the culture to expand NK cell progenitors. The stromal cells from LT$\alpha^{-/-}$ mice treated with anti-LTβR Ab supported NK cell progenitors more efficiently than untreated stromal cells (Fig. 5*C*). Therefore, the signaling via the LTβR on BM stromal cells is important for their ability to support NK cell progenitors.

Interestingly, introduction of LT-expressing BM cells into irradiated LT$\alpha^{-/-}$ mice failed to restore NK cell development within 6 wk (13) while LT-deficient BM cells cultured with anti-LTβR Ab could restore their ability to support NK cell development (Fig. 5*C*). Furthermore, repeated LTβR-Ig treatment inhibits NK cell development in vivo. Although the addition of anti-LTβR Ab in the culture promotes the growth of LT-deficient NK cells in vitro, the action of the Ab in vivo is not easy to evaluate due to short half-life (only 1 day). Our preliminary data showed that repeated treatment of LT$\alpha^{-/-}$ mice with anti-LTβR Ab up to 14 days failed to restore the number of NK cells in the spleen (data not shown). It is possible that the defects in BM microenvironment of LT$\alpha^{-/-}$ mice are profound and complex and that some components in BM of LT$\alpha^{-/-}$ mice could not be completely restored without a prolonged BMT. Alternatively, some elements in BM of LT$\alpha^{-/-}$ mice could not be readily restored by LT-expressing cells in vivo.

Thus, our study supports the hypothesis that the signal via LTβR on BM stromal cells after direct contact with membrane LT-expressing cells is required at several stages of NK cell development. 1) Before the IL-15 dependent stage, LT-activated stromal cells are required for the differentiation of NK cell precursor into IL-15 responsive NK progenitor cells. 2) These stromal cells are also required for the differentiation of NK1.1$^+$ NK cells into NK1.1$^+$ Ly49$^+$ NK cells. In the absence of stromal cells, a set of cytokines is sufficient to drive early NK cell precursor into NK1.1$^+$ NK cells but these cells could not further express Ly49 (5). These steps may depend on cell-cell contact. 3) Undefined factors from stromal cells may also be important for NK cell development. 4) The direct action of membrane LT likely depends on cell-cell contact. Administration of LTβR-Ig into adult mice impairs NK cell development, suggesting a constant activation of stromal cells by LT is required to maintain a dynamic BM microenvironment. Membrane LT is expressed not only on activated T, B, and NK cells but also on some NK precursors, such as the CD4$^+$, CD3$^-$ cells (16, 26). RAG-1$^{-/-}$ mice have a high percentage of NK cells in lymphoid tissues, suggesting that LT-expressing cells of the NK lineages, such as early NK cell precursors, may be sufficient to activate LTβR-expressing stromal cells, which then play a role in NK cell development (13).

Reciprocal BM transfer experiment is a useful tool to dissect the role of donor BM-derived cells vs recipient-derived stromal cells for the development of a subset of hemopoietic cells in vivo. We have previously shown that transfer of LT-deficient BM cells into irradiated wt mice led to a reduction of splenic NK cells (13). In such a system, it is difficult to assess whether the reduced number of NK cells is primarily caused by impaired NK progenitors or by impaired stromal cell development. The lack of LT-expressing cells after BMT can contribute to either defect. We have previously demonstrated that lack of LT in such reconstitution could lead to alteration of stromal microenvironment in the wt recipient, such as the loss of follicular dendritic cell (FDC) clustering (16). To further dissect that, we transferred BM cells from LTβR$^{-/-}$ mice to wt mice because these BM cells contain both potentially impaired NK cell lineage and LT-expressing cells. The ability to restore NK cell development by BM cells from LTβR$^{-/-}$ mice in this experiment suggests that there is no severe defect in NK progenitors in LTβR$^{-/-}$ mice. Furthermore, we have demonstrated that progenitors from LT$\alpha^{-/-}$ mice develop into mature NK cells in the presence of mature stromal cells. In contrast, lack of LTβR

in the stromal cells, even with reconstitution of normal progenitors, prevents the efficient restoration of NK cell development. Finally, our series of in vitro experiments (Figs. 4 and 5) demonstrated that there is a defect in BM stromal cells from LT$\alpha^{-/-}$ mice or LTβR$^{-/-}$ mice. Together, the data suggest that the defect of NK cell development in either LTβR$^{-/-}$ mice or LT$\alpha^{-/-}$ mice is primarily attributed to the impaired stromal cells.

IL-15 is an essential cytokine for NK cell development in vivo and in vitro (5, 9). Recent studies showed that the IL-15 gene expression in radiation-resistant cells (stromal cells) was regulated by IFN-regulatory factor-1 (IRF-1), a transcription factor. IL-15 production by various BM cells, including stromal cells, was associated with the expression of IRF-1. Lack of IRF-1 impaired the ability of the BM microenvironment to support NK cell development in an IL-15-dependent manner (11, 27). However, we found that BM cells from LT$\alpha^{-/-}$ mice produced normal amounts of IL-15 and that LT-deficient NK cell progenitors responded to IL-15 (13). Therefore, the action of LT in NK development is likely independent from the IL-15/IRF-1 pathway or is upstream of the action of IL-15. We noticed that only a few hemopoietic cells that were in close contact with stromal cells remained alive in the culture deprived of exogenous IL-15 for 1 wk. It seems that the development of early NK cell progenitors does rely on cell-cell contact possibly via membrane ligands, such as membrane LT, with their receptors. Such contact may activate stromal cells to produce various stromal cell-derived cytokines and express other membrane molecules, which then promote NK cell progenitors to express IL-15R. The nature of these molecules is unknown.

Therefore, we propose the following model (Fig. 6): signal via LTβR on stromal cells after contact with cells expressing membrane LT is required for the development or activation of stromal cells, which in turn promote the expression of IL-15R on NK precursors. IL-15-responsive NK cells will further differentiate into NK1.1$^+$ NK cells in response to IL-15 from various sources and stromal cells are not essential at this latter stage. However, stromal cells are also required for the further differentiation of NK1.1$^+$ NK cells into NK1.1$^+$ Ly49$^+$ cells. The development and activation of stromal cells are likely to play a critical role in the development and function of other lymphoid cells. Increasing evidence indicates that membrane LT-dependent signaling by BM-derived cells to the supporting cells (such as stromal cells) in the microenvironment may be necessary for their maturation or activation (16, 18, 19). These mature stromal cells then support the development and function of lymphoid cells. For example, LT on B cells is required for the development of FDCs that in turn support the formation of the germinal center (16, 28). Lack of LT in wt after BMT using LT-deficient BM or after LTβR-Ig blockage could lead to impaired development of normal stromal cells in BM for NK cell development or in peripheral lymphoid tissues for FDC development. Further investigation of such reciprocal cellular collaborations resulting from direct ligand-receptor interactions will improve our understanding of the molecular mechanisms by which stromal cells support lymphoid cell development and allow us to design better medications for BM reconstitution and regeneration of immune system.

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