Expansion of Functional NK Cells in Multiple Tissue Compartments of Mice Treated with Flt3-Ligand: Implications for Anti-Cancer and Anti-Viral Therapy[1]

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The generation and activity of NK cells appear to be regulated by a particular set of cytokines. We examined the in vivo effects of recombinant human Flt3 ligand (Flt3-L), a recently cloned potent hemopoietic cytokine, on NK cell development in mice. Daily i.p. administration of Flt3-L consistently induced striking increases in both the absolute number and the total cytotoxic activity of mature nonactivated NK cells within various tissues. Dose- and time-dependent increases were observed in the bone marrow (~2- and ~11-fold, respectively), thymus (~2.8- and ~2.0-fold), blood (~11- and ~15-fold), spleen (~10- and ~9-fold), and liver (~15- and ~39-fold). In addition, IL-2 induced a rapid increase in NK activity, NK cell proliferative responses, generation of lymphokine-activated killer activity, and development of activated adherent NK cells, which were all significantly increased by Flt3-L treatment. Thus, in addition to its recently reported capacity to stimulate dendritic cell production, Flt3-L has a prominent biologic role in NK cell generation in vivo. This is probably a result of selectively induced expansion of NK cell progenitors (pro-NK cells), because Flt3-L stimulates in vitro proliferation of pro-NK cells without affecting the cytotoxicity of mature NK cells. The results also indicate that either alone or in combination with a potent activator of NK cells, such as IL-2, Flt3-L could be used to markedly augment the number and activity of NK cells, especially in the liver. Flt3-L appears to have considerable potential for therapy of both cancer and viral infection. *The Journal of Immunology,* **1998, 161: 2817–2824.**

NK cells represent a distinct population of lymphocytes in terms of both phenotype and function (1). They have a large granular lymphocyte morphology and express characteristic cell surface receptors, such as the NK cell receptor protein-1 (termed NK1.1 in mice), the low affinity receptor for the Fc portion of IgG (FcγRIII; CD16), and the killer cell inhibitory receptors (1–3). The absence of both rearrangement of the TCR and expression of cell surface markers of T cells, B cells, and monocytes/macrophages represents an additional important phenotypic characteristic of NK cells (1). NK cells exhibit spontaneous non-MHC-restricted cytotoxic activity against virally infected and tumor cells, and mediate resistance to viral infections and cancer development in vivo (1, 4, 5). Thus, NK cells represent major effector cells of innate immunity. In addition, NK cells possess a variety of other functions, including the ability to secrete cytokines and to regulate adaptive immune response and hemopoiesis (1, 6–8).

NK cells differentiate from $CD34^+$ primitive hemopoietic progenitor cells under the influence of various cytokines produced by bone marrow stromal cells and/or immune cells (such as c-*kit* ligand, IL-2, and IL-15) (9–12). The intact bone marrow is necessary for NK cell generation (13). Following their differentiation (most likely in the bone marrow), NK cells become distributed within a variety of lymphoid and nonlymphoid tissues, including blood, spleen, liver, lungs, intestines, and decidua (1, 4, 14–16). During postnatal development, the number of NK cells increases gradually at strategically important positions, such as in the circulation, within small vessels (attached to endothelial cells), and/or in the surrounding tissue (4, 17, 18). In addition, NK cells have been found in significant numbers in tumors, where they may exert antitumor activity (4). Upon systemic treatment with various biologic response modifiers, particularly IL-2, the number of activated NK cells and their antiviral and antimetastatic activities have been found to increase dramatically in various tissues (4, 5, 19–21). These findings have led to the development of promising therapeutic strategies based on the activation and expansion of NK cells (22). However, only limited success has been achieved in cancer treatment, restricted mostly to transient remission and prolongation of survival in about one-third of treated patients with metastatic melanoma or renal cell carcinoma (23). A significant finding is that the total number and activity of NK cells may be decreased substantially in virally infected and/or cancer patients (4, 5). Under these circumstances, immunotherapy based on the activation of NK cells might not be effective. Therefore, correction of the immunologic defect by augmenting the number and the function of tissue NK cells, followed by their activation and further expansion, might provide a novel and more efficient therapeutic approach. Development of such an approach, however, is dependent on the identification of cytokines able to efficiently induce the expansion and differentiation of functionally mature NK cells in vivo.

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Flt3 ligand (Flt3-L)[4] is a recently cloned cytokine that plays a role in normal hemopoiesis and mobilization of hemopoietic stem cells (24, 25). An additional important activity of Flt3-L is its capacity to induce striking expansion of functional dendritic cells (DC) in lymphoid and nonlymphoid tissues (26–28). Limited results to date indicate that Flt3-L induces a slight increase in the number of $NK1.1^+$ cells in the bone marrow and spleen of mice (25). This suggests that Flt3-L may induce the expansion and differentiation not only of DC, but also of NK cells and/or $CD3^+NK1.1^+$ (atypical) T cells. In the present study we addressed this possibility by examining the number and function of NK cells in bone marrow, thymus, blood, spleen, and liver of mice treated with Flt3-L. We show that Flt3-L induces a dramatic increase in the number and function of $CD3^-NK1.1^+$ cells, the activity of which can be further augmented by IL-2.

Materials and Methods

Animals

C57BL/6 ($H2^b$, $I\text{-}A^b$) mice, 6 to 12 wk old, were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in the central specific pathogen-free facility of the University of Pittsburgh Medical Center (Pittsburgh, PA).

Abs and cytokines

Phycoerythrin(PE)-conjugatedanti-NK1.1,FITC-conjugated anti-CD3,anti-CD11c, anti-CD80 (B7-1), anti-CD86 (B7-2), anti-MHC class II, anti-CD45, anti-B220, anti-CD11b, and corresponding isotype-matched nonreactive control mAbs were obtained from PharMingen (San Diego, CA). Chinese hamster ovary cell-derived human Flt3-L was provided by Immunex Research and Development (Seattle, WA). Human rIL-2 (18×10^6 IU/mg) was a gift from Chiron-Cetus (Emeryville, CA).

Tumor cell lines

An NK cell-susceptible, Moloney virus-induced YAC-1 lymphoma of A/Sn mouse origin was used as a source of tumor target cells for testing NK activity. NK cell-resistant P815 mastocytoma cells of DBA/2 mouse origin were used as the target cells for detection of LAK activity. The cell lines were grown in RPMI 1640 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated FCS, all purchased from Life Technologies (Grand Island, NY).

Treatment of mice

In most experiments, animals (two per group) received once daily i.p. injections of 10 μg of Flt3-L in 200 μl of sterile HBSS. In addition, in experiments performed to test the dose dependence of Flt3-L treatment, 2, 4, 8, or 16 μg of Flt3-L was injected. Control animals received once daily i.p. injections of 200 μl of sterile HBSS. Mice were sacrificed 24 h after the last injection. The regimen of injections is indicated for each experiment.

Isolation of lymphoid cells

Animals were anesthetized with Metofane (Pitman-Moore, Mundelein, IL). Blood was obtained by cardiac puncture using a heparinized syringe. Blood mononuclear leukocytes were separated by centrifugation on Ficoll-Hypaque gradients (density = 1.077) at $300 \times g$ for 20 min at room temperature. Blood mononuclear leukocytes were collected from the gradient interface. The perfusion of whole animals was performed in situ via the left ventricle with 25 ml of HBSS followed by 5 ml of 1 mg/ml collagenase (grade IV, Sigma, St. Louis, MO). Spleens and thymuses were removed under sterile conditions, and single cell suspensions were prepared in RPMI 1640 medium as described. Bone marrow cells were obtained from two tibias and femurs per animal by flushing the bone channel with RPMI 1640 using a syringe. Liver nonparenchymal cells (NPCs) were isolated by digestion of livers with collagenase (1 mg/ml) followed by Percoll gradient centrifugation as previously described (29). The mononuclear leukocytes were treated for 10 s with ice-cold distilled water to lyse erythrocytes, washed three times in RPMI 1640, then resuspended in RPMI 1640 containing 10% FCS. Their number and viability were determined after mixing with 0.4% (w/v) trypan blue solution in PBS and counting in a hemocytometer.

Proliferation assays

[^{3}H]Thymidine incorporation assays were performed as described previously (30). Briefly, lymphoid cells were plated in triplicate at a concentration of 2×10^4 cells/well in 0.2 ml RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine (Life Technologies), 5×10^{-5} M 2-ME, and antibiotics (streptomycin plus penicillin; hereafter referred to as complete tissue culture medium (TCM)) in U-bottom wells of 96-well plates (Corning Costar, Cambridge, MA). IL-2 was added at final concentrations ranging from 0.022 to 22 nM (6–6000 IU). Control wells contained cells in medium alone. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 5 days. On day 4 of the culture, the cells were pulsed with 1 μCi of [^{3}H]thymidine/well, and on day 5 they were harvested using a semiautomatic cell harvester (Skatron Instruments, Sterling, VA). The radioactivity of incorporated [^{3}H]thymidine in the cell DNA was determined in a scintillation counter.

IL-2-induced generation of LAK activity

Lymphoid cells were cultured in horizontally positioned T25 flasks (Corning Costar), at an optimal density of 2×10^6 viable cells/ml (5 ml/flask) in TCM containing 22 nM rIL-2 at 37°C in a humidified atmosphere of 5% CO_2 in air for 5 days.

Generation of adherent NK (A-NK) cells

A-NK cells were generated using a modification of a previously described technique (31). Briefly, 10×10^6 lymphoid cells were incubated for 2 h in 5 ml of TCM in horizontally positioned T25 flasks at 37°C to eliminate plastic adherent macrophages and B cells. The suspension of nonadherent cells was then transferred into new T25 flasks, supplemented with 22 nM IL-2, and cultured for 48 h at 37°C in humidified atmosphere of 5% CO_2 in air. Thereafter, the nonadherent cells were decanted, and the A-NK cells were washed three times with warm (37°C) TCM to completely eliminate nonadherent cells. The A-NK cells were supplemented with cell culture-conditioned medium (from 48-h IL-2-induced splenocytes), counted per unit surface area (grid) under an inverted microscope (magnification, ×200), and cultured for an additional 3 days. Expansion of A-NK cells was determined on the basis of the number of A-NK cells at the beginning and that at the end of culture.

Immunofluorescence staining and flow cytometry

Immunofluorescent staining of cell surface Ags was performed as described previously (30). Briefly, lymphoid cells (0.2×10^6/0.2 ml) were suspended in PBS containing 0.1% (w/v) sodium azide and 1% (v/v) FCS, then two color stained by 30-min incubation in the presence of PE- and/or FITC-conjugated mAbs. Flow cytometric analysis was performed using a Coulter Elite flow cytometer (Coulter, Hialeah, FL). Appropriate isotype-matched nonreactive mAbs were included as controls in each experiment. Calculation of the absolute number of phenotypically distinct leukocytes within tissues or organs was based on the absolute cell count per 1 ml of blood, two tibias and femurs, or whole spleen or liver in the pool of cells obtained from two animals per group.

Cell sorting

To obtain highly purified populations of spleen DC, splenocytes obtained from Flt3-L-treated mice were double stained with FITC-conjugated anti-CD11c and PE-conjugated anti-NK1.1 mAbs (10 μg/ml/10^7 splenocytes), then sorted in a Coulter Elite flow cytometer. Bright, single-stained cells with anti-CD11c (DC) were sorted (>95% purity) from the rest of the splenocytes, washed, and used in cytotoxicity assays.

Cytotoxicity assays

NK activity was measured against YAC-1 tumor cell targets, and LAK activity was measured against P815 tumor cell targets using a standard 4-h ^{51}Cr release cytotoxicity assay as previously described (30). Briefly, the target cells were labeled with 100 μCi of [^{51}Cr]sodium chromate (sp. act., 5 μCi/mM; DuPont-New England Nuclear, Boston, MA) at 37°C for 1 h, washed, and incubated with effector cells at four different E:T cell ratios in U-bottom 96-well plates (Corning Costar). Spontaneous release and maximum release were determined by incubating target cells without effectors in medium alone or in 5% Triton X-100, respectively. The spontaneous release was always <10%. The assay was performed in triplicate. Radioactivity was counted in a gamma counter, and the percentage of specific lysis was determined according to the formula: % specific lysis = [(mean

[4] Abbreviations used in this paper: Flt3-L, Flt3 ligand; DC, dendritic cell; PE, phycoerythrin; LAK, lymphokine activated killer; NPC, nonparenchymal cell; TCM, tissue culture medium; A-NK, activated adherent natural killer cells; LU, lytic units.

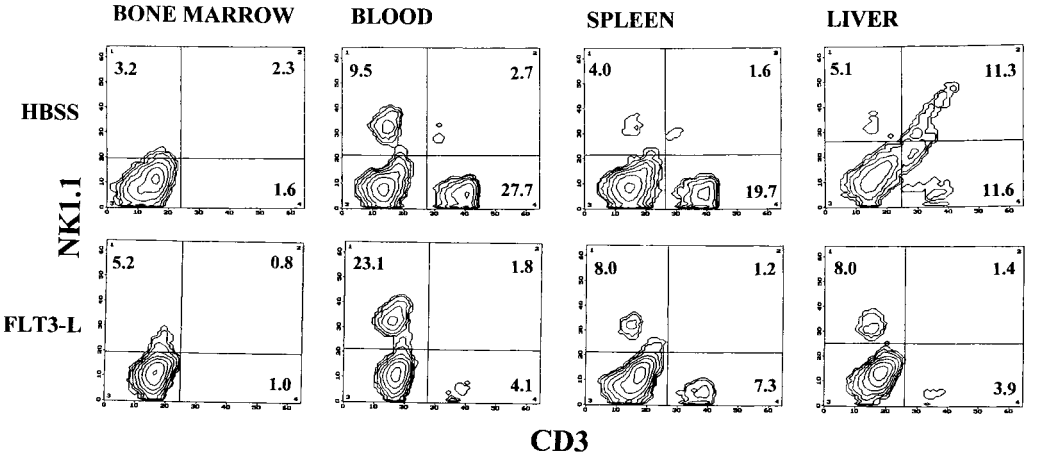

FIGURE 1. Flt3-L induces in vivo increases in the proportion of $CD3^-NK1.1^+$ NK cells in various tissues. Mononuclear leukocytes were isolated from various tissues of C57BL/6 mice treated once daily for 10 days with HBSS or Flt3-L (10 μg/mouse/day). The cells were stained simultaneously with FITC-conjugated anti-CD3 and PE-conjugated anti-NK1.1 mAbs and analyzed by flow cytometry. Two-color contour plots and percentages of positive cells are presented. The results are from one representative experiment of three performed, with two animals per treatment group.

experimental cpm release − mean spontaneous cpm release)/(mean maximal cpm release − mean spontaneous cpm release)] × 100. Lytic units $(LU)_{20}$ per 10^7 effector cells were calculated using a computer program (30). One lytic unit was defined as the number of effector cells needed to lyse 20% of 5×10^3 target cells. LU per 10^7 NK1.1 cells were calculated on the basis of the following formula: $[(LU_{20}/10^7$ lymphocytes)/% $CD3^-NK1.1^+$ cells)] × 100.

The total LU of NK cells per tissue or organ were calculated based on the formula: (total number of $CD3^-NK1.1^+$ cells/10) × $LU_{20}/10^7$ $CD3^-NK1.1^+$ cells.

Results

Flt3-L increases the number of functionally mature NK cells

To test the possibility that Flt3-L induces NK cells, mice were injected once daily for 10 days with 10 μg of Flt3-L, a regimen described previously for optimal induction of DC (26). At the end of this treatment, various tissue compartments were analyzed by flow cytometry and cytotoxicity assays for the presence and cytotoxic activity of NK cells. Flow cytometric analyses demonstrated that Flt3-L treatment, compared with HBSS treatment, induced an increase in the proportion of $CD3^-NK1.1^+$ cells in the bone marrow, thymus, blood, spleen, and liver: 1.6-, 2.8-, 2.4-, 2.0-, and 1.6-fold, respectively (Fig. 1). However, Flt3-L treatment more dramatically increased the absolute number of NK cells calculated per bone marrow of two tibias and femurs (1.8-fold), whole thymus (2.0-fold), 1 ml of blood (10.7-fold), whole spleen (6.8-fold), and whole liver (14.9-fold; means of three experiments, two animals per group). In contrast, the relative number of $CD3^+$ T cells decreased (Fig. 1), while their absolute number did not significantly change in all tested tissues (data not shown). That was mostly due to a striking and selective increase in the number of both DC (data not shown) (25–28) and NK cells in these sites. NK cells from Flt3-L-treated animals were able to kill YAC-1 targets efficiently (Table I), but not P815 tumor cell targets (data not shown). Flt3-L treatment significantly increased NK cytotoxic activity in all tested tissues compared with that in control animals. The total LUs of $CD3^-NK1.1^+$ cells per bone marrow of two tibias and femurs, 1 ml of blood, whole spleen, and whole liver were highly increased in Flt3-L-treated animals compared with those in sham-injected controls (2.2-, 15.1-, 5.5-, and 18.4-fold,

Table I. *NK cell killing capacity of mononuclear leukocytes in various mouse tissues following treatment with Flt3-L*[a]

		% Cytotoxicity						
Tissue	Treatment	200:1	100:1	50:1	75:1	$LU_{20}/10^7$	$LU_{20}/10^7$ NK	Total LU
Bone marrow	HBS	7.4	4.9	4.2	1.9	3.6	101.4	21.3
	Flt3-L	9.8	7.2	5.5	3.4	5.0	129.0	46.8
Spleen	HBSS	22.6	14.8	9.3	6.6	13.0	420.3	202.6
	Flt3-L	35.6	27.3	20.3	10.5	31.7	1058.0	1112.5
		40:1	20:1	10:1	5:1			
Blood	HBSS	12.4	3.3	0.4	NT	11.3	119.6	1.1
	Flt3-L	36.2	19.9	3.1	NT	50.5	218.4	33.4
Liver	HBSS	13.4	7.9	5.1	2.1	25.6	192.3	26.3
	Flt3-L	16.7	10.5	5.3	3.1	67.7	406.3	485.1

[a] C57BL/6 mice were treated as described in Figure 1. Mononuclear leukocytes were isolated from different tissues and tested using cytotoxicity assays against ^{51}Cr-labeled YAC-1 targets. The cytotoxicity was assessed at three to four different E:T ratios. Data are from one representative experiment of three performed. % Cytotoxicity are means of triplicates. SDs were consistently less than 10% of means. NT, not tested.

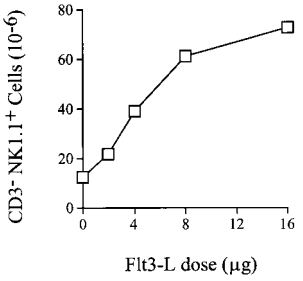

FIGURE 2. Dose dependence of Flt3-L-induced increase in the number of NK cells. C57BL/6 mice were treated once daily for 10 days with the indicated doses of FLT3-L. After this treatment, the animals were sacrificed, and their splenocytes were isolated and analyzed for the presence and absolute number of $CD3^-NK1.1^+$ per organ, using flow cytometry and absolute number of splenocytes. The absolute numbers of $CD3^-NK1.1^+$ cells per spleen are shown. The results are from one experiment, with two animals per treatment group.

respectively). Therefore, using an established protocol that is optimal for the induction of DC, we demonstrated that Flt3-L also significantly increased the number of functionally mature, nonactivated NK cells in mouse lymphoid tissues, blood, and liver.

Dose dependence of Flt3-L-induced NK-cell generation

To confirm the above findings and to define the optimal dose of Flt3-L for induction of NK cells, we then examined the number of NK cells and NK cytotoxic activity in the spleens of mice treated for 10 days with graded doses of Flt3-L. It was found (Figs. 2 and 3) that Flt3-L augmented the number of $CD3^-NK1.1^+$ cells as well as the total LU of $CD3^-NK1.1^+$ cells per organ/tissue in a dose-dependent manner. The optimal dose of Flt3-L for NK cell induction ranged between 8 and 16 μg/day, similar to that for DC induction (26).

Kinetics of Flt3-L-induced generation of NK cells

To determine the time course of Flt3-L-mediated induction of NK cells in various tissues, we examined the cellularity, number of $CD3^-NK1.1^+$ cells, and NK cytotoxicity in bone marrow, spleen, and liver following different durations of Flt3-L treatment. Changes in the number of $CD3^+NK1.1^+$ T cells, $CD3^+NK1.1^-$ T cells, and $CD11c^+$ DC were also evaluated in parallel.

Changes in the number of lymphoid cells. The absolute number of lymphoid cells increased dramatically in the spleen (3.4-fold)

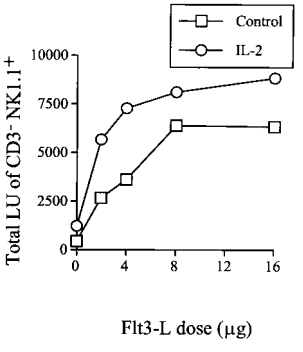

FIGURE 3. Dose dependence of Flt3-L-induced increase in NK activity. Splenocytes were isolated from mice treated as described in Figure 2, and their NK activities were analyzed against YAC-1 targets using 4-h ^{51}Cr release assays. The assays were performed in the absence or the presence of 22 nM IL-2. Data are the total LU of $CD3^-NK1.1^+$ cells per spleen. The results are from one experiment, with two animals per treatment group.

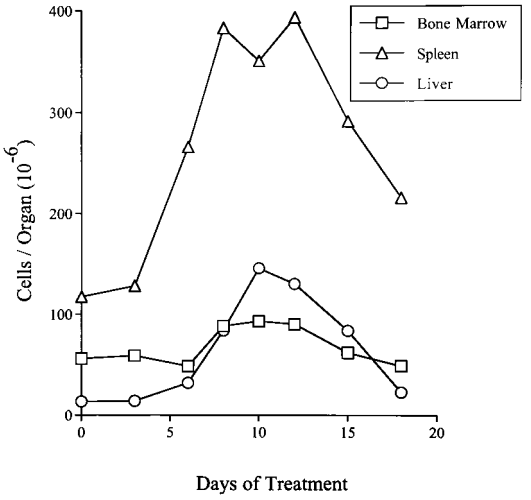

FIGURE 4. Kinetics of the Flt3-L-induced increase in cellularity of various tissues. C57BL/6 mice were treated once daily with Flt3-L (10 μg/animal/day) for the indicated period. Following this treatment, the mice were sacrificed, and bone marrow, spleen, and liver mononuclear leukocytes were isolated and counted. The absolute numbers of mononuclear leukocytes per organ $\times$ 10^6 are presented. The results are from one representative experiment of two performed, with two animals per treatment group.

and liver (10.9-fold) and moderately in the bone marrow (1.7-fold; Fig. 4). In the spleen and liver, the increase was detected on day 6 of Flt3-L treatment. The peak of the response in the bone marrow and spleen was reached on day 8 and in the liver on day 10. Following this point, a decrease in the response was detected from day 12 in the liver and from day 15 in the bone marrow and spleen, which was continued in each tissue to day 18, when values returned to control levels.

Changes in the number of NK cells, T cells, and DC. The absolute number of $CD3^-NK1.1^+$ NK cells was increased strikingly in the spleen and liver and was moderately augmented in the bone marrow of Flt3-L-treated mice (Fig. 5). However, a slight decrease in the number of $CD3^-NK1.1^+$ NK cells compared with that in normal controls was observed in the bone marrow from days 3 to 6. In contrast, the total number of NK cells started to rise simultaneously in both the spleen and the liver (Fig. 5). These findings indicate that an early increase in mobilization in and movement of $CD3^-NK1.1^+$ NK cells from the bone marrow to the other tissues occurs during Flt3-L treatment. The number of $CD3^-NK1.1^+$ cells increased in the bone marrow from day 6 and reached its peak on day 8 (2-fold increase). In contrast, the increase in number of NK cells was much more striking in the liver and spleen and peaked, respectively, there on day 10 (15-fold increase) and day 12 (10-fold increase). After these time points, the number of NK cells remained elevated in the bone marrow, but decreased gradually in the spleen and liver by day 18. The total number of $CD11c^+$ DC (Fig. 5) exhibited similar changes in the bone marrow and liver, but in the spleen, they peaked 2 days earlier than that of NK cells. In contrast to these findings, the number of $CD3^+NK1.1^+$ T cells did not change significantly in the bone marrow, was increased slightly in the liver on days 6 and 12, and was continuously and highly increased (6-fold) in the spleen from days 6 through 18 (Fig. 5). In addition, a significant increase in $CD3^+NK1.1^-$ T cells was observed only in the spleen from days 3 to 12.

Changes in NK cell cytotoxicity. During the course of Flt3-L treatment, NK activity was strikingly increased in all three tissues tested, but strictly followed the phenotypic changes only in the

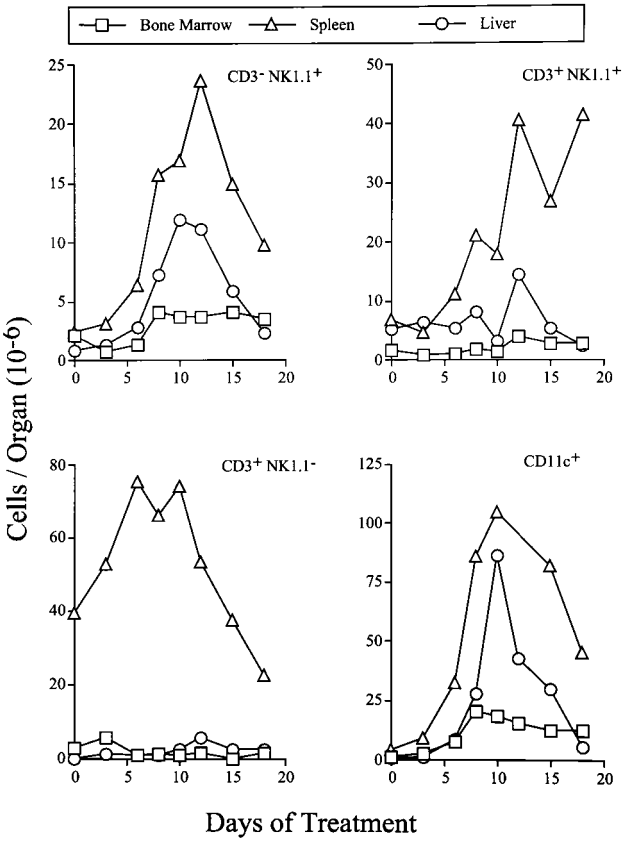

FIGURE 5. Kinetics of Flt3-L-induced changes in the number of NK cells, T cells, and DC in various tissues. C57BL/6 mice were treated, and mononuclear leukocytes were isolated from tissues as described in Figure 4. The cells were double stained with FITC-conjugated anti-CD3 and PE-conjugated anti-NK1.1 mAbs, single stained with FITC-conjugated anti-CD11c, and analyzed using flow cytometry. Based on the absolute number of mononuclear leukocytes per organ and percentages of marker positive cells, the absolute numbers ($\times 10^6$) of $CD3^-NK1.1^+$ (NK) cells, $CD3^+NK1.1^+$ (T) cells, $CD^+NK1.1^-$ (T) cells, and $CD11c^+$ (DC) cells were calculated and presented in the figure. The data are from one representative experiment of two performed, with two animals per treatment group.

liver (Figs. 5 and 6). Thus, in the bone marrow, NK activity increased from day 3 and peaked in three waves, with the first, sharp, and most prominent peak on day 6 (7-fold increase), followed by second and third smaller peaks on days 12 and 18, respectively. In the spleen, a biphasic response, with two sharp and prominent peaks on day 8 (8-fold increase) and day 15 (9-fold increase), was demonstrated. However, the two peaks in the spleen were delayed by 2 and 4 days, respectively, compared with the first two peaks in the bone marrow. In the liver, a very large increase in total NK activity (39-fold) developed from days 6 to 12, followed by a gradual decrease in cytotoxicity up to day 18 of Flt3-L treatment (Figs. 5 and 6). The peak of NK activity was delayed in the liver compared with the first two peaks of NK activity in the bone marrow and spleen. Presentation of these data as LU_{20} per 10^7 $CD3^-NK1.1^+$ cells produced curves similar to those shown in Figure 6, but the increase in NK activity after treatment with Flt3-L was approximately 15-fold in bone marrow (day 6 of treatment), approximately 2-fold in spleen (days 8 and 15 of treatment), and approximately 2-fold in liver (day 10 of treatment), indicating that the $CD3^-NK1.1^+$ population of lymphocytes contained more cytotoxic cells in all tested tissues, particularly in bone marrow (data not shown). The observed early and sharp increase in NK activity in bone marrow and the delays in the increase in NK activity in the spleen and liver compared with that in the bone marrow, and in the liver compared with that in the spleen, are consistent with generation of NK cells in the bone marrow and their sequential movement into the spleen and then to the liver. The absence of correlation between $CD3^-NK1.1^+$ NK cells and NK activity in the bone marrow and the partial correlation between these parameters in the spleen may indicate that Flt3-L induces generation and differentiation of NK cells in these tissues from marker-negative noncytotoxic precursor cells by sequential development of NK activity and cell surface NK1.1 expression. It is also possible that Flt3-L induced an increase in cytotoxic activity in mature NK cells, and that this created the discrepancy between the number of $NK1.1^+CD3^-$ cells and NK activity. Alternatively, some other effector cells of a non-NK cell lineage could acquire cytotoxic activity against YAC-1 target cells in the course of Flt3-L treatment. The second and third possibilities appear unlikely, because the discrepancy was selective for bone marrow and partial for spleen, but was absent in liver, and incubation of normal splenocytes with Flt3-L did not increase NK activity (data not shown). The first possibility is considered the most likely, because it has been demonstrated in a defined, in vitro, long term mouse bone marrow culture system that during generation of NK cells from NK cell precursors, NK activity develops before expression of NK1.1 (10). The finding of a strict correlation between $CD3^-NK1.1^+$ NK cells and NK activity in the liver confirms that liver is populated predominantly by functionally mature NK cells that express NK1.1.

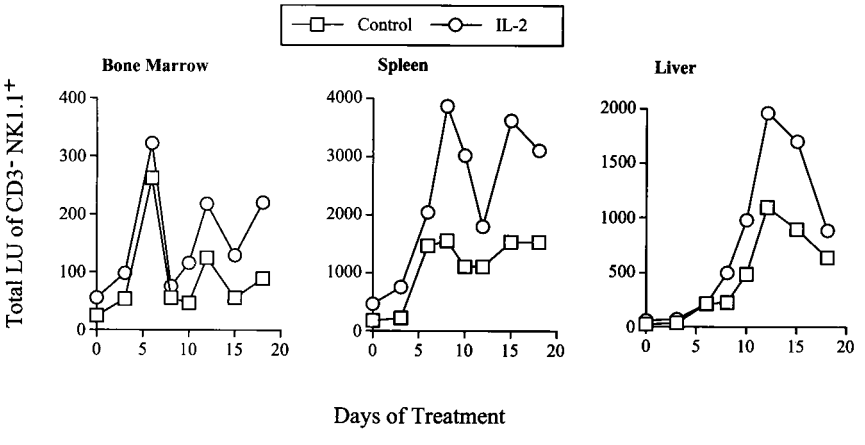

FIGURE 6. Kinetics of Flt3-L-induced changes in NK activity. C57BL/6 mice were treated, and mononuclear leukocytes were isolated from tissues as described in Figures 4 and 5. The NK activity of the isolated leukocytes was assessed against YAC-1 targets using 4-h ^{51}Cr release assays. The assays were performed in the absence or the presence of 22 nM IL-2. Data are total LU of $CD3^-NK1.1^+$ cells per organ. The results are from one representative experiment of two performed, with two animals per treatment group.

Responsiveness of NK cells to IL-2 after in vivo Flt3-L treatment

We next determined whether newly generated NK cells that arise during Flt3-L treatment could be induced with NK cell-activating cytokines to proliferate and augment their activities. The induction of increased NK activity, proliferation, generation of LAK activity, and expansion of A-NK cells was tested in IL-2-stimulated cell cultures prepared from bone marrow, spleen, or liver lymphoid cells of Flt3-L-treated mice.

Induction of increase in NK activity. The rapid IL-2-induced increase in NK activity was elevated in the splenocyte and liver NPC cultures from Flt3-L-treated mice, particularly at the peaks of the Flt3-L-induced increase in NK activity, compared with that in controls (Figs. 3 and 6). In contrast, the IL-2-induced increase in NK activity in the bone marrow-derived cells was not apparent until day 8 of Flt3-L treatment and was similar to that in control animals. After that time point, IL-2-induced enhancement of NK activity became notable in the bone marrow of Flt3-L-treated mice, but was less prominent than that in the spleen or liver.

Induction of NK cell proliferation. IL-2-induced lymphocyte proliferation was markedly increased in 5-day cultures of liver-derived NPC obtained from Flt3-L-treated mice after 10 days of treatment compared with that in control animals. Similar results were obtained with splenocytes, but the effect was not as prominent as with the liver NPC. In contrast, bone marrow cells from Flt3-L-treated mice responded less well to IL-2 than those from control animals (data not shown).

Induction of LAK activity. Similar results were obtained in the assays measuring induction of LAK activity. Thus, in vitro generation of LAK activity by 5-day IL-2 stimulation of splenocytes or liver NPC was increased substantially by in vivo Flt3-L treatment. This increase was more pronounced with liver NPCs (10.1-fold increase in P815 killing) than with splenocytes (2.2-fold increase in P815 killing). In contrast, bone marrow cells from Flt3-L-treated mice generated less LAK activity than those from control animals (data not shown). These findings indicate that there are important changes in IL-2-inducible NK cell functions in all three lymphoid tissues during the course of Flt3-L treatment. Thus, bone marrow cells became less responsive to IL-2 despite the increase in the proportion of $CD3^-NK1.1^+$ cells. In contrast, spleen and, in particular, liver NK cells from Flt3-L-treated mice became more responsive to IL-2, and the increased activity was correlated with the increase in the proportion of $CD3^-NK1.1^+$ cells found in these tissues. These findings are possibly a consequence of differential changes in size and maturation stage of NK cell populations in different mouse tissues during Flt3-L treatment.

Generation of A-NK cells. In addition, IL-2 stimulation of splenocytes from Flt3-L-treated mice, compared with those from controls, induced development of a 4.8-fold larger number of A-NK cells (from days 0–2 of culture) as well as an 8.3-fold increase in their expansion (from days 2–5 of culture). A-NK cells generated from splenocytes of either control or Flt3-L-treated mice were significantly enriched (62 and 68% $CD3^-NK1.1^+$ cells, respectively). These findings demonstrate that in addition to its potent ability to induce generation and expansion of mature NK cells in mice, Flt3-L enhances their ability to respond to IL-2 by proliferation and generation of effector activities.

Discussion

Several cytokines with potent activating ability for murine and human NK cells are now available. These include IFNs, IL-2, IL-12, and IL-15 (32–34). IL-2 and IL-15 have similar activities due to the shared use of common receptor subunits (β- and γ-chains of IL-2R) (34). IL-2 and IL-15 not only induce activation and expansion of mature NK cells, but also stimulate their differentiation from primitive hemopoietic progenitors (9–12, 35). However, this differentiation is followed by NK cell activation and generation of LAK activity (9, 11, 35). To date, no information is available for cytokines that can selectively induce in vivo the generation of functionally mature NK cells without induction of their activation. Here, we demonstrate that in various mouse tissues, Flt3-L induces a striking increase in the number of $CD3^-NK1.1^+$ lymphocytes with cytotoxic activity of mature nonactivated NK cells.

Recent in vitro studies have demonstrated that Flt3-L can induce the expansion of fetal liver, bone marrow, or thymic NK cell progenitors and can costimulate (with either IL-15 alone or a combination of IL-6, IL-7, and IL-15) the in vitro generation of NK cells from their progenitors (36–38). On the other hand, it has been shown that NK cell lines lack the expression of Flt3 (39), and we determined that coculture of mouse splenocytes (containing mature NK cells) with Flt3-L had no effect on NK cell function (data not shown). Therefore, it appears that pro-NK cells, but not mature NK cells, express Flt3 and are able to respond to Flt3-L. In this context, our data may indicate that Flt3-L is also a potent in vivo growth factor for pro-NK cells that increases the frequency of pro-NK cells and thus augments the level of NK cell generation. Other cytokines, either preformed or induced by Flt3-L, might be responsible for the increased generation of NK cells from the enlarged pool of pro-NK cells. The precise mechanisms underlying this activity as well as the stage of NK cell differentiation where this expansion occurs remain to be determined.

In the present study, NK cells were functionally defined as effector cells that killed NK sensitive (YAC-1) tumor cell targets. However, it has been shown recently that rat DC express the NK cell receptor protein-P1, and can kill NK-sensitive targets, including YAC-1, via a Ca^{2+}-dependent mechanism (40). Therefore, in a mixed population of mononuclear leukocytes from Flt3-L-treated mice containing a large number of DC, it is conceivable that at least some of the tested cytotoxic activity might be mediated by DC, if they express NK1.1. However, we were able to exclude this possibility by determining 1) that mouse DC ($CD11c^+$ cells) tested in various organs did not express NK1.1; and 2) that highly purified, sorted spleen $CD11c^+$ cells (DC) were not significantly cytotoxic against YAC-1 targets, in contrast to $NK1.1^+CD11c^-$ (NK) cells (data not shown). In addition, previous studies in rodents and humans have demonstrated that most induced activities during the first week of IL-2 stimulation (including early increases in NK activity, proliferation, generation of LAK activity, and development of A-NK cells) are due to the response of NK cells (32). Therefore, IL-2-induced functions assessed in this study are believed to reflect only the abilities of NK cells and provide further evidence that lytic activity was mediated by NK cells rather than by DC.

It has been established that NK cell development occurs sequentially in the bone marrow stromal microenvironment from noncytotoxic and NK cell marker-negative pro-NK cells (9–12). However, conflicting published data are available regarding the order of NK1.1 expression and the appearance of cytotoxic activity during differentiation of pro-NK cells into NK cells (10, 37, 41). Thus, development of NK activity before expression of the specific cell surface lineage marker (NK1.1) (10), expression of NK1.1 before the development of NK activity (41), and simultaneous acquisition of both NK1.1 and NK activity (37) have been described. The present study demonstrates that Flt3-L induces an early dramatic increase in NK activity in the bone marrow in the absence of a

simultaneous increase in the number of $CD3^-NK1.1^+$ cells. These findings indicate that Flt3-L might first stimulate noncytotoxic, marker-negative pro-NK cells in the bone marrow to differentiate into NK cell marker-negative cytotoxic, immature NK cells. Our experiments also determined that Flt3-L treatment sequentially increased NK activity in the bone marrow, spleen, and liver and elevated this activity in the blood. Furthermore, the correlation between the increase in NK activity and number of $CD3^-NK1.1^+$ cells was partial in the spleen and complete in the liver. These findings suggest that immature NK cells, which begin their differentiation in the bone marrow, sequentially continue to differentiate in the blood and/or spleen, where they begin to express the lineage marker NK1.1. Finally, when fully mature, NK cells move into the liver. In support of this interpretation, we also showed that responsiveness of NK cells to IL-2 was manifested differently by Flt3-L induction in different tissues. Thus, it was low in the bone marrow, intermediate in the spleen, and high in the liver. These findings suggest that during Flt3-L treatment, NK cells in the bone marrow are less mature than those in the spleen, and those in the spleen are less mature than NK cells in the liver.

During the first 10 or 12 days of continuous Flt3-L treatment, we found a dramatic increase in the number and function of NK cells and in the number of DC in the spleen and liver. However, this increase was followed by a steady decrease to the end of treatment on day 18. These findings indicate that a potent negative regulatory mechanism(s) may be engaged during prolonged Flt3-L treatment. It is possible that the proliferative capacity of bone marrow stem cells may be exhausted, or that cytokines that regulate hemopoiesis, such as TGF-β, are induced (42) during Flt3-L treatment. It might also be possible that during chronic Flt3-L treatment, NK cells and DC could become susceptible to the induction of apoptosis, die, and thus be eliminated. Alternatively, NK cells could move to the other tissues that were not examined, including the intestine.

Recent demonstrations that Flt3-L induces the regression of both immunogenic (43) and nonimmunogenic (MHC class I-deficient) (44, 45) tumors could be related, respectively, not only to enhanced induction of T cell-mediated specific antitumor immune response by DC, but also to the striking expansion of NK cells and their lytic activity, as shown here. As Flt3-L induces a dramatic increase in the number of both immature and mature NK cells, treatment with Flt3-L followed by cytokines (e.g., IL-2 and/or IL-15) able to induce both differentiation of immature NK cells to mature NK cells and activation of mature NK cells, might provide a powerful new strategy for NK cell-based therapies. In support of this possibility, the present study demonstrates that mononuclear leukocytes from the spleen and liver of Flt3-L-treated mice have an increased ability to respond to IL-2 by rapid augmentation of NK activity, proliferation, and generation of LAK activity, as well as A-NK cells. As Flt3-L and IL-2 have important abilities to increase the number of mature, nonactivated, and activated NK cells, respectively, particularly in the liver, our data indicate that combined sequential treatment with Flt3-L and IL-2 or other NK cell-activating cytokines might lead to the development of improved NK cell-based strategies for the therapy of liver cancer and viral hepatitis.

In conclusion, we show that in addition to its previously demonstrated potent capacity to augment functional DC, Flt3-L is a powerful inducer of functional NK cells in vivo. It stimulates the generation and expansion of mature, nonactivated NK cells in multiple tissues. These Flt3-L-induced NK cells show increased responsiveness to IL-2.

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