Interleukin 18 Induces the Sequential Activation of Natural Killer Cells and Cytotoxic T Lymphocytes to Protect Syngeneic Mice from Transplantation with Meth A Sarcoma

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ABSTRACT

We have recently reported that interleukin 18 (IL-18) pretreatment induces immunologically mediated antitumor effects in BALB/c mice injected i.p. with syngeneic Meth A sarcoma. In this study, mice were pretreated with IL-18 before Meth A transplantation, and immunocompetence in pretreated or untreated tumor-bearing mice (TBM) 3, 9, and 15 days after transplantation was compared with that of normal mice. On day 3, pretreated TBM mitogen-stimulated spleen cells produced significantly decreased levels of IL-2 and IFN-γ during 24-h culture. In contrast, IL-10 and granulocyte macrophage colony-stimulating factor productions were significantly enhanced in pretreated TBM cultures, and natural killer (NK) cell activity was also significantly augmented. Spleenomage was also observed in the pretreated TBM on day 3, and the proliferating cells were identified as asialo GM1+ cells by flow cytometry. Cytotoxic activity of pretreated TBM spleen cells after a 5-day mixed lymphocyte-tumor cell culture did not differ from that of untreated TBM and normal mice on day 3 but was significantly enhanced on days 9 and 15 compared with that observed in normal mice and untreated TBM. Concurrently, the production of IL-2 and of IL-10 recovered and decreased, respectively, and NK activity dropped to normal levels. The effects of IL-18 on cytokine production and NK activity observed on day 3 treated TBM were also reproduced in normal mice. In conclusion, IL-18 seems to enhance the generation of NK activity early after tumor transplantation and simultaneously induces an increase and a decrease in the production of IL-10 and IL-2, respectively. As NK activity subsides to normal levels and IL-10 synthesis decreases, IL-2 synthesis is restored, and cytolytic cell activity is significantly enhanced. These results provide new insight into the immunologically mediated antitumor effects of IL-18.

INTRODUCTION

A functioning immune system not only protects the host from invasion by opportunistic foreign microorganisms but is also a system of defense against syngeneic cells that have departed from cell growth control networks that normally function to prevent uncontrolled cell growth and the evolution of neoplastic disease. Studies have shown that malignant cells can develop mechanisms that prevent the induction of an effective antitumor immune response, leading to in vivo selection and the emergence of malignant cells, which are hardy and more difficult for the immune system to eliminate (1, 2). Models that explain how malignant cells escape from immunological surveillance networks and factors that stimulate an effective antitumor immune response present new opportunities and target molecules for the immunotherapy of malignant disease (3–5).

IL-18,3 which was previously known as IFN-γ-inducing factor, is an 18.3-kDa protein that induces the production of IFN-γ by mouse cells in vivo and in vitro (6), augments mouse and human NK cell activity (6, 7), enhances IL-2-mediated mouse and human T-cell proliferation, and has a tendency to augment the production of Th type 1 cytokine production by murine Th 1 clones and enriched human T cells in vitro (8, 9). Murine NK cell clones established from the liver have also been shown to exhibit enhanced Fas-mediated cytotoxic effects when stimulated with murine IL-18 (10). Some of the immunological effects induced by this molecule are similar to those of IL-12, a cytokine that is undergoing clinical trials in patients with malignant disease; likewise, IL-18 was also considered a potential agent for the induction of immunologically mediated antitumor responses in vivo.

We have recently reported that IL-18 exhibits significant antitumor effects in BALB/c mice transplanted i.p. with the syngeneic sarcoma Meth A, especially when administered before tumor transplantation, and also induces immunological memory in the surviving mice (11). According to this treatment schedule, at least 90% of treated mice survived long term after treatment. The in vivo antitumor effects of pretreatment with IL-18 in the Meth A model were abrogated by eliminating NK cell activity after treatment with anti-asialo GM1 polyclonal antibody. Mice that survived injection with Meth A after pretreatment with IL-18 spontaneously rejected a second transplantation of the tumor cells about 3 months after the first tumor injection. Interestingly, the effector cells responsible for immunological memory in MLTCs during in vitro stimulation of mouse spleen cells with MMC-treated Meth A cells were found to be CD4+ T cells by depletion with antibodies and rabbit complement. The immunological events that occur between the induction of NK activity and the induction of CD4+ memory T cells are the subject of the present study.

MATERIALS AND METHODS

Experimental Animals. Eight-week-old female BALB/c mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan) and used in our experiments 2–3 weeks after procurement. Mice were kept under conventional conditions in a specific pathogen-free environment and provided with standard feed and water ad libitum.

Reagents. Murine IL-18 is a product of Hayashibara Biochemical Laboratories (Okayama, Japan) and was produced and purified as described previously (6). Cell culture was performed in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Whittaker, Walkersville, MD), penicillin (100 units/ml), and streptomycin (50 μg/ml), referred from here onward to as complete medium. Cells were incubated at 37°C in a 5% CO2 humidified atmosphere. Meth A sarcoma grows aggressively in syngeneic BALB/c mice and was obtained from American Type Culture Collection (Rockville, MD). These cells were maintained in culture in vitro and occasionally passaged i.p. in syngeneic mice to ensure that the tumorigenic potential of the cells did not diminish with culture.

In Vivo Treatment Protocols. Mice were injected i.p. with 1 μg of IL-18 or vehicle twice, 3 days and 6 h before being transplanted i.p. with 1 × 106 Meth A cells. We have previously reported that this IL-18 pretreatment schedule is very effective at preventing progressive tumor growth in vivo (11). Control normal mice were included in all our experiments. Untreated TBM seemed to be normal for the first week after i.p. transplantation of Meth A...
cells; however, from about day 10, the abdomens of the mice seemed to expand, and a corresponding increase in body weight of the untreated mice was recorded. Mice pretreated with IL-18 did not accumulate malignant ascites. The increase in body weight of the untreated TBM correlated well with an increase in the accumulation of malignant ascites and is represented in Fig. 1 as an indicator of when immunocompetency was examined in this study. On day 15 after tumor transplantation, untreated TBM were still feeding well and moved about freely. About 18 days after transplantation of the tumor cells, mice became morbid, and before the mice died of ascites, a decrease in body weight was recorded. Based on this data, days 3, 9, and 15 were chosen to determine changes in immunocompetency in the treated and untreated TBM as compared with control normal mice.

In Vitro Proliferation and Cytokine Production Assays. On days 3, 9, and 15 after injecting mice with Meth A cells, spleens were removed from the TBM treated with IL-18 or untreated, and control non-tumor-bearing mice (n = 3 mice/group). Single cell suspensions were obtained in loose-fitting ground glass homogenizers. Erythrocytes were removed by hypoosmotic shock, and the remaining spleen cells were washed twice with cold 0% FBS RPMI 1640 and once with complete medium. Cells were resuspended in complete medium containing 2.5 µg/ml Con A and adjusted to a density of 1 \times 10^5 cells/ml. To determine proliferation in the presence of a T-cell mitogen, 100-µl aliquots of the cell suspensions were seeded per well in triplicate in flat-bottomed 96-well plates, and 100-µl aliquots of medium only or medium containing various concentrations of Con A were added. Plates were incubated at 37°C for 72 h and pulsed with 0.5 µCi of [3H]thymidine/well for the last 18 h of culture. Cells were harvested, and incorporated thymidine was determined on an automated direct beta counter (Becton Dickinson, Bedford, MA). To determine cytokine production, 2-ml volumes of the same cell suspensions were seeded in triplicate in 12-well culture plates, and the cells were cultured for 24 h. Culture supernatants were harvested, passed through 0.22-µm filters, and stored at -20°C until assay for cytokine levels by ELISA.

Flow Cytometric Analysis. Erythrocyte-free spleen cell suspensions of the treated and untreated normal and tumor-bearing groups were obtained as described elsewhere in this study. Cells were washed twice with cold PBS and incubated with antibodies against mouse CD3 (Cedarlane, Ontario, Canada), CD4 (Cedarlane), CD8 (Cedarlane), and asialo GM1 (Wako Fine Chemicals, Osaka, Japan) for 45 min on ice. Cells were washed again with cold PBS and incubated with FITC-conjugated secondary antibodies in the dark. After washing again with PBS, cells were examined for positive staining by flow cytometry (Epics Profile, Hialeah, FL), and the percentage of positively stained cells was determined by the cytometer.

MLTC and Cytotoxicity Assays. To determine specific CTL activity, Meth A cells were washed and suspended in medium at a density of 1 \times 10^6 cells/ml in complete medium. MMC was added to the culture at a concentration of 100 µg/ml, and the cells were incubated for at least an hour, after which cells were washed three times with medium and resuspended at 4 \times 10^5 cells/ml. One-ml volumes of the treated Meth A cell suspensions were seeded in 12-well plates. Next, erythrocyte-free spleen cells were obtained as described above and added to the wells at a cell density of 2 \times 10^7 cells/ml in 1-ml volumes of complete medium containing 20 units/ml IL-2 (final concentration, 10 units/ml). Plates were incubated for 5 days, and spleen cells were collected, washed, and resuspended as 1-ml volumes of 1 \times 10^6 cells/ml. Cells were also treated with either anti-CD4 or anti-CD8 monoclonal antibodies for 30 min on ice and then treated with a final 10-fold dilution of low-toxicity rabbit complement for 30 min at 37°C before washing. To determine cytotoxicity activity, the JAM test was used (12). Briefly, Meth A cells were labeled at 2 \times 10^5 cells/ml with 5.0 µCi/ml [3H]thymidine for 4 h and washed free of the isotope. Effector and target cells were seeded in triplicate in a final volume of 200 µl in round-bottomed well plates at various E:T ratios, and plates were incubated for 18 h before cell harvest and measurement of thymidine incorporation. The percentage of DNA fragmentation was calculated from triplicate wells according to the formula below:

\[
\text{DNA fragmentation} = \frac{(\text{Maximum cpm} - \text{sample cpm})}{\text{Maximum cpm}} \times 100\%
\]

where maximum cpm is the amount of label retained by Meth A cells cultured alone, and sample cpm is the amount of label that was retained by Meth A cells cocultured with effector cells.

Kinetics of NK Cell Activity. Erythrocyte-free spleen cell suspensions were obtained from normal mice and treated or untreated TBM on the indicated days and seeded in 96-well round-bottomed culture plates containing 3Cr-labeled YAC-1 cells at various E:T ratios. Plates were incubated for 4 h, after which three control wells containing labeled YAC-1 cells received 2x HCl to lyse the cells (maximum release), and three control wells also containing labeled YAC-1 cells were left undisturbed (spontaneous release). Plates were spun on a centrifuge to sediment the cells, and 100 µl of cell-free supernatants were collected, and the amount of label released in the supernatants was determined by an automated counter. The percentage of cytotoxicity was determined according to the following formula:

\[
\text{Percent cytotoxicity} = \frac{(\text{Sample cpm} - \text{spontaneous cpm})}{(\text{Maximum cpm} - \text{spontaneous cpm})} \times 100\%
\]

Effects of In Vivo Treatment with IL-18 on Normal Mice. To determine whether the effects observed in TBM pretreated with IL-18 can also be observed in normal mice, we treated mice with 1 µg of IL-18 i.p. once and again 3 days later, and we harvested spleens from the treated mice and normal mice 6 days after the first injection of IL-18. Erythrocyte-free spleen cell suspensions were prepared, and proliferative responses, cytokine production, and NK activity were examined as described in the preceding sections of this paper.

Statistical Evaluations. Degrees of significance between the groups tested were determined by Student's t test. All experiments were performed at least twice and were reproducible.

RESULTS

Changes in the Proliferative Response to Con A of Untreated and IL-18-treated TBM. Proliferation of mitogen-stimulated TBM spleen cells did not differ greatly from that of normal mouse spleen cells on days 3, 9, and 15 after tumor transplantation; however, in the absence of mitogen, day 3 pretreated TBM spleen cells proliferated about 13-fold of the levels observed in untreated TBM and normal mice. On day 9 after tumor transplantation, pretreated TBM spleen cell proliferation was still significantly enhanced in the absence of mitogen when compared to that of normal controls (Fig. 2). Untreated
TBM spleen cells also exhibited enhanced proliferation, but this was lower than that observed in pretreated TBM. On day 15, proliferation of both treated and untreated TBM spleen cells was significantly greater than that observed in controls; however, infiltration of untreated TBM spleens by Meth A cells could be observed, and the enhanced proliferation in untreated TBM cultures on day 15 may be partly due to infiltrating tumor cells.

**Changes in in Vitro Cytokine Production by Mitogen-stimulated Spleen Cells.** Con A-stimulated spleen cells from day 3 TBM pretreated with IL-18 showed suppressed production of IL-2 and IFN-γ and a simultaneous significant increase in the production of IL-10 during 24 h of culture as compared with normal mouse spleen cell cultures (Fig. 3). The production of IL-2 almost recovered by day 9 and was fully recovered on day 15 in the pretreated group. The production of IL-10 by pretreated TBM spleen cells decreased on days 9 and 15 in inverse correlation with an increase in IL-2 production but remained above control levels. There was also a moderate but significant increase in the production of IL-10 in untreated TBM Con A-stimulated spleen cell cultures as compared to that of controls on days 9 and 15 and a 25% decrease in the production of IL-2 that lasted throughout the observation period. The production of IFN-γ was decreased in treated and untreated TBM cultures after tumor transplantation, whereas the production of GM-CSF in the pretreated group was significantly increased above control levels (Fig. 3). The average concentrations of cytokines produced by mitogen-stimulated normal mouse spleen cells during 24 h in culture are as follows: IL-2, 16.5 ng/ml; IL-10, 600 pg/ml; IFN-γ, 72 IU/ml; and GM-CSF, 133 pg/ml.

**Changes in the Percentages of Spleen Cell Subsets Induced by IL-18.** After staining for different mouse spleen cell subsets, a decrease in the percentages of normal or day 3 TBM spleen cells expressing CD3 was observed by flow cytometry after pretreatment with IL-18 in vivo when compared with control untreated cells (Table 1), which was also reflected in the percentages of CD4- and CD8-expressing cells. A concurrent increase in the percentage of asialo GM1+ cells was also observed in the treated mouse spleens, indicating that the proliferating cells causing splenomegaly on day 3 after in vivo treatment with IL-18 were probably NK cells. On days 9 and 15 after transplantation, the percentages of asialo GM1+ cells in the treated TBM group returned to approximately normal levels, as did those of other spleen cell subsets (data not shown).

**Kinetics of CTL Activity.** On day 3 after transplantation of the tumor cells, the spleen cell cytotoxic activities of untreated TBM and TBM treated with IL-18 were approximately equal to that observed in normal mouse cultures (Fig. 4). The cytotoxic activity observed in untreated and pretreated TBM could be augmented by treating the cells after MLTC with anti-CD8 monoclonal antibody and complement, indicating that CD8+ T cells may be exerting a suppressive effect on cytotoxic antitumor spleen cells in TBM. The cytotoxic activity in pretreated TBM spleens was significantly enhanced on day 9 after tumor injection and was about double that observed in normal mice. Even untreated TBM exhibited a cytotoxic activity higher than that observed in normal mouse spleen cell cultures on day 9. Results obtained for day 15 were similar to those obtained on day 9 after tumor challenge. Subset depletion experiments showed that on days 9 and 15, the cytotoxic activity in pretreated TBM spleens was due to both CD4+ and CD8+ cells. The data shown in Fig. 4 represent results obtained at E:T ratios of 100:1 for all experiments.

**Kinetics of NK Activity.** Our previous study has shown that when TBM pretreated with IL-18 were depleted of asialo GM1+ cells with antibody early after tumor transplantation, the antitumor effects of pretreatment with IL-18 were abolished. NK activity of pretreated TBM on day 3 was significantly enhanced as compared to that of normal mice and untreated TBM (Fig. 5). NK activity of the pretreated mice decreased to normal levels on days 9 and 15 after transplantation, indicating that the augmentation of NK activity in vivo is short-lived. In our experiments, spontaneous release of 51Cr was always less than 10% of the maximum release.

**Changes in Proliferation, Cytokine Production and NK Activity of Normal Mouse Spleen Cells after Treatment with IL-18 in Vivo.** The proliferation of spleen cells from normal mice treated with IL-18 in vivo was significantly enhanced in the absence of mitogen when compared with that observed in control mouse spleens (Fig. 6). This enhanced proliferation seemed to be a maximum, because addition of increasing amounts of the mitogen Con A could not increase proliferation of the cells. Mitogen-stimulated normal mouse spleen cells also exhibited significantly increased productions of IL-10 and GM-CSF and decreased production of IL-2 (Fig. 7). There was no difference in the production of IFN-γ between the treated and control...
groups. As shown in Fig. 8, spleen cells of normal mice treated with IL-18 in vivo also exhibited augmented NK activity against YAC-1 cells in vitro. These results were basically similar to those observed in TBM pretreated with IL-18 but are less pronounced in normal mice and indicate that the immunomodulating effects of IL-18 in this group are intrinsic to IL-18 but are possibly amplified by the transplantation of Meth A cells.

DISCUSSION

We have previously shown that pretreating mice with IL-18 protects them from the lethal effects of i.p. injection with syngeneic Meth A sarcoma (11). In the same report, we showed that NK cells play an early and indispensable role in the protective antitumor effects induced by IL-18, and this NK activity was later followed by the induction of CD4+ T-cell-mediated memory specific to Meth A. In this study, we have further examined the effects of IL-18 in Meth A TBM during the early stages of tumor rejection and compared these with the same immunological parameters in untreated TBM, which succumb to progressive tumor growth within about 3 weeks after transplantation, and normal untreated mice.

NK activity was significantly enhanced in the pretreated group early after the administration of IL-18 and transplantation of the tumor but decreased to normal levels by day 9 after tumor injection. On day 3, no specific CTL activity could be observed in pretreated TBM, but depletion of CD8+ cells from the spleen cell cultures enhanced spleen cell cytolytic activity in the tumor-bearing groups. Production of IL-2 by Con A-stimulated pretreated TBM spleen cells was significantly inhibited on day 3, and because several reports have shown that CD8+ suppressor T-cell-derived IL-10 can suppress IL-2 production (13, 14), we next examined the production of IL-10 in mitogen-stimulated pretreated TBM spleen cell cultures. Indeed, the production of IL-10 in these cultures was found to be enhanced 10-fold as compared to normal control levels, whereas the levels of the cytokine in untreated TBM cultures also significantly increased. IL-10 production was also found to be significantly increased in MLTC culture supernatants of treated and untreated TBM spleen cells compared to those containing normal mouse spleen cells (data not shown). This indicates that stimulation of TBM spleen cells with Meth A cells is enough to induce IL-10 production that is enhanced by treatment in vivo with IL-18. However, the cellular origin of the IL-10 observed in our model is still unknown. The production of IFN-γ by mitogen-stimulated mouse spleen cells, which is greatly enhanced after exposure to IL-18 in vitro (6), was inhibited in treated and untreated TBM spleen cell cultures after tumor transplantation. GM-CSF production was enhanced in the pretreated TBM cultures to a peak level on day 3 and started to decline on days 9–15 after transplantation. Mitogen-
increased the percentage of asialo GM1+ cells but slightly decreased the percentage of CD8-expressing cells on day 3 after challenge. Therefore, we consider that IL-18 probably induces the proliferation of asialo GM1+ CD8- cells in the early stages after tumor challenge. The untreated TBM spleens also contained proliferating cells on days 9 and 15; however, on day 15, TBM spleens were found to be infiltrated with Meth A cells, therefore the results on spleen cell proliferation in this group cannot be interpreted with certainty.

The enhanced NK activity, cytokine production profile, and enhanced unstimulated spleen cell proliferation observed in the pretreated TBM group were also observed in normal mice treated twice with IL-18 and sacrificed 3 days after the last administration of IL-18. Untreated mitogen-stimulated as well as MMC-treated Meth A-stimulated untreated TBM spleen cells (5-day MLTC cultures) also produce increased amounts of IL-10 when compared to control normal mouse spleen cell cultures (Fig. 3 and data not shown), indicating that

stimulated spleen cells from untreated TBM produced slightly increased amounts of GM-CSF on day 9 after transplantation, which decreased to below control levels on day 15.

Spleen cells from the pretreated mice exhibited enhanced proliferation in vitro in the absence of mitogen when compared to normal mouse spleen cells. This enhanced proliferation did not diminish up to 2 weeks after treatment with IL-18 in vivo. The normal mouse and TBM spleen cells causing splenomegaly in the early stages after treatment with IL-18 were identified as asialo GM1+ cells by flow cytometry. Spleen cell subsets were found to return to normal levels on days 9 and 15 after treatment with IL-18 (data not shown). A recent report has shown that IL-12 preferentially stimulates a subset of asialo GM1+ CD8+ mouse spleen T cells (15). In our system, IL-18 in-
the immune system is activated in the Meth A tumor-bearing state and enhanced by treatment with IL-18. Because IL-10 is also known to induce NK activity in mice (16), it is possible that IL-18, which is also known to directly augment NK activity, cooperates with IL-10 to produce a stronger antitumor effect. Cooperation between these two cytokines can take the form of an additive effect on NK cell activation or a direct effect on the tumor cells, because IL-10 has been reported to confer sensitivity on NK-resistant tumor cells (17, 18). We have not attempted to use neutralizing antibodies against IL-10 to determine whether this cytokine is involved in the antitumor effects induced by IL-18, mainly because of the large amounts of antibody reportedly required to neutralize the cytokine in vivo (19). It is interesting, however, that a decrease in the production of IL-10 by treated TBM spleen cells is accompanied with a simultaneous drop in NK activity to normal levels.

In a previous in vitro study on the stimulatory effects of human IL-18, we demonstrated that IL-18 inhibits the production of IL-10 in Con A-stimulated cultures of human peripheral blood mononuclear cells, even though NK activity was stimulated in similar cultures in the absence of mitogen (7). We also showed in a related study that IL-18 enhances IL-2 production by enriched human T cells stimulated with anti-CD3 antibody (9). The apparent discrepancies between our previous in vitro results and the results revealed in this study are probably due to the different environments encountered in the in vivo and in vitro systems. These discrepancies are not a result of the different species examined in these studies, because Kohno et al. (8) have also recently shown that murine IL-18 enhances IL-2 production by Th type 1 clones in vitro. Therefore, we think that the complex interacting immune networks active in vivo are the cause for the discrepancies between results obtained in vitro and in vivo.

In conclusion, IL-18 stimulates NK activity in vivo, possibly in cooperation with other cytokines such as IL-10, followed by the generation of CTL. The sequential generation of a nonrestrictive effector (NK) cell response followed by a specific (CTL) response induced by IL-18 in vivo provides a useful model for experimental treatment protocols with emphasis on the timing of treatment. This study will help identify novel molecules involved in in vivo networks of antitumor responses and immunological parameters that indicate the possibility of a beneficial outcome after treatment with immunostimulatory cytokines such as IL-18.

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REFERENCES

7. Ushio, S., Namba, M., Okura, T., Hattori, K., Nukada, Y., Akita, K., Tanabe, F.,
ANTITUMOR KILLER CELL ACTIVATION BY IL-18


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