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In Vivo Antitumor Activity of NKT Cells Activated by the Combination of IL-12 and IL-18

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Interleukin-12 and IL-18 have been demonstrated to potentiate innate immunity in a variety of experimental tumor models, but the functional roles of NK and/or NKT cells and their mechanism of action in these models have not been fully addressed. Through adoptive transfer of NKT cells activated in vitro with a combination of IL-12 plus IL-18 (IL-12/IL-18 NKT) into syngeneic animals, we demonstrate in this study that IL-12/IL-18 NKT cells are essential and collaborate with the host’s own NK cells in natural host immunity against the growth of ALC and MC57X syngeneic tumors. The relative roles of the adoptively transferred IL-12/IL-18 NKT cells and endogenous NK cells in host protection were first shown in normal C57BL/6 (B6) mice treated with anti-asialo GM1 Ab that selectively depletes NK cells; second, in B6.TCRJα281−/− mice specifically deficient for NKT cells; and third, in B6.scid mice that also lack NKT cells. Furthermore, by injecting normal B6 mice with anti-IL-2 and/or anti-IFN-γ mAb, we could demonstrate that effective innate immunity against both types of syngeneic tumors was dependent on the production of IL-2 and IFN-γ by the adoptively transferred NKT cells. In vitro studies confirmed both the secretion of IL-2 and IFN-γ by the IL-12/IL-18-activated NKT cells and their collaborative role with NK cells for lysis of ALC and MC57X syngeneic tumor targets. This is the first description of an antitumor function of IL-12/IL-18 NKT cells adoptively transferred into syngeneic hosts that provides the basis for a new modality in the cellular immunotherapy of cancer. The Journal of Immunology, 2003, 171: 2953–2959.
mechanisms including activation of NK and CTL cytotoxicities (41), and combined systemic administration of IL-18 and IL-12 has been demonstrated to stimulate the antitumor activity of dendritic cells pulsed with tumor cDNA (42). Finally, in a most recent work, Hashimoto et al. (43) using NK-depleted mice or V6 NK T cell-deficient animals indicated that conventional NK, but not NKT cells are major effectors in IL-18-induced innate immunity.

Detailed analysis of the in vivo cellular mechanisms induced by IL-18 alone or in combination with IL-12, resulting in inhibition of tumor growth, has been hampered by the fact that these cytokines were administered exogenously or were secreted locally by genetically modified tumor cells, thereby influencing several immune pathways of which only few were analyzed (i.e., those concerning cytotoxic responses). In the present study, we activated in vitro highly purified NK or NKT lymphocytes by a mixture of IL-12 and IL-18, which were subsequently transferred into B6 normal, NKT-deficient (NKT–/–), or SCID mice inoculated with syngeneic tumors. By performing such experiments, we were able to demonstrate that NKT cells when activated in vitro with a combination of IL-12 and IL-18 are sufficient, via an IL-2- and IFN-γ-dependent collaboration with the host’s own NK cells, to inhibit tumor progression in vivo.

Materials and Methods

Mice

Inbred (B6) mice were purchased from the animal breeding facility of the Hellenic Pasteur Institute (Athens, Greece), and B6.Jrscid/scid mice from M. Taniguchi (Chiba University School of Medicine, Chiba, Japan). B6.CB17-PrkdΔ−Δluc (B6,scid) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice aged 6–8 wk old were used in all experiments, which were performed according to animal experimental ethics committee guidelines.

Cell lines

The two tumors used in this study are of B6 origin. MC57x is a methylcholanthrene-induced fibrosarcoma, and ALC is a T cell lymphoma induced by radiation leukemia virus D-RadLV. Both cell lines were provided by R. Kiessling (Department of Oncology and Pathology, Karolinska Hospital, Stockholm, Sweden). ALC was propagated by i.p. transfer in B6 mice, and it grew as an ascites tumor. MC57x was propagated by s.c. injection of cell suspensions prepared by passing the tumor tissue through a wire mesh in RPMI 1640 medium (Life Technologies, Grand Island, NY). In the experiments described below, ALC or MC57x cells were inoculated s.c. in the right flank and grew as solid tumors at the injection site.

Cell preparation

Mononuclear cells were obtained from spleens and liver of mice by purification over Histopaque-1083 (Sigma-Aldrich, St. Louis, MO). DX-5 (NK.1.1) cells were purified from murine mononuclear cells by positive selection using anti-DX-5 microbeads, according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). We targeted the DX-5 Ag for isolating total NK cells because this is much less mouse strain specific than the NK cell marker NK1.1. DX-5 NK cells isolated by the same procedure were successfully used to study NK cell-mediated cytotoxicity in normal and immunodeficient mice in vitro (44) or by adoptive transfer (45). NK1.1 CD3+ (NKT) and NK 1.1 CD3+ (NK) were further separated from total DX-5 cells by cell sorting on an ALTRA cell sorter ( Coulter, Hialeah, FL) using FITC-conjugated anti-CD3 mAb (clone 145-2C11) and PE-conjugated anti-NK 1.1 mAb (clone PK 136), both purchased from BD PharMingen (San Diego, CA). After sorting, NKT cells were >98% NK 1.1 and >98% CD3+, whereas NK cells were >98% NK1.1 and <1% CD3+. By applying this method, we have been able to isolate 2–2.5 × 106 NKT cells and 4–5 × 106 NK cells per mouse.

Cell cultures

Highly purified NKT or NK cells (1 × 106 cells/ml) were cultured in 14-ml conical tubes (Costar, Cambridge, MA) in RPMI 1640 culture medium supplemented with 10% FCS (Life Technologies), 2 mM l-glutamine (Sigma-Aldrich), 5 × 10−3 M 2-ME (Sigma-Aldrich), and 1 mM sodium pyruvate (Sigma-Aldrich) (complete medium). Cytokines were added at the following final concentrations: murine IL-18 at 100 ng/ml (Peprotec EC, London, U.K.) and murine rIL-12 at 10 ng/ml (R&D Systems, Abingdon, U.K.). The doses of each of the cytokines used in this study have been assayed for optimal stimulation of NKT cells in preliminary experiments (data not shown). One day later, supernatants were removed for cytokine (IL-2 and IFN-γ) determination using specific ELISA kits (R&D Systems). The cultured NKT cells were then washed once in RPMI 1640, resuspended in fresh complete medium, placed in 50-μl aliquots into wells of 96-well U-bottom plates (Costar), and incubated in CO2 incubators for 48 h with 50 μl containing equal number of freshly isolated syngeneic NK cells or NKT cells (as a control). In some experiments, NK cells were added in the IL-12/IL-18 NKT cell cultures with mixtures of anti-IL-2 plus anti-IFN-γ mAbs, each at 10 μg/ml final concentration (included in the 50-μl aliquots). The same mAbs were used for in vivo IL-2 and IFN-γ neutralization (see below).

At the end of the incubation, 100-μl aliquots containing 5 × 105 51Cr-labeled tumor targets were added to each culture at the indicated E:T ratios, and incubation was prolonged for another 12 h. Labeling of tumor targets and estimation of percentage of cytotoxicity have been described in detail elsewhere (46).

Tumor regression models

Mice were inoculated s.c. with 2 × 106 MC57x or ALC tumor cells, each in 0.5 ml PBS. Injections with IL-12/IL-18 NKT cells, which have been preincubated for 24 h with IL-12/IL-18, were administered i.p. simultaneously with tumor cells on day 0 and on days 1 and 2 thereafter (0.5 × 106 cells/injection/mouse). Tumor growth in mice receiving only tumor cells was first observed 3 wk after tumor inoculation (painless tumor), and mice were monitored for another 5 wk. The observation was terminated with the euthanization of mice when the tumor mass grew up to 1.5 cm in diameter (usually between 60 and 70 days after tumor inoculation). Mice treated with cytokine-derived NKT effector cells were considered free of tumor if there was no palpable tumor at the site of injection during the time period of 8 wk. In vivo depletion of NK cells only was performed by injections of anti-asialo GM1 Ab (Cedarlane Laboratories, Ontario, Canada) (20–30 μl per injection) on days 1 and 0 (day of tumor inoculation) and weekly thereafter (47). Assessment of cell depletion was performed following preparation of cell suspensions from spleen and liver, as described (14, 47). This protocol was shown to maintain significant depletion of the NK population: in a total of seven mice tested, the percentage of NK cells was reduced from 3.5–5.0% in the spleens of untreated mice to 0.2–0.4%, and from 6–8% in their livers to 0.5–1.0% after treatment, whereas the levels of NKT cells remained unaffected (0.8–1.3% in the spleen and 20–30% in the liver). For in vivo IL-2 and/or IFN-γ neutralization, mice received per injection 100 μg anti-mouse IL-2 mAb (clone JES6-1A12, IgG1) and/or 100 μg anti-mouse IFN-γ mAb (clone 37895/1/1, IgG2a), both purchased from R&D Systems. Injections were given i.v. in 0.2 ml PBS on day 0 and weekly thereafter. Rat IgG1 clone R3-34) and rat IgG2a clone (clone R35-95), both from BD Pharmingen, were administered as isotype-matched controls for anti-IL-2 and anti-IFN-γ mAb, respectively.

Results

NK1.1+CD3+ (NKT) cells activated in vitro with IL-12 plus IL-18 and adoptively transferred into syngeneic mice induce protection against the growth of ALC and MC57x tumors

We (5) and others (45) have shown that both ALC lymphoma (H-2b) and MC57x fibrosarcoma (H-2b) grow progressively slowly when injected into syngeneic animals at relatively low numbers (i.e., 1 × 103 to 2 × 103 cells), developing large solid tumors (1.5–2.0 cm diameter) after 6–7 wk. Adaptive immunity against these tumors can be generated following active immunization with irradiated ALC or MC57x tumor cells, whereby C57BL/6 mice develop T cell-mediated antitumor immune responses resulting in tumor rejection (48, 49). However, a clear picture as to how innate immunity can control the growth of these tumors has been lacking. Considering the important role of NKT cells in immunosurveillance of sarcomas (50) as well as the potential of IL-12 plus IL-18 to activate NKT cells independently of TCR engagement (38), we sought to induce protection in B6 recipients inoculated with ALC or MC57x tumors by adoptive transfer of NKT cells, preincubated in vitro with a combination of IL-12 and IL-18. The vast majority of ALC-injected B6 mice and all B6 mice inoculated with MC57x syngeneic tumors demonstrated...
complete inhibition of tumor growth when treated with syngeneic NKT cells (Fig. 1). Single cytokine incubation (i.e., IL-12 or IL-18) did not induce significant protection (Fig. 1). In contrast to normal mice, B6 mice specifically depleted of NK cells using anti-asialo GM1 injections were not protected against ALC or MC57X tumor growth upon treatment with IL-12/IL-18 NKT cells (Fig. 2). This suggested that host NK cells in synergy with the adoptively transferred syngeneic IL-12/IL-18 NKT cells were indispensable for innate protection from ALC- and MC57X-induced tumor formation. To investigate the role of endogenous NKT cells in these tumor models, we have used B6/Ja 281/−/− and B6.scid mice, both of which lack NKT cells. As shown in Fig. 2, both types of strains were protected upon transfer of IL-12/IL-18 NKT cells. As with normal B6 mice, treatment with anti-asialo GM1 rendered B6/Ja 281/−/− and B6.scid mice susceptible to tumor growth. These data suggested that neither endogenous NKT nor T cells (which are also absent in B6.scid mice) played a significant role in tumor rejection.

**FIGURE 1.** Adoptive transfer of NKT cells cultured in vitro with IL-12 plus IL-18 (IL-12/IL-18 NKT) protects B6 mice against ALC and MC57X tumor growth. Groups of B6 mice were inoculated s.c. with 2 × 10⁶ ALC or MC57X tumor cells (day 0). Subgroups of B6 mice received then purified NKT cells (0.5 × 10⁶ cells/mouse) preincubated for 24 h either in plain medium (NKT), or with 10 ng/ml rIL-12 (IL-12/NKT), 100 ng/ml rIL-18 (IL-18/NKT), or 10 ng/ml IL-12 plus 100 ng/ml IL-18 (IL-12/IL-18 NKT) via i.v. adoptive transfer (i.e., three injections per mouse starting on day 0 and for the following 2 days). Tumor development was recorded at 8 wk as a percentage of the mice in each group (in parentheses). Significant difference from the PBS-treated groups (B6) was determined using a Fisher’s exact test. *, p < 0.01. Mean values (±SD) from two independently performed experiments are shown.

**FIGURE 2.** Depletion of NK cells in vivo by anti-asialo GM1 (anti-GM1) treatment abrogates the protective effect of the adoptively transferred IL-12/IL-18 NKT cells. Wild-type mice (B6), B6.Ja281/−/− mice, or B6.scid mice inoculated with ALC or MC57X tumor cells received injections with anti-GM1 during and after treatment with IL-12/IL-18 NKT cells, as described in Materials and Methods. Significant difference from mice that received no anti-GM1 injections was determined using a Fisher’s exact test. *, p < 0.01. Mean values (±SD) from two independently performed experiments are shown.

**IL-12/IL-18 NKT cells produce IFN-γ and IL-2 to activate NK cells for antitumor activity**

Recent reports have demonstrated the capacity of NKT cells to mediate antitumor cytotoxicity directly through interaction with the target cells or indirectly by producing IFN-γ or IL-2 to activate NK cells (12, 13). To test the potential mechanisms regulating potent antitumor innate immunity mediated by IL-12/IL-18 NKT cells in our experimental tumor models, we injected tumor cell-inoculated normal B6 mice with IL-2- and/or IFN-γ-neutralizing mAb during and after treatment with IL-12/IL-18 NKT cells, as described in Materials and Methods. Significant difference from mice that received no anti-GM1 injections was determined using a Fisher’s exact test. *, p < 0.01. Mean values (±SD) from two independently performed experiments are shown.
both IL-2 and IFN-γ (Fig. 4). Significantly lower levels of both IL-2 and IFN-γ were produced when NKT cells were incubated with each of the cytokines alone (data not shown). The same cells when coincubated for another 48 h with freshly isolated syngeneic NK cells induced lysis of both ALC and MC57X tumor targets (Fig. 4) at fairly high levels (50–60% cytotoxicity against both tumor targets at an E:T = 40), which were drastically reduced when mixtures of anti-IL-2 plus anti-IFN-γ mAb were present throughout the 48-h incubation (Fig. 3). In addition, only low levels of tumor cell lysis were observed when NKT cells were added to the IL-12/IL-18 NKT cells (25 and 19% cytotoxicity at an E:T = 40 against ALC and MC57X tumor targets, respectively). These data suggested that IL-12/IL-18 NKT cells mainly act as regulatory cells producing IL-2 and IFN-γ to induce NK cell-mediated cytotoxicity, although they themselves may also act as effectors, by lysing their targets, however, at significantly lower levels.

Discussion

The data presented in this work demonstrated for the first time immunotherapy of syngeneic tumors by adoptively transferred NKT cells activated in vitro by a combination of IL-12 plus IL-18. They also show that in vivo collaboration between the transferred IL-12/IL-18 NKT cells with the host’s own NK cells is required for effective host protection from tumor growth. Furthermore, we demonstrate that IL-2 and IFN-γ produced by the IL-12/IL-18-activated NKT cells are critical components in natural antitumor immunity.

To date, most studies addressing the role of NKT cells in various experimental models of immune response have investigated pathways leading to their activation upon TCR engagement (16). However, only a restricted set of Ags has been found to stimulate NKT cells via TCR in a CD1-dependent manner (11, 12). In apparent contrast with the above studies, NKT cells have also been demonstrated to promote tumor rejection in response to exogenous factors in a TCR-independent manner. In a recently published work, Leite-de-Moraes et al. (38) demonstrated that NKT cells can be fully activated in the absence of TCR cross-linking, in response to IL-18 associated with IL-12. In the same report, it was found that NKT cells stimulated with IL-12 and IL-18 produced high levels of IFN-γ and expressed increased cytotoxic potential against Fas-positive targets. No TCR engagement in NKT cells has also been demonstrated in IL-12-induced tumor rejection models (29, 50) in which NKT cells played an essential role. In these tumor models, IFN-γ and/or perforin were required for IL-12-induced and NKT cell-mediated antitumor immunity. These results agree with the TCR-independent stimulation of NKT cells in our model and imply that several death-inducing ligands must be involved in the capacity of NKT cells to induce target cell apoptosis.

Although NKT cell activation in our system does not require treatment with an exogenous CD1d-binding ligand, we still cannot exclude the possibility that the adoptively transferred IL-12/IL-18 NKT cells may recognize tumor-derived material presented to them in the context of CD1d expressed either by the tumor cell themselves or by host APCs. Crowe et al. (50) have most recently shown that NKT cells in the absence of exogenous stimulatory factors play a significant role in immunosurveillance of 3-methylcholanthrene (MCA)-induced sarcomas, and that this effect depends on CD1d recognition and requires additional involvement of NK cells. Although NK cells are known to be activated following stimulation of NKT cells by α-galactosylceramide (αGalCer) (51–53), no evidence for activation of NKT cells by α-GalCer was provided in the above-mentioned study (50). Thus, it seems that in addition to activation with α-GalCer or IL-12, NKT cells can be stimulated to exert potent antitumor effectors by a variety of TCR-dependent or TCR-independent stimuli that may not be mutually exclusive, that is, NKT cells can be activated both through recognition of CD1d-bound ligands and through the action of a single cytokine or cytokine combination. With respect to this, several studies suggest that dendritic cells upon contact with NKT cells via TCR in a CD1-dependent manner (11, 12). In apparent contrast with the above studies, NKT cells have also been demonstrated to promote tumor rejection in response to exogenous factors in a TCR-independent manner.

Certainly, the data described in this report do not necessarily ascribe a direct in vivo antitumor effect to the transferred IL-12/IL-18 NKT cells, because tumor growth inhibition was not observed when mice were depleted of endogenous NK cells. In addition, our in vitro data clearly demonstrate efficient tumor cell lysis only when the IL-12/IL-18 NKT cells are coincubated with

3 Abbreviations used in this paper: MCA, 3-methylcholanthrene; α-GalCer, α-galactosylceramide.
syngeneic NK cells. Thus, we could assume that the cytokine-induced NKT cells mediate antitumor cytotoxicity indirectly by producing IFN-γ and IL-2 to activate NK cells. A dual function of NKT cells was recently reported by Metelitsa et al. (12), who found that NKT cells recognizing CD1d-bound ligands on tumor targets are stimulated to perform cytotoxicity and also to produce IL-2 that is required for inducing cytotoxic responses in NK cells. In the same report, it was shown that IFN-γ synergistically acted with IL-2 to enhance NK cell cytotoxicity. The likelihood that IFN-γ produced by NKT cells may also lead to NK cell activation was shown in earlier studies in which in vivo injection of α-GalCer in wild-type mice resulted in IFN-γ production and CD69 induction by NK cells. This, however, was not the case when α-GalCer was injected in RAG- or CD1-deficient mice or in wild-type mice pretreated with anti-IFN-γ Abs (56), which suggested that NK cell activation was a secondary event that depended on IFN-γ release by NKT cells. Given the fact that upon a 24-h incubation with IL-12/IL-18 our NKT cells produce both IL-2 and IFN-γ, we suggest that these may have a putative regulatory role by activating syngeneic NK cells. Of course, we cannot rule out the possibility that the critical contribution of IL-12/IL-18 NK cells in vivo may be direct tumor cell lysis. Our in vitro data demonstrated low cytotoxicity levels against both ALC and MC57X tumor targets mediated by the IL-12/IL-18 NK effectors. However, the in vitro data do not necessarily reflect the functional status of effector cells in vivo. For example, cytokine-induced CD3+CD56+ killer cells were demonstrated to mediate superior antitumor effects in vivo, although their in vitro cytotoxicity levels against the same tumor cells were somehow lower compared with those mediated by lymphokine-activated killer cells (57). This is not to say that the IL-12/IL-18 NKT cells protect mice from tumor development by direct lysis, as our data indicate that most of the antitumor cytotoxicity is conducted by NK cells. However, direct tumor cell lysis in vivo by the cytokine-induced NKT cells through feedback stimulation by the already activated NK cells or through TCR-CD1d interactions cannot be excluded. In this respect, it would also be important to examine the in vivo antitumor activity of IL-12/IL-18 NK cells in relation to trans activation of NKT cells. Work is now in progress in our laboratory to address these points.

Sequential production of IFN-γ by α-GalCer- or IL-12-activated NKT cells and NK cells has to date been reported to be essential for the innate control of MCA-induced sarcoma (50). In this study, we provide additional information on how tumors can be innately controlled by both NKT and NK cells by demonstrating that upon IL-12/IL-18 activation, NKT cells produce both IL-2 and IFN-γ, which synergistically act to induce NK cells to mediate antitumor cytotoxicity. At present, we have not determined whether IFN-γ alone could have a direct effect, or, as in agreement with earlier reports (58, 59), only enhances NK cytotoxicity induced by IL-2. IL-2 is well known to directly activate NK cell proliferation and cytotoxicity (60, 61), and, combined with IL-2, IFN-γ may also augment NK cell activation (62, 63). Moreover, IFN-γ produced by NKT cells in vivo may indirectly stimulate NK cell mobilization from blood (60) and induce NK cells themselves to produce IFN-γ (56). However, IFN-γ is not the sole effector molecule produced by the activated NK cells to confer antitumor immunity. It has been reported that NKT cell-dependent activation of NK cells may also result in enhanced perforin and TNF-α production (4, 13, 64), both of which may, additionally to IFN-γ, confer antitumor immunity (5, 13). Therefore, it will be important not only to determine the nature of signals that activate NKT cells, i.e., whether they are either tumor cell dependent (i.e., glycolipid ligands) or independent (e.g., cytokines), but also the signals that regulate the outcome of NKT function to be able to manipulate immune responses toward effective tumor destruction.
Cytokine-induced NKT cell activation has been performed to date in cultures supplemented with 10% FCS or 10% human AB serum (3, 38, 39), thus raising the possibility that serum factors might interfere in these activation pathways. To address this, we repeated our in vitro experiments also in protein-free hybridoma medium. NKT cells grown in this medium in the presence of IL-12/IL-18 produced comparable high levels of IFN-γ and IL-2 in vitro and were as effective in vivo (data not shown) as the IL-12/IL-18-activated NKT cells described in this work that were grown in RPMI 1640 supplemented with 10% FCS. These data suggested that the activation of our NKT cells was dependent solely on the synergistic action of these cytokines.

NK and NKT cells have been shown to contribute to IL-12-induced immunity against tumors. For example, metastasis of the EL4-S3 lymphoma was strictly controlled by NK cells following therapy with high-dose IL-12 (29). Intriguingly, in melanoma B16F10 and lung tumor RM-1 models, both NK and NKT cells contributed to natural protection from tumor metastasis (29). In those 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. A similar situation apparently also applies for the IL-12-induced antitumor immunity. Thus, although NK, but not NKT, cells were demonstrated to play an essential role in the reduction of B16 pulmonary metastases (43), a preceding report suggested that perforin-dependent cytotoxicity of liver NKT cells is augmented in vitro by IL-12 stimulation (65). Thus, it appears that the relative roles of each population are tumor and therapy dependent. In addition, the activation of NK and NKT by IL-12 or IL-18 may follow different kinetics depending on the expression of the cytokine-specific receptors on either subset. To this end, naive NK cells were found to express at high levels the IL-18R, whereas NKT cells did so only after IL-12 stimulation (43). Thus, our in vitro model for NKT cell activation with the combination of IL-12 plus IL-18 is in line with those reports. At present, it is not known whether the observed collaboration between IL-12/IL-18-activated NKT cells with syngeneic NK cells would be as effective in metastatic tumor models. Smyth et al. (29) recently reported that in the i.v. RM-1 lung tumor model, NKT (in addition to NK) cells appeared to also contribute to regression of metastatic lesions. We are currently working on the establishment of lung and/or liver metastatic tumor models via i.v. injections of ALC or MC57X tumor cells to investigate this point.

Our work provides the first evidence that NKT cells activated in vitro with a combination of IL-12 plus IL-18 may be critical in natural immune responses against the growth of the MCA-induced MC57X fibrosarcoma and the ALC lymphoma. These results demonstrate the significance of a novel therapeutic mechanism and encourage clinical investigation of the immunoregulatory and antitumor activities of IL-12/IL-18-activated NKT cells.

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References


