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The Anti-Tumor Activity of IL-12: Mechanisms of Innate Immunity That Are Model and Dose Dependent[1]

Mark J. Smyth,[2]* Masaru Taniguchi,[†] and Shayna E. A. Street*

IL-12 has been demonstrated to have potent anti-tumor activities in a variety of mouse tumor models, but the relative roles of NK, NKT, and T cells and their effector mechanisms in these responses have not been fully addressed. Using a spectrum of gene-targeted or Ab-treated mice we have shown that for any particular tumor model the effector mechanisms downstream of IL-12 often mimic the natural immune response to that tumor. For example, metastasis of the MHC class I-deficient lymphoma, EL4-S3, was strictly controlled by NK cells using perforin either naturally or following therapy with high-dose IL-12. Intriguingly, in B16F10 and RM-1 tumor models both NK and NKT cells contribute to natural protection from tumor metastasis. In these models, a lower dose of IL-12 or delayed administration of IL-12 dictated a greater relative role of NKT cells in immune protection from tumor metastasis. Overall, both NK and NKT cells can contribute to natural and IL-12-induced immunity against tumors, and the relative role of each population is tumor and therapy dependent. *The Journal of Immunology,* 2000, 165: 2665–2670.

Interleukin-12 was first identified and isolated as an NK cell stimulatory factor (1). Compared with other cytokines, it has a unique 70-kDa heterodimeric structure composed of two covalently linked p35 and p40 subunits, both of which are required for biological activities (1, 2). IL-12 is produced principally by APC, such as monocytes, macrophages, and dendritic cells. In addition to a stimulatory effect on NK cells, IL-12 activates cytotoxic T cells (3, 4), differentiates $CD4^+$ lymphocytes (5, 6), plays an important role in regulating the balance between type I and type 2 responses of Th lymphocytes (7, 8), primes macrophages for NO production (9), and possesses IFN-γ-dependent anti-angiogenic activity (10, 11).

IL-12 has been shown to possess potent anti-tumor activity in a wide variety of murine tumor models (2, 12–15). Activity has been demonstrated against tumors of various histologies, including carcinomas arising from the colon (CT26, MC38), kidney (Renca), and lung (3LL); carcinogen-induced sarcoma lines (including the methylcholanthrene-induced series); and melanoma (B16F10 and derivatives) (12, 14, 16–19). Significant IL-12-induced anti-tumor activity has been demonstrated against established cutaneous deposits, experimental metastases (12, 16), and spontaneous metastases (18, 20).

The mechanisms through which IL-12 elicits its potent antitumor activity remain unclear. IL-12 has been reported to promote anti-angiogenic activity, a process thought to occur by the IFN-γ-mediated induction of CXC chemokines, IFN-γ-inducible protein-10 and Mig (21, 22). Furthermore, IFN-γ production leads to macrophage activation and the induction of inducible NO synthase

(23), thus raising the possibility that reactive oxygen metabolites contribute to the anti-tumor activities of IL-12 (24, 25). In many experimental model systems, depletion of T cells can prevent IL-12-induced tumor regressions (12, 16). Additional studies have implicated $NK1.1^+$ T cells as essential in IL-12-induced anti-tumor responses (26, 27). In contrast, in some tumor models, IL-12 retained partial activity in athymic nude mice or RAG-deficient mice (12, 28). Therefore, depending upon the tumor system employed, T cells, NK cells, and/or macrophages may be involved in the anti-tumor immune responses generated by IL-12. In this study, the anti-tumor effector mechanisms employed by the innate immune system in response to IL-12 were examined in three different tumor models. We have already characterized the role of natural immunity in these models and demonstrated a strong correlation between the anti-tumor activity of NKT cells and a requirement for endogenous IL-12 (29). Herein, we demonstrate that the relative contributions of NK and NKT cells depend upon the dose and time of IL-12 administration and that in these tumor models IFN-γ and/or perforin (pfp)[3] are required for IL-12-mediated anti-tumor immunity.

Materials and Methods

Mice

Inbred C57BL/6 mice were purchased from The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The following gene-targeted mice were bred at the Austin Research Institute Biological Research Laboratories: C57BL/6 IFN-γ-deficient mice (B6.IFN-$\gamma^{-/-}$; provided by Genentech, South San Francisco, CA) (30); C57BL/6 perforin-deficient (B6.Pfp$^{-/-}$) mice (31) (from Dr. Guna Karupiah, John Curtin School of Medical Research, Canberra, Australia); C57BL/6 IL-12p40-deficient mice (B6.IL-12p40$^{-/-}$) (32) (from Hoffmann-La Roche, Nutley, NJ); C57BL/6.RAG-1-deficient mice (B6.RAG-1$^{-/-}$; from Dr. Lynn Corcoran, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia); and C57BL/6 TCRJα281-deficient (B6.Jα281$^{-/-}$) mice (29). Mice 4–8 wk of age were used in all experiments that were performed according to animal experimental ethics committee guidelines.

Tumor surveillance in vivo

B16F10 mouse melanoma and other tumor cell lines used in this study were maintained as previously described (29, 33). Effector function was

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[3] Abbreviations used in this paper: pfp, perforin; RAG, recombinase-activating gene.

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examined in three different mouse tumor models (EL4-S3 (β_2-microglobulin-deficient EL4) lymphoma, B16F10 melanoma, and RM-1 prostate carcinoma) as previously described, using gene-targeted mice or mice depleted of lymphocyte subsets (32–34). For RM-1 experimental metastasis, mice were injected s.c. with RM-1 tumor cells (2×10^6; prepared from an in vitro stock passage), and tumors were established for 9 days. At this time, s.c. tumors were surgically resected, and RM-1 cells were injected via the dorso-lateral tail vein. Mice were euthanized 14 days later, the lungs were removed and fixed in Bouin's solution, and surface lung metastases were counted with the aid of a dissecting microscope (33, 34). Mouse IL-12 was provided by Genetics Institute (Cambridge, MA). The preparation of IL-12 was diluted in PBS immediately before use. In all tumor metastasis models mice received IL-12 as follows: high, 250 U i.p. on days −5, −4, −3, −2, −1, 1, 2, 3, 4, and 5 (where day 0 was the day of i.v. tumor inoculation); low, 50 U i.p. on days −5, −4, −3, −2, −1, 1, 2, 3, 4, and 5; and delayed, −250 U i.p. on days 3–12. Mice were administered anti-NK1.1 mAb as previously described (29, 34), i.e., 100 μg i.p. on days −2, 0, and 7 (where day 0 was the day of i.v. tumor inoculation). For metastasis experiments, the data were recorded as the mean number of metastases ± SE. Significance was determined by a nonparametric Mann-Whitney test.

Results and Discussion

B16F10:NK cells and pfp are sufficient for natural anti-tumor immunity

Initially we examined natural tumor immunity in a B16F10 melanoma lung metastasis assay. We employed RAG-1$^{-/-}$ and Jα281$^{-/-}$ mice (to assess the role of NKT cells), pfp$^{-/-}$ and IFN-$\gamma^{-/-}$ mice (to determine effector function), and NK cell-depleted mice (to assess the role of NK cells). A number of doses of B16F10 tumor cells were administered i.v., because it had been previously shown that greater inoculums ($>10^5$ cells) metastasized just as effectively in immunocompetent C57BL/6 mice, as in NK cell-depleted mice (12, 26, 28). As shown in Fig. 1a, natural anti-tumor immunity could only be demonstrated at low doses of B16F10 tumor cells (5×10^4), and that immunity was consistently mediated by NK cells in a pfp-dependent manner. There appeared to be no role for IFN-γ in natural host protection, and only at the lowest dose of B16F10 could a minor, but significant, role for NKT cells be demonstrated ($p < 0.05$).

B16F10:NK cells and pfp are sufficient for the anti-metastatic effect of high-dose IL-12

Several reports have demonstrated the activity of IL-12 against the B16 melanoma and its metastatic variant B16F10 (12, 26, 28). The outcomes of these studies were dramatically different. Data reported by Brunda et al. (12) and Cui et al. (26) in *bg/bg* and Jα281$^{-/-}$ mice, respectively, suggested that NK cells were not the primary cell type protecting against s.c. B16F10 tumor growth or metastasis to the lung. By contrast, Kodama et al. (28), using mice doubly deficient for pfp and RAG-2, clearly showed that the anti-metastatic effect of IL-12 on B16 melanoma was mediated by pfp and NK cells. Particularly striking was the discrepancy between the studies of Kodama and Cui, because both studies used similar models and conditions. The possibility that the dose of IL-12 may influence the relative roles of NK and NKT cells was raised, but not tested, by Kodama et al. (28). We decided to examine this issue further using the B16F10 metastasis model and including RAG-1$^{-/-}$, Jα281$^{-/-}$, pfp$^{-/-}$, IFN-$\gamma^{-/-}$, and NK cell-depleted mice. A cell number was chosen (5×10^5) such that all groups of untreated mice displayed similar tumor burdens. As shown in Fig. 1b, anti-tumor immunity induced by high-dose IL-12 (total, 2500 U over 10 days) was mediated by NK cells in a pfp-dependent manner. At this dose of IL-12 there was no apparent role for NKT cells. While these data were in concert with those reported by Kodama et al. (28), the observation that IL-12 still retained significant anti-tumor

activity in B6.IFN-$\gamma^{-/-}$ mice further confirmed that high-dose IL-12 mediated its effect strictly through pfp.

### B16F10:NKT cells partially contribute to the anti-metastatic effect of low-dose IL-12

We next examined the relative roles of NK and NKT cells at a low dose of IL-12 (total, 500 U over 10 days) administered using the same schedule (Fig. 1c). By contrast to the observations with high-dose IL-12, we detected a partial role for NKT cells in the anti-tumor immunity induced by low-dose IL-12. This was evidenced by the reduced effect of low-dose IL-12 in NKT cell-deficient TCR Jα281$^{-/-}$ mice ($p < 0.005$).

Another distinction between previous experiments examining B16F10 melanoma metastasis was the timing of IL-12 administration (26, 28). Therefore, we examined the possibility that pretreatment with high-dose IL-12 before tumor inoculation may be sufficient to activate effector NK cells in the absence of NKT cells, whereas delayed treatment with high-dose IL-12 (i.e., commencing on day 3 after tumor inoculation (26)) may require NKT cells to amplify NK cell effector function, particularly in a tumor-burdened host. When mice received 2500 U of IL-12 over 10 days from 3 days after B16F10 tumor inoculation (delayed), treatment was less effective, and the relative role of NKT cells was enhanced (Fig. 1c). With mice that received either low-dose IL-12 or delayed treatment with IL-12, IL-12 mediated anti-tumor immunity via both pfp and IFN-γ (Fig. 1c). This contrasts with an exclusive role for pfp in anti-tumor activity mediated by high-dose IL-12 (Fig. 1b) and correlates with a greater role for NKT cells at lower doses of IL-12.

EL4-S3:NK cells and pfp are sufficient for the anti-metastatic effect of IL-12

To follow up the above observations in another model of experimental tumor metastasis (involving another organ site), we next examined hepatic metastasis of the MHC class I-deficient EL4-S3 lymphoma. We have previously demonstrated that the experimental hepatic metastasis of EL4-S3 lymphoma cells was naturally controlled by NK cells in a pfp-dependent and IL-12-independent manner regardless of the cell number inoculated (29, 34). These data were confirmed at a dose of 5000 cells as shown in Fig. 2a. Furthermore, IL-12 had been shown to control the metastasis of parental EL4 in both B6 and *nu/nu* mice (35). Therefore, it remained to be established whether exogenous IL-12 could control hepatic metastasis of EL4-S3 tumor cells and what immune cells and effector mechanisms were responsible for protection. As shown in Fig. 2b, high-dose IL-12 therapy (2500 U over 10 days) had a profound inhibitory effect on the development of hepatic metastases in B6, B6.RAG-1$^{-/-}$, and B6.Jα281$^{-/-}$ mice. A lack of effect of IL-12 on the control of EL4-S3 hepatic metastasis in NK cell-depleted mice further confirmed that NK cells ($p < 0.005$), but not NKT or T cells, mediated the anti-tumor effects of IL-12 against this MHC class I- and CD1-deficient tumor. Treatment of B6.pfp$^{-/-}$ and B6.IFN-$\gamma^{-/-}$ mice with IL-12 suggested that pfp-dependent NK cell cytotoxicity was sufficient for the anti-metastatic effect of IL-12. Surprisingly, when a low dose of IL-12 was administered (500 U over 10 days) or a high-dose delayed, NK cells were again critical for the IL-12-mediated control of EL4-S3 tumor metastasis (Fig. 2c). Thus, while it has been shown that administration of IL-12 can elevate the cytotoxic activity of liver NK1.1$^+$ T cells (35) and that adoptively transferred NKT cells can participate in the clearance of the EL4 lymphoma (27), clearly NK cells alone or upon IL-12 administration control the metastasis of the EL4-S3 lymphoma. It is possible that EL4-S3 lymphoma cells may elicit a distinct immune response to parental EL4 because of

FIGURE 1. The dose and timing of IL-12 treatment dictate the relative roles of NK cells and NKT cells in its anti-metastatic effect against B16F10 melanoma. *a*, Groups of five B6, B6.pfp$^{-/-}$, B6.IFN-$\gamma^{-/-}$, or B6.Jα281$^{-/-}$ mice or B6 mice treated with anti-NK1.1 mAb on days -2, 0 (the day of B16F10 tumor inoculation), and 7 were inoculated i.v. with decreasing numbers (5×10^5, 1×10^5, 5×10^4) of B16F10 tumor cells as indicated. *b*, Groups of 5–10 mice, as described above, including B6.RAG-1$^{-/-}$ mice, were inoculated i.v. with 5×10^5 B16F10 tumor cells (untreated). Similar groups of mice were treated i.p. with 250 U of IL-12 on days -5, -4, -3, -2, -1 before and days 1–5 after B16F10 tumor inoculation (high). *c*, Groups of five B6, B6.Jα281$^{-/-}$, B6.pfp$^{-/-}$, and B6.IFN-$\gamma^{-/-}$ mice or B6 mice treated with anti-NK1.1 mAb were inoculated i.v. with 5×10^5 B16F10 tumor cells (untreated). Similar groups of mice were treated i.p. with 250 U of IL-12 on days 3–12 after B16F10 tumor inoculation (delayed) or with 50 U of IL-12 on days -5, -4, -3, -2, and -1 before and days 1–5 after tumor inoculation (low). In all experiments, 14 days after tumor inoculation the lungs of these mice were harvested, and colonies were counted and recorded as the mean number of colonies $\pm$ SE. Asterisks indicate the groups in which IL-12 treatment significantly enhanced that group's number of lung metastases above that in the same treated control wild-type B6 mice (by Mann-Whitney test: *, $p < 0.05$; **, $p < 0.01$).
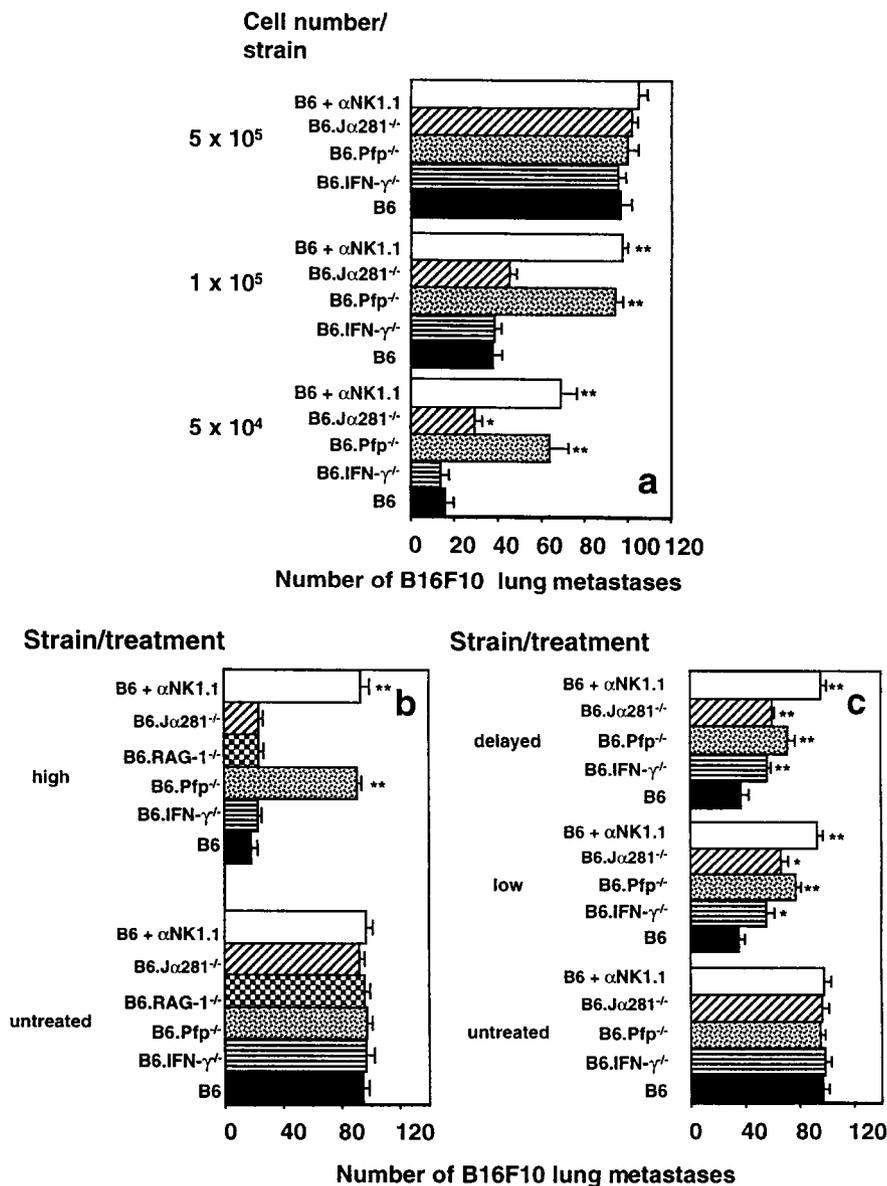
their lack of MHC class I or CD1. Certainly, it has been shown that class I status can greatly influence NK cell recruitment to tumors (36, 37). Regardless, these data suggest that IL-12 treatment controls at least one tumor quite independently of NKT cells and the dose of IL-12 administered.

RM-1:NK cells, NKT cells, pfp, and IFN-γ contribute to the anti-metastatic effect of IL-12

Because distinct differences were observed between B16F10 and EL4-S3 tumor models, we elected to examine another model of tumor metastasis in which we had previously demonstrated a clear role for NKT cells in natural protection. At low cell numbers inoculated, innate protection from RM-1 tumor metastasis has previously been shown to be mediated by both NK and NKT cells (29, 33). Confirming these previous reports, B6 mice depleted of NK1.1$^+$ cells displayed a significantly higher number of RM-1 lung metastases than untreated B6 mice or B6 mice deficient for T cells (RAG-1$^{-/-}$) or NKT cells (Jα281$^{-/-}$; Fig. 3*a*). The major effector molecules involved in host protection were pfp and, to a lesser extent, IFN-γ, as evidenced by the significant increase in RM-1 lung metastases in mice deficient for each of these mole-

cules (Fig. 3*a*). We have previously shown that there was no apparent role for Fas ligand or TNF effector molecules in this model of metastasis (29). As shown in Fig. 3*b*, high-dose IL-12 did have a significant therapeutic effect against experimental RM-1 tumor metastasis to the lung. Furthermore, IL-12-induced anti-tumor immunity was mediated by NK1.1$^+$ cells, because clearly IL-12 was without effect in anti-NK1.1 mAb-treated mice. High-dose IL-12 was also very effective in B6.Jα281$^{-/-}$ and B6.RAG-1$^{-/-}$ mice, although some reduction in IL-12 activity was noted (Fig. 3*b*). Consistent with the B16F10 model, these data suggested that the relative role of NKT cells was minor following high-dose IL-12. High-dose IL-12 only partially protected B6.Pfp$^{-/-}$ mice, suggesting that Pfp was the major effector molecule responsible for both IL-12-induced and natural anti-tumor immunity against RM-1 tumor cells. In concert with our previous observations in the B16F10 model (Fig. 1*c*), the relative role of NKT cells in anti-tumor immunity was greater in mice receiving low-dose IL-12 or delayed treatment with IL-12 (Fig. 3*c*), and both pfp and IFN-γ controlled tumor metastasis (data not shown). Overall, in three different models of experimental tumor metastasis we have demonstrated that the level of IL-12 administered dictated the relative roles of NK
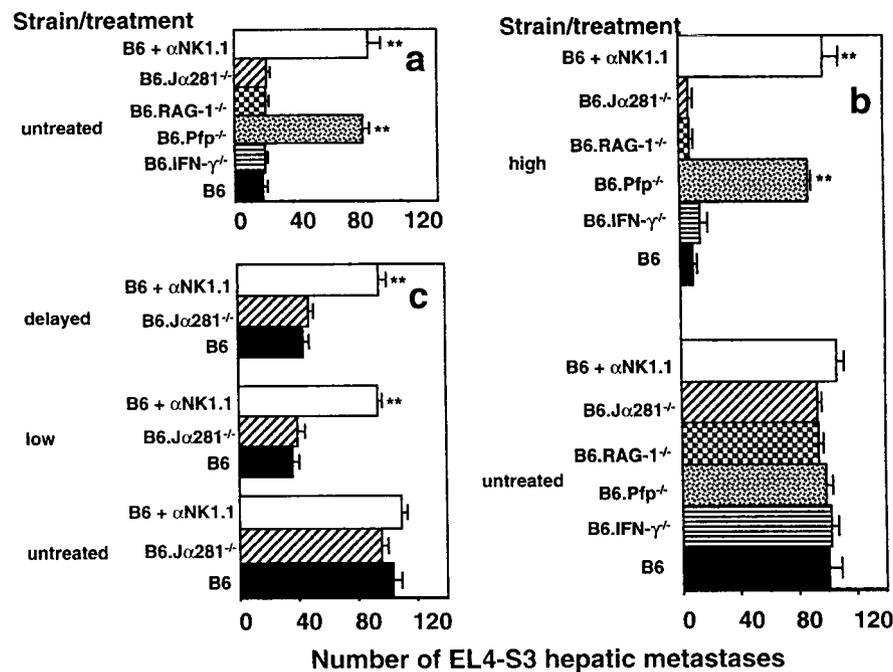
FIGURE 2. NK cells and pfp are sufficient for the anti-metastatic effect of IL-12 against EL4-S3 lymphoma. *a*, Groups of 10 B6, B6.Pfp$^{-/-}$, B6.IFN-$\gamma^{-/-}$, B6.RAG-1$^{-/-}$, or B6.Jα281$^{-/-}$ mice or B6 mice treated with anti-NK1.1 mAb on days −2, 0 (the day of B16F10 tumor inoculation), and 7 were inoculated i.v. with 5 × 10^3 EL4-S3 tumor cells as indicated. *b*, Groups of mice as described above were inoculated i.v. with 5 × 10^4 EL4-S3 tumor cells (untreated). Similar groups of mice were treated i.p. with 250 U of IL-12 on days −5, −4, −3, −2, and −1 before and days 1–5 after EL4-S3 tumor inoculation (high). *c*, Groups of 5–10 B6 and B6.Jα281$^{-/-}$ mice or B6 mice treated with anti-NK1.1 mAb were inoculated i.v. with 5 × 10^4 EL4-S3 tumor cells (untreated). Similar groups of mice were treated i.p. with 250 U of IL-12 on days 3–12 after EL4-S3 tumor inoculation (delayed) or with 50 U of IL-12 on days −5, −4, −3, −2, and −1 before and days 1–5 after tumor inoculation (low). In all experiments, 14 days after tumor inoculation the lungs of these mice were harvested and fixed, and colonies counted and recorded as the mean number of colonies ± SE. Asterisks indicate the groups in which IL-12 treatment significantly enhanced that group's number of lung metastases above that in control wild-type treated B6 mice (by Mann-Whitney test: *, *p* < 0.05; **, *p* < 0.01).

cells and NKT cells in host immune protection from tumor metastasis. Higher doses of IL-12 preferentially employ NK cells, while lower doses of IL-12 favor a greater role for NKT cells in IL-12-induced anti-tumor immunity. Nevertheless, our studies also indicate that the relative importance of NK and NKT cells in both natural and IL-12-mediated immunity is tumor model dependent, and thus in some tumor models IL-12 may exert its anti-tumor activities independently of NKT cells.

Concluding remarks

In summary, both NK and NKT cells can contribute to natural and IL-12-induced immunity against tumor initiation and metastasis; however, the relative role of each is tumor and therapy dependent. It is clear that the role of NKT cells in natural anti-tumor immunity varies from critical to nonexistent depending upon the tumor model examined. In tumor models in which both NK and NKT cells clearly contributed to tumor immunity (B16F10 and RM-1), NKT cells were principally required for the low-dose activities of IL-12. It is not surprising that NKT cells are more sensitive to low doses of IL-12 in vivo, given that NKT cells express higher levels of IL-12R than NK cells (38). Thus, defects in tumor cell rejection were observed in NKT cell-deficient mice when the IL-12 administered was limiting. NKT cells were also required when high-dose IL-12 treatment was delayed, suggesting that tumor burden may influence the relative roles of NK and NKT cells. Carnaud et al. (39) have demonstrated that NKT cells may provide a critical initial stimulation of NK cells via IFN-γ, and IFN-γ can, in turn, up-regulate IL-12R on NK cells (40). This networking between NKT and NK cells may be an important factor in the relative

contribution of each of these populations to natural anti-tumor immunity. In this light it was interesting to note a correlation between the relative roles of NKT cells and IFN-γ in the B16F10 and RM-1 tumor models.

IL-12 may also be a critical component of NKT cell activation in natural anti-tumor immunity. Data provided herein with RM-1 and previously with RM-1 (29, 33, 34) and methylcholanthrene-induced fibrosarcomas (33) suggests that there is a strong correlation between the activities of endogenous IL-12 and NKT cells. If it was simply that the level of IL-12 dictated whether NKT cells or NK cells were critical for an effective anti-tumor response, then one might expect NKT cells to be more responsive than NK cells to local and limiting concentrations of endogenous IL-12 triggered in a natural response. This does not seem to be the case in every tumor model. What remains to be determined is why some tumors are naturally inhibited by NKT cells and others are not. All three tumors used in this study are susceptible to direct cytotoxicity mediated by NK and NKT cells (26–28, 29, 33, 35), and thus, the differential roles of NK/NKT cells in each model are probably not explained by target cell sensitivity. B16F10 and RM-1 tumor models evaluated lung metastasis, whereas EL4-S3 was examined in a hepatic metastasis model. However, it is unlikely that the site of tumor colonization explains the differential roles of NK/NKT cells in each model, because lung contains NKT cells, indeed considerably fewer than in liver (41). Furthermore, studies by Cui et al. (26) using B16 melanoma suggested that IL-12 mediated its anti-tumor effect in both the liver and lung via NKT cells. All three tumors lack significant MHC class I or CD1d expression (33, 42,
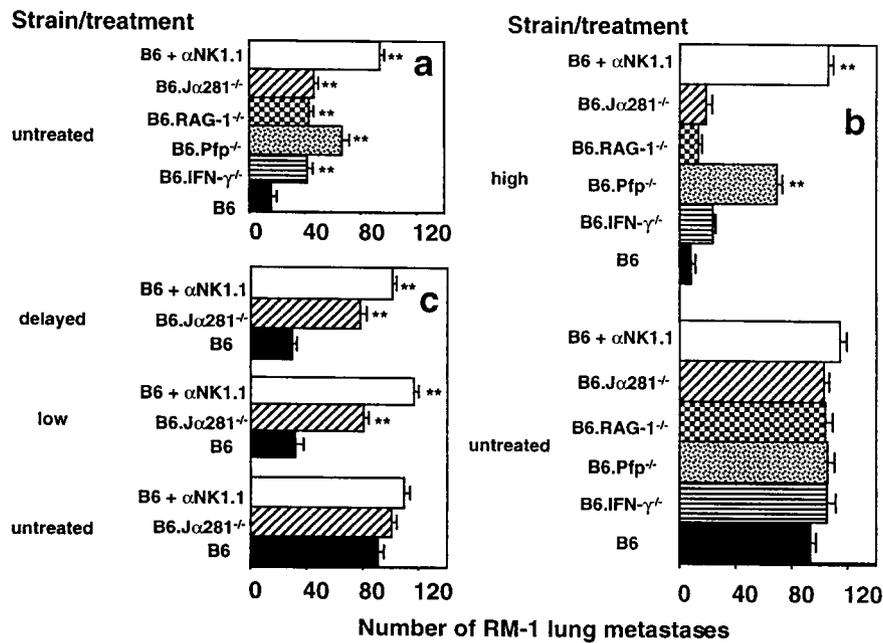
FIGURE 3. RM-1: NK cells, NKT cells, pfp, and IFN-γ contribute to the anti-metastatic effect of IL-12. *a*, Groups of 5–10 B6, B6.pfp$^{-/-}$, B6.IFN-$\gamma^{-/-}$, B6.RAG-1$^{-/-}$, and B6.Jα281$^{-/-}$ mice or B6 mice treated with anti-NK1.1 mAb (on days -2, 0 (the day of i.v. RM-1 tumor inoculation), and 7) were inoculated s.c. between the shoulder blades with RM-1 tumor cells (2×10^6), and tumors were allowed to establish for 9 days. Subcutaneous tumors were then resected, and 1×10^4 RM-1 cells were injected i.v. via the tail vein. *b*, Groups of mice as described above were inoculated i.v. with 1×10^5 RM-1 tumor cells (untreated). Similar groups of mice were treated i.p. with 250 U of IL-12 on days -5, -4, -3, -2, and -1 before and days 1–5 after RM-1 tumor inoculation (high). *c*, Groups of 5–10 B6 and B6.Jα281$^{-/-}$ mice or B6 mice treated with anti-NK1.1 mAb were inoculated i.v. with 1×10^5 RM-1 tumor cells (untreated). Similar groups of mice were treated i.p. with 250 U of IL-12 on days 3–12 after RM-1 tumor inoculation (delayed) or with 50 U of IL-12 on days -5, -4, -3, -2, and -1 before and days 1–5 after tumor inoculation (low). In all experiments, 14 days after tumor inoculation the lungs of these mice were harvested and fixed, and colonies were counted and recorded as the mean number of colonies $\pm$ SE. Asterisks indicate the groups in which IL-12 treatment significantly enhanced that group's number of lung metastases above that in control wild-type treated B6 mice (by Mann-Whitney test: *, $p < 0.05$; **, $p < 0.01$).

43); however, RM-1 and B16F10 do express MHC class I following IFN-γ stimulation in vitro (42). EL4-S3 is a β_2-microglobulin-deficient variant of EL4 and cannot express MHC class I or CD1d. While it is unlikely that up-regulated MHC class I expression influences relative NK/NKT cell activities, it remains to be determined whether CD1d expression may be regulated and relevant in vivo following IL-12 administration. Tumor phenotype and location and corresponding NK/NKT cell phenotypes are factors that must now be addressed in greater detail.

Acknowledgments

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