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FLT3-Ligand Administration Inhibits Liver Metastases: Role of NK Cells[1]

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FLT3-ligand (FL) is a recently described cytokine that stimulates the proliferation and differentiation of hematopoietic progenitors both in vivo and in vitro and, when administered to mice, induces an accumulation of dendritic cells (DC) in different lymphoid and nonlymphoid organs and tissues, including the liver. We have studied the antitumor effect of FL administered alone or in combination with IL-12 in a day 3 murine liver metastasis model. FL significantly reduced the number of hepatic metastases (36.00 ± 11.00 vs 92.00 ± 10.19 in control group, $p < 0.05$). Histologic evaluation of the livers revealed that FL induced a significant infiltration of the tumor border by lymphocytes and DC associated with increased number of apoptotic figures. Immunohistochemical analysis demonstrated that FL significantly enhanced the number of DC in the liver parenchyma and within the liver metastases, as well as the number of $CD4^+$ and $CD8^+$ T lymphocytes. These data support the suggestion that DC may be directly involved in the antitumor effect of FL. Interestingly, the antitumor effect of FL was greatly reduced by the NK depletion. Combination of FL and IL-12 resulted in greater antitumor efficacy than these cytokines alone. In summary, we have shown that FL has significant antitumor effect on preexisting murine C3 liver tumors that is mediated by NK cells. We have also demonstrated that the FL/IL-12 combination has an enhanced antitumor activity in the same murine tumor model. ***The Journal of Immunology,*** **1998, 161: 6164–6170.**

The FLT3-ligand (FL)[3] is a recently identified cytokine (1, 2) that plays an important role in the proliferation, survival, and differentiation of early murine and human hematopoietic precursor/stem cells (3). Two recent studies reported that the in vivo administration of FL resulted in a time-dependent and reversible accumulation of dendritic cells (DC) in a variety of lymphoid and nonlymphoid tissues including the liver. Also, these cells were shown to be functionally active as APCs inducing Ag-specific T cell responses both in vitro and in vivo (4, 5). Moreover, FL increases the relative number of NK cells in the spleen of tumor-free animals in vivo (6). Lynch et al. (7) recently showed that FL treatment generated antitumor immune responses in a murine s.c. tumor model and that the specific immunity was mediated in part by $CD8^+$ T cells.

IL-12 promotes the activation and generation of CTL, stimulates NK activity and macrophage function, and regulates Th1 cell generation (8–10). The antitumor activities of IL-12 have been studied extensively in a number of animal models with promising results (11), but its clinical application was made difficult by the occurrence of unusual systemic toxicity observed at high doses of the cytokine (12, 13). Thus, alternative strategies of IL-12 application will be important for the future development of IL-12-based immunotherapies. Moreover, IL-12 has been shown to have a synergistic interaction with FL in stimulating the growth and expansion of early murine bone marrow progenitor cells in vitro (14), suggesting the possible use of combination of these cytokines in a tumor immunotherapy.

We have examined the effect of FL treatment on the growth of liver metastases in a murine model following the intraportal administration of C3 sarcoma cells. Splenectomy, widely used in murine liver tumor models, has been shown to modify the host's immune responses (15–17). We therefore performed direct portal vein injection of tumor cells rather than the conventional splenic injection of tumor cells that requires a secondary splenectomy. We found a significant antitumor effect of FL-based immunotherapy. It is possible that this effect was mediated by several different mechanisms since different populations of the immune effector cells were involved. Our data showed that NK cells played an important role because NK depletion resulted in the inhibition of the antitumor activity of FL. In addition, we have observed an increased infiltration of liver tumors by DC and T lymphocytes, suggesting the possible involvement of DC-mediated stimulation of specific T cell responses. We have also demonstrated an enhanced antitumor effect of the FL/IL-12 combination when compared with the cytokines alone using the described murine tumor model. Thus, these data support the further development of preclinical and clinical studies design to evaluate the efficacy of FL-based immunotherapies, including FL in combination with IL-12 for the treatment of cancer.

Materials and Methods

Mice

Male C57BL/6 (B6, H-2^b) mice, 6–8 wk old, were obtained from Taconic (Germantown, NY). The animals were housed in a pathogen-free animal facility, four mice per cage, under controlled temperature and humidity, and a 12-h light/dark cycle with food and water available ad libitum. All

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[3] Abbreviations used in this paper: FL, FLT3-ligand; DC, dendritic cell(s); MNC, mononuclear cell(s).

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animals were acclimatized for at least 2 wk before the initiation of experiments. All experimental procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Tumor cell lines

C3 sarcoma is a tumor cell line obtained by tranfecting early mouse embryos with a plasmid containing the entire genome of the human papilloma virus type 16 (18). MC38 is a murine colon adenocarcinoma cell line kindly provided by Dr. S. Rosenberg (Surgery Branch, National Cancer Institute). These tumor cell lines are C57BL/6 syngeneic. They were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Both cell lines were regularly passed through animals and were mycoplasma-free.

Tumor models

Liver metastases were obtained by direct portal injection of MC38 or C3 tumor cells. Mice were anesthetized by methoxyflurane (Mallinckrodt Veterinary, Mundelein, IL) inhalation. The portal vein was exposed through a small midline incision and 300 μl of a suspension of 2×10^5 C3 cells or 1×10^4 MC38 cells in HBSS was injected. Hemostasis was obtained by a gentle compression of the inoculation site with a cotton swab. The animals were sacrificed by cervical dislocation 17 days later. In our preliminary experiments, 17–20 days was found to be the optimal time to count distinct nonconfluent liver metastases in control animals. The spleens and livers were collected and weighed. The liver metastases were counted in a blinded fashion. Blood was either collected by cutting the tail vein for flow cytometry analysis or by direct intracardiac puncture to measure the aminotransferase activity (data presented as international units (IU)) using a Technicon RA-5-00 (Miles, Tarrytown, NY).

Treatment protocols

Mice were randomized and injected with 100 μl i.p. daily for 10 days, starting on day 3 after tumor cell administration of either 0.01% mouse serum albumin (MSA; Sigma, St. Louis, MO) alone (control group) or 10 μg FL in 0.01% MSA. Murine IL-12 (0.2 μg in 0.01% MSA) was administered daily for 5 consecutive days starting on day 10 after tumor cell injection. The animals receiving a combination of FL and IL-12 were injected into both flanks according to the same schedule.

NK depletion

Twenty microliters of anti-Asialo-GM1 antiserum (Wako, Richmond, VA) were injected i.p. every 4 days starting 2 days after the intraportal injection of tumor cells, which is 1 day before FL injections were initiated. Depletion of NK cells was confirmed by FACS analysis of blood and spleen samples.

Morphologic analysis

The entire liver was harvested and examined before and after fixation with neutral buffered formalin (JT Baker, Phillipsburg, NJ) for 4–5 days. From each lobe of the liver, 2–3 slices were routinely processed and embedded in paraffin. Four-micron paraffin sections were stained with hematoxylin/eosin and utilized for pathologic analysis. Morphologic, as well as morphometric analysis of immunohistochemical slides, were performed by an experienced pathologist blinded to sample codes as well as experimental design. All samples were analyzed randomly and at least twice.

Immunohistochemistry

A part of each lobe of the liver was embedded in OCT Compound (Miles, Elkhart, IN), frozen, and stored at −80°C. Cryostat sections (6 μm) were used for immunohistochemical evaluation. The following primary mAbs were used: rat anti-mouse CD8a (Ly-2), rat anti-mouse CD11b (Mac-1á chain), rat anti-mouse IgG2bκ, rat anti-mouse IgG2aκ (PharMingen, San Diego, CA), and rat anti-mouse NLDC-145 (Serotec, Washington DC). Each primary Ab was applied for 1 h at room temperature. After two washes in PBS, the sections were incubated with the biotinylated secondary mouse anti-rat IgG $F(ab')_2$ Ab (Jackson ImmunoResearch, West Grove, PA) for 45 min followed by an avidin-biotin-peroxidase complex method using Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The color reaction was developed for 1–8 min using a peroxidase chromogen kit (AEC, Biomega, Foster City, CA). Negative controls included staining with the corresponding isotype for each Ab and staining with secondary Ab alone. Positive controls included immunostaining of known positive tissues.

Table I. *Effect of FL immunotherapy on the liver metastases, liver, and spleen in tumor-bearing mice*[a]

	FL-Treated Mice[b]	Control mice[c]	*p* Value[d]
Number of metastases[e]	36.00 ± 11.00	92.00 ± 10.19	<0.05
Spleen weight (g)	0.29 ± 0.05	0.08 ± 0.01	<0.01
Liver weight (g)	1.49 ± 0.10	1.30 ± 0.03	NS
SGPT[f] (IU)	36.20 ± 5.17	60.80 ± 24.61	NS

[a] A total of 19 mice in 4 independent experiments were analyzed. The animals were sacrificed 17 days after the portal injection of C3 tumor cells and the number of liver metastases, liver and spleen weights, and liver enzymes were determined.
[b] Nine mice were treated with FL starting on Day 3 after injection of C3 sarcoma cells.
[c] Ten control mice received MSA injections using similar treatment schedule.
[d] NS, non significant.
[e] The mean number of metastases, spleen and liver weight and SGPT activity are shown ± SEM.
[f] SGPT, serum glutamic pyrovic transaminases.

Flow cytometry

The following mAbs were used: phycoerythrin-conjugated rat anti-mouse NK1.1 (clone PK136) and FITC-conjugated rat anti-mouse B220/CD45R Ab (clone RA3-6B2) (PharMingen). The splenic cells were dissociated and filtered through a 70-μm pore size nylon cell strainer; RBCs were lysed, and the remaining cells were stained as previously described (5). After tail vein blood collection in vacutainer tubes containing heparin, the blood samples were diluted with medium, layered over Lympholyte-M (Cedarlane, Hornby, Ontario, Canada), and centrifuged at 200 × *g* for 30 min. The mononuclear cells (MNC; buffy coat interface) were then harvested and stained as previously described (5).

Reagents

PBS, HBSS, L-glutamine, gentamicin, penicillin, streptomycin, nonessential amino acids, and sodium pyruvate were purchased from Life Technologies (Grand Island, NY). Paraformaldehyde was obtained from Sigma. Human Chinese hamster ovary cell-derived FL was supplied by Immunex R&D Corp. (Seattle, WA). Recombinant murine IL-12 was a gift from Hoffman La-Roche (Nutley, NJ).

Statistical analysis

SigmaStat statistical software package (Jandel Scientific, San Rafael, CA) was used for data analysis. One-way analysis of variance (ANOVA) was performed to evaluate the significance of differences between the experimental groups. All pairwise comparisons of every combination of group pairs utilized the Student-Newman-Keuls method. For a single comparison of two groups, the Student's *t* test was applied. To compare the effect of different treatments on liver metastases number divided into three different categories, the χ^2 analysis of contingency tables was used. For all analysis, the level of significance was set at a probability of 0.05 to be considered significant. Data are presented as means ± SEM.

Results

FL administration significantly inhibits the growth of liver metastases in a therapy model

To study the efficacy of FL in the treatment of C3 sarcoma liver metastases, we treated a total of 19 mice in four independent experiments (Table I). The number of liver metastases was reduced 3-fold in the FL-treated group relative to the control group. There was no difference in the size of the metastases between the FL and control groups at gross examination. The average spleen weight was increased up to 3-fold in the FL-treated group, although the average liver weight was not significantly changed. No overt toxicity or weight loss was noted in any of the FL-treated mice. Liver enzymes (serum glutamic pyruvic transaminases) were lower in the treated group without reaching statistical significance. These data suggest that FL therapy has a strong inhibitory effect on the growth of liver metastases without significant effect on liver function.

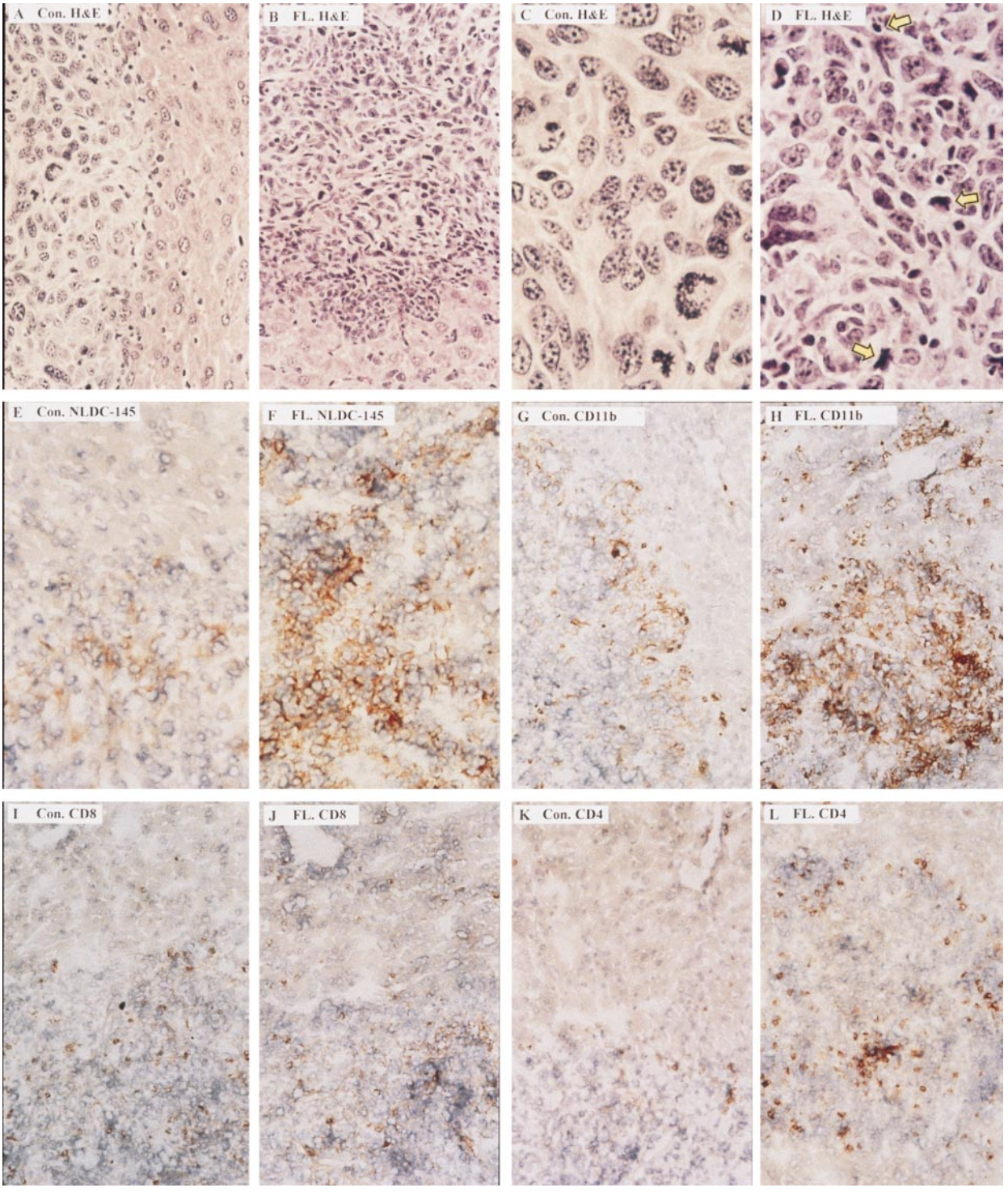

FIGURE 1. Hematoxylin/eosin staining and immunohistochemical evaluation of liver sections from FL-treated and nontreated tumor-bearing animals revealed macrophage, lymphocyte and DC infiltration. *A* and *B*, Hematoxylin/eosin staining (magnification, ×200). *A*, Control mouse: the border between tumor and liver parenchyma is sharp. *B*, FL-treated mouse: the border between the metastasis and parenchyma is infiltrated by MNC and nonparenchymal cells-like cells. *C* and *D*, Hematoxylin/eosin staining (magnification, ×1000). *C*, Control mouse: no apoptotic figures. *D*, FL-treated mouse: frequent apoptotic figures (arrows). *E* and *F*, NLDC-145$^+$ staining (magnification, ×400). *E*, Control mouse. *F*, FL-treated mouse. *G* and *H*, CD11b$^+$ staining (magnification, ×200). *G*, Control mouse. *H*, FL-treated mouse. *I* and *J*, CD8$^+$ staining (magnification, ×200). *I*, Control mouse. *J*, FL-treated mouse. *K* and *J*, CD4$^+$ staining (magnification, ×200). *K*, Control mouse. *L*, FL-treated mouse. FL enhanced the number of NLDC-145$^+$, CD11b$^+$, CD8$^+$, and CD4$^+$ cells in the tumor and liver parenchyma, seen at the upper part of samples *E–L*.

FL administration induces the infiltration of the liver metastasis by DC and lymphocytes

Livers from both control and FL-treated animals were analyzed 10 days after the initiation of treatment (Fig. 1). Examination of tissue samples obtained from the nontreated mice and stained with hematoxylin/eosin revealed that the tumor-free liver parenchyma had an unmodified architecture and normal morphology as expected. The border between metastases and liver parenchyma in the control samples was clearly indicated, sharp, well demarcated, and

characterized by insignificant MNC infiltration within both tissues. Apoptotic figures were a rare event within the tumor or the liver parenchyma. In contrast, liver samples from FL-treated animals showed a significant infiltration of the border between the metastases and liver parenchyma by two types of cells. A significant MNC infiltration in association with small parenchymal necrosis was noted in tumor-free parenchyma. Furthermore, a marked accumulation of nonparenchymal cells with irregular shaped nuclei was pronounced at the border of the metastases. MNC infiltration was also present within the metastases. Importantly, high numbers of apoptotic figures were found within the liver metastasis. These observations were also confirmed using FL immunotherapy and another tumor cell line, MC38, a murine colon adenocarcinoma (data not shown).

It is important to note that one mouse from the FL-treated group demonstrated complete regression of the tumor. Histologic examination of the liver obtained from this animal revealed no metastases in any liver sections examined. Large numbers of necrotic areas in association with MNC and neutrophilic infiltration characterized this liver. These results suggest that FL treatment induces the appearance in the liver of a large number of nonparenchymal cells having the histologic features of DC as well as the infiltration of liver tumors by MNC and DC.

FL enhances the number of DC, macrophages, CD4$^+$, and CD8$^+$ lymphocytes in metastases and liver parenchyma

Livers from both control and treated mice were analyzed 10 days after initiation of treatment using immunohistochemical techniques. Examination of livers metastasized by C3 tumors revealed a moderate increase of CD11b$^+$ (Mac1) cells, presumably macrophages, in the liver parenchyma and significant increases at the tumor site. We have also found a moderate but significant increase in both CD8$^+$ and CD4$^+$ cells in the liver parenchyma and tumor mass in the samples obtained from the FL-treated animals compared with the control samples (Fig. 1). Importantly, FL markedly increased the number of NLDC-145$^+$ DC in the surrounding parenchyma and moderately enhanced the number of NLDC-145$^+$ cells within the liver metastasis (Fig. 1). Analysis of MC38 liver metastases revealed similar results (data not shown). Thus, we concluded that FL therapy in mice significantly enhanced the number of DC and macrophages and to a lesser extent the number of cytotoxic and helper T lymphocytes, in the liver parenchyma, and within the liver tumor itself.

NK cells play an important role in the antitumor effect of FL

The percentage of NK cells in the spleens and blood of FL-treated and nontreated tumor-bearing animals was determined by flow cytometry 1 day after the last administration of FL (day 13). B220 is known to be expressed on lytically active subsets of lymphokine-activated killer cells, such as NK cells and non-MHC-restricted CTL. Using this Ab we wanted to demonstrate that both populations of NK cells, B220$^-$ and B220$^+$ might be affected by the treatment of mice with FL in vivo. In addition, we and others (4, 5) have recently demonstrated that murine B lymphocytes were less affected by the FL administration than T cells, DC, and Gr-1$^+$ cells in vivo. Based on this observation, we used B220 staining as an additional control which served to demonstrate the relative specificity of the FL effect on the generation of NK cells in treated animals. As shown in Fig. 2, FL therapy markedly increased the percentage of NK1.1$^+$ cells by 3-fold (8% vs 25%) in the spleen and 2-fold (5% vs 10%) in the blood. Interestingly, that both populations of NK cells, B220$^+$ and B220$^-$, were significantly increased, although the increase in percentage of NK1.1$^+$B220$^-$ cell was substantially higher when compared with the percentage of

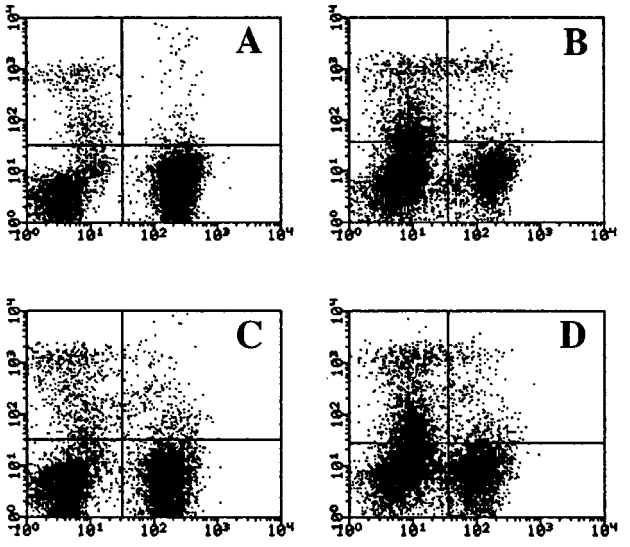

FIGURE 2. Flow cytometry analysis of cell populations separated from blood (*A* and *B*) or spleen (*C* and *D*) and obtained from control (*A* and *C*) or FL-treated (*B* and *D*) animals. Double staining with anti-B220 (*x*-axis) and anti-NK1.1 (*y*-axis) Abs revealed an increase in NK1.1$^+$ cells from 5% to 10% in the blood, and from 8% to 25% in the spleen following the FL-based immunotherapy.

NK1.1$^+$B220$^+$ cells. To further evaluate the role of NK cells in the antitumor effect of FL, we depleted NK cells in the treated and control animals. NK cell depletion was initiated 2 days after the portal vein injection of C3 tumor cells (one day before the initiation of the FL-based therapy). Twenty-two mice in three independent experiments were randomized into four groups (Fig. 3). We confirmed that FL-treated mice had fewer metastases than the control mice (45.33 $\pm$ 17.69 vs 166.00 $\pm$ 22.47, $p < 0.05$). The NK cell-depleted and FL-treated mice had significantly more metastases than the nondepleted ones (160.80 $\pm$ 16.02 vs 45.33 $\pm$ 17.69, $p < 0.05$). The NK cell-depleted mice in the control group also had more metastases than the nondepleted ones without reaching statistical significance (176.20 $\pm$ 23.80 vs 166.00 $\pm$ 22.47). There was no difference in the number of liver metastases between the NK cell-depleted mice in the FL-treated group and the control

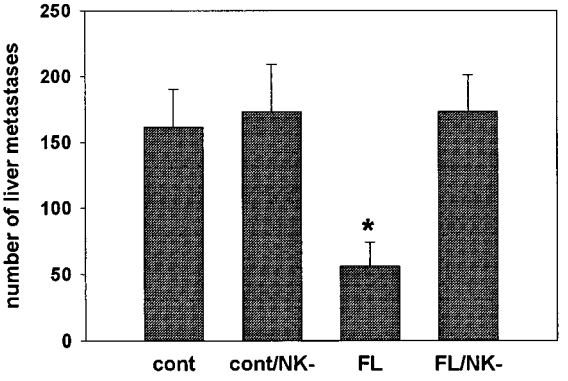

FIGURE 3. NK cell depletion abrogated the antitumor effect of FL. NK cell depletion was started 2 days after the portal vein injection of C3 tumor cells (i.e., 1 day before the initiation of the FL-therapy). Twenty-two mice in three independent experiments were randomized into four groups: control nondepleted (con), control NK-depleted (con/NK-), FL-treated nondepleted (FL) and FL-treated NK-depleted (FL/NK-). Data presented as mean $\pm$ SEM. *, Number of hepatic metastases was significantly reduced in the FL group compared with the FL/NK- group ($p < 0.05$).

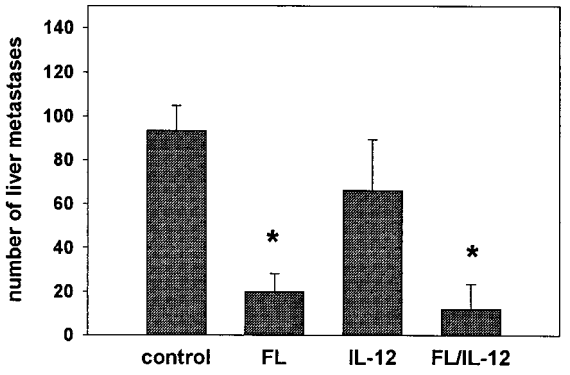

FIGURE 4. Treatment of tumor-bearing mice with FL in combination with IL-12 significantly reduced the number of hepatic metastases compared with the therapy with IL-12 alone. A total of 23 mice were seeded in three independent experiments. Data presented as mean ± SEM. *, Number of hepatic metastases was significantly reduced in the FL and FL/IL-12 groups compared with the control and IL-12 monotherapy groups ($p < 0.05$).

group (160.80 ± 16.02 vs 166.00 ± 22.47). Thus, these data suggest that NK depletion in vivo abrogated the FL-mediated antitumor effect in a day 3 murine liver tumor model.

Antitumor effect of the FL/IL-12 combination therapy

To evaluate the antitumor efficacy of FL/IL-12 combination therapy, 23 mice received injection of tumor cells in three independent experiments. As shown in Fig. 4, the number of hepatic metastases was significantly reduced in the FL- and FL/IL-12-treated groups compared with the control nontreated group (19.00 ± 8.29 and 13.52 ± 11.33, respectively, vs 83.43 ± 11.36, $p < 0.05$) and to the IL-12 monotherapy group (66.00 ± 23.28, $p < 0.05$). There were fewer liver metastases in the FL/IL-12 combination group than in the FL monotherapy group, but the difference did not reach statistical significance. The reason of this was a single mouse in the FL/IL-12 group, which developed a large number of metastasis. Statistical analysis of combined results did not reveal significant differences between FL and FL/IL-12 groups, although analysis of individual experiments has demonstrated a statistically significant difference in two of three experiments (the last one failed because of a single unusual mouse). To compare the effect of different treatments on liver metastases number divided into three different categories (see Table II), we used a χ^2 analysis of contingency tables. Statistical analysis of these data revealed that $\chi^2 = 239.292$ with 6 degrees of freedom, suggesting that the distribution of the experimental groups within the chosen categories is significantly different ($p < 0.001$). As can be seen from Table II, the percentage of mice with a limited number of metastases was highest in the FL/IL-12 group. Indeed, all animals in the FL/IL-12-treated group, except one, had less than 10 metastases. One mouse had 70 metastases, but the metastases were markedly smaller by gross examination than the metastases found in other groups. Thus, these results suggest that the FL/IL-12 combination immunotherapy of liver metastases is more effective treatment than either one cytokine alone in the established murine liver tumor model.

Table II. *Effect of FL, IL-12, and FL/IL-12 combination on the number of liver metastases in mice*

Treatment groups (n = 23)	Percentage of Mice with < 10 Metastases	Percentage of Mice with 10–100 Metastases	Percentage of Mice with >100 Metastases
Control	0	43	57
FL	33	67	0
IL-12	25	50	25
FL/IL-12	83	17	0

Discussion

In vitro and in vivo Ag-pulsed DC can directly sensitize T cells and stimulate the development of both protective and therapeutic specific antitumor immune responses (19–21). One of the limiting factors in using these professional APCs for human DC-based immunotherapy is the relatively low number of functionally active DC available for use. DC have been identified in both human and murine livers and are located in the portal triads and around the hepatic veins (22–24). We have recently shown that FL greatly enhances the number of DC in the liver parenchyma in vivo (5). The aim of this study was to evaluate whether FL administration could induce an antitumor effect in a murine liver metastasis therapy model and to determine primary mechanisms involved in this effect.

We used direct intraportal injection of C3 sarcoma cells to induce liver metastases. This method was preferred over injection of tumor cells into the spleen or tail vein, because it provided an animal model which is more clinically relevant to study liver metastases. Tail vein injection of tumor cells induces a diffuse metastatic disease that includes overt lung metastases. Splenic injection of tumor cells requires splenectomy which has been shown to be beneficial in tumor-bearing rodents by removing a source of suppressive factors and by eliminating suppressor cell activity (15–17). Moreover, the spleen has been shown to enlarge due to the extramedullary hematopoiesis during FL administration and may play an important role in FL-based therapy models (5). In addition, a deleterious role of splenectomy has been demonstrated in some human clinical settings of cancer (25). Thus, intraportal administration of tumor cells should serve as a more appropriate way to induce liver metastases to study the relationship between the tumor and immune cells in the liver. FL induces regression of s.c. tumor when administered to animals bearing an Methylcholanthzene-induced fibrosarcoma of B10 origin (7) or C3, MC38, and TS/A tumors (26, 27). Chen et al. (28) have recently reported a similar effect of FL using murine breast cancer cell line C3L5. We have also demonstrated an antitumor efficacy of FL in the murine B16 and CL8-1 melanoma and EL-4 lymphoma models (29). However, there is no information available about the effect of FL immunotherapy during liver metastasis disease.

We showed here that FL had a significant antitumor effect on liver metastases and increased the number of DC and T cells at the tumor site. FL does not have a direct effect on tumor cell lines in vitro (7), but in vivo greatly enhances the number of DC in the spleen and other organs (4, 5). Taken together with the observation that the number of DC at the tumor site positively correlates with a better prognosis of cancers (30), our data support the hypothesis that DC play an important role in the induction of antitumor responses. However, we have recently demonstrated that tumor infiltration with DC may reflect the immunogenicity of murine tumor (29). Because DC play a pivotal role in the induction of immune responses, it is possible to speculate that an increased number of functionally active DC in tumor-bearing host could stimulate a higher level of specific antitumor immune responses. Others have recently made a similar suggestion (31). It is also possible that tumor infiltrating DC play a role in the regulation of T cell survival, including CTL, in the local tumor microenvironment. DC may protect T lymphocytes from tumor-induced apoptotic death (M. T. Lotze, unpublished observation). In fact, we have observed increased number of both $CD4^+$ and $CD8^+$ T cells within liver

tumors in FL-treated mice. IL-12 produced by activated DC might, at least in part, be responsible for the alteration of T cell survival by DC.

Furthermore, FL seems to augment the IL-12 production by DC (M. R. Shurin, unpublished data), which additionally supports this hypothesis. The metastases in the FL-treated mice were also infiltrated by lymphocytes, suggesting a role for CTL in the effector phase of antitumor immunity. Indeed we have previously shown that animals that have rejected the s.c. C3 tumor due to the FL treatment, developed specific long-term immune memory and effectively rejected rechallenge with the same tumor cells (26).

We have also shown here that FL induces a strong augmentation of NK cells in the spleen and blood. Similar results were recently reported by Shaw et al. (39) who demonstrated that FL treatment sequentially increased NK activity in the bone marrow, spleen, and liver in normal mice. NK cells kill target cells without the need for prior sensitization and without class I MHC restriction. They are also involved in surveillance and control of hematogenous spread of tumor cells, which makes them the earliest cellular effector mechanism against dissemination of metastases (32). Moreover, patients with advanced metastatic disease often have abnormalities in NK cell function and/or number (33). The increased number of NK cells in the spleen of FL-treated animals was still observed 4 days after completing FL therapy. The percentage of NK cells in the spleens of the animals bearing liver metastases was higher than previously reported for tumor-free animals (6). This difference may reflect the effect of tumor-derived factors on the generation of NK cells, as discussed above, or the development of antitumor immunity in tumor-bearing mice at early stages of tumor growth. NK cell depletion significantly abrogated the antitumor effect of FL in our murine tumor model (Fig. 3), suggesting that NK cell activity was necessary for the inhibition of tumor growth induced by FL in early therapy model. A similar mechanism has been observed for the IL-12-based therapy. Early antitumor effects of IL-12 has been suggested to be mediated by NK cells while late antitumor effects seem to be mediated by T cells (11). In fact, the mouse liver contains a large number of NK cells that have been shown to be potent effector cells against tumors after the stimulation with IL-12 (34). FL may act similarly to IL-12 or may stimulate IL-12 production by immune cells, in particular by DC. We hypothesized that the stimulation of both DC and NK generation and activity with FL and IL-12 could be a better activator of antitumor immune responses than either of cytokine alone. In addition, the interaction between DC and NK cells within the tumor microenvironment may also play an important role in the effector phase of antitumor immune responses. We are currently investigating this possibility.

Even though the number of metastases was reduced in the FL-treated animals, there was no significant liver weight loss in this study. On the contrary, there was a trend toward higher liver weights in the FL-treated animals, although it did not reach a statistically significant level. This is in agreement with previously published data (5) demonstrating that in tumor-free animals the liver weight was higher in the FL-treated animals due to newly arrived nonparenchymal cells, a significant number of which were DC. Here, we have confirmed the presence of DC in the livers of tumor-bearing animals.

IL-12 is a cytokine with a potent antitumor activity. It has been shown that FL synergizes with IL-12 to stimulate the growth and differentiation of early hematopoietic progenitors in vitro (14). In this study, we evaluated the antitumor efficacy of FL/IL-12 combination therapy in the setting of liver metastases in vivo. Our data suggest that FL/IL-12 therapy was more effective than IL-12 or FL alone for the treatment of murine liver metastasis. There were more mice with fewer metastases in the FL/IL-12 group when compared with the FL monotherapy group (Table II). In fact, only one mouse in this group had more than 10 metastases, although the sizes of these metastases were significantly smaller than that of the other groups. The antitumor effect of IL-12 appears to be mediated through early NK stimulation and later promotion of both CD4- and CD8-mediated responses to tumor (35, 36). The central role of IFN-γ in the antitumor effects of IL-12, promoting the development of a Th1 response, has been demonstrated by its neutralization (37). IL-12, in the setting of FL administration, may also promote T cell survival at the tumor site. In addition, IL-12 plays an important role in the mechanism of DC-mediated antitumor activity. It has been shown that in vivo neutralization of IL-12 in mice immunized with tumor peptide-pulsed DC blocks the induction of specific immune response to tumor peptide (38).

In conclusion, we have shown that FL administration induces a significant antitumor effect in a murine C3 liver metastases model and that it is likely mediated by both DC and NK cells. Enhanced antitumor effects of the FL/IL-12 combination were also observed in this tumor model suggesting its consideration for further preclinical and clinical trials.

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