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Rapid Generation of Potent and Tumor-specific Cytotoxic T Lymphocytes by Interleukin 18 Using Dendritic Cells and Natural Killer Cells[1]

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ABSTRACT

We hypothesized that antitumor-specific immunity, which is induced by interleukin (IL) 18 treatment in murine tumor models, is promoted by enhancing natural killer (NK)-mediated destruction of tumor and delivery to dendritic cells (DCs). These activated and antigen-pulsed DCs then critically and optimally induce an adoptive immune response, positioning IL-18 as an important bridge between the innate and adoptive immune response. The effect of IL-18 added to cultures of live tumor cells (MCA205, a mouse sarcoma cell line), NK cells, DCs, and T cells was assessed. When recombinant (r) mIL-18 protein was added to this culture, potent NK cytolytic activity with subsequent generation of CTLs was observed in a dose-dependent manner. Without introduction of either rmIL-18 or NK cells into this culture, systemic cytolytic activity was significantly decreased. Following the absence of direct contact of either NK cells or DCs with other cells in this cooperative coculture system using transwell, the systemic cytolytic activity of both NK cells and CTLs was greatly suppressed. The cytolysis mediated by effector cells harvested after completion of the culture was primarily restricted to MHC class I and highly specific for the tumor cells used in the coculture. Furthermore, we examined the efficiency in the induction of cytolytic T cells of other established IFN-γ inducing T-cell growth factors, IL-2, and IL-12 in this culture system and compared them with that mediated by IL-18. Neither IL-2 nor IL-12 induced tumor-specific cytolytic T cells to the same degree as that mediated by IL-18. Efficacy of this system in induction of tumor-specific CTLs was also observed in the system using MC38 adenocarcinoma cells. These results are consistent with the notion that IL-18 induces tumor-specific immunity through enhancing NK activity, which in turn mediates tumor cell death and activates and primes DCs.

INTRODUCTION

IL[3]-18, initially termed "IFN-γ-inducing factor," was cloned from mice with fulminant hepatitis induced by the administration of *Propionibacterium acnes* and lipopolysaccharide (1). IL-18 stimulates NK cells, T cells, B cells, and cells of the monocyte lineage to express IFN-γ at high levels (1, 2). Furthermore, IL-18 plays an important role in T-cell proliferation (1), CTL activation (3), and enhancement of NK cell activity primarily through Fas-FasL mechanism (2, 4–6). Systemic administration of rIL-18 is associated with significant *in vivo* antitumor effects that seem to be primarily mediated by enhanced NK activity (7, 8). We also have reported that established tumors could be successfully treated by i.t. injection of recombinant adenoviral vectors expressing biologically active mIL-18 (Ad.PTH.IL-18; Ref. 9). Although the mechanism of the antitumor effect has not been fully explained, we have shown that this antitumor effect is completely abrogated by depletion of asialo GM-1-positive cells, most likely NK cells (9).

Recent studies, including one using IL-18-deficient mice, support the notion that IL-18 plays an important role in the development of cellular immunity (Th1 response) following antigen presentation (10, 11). We have reported that mice treated with i.t. injection of Ad.PTH.IL-18 rejected a subsequent rechallenge with tumor cell, suggesting induction of effective tumor immunity with this treatment (9). In the present study, we analyzed the role of NK cells stimulated with IL-18 in CTL induction against tumor cells in an *in vitro* system consisting of live tumor cells, NK cells, DCs, and T cells with or without rmIL-18. This *in vitro* analysis system suggests that NK cells stimulated by IL-18 play a significant role in inducing tumor-specific immunity through activation and antigen-loading DCs, which are the primary antigen-presenting cells eliciting an effective adoptive immune response (12, 13).

MATERIALS AND METHODS

Recombinant Cytokines. Murine rIL-18 was supplied by Hayashibara Biochemical Laboratories (Okayama, Japan). Murine rIL-4 and murine GM-CSF were generously provided by Dr. Satwant Narula (Schering-Plough Research Institute, Kenilworth, NJ). rhIL-2 and rmIL-12 were kindly provided by Chiron Corp. (Emeryville, CA) and Genetics Institute (Cambridge, MA), respectively.

Tumor Cell Lines and Animals. MCA205, a methylcholanthrene-induced murine fibrosarcoma cell line, and MC38, a murine colon adenocarcinoma, were generous gifts from Dr. S. A. Rosenberg (National Cancer Institute, Bethesda, MD). The D122 highly metastatic variant of 3LL tumor cells was kindly provided by L. Eisenbach (Weizmann Institute of Science, Rehovot, Israel). YAC-1 was a generous gift of W. Chambers (University of Pittsburgh, Pittsburgh, PA). These cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 mg/ml streptimycin, 100 IU/ml penicillin, and 5×10^{-5} M 2-ME (all from Life Technologies, Inc., Grand Island, NY), referred to henceforth as CM. Primary cultures of syngeneic fibroblasts were obtained from the lungs of C57BL/6 mice (14). Wild-type C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). The *scid/scid* C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, MA). These animals were maintained in the animal facility at the Center for Biotechnology and Bioengineering, University of Pittsburgh, and were used for experiments when they were 7–12 weeks of age. All of the animals were ear-tagged, randomized before experiments, and treated and examined in a blinded fashion.

Cell Preparation. Bone marrow-derived DCs cultured with GM-CSF and IL-4 for 6 days and hepatic MNCs and were prepared as described previously, respectively (14, 15).

Flow Cytometry. For phenotypic analysis, DCs were stained with PE- or FITC-conjugated mAbs against murine cell surface molecules (CD11c, CD80, CD86, Gr-1, H-2Kb, I-Ab, and appropriate isotype controls; all were from PharMingen, San Diego, CA). Lymphoid cells harvested from lymph nodes were stained with CD4 and CD8. They were examined with the FACScan (Becton Dickinson, Sunnyvale, CA).

Cytokine Release or CTLs Assay *in Vitro*. To examine the role of NK cells in CTL induction *in vitro*, splenocytes from C57BL/6 SCID mice (NK-rich cells) were dissociated in CM to yield a single-cell suspension. The

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[3] The abbreviations used are: IL, interleukin; NK, natural killer; DC, dendritic cell; i.t., intratumoral; CM, complete medium; MNC, mononuclear cell; CCS, cooperative coculture system; GM-CSF, granulocyte machrophage-colony-stimulating factor; r, recombinant; mAb, monoclonal antibody.

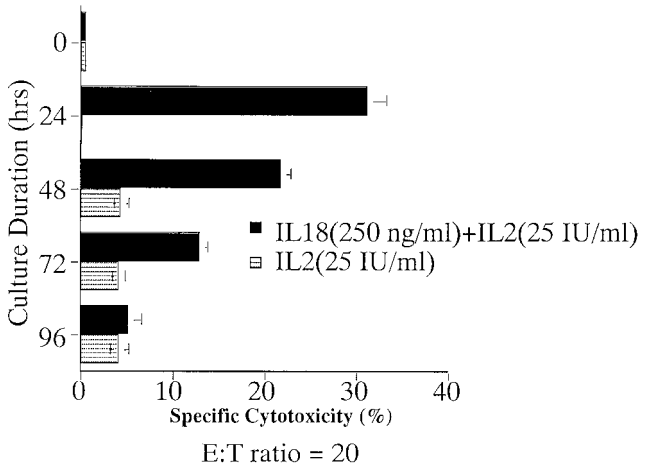

Fig. 1. IL-18 stimulates NK cytolytic activity against MCA205 cells. Cells mainly consisting of NK cells (splenocytes from SCID mice) were cultured with or without 250 ng/ml rIL-18 in the presence of 25 IU/ml IL-2. The cytolytic activity was assessed against MCA205 cells at an E:T ratio of 20. Data represent the mean ± SD of cytotoxicity.

percentage of NK cells was determined using mAb (41–50%). After erythrocyte lysis, cells of 4×10^5/ml were plated into 24-well plates. Inguinal lymph nodes, abdominal wall lymph nodes, and axial lymph nodes of naive C57BL/6 mice were collected and suspended in CM. These cells of 2×10^6/well were added to NK cells. The percentage of T cells was determined using antimouse CD8 and antimouse CD4 mAbs (31–40% and 37–51%, respectively). Day 6 DCs and tumor cells were resuspended and plated 2×10^5/well and 2×10^4/well, respectively. On day 4, the number of tumor cells reached 1×10^6. rhIL-2 was added to the culture medium at a very low final concentration of 25 IU/ml. The rmIL18 was added at graded doses of 0–250 ng/ml in a final volume of 2.4 ml/well. In some experiments, NK cells were cultured with rIL-18 (250 ng/ml) and rIL-2 (25 IU/ml) for 4 days to analyze the cytolytic activity against MCA205 cells. In some experiments, MCA205 cells were treated with UV at 1.52 mW/cm^2 for 30 min and added at 2×10^6/ml (0.5 ml) immediately after treatment. Forty-eight h after UV treatment, 73% of the MCA205 cells were confirmed to be apoptotic by Annexin V expression. In some experiments, either NK cells or DCs were plated in a well using a 0.4-μm pore size transwell (Corning Coster, Cambridge, MA) to prevent direct cell-to-cell contact. Forty h after the culture, 0.4 ml of supernatant was collected for ELISA analysis for IFN-γ (PharMingen). The lower detection limit of these assays was 15 pg/ml. For cytotoxic assay, cells were cultured for 4 days and collected and tested for cytotoxic activity. Viable lymphoid cells were counted using trypan blue-exclusion method and used as effector cells for the standard 4-h ^{51}Cr release assay against target cells. In brief, 10^6 cells of each target were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ for 1 h, rinsed twice, and plated with target cells at an appropriate E:T ratio in 96-well round-bottomed plates. The supernatant (100 μl) was collected after a 4-h incubation, and the radioactivity was counted with in gamma counter. The percent-specific lysis was calculated using the following formula: % specific lysis = 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release). In some experiments, ^{51}Cr release assay was performed with 50 μl of blocking antibodies against H-2K^b (HB41/B896) and H-2K^d (HB159) to examine class I restriction in cell killing [both HB41/B896 and HB159 were kindly provided by E. Gorelik (University of Pittsburgh Cancer Institute, Pittsburgh, PA)].

Cell Sorting. After preincubation with antimouse CD32/16 (2.4G2) mAb, liver MNCs were stained with FITC-conjugated antimouse CD3ϵ (145–2C11) and phycoerythrin-conjugated antimouse NK1.1 (PK136). All mAbs were obtained from PharMingen. For sorting, a FACS Vantage (Becton Dickinson Co., San Jose, CA) was used. In experiments to evaluate CTL induction, sorted NK cells or NKT cells in this way were used in place of splenocytes harvested from SCID mice as described above.

Statistical Evaluation. Statistical analysis was performed using a repeated measure ANOVA method when comparing the *in vivo* tumor growth and cytotoxic activity in an individual group. The unpaired two-tailed Student's *t* test was used to compare cytokine expression. Differences were considered significant when the P was <0.05.

RESULTS

IL-18 Promotes NK Cell Killing, IFN-γ Expression, and Induction of a Potent and Specific CTL Response *in Vitro*. To analyze the NK cell activity enhanced with IL-18, the NK cell population was cultured with or without IL-18 in the presence of 25 IU/ml rIL-2. As shown in Fig. 1, the cytolytic activity against MCA205 cells were induced 24 h after culture in the presence of rIL-18 and gradually reduced afterward. On day 4, the cytolytic activity of the effector cells cultured with IL-18 was the same as those without IL-18. These results indicate that the NK cytolytic activity against MCA205 cells was rapidly enhanced following stimulation with IL-18.

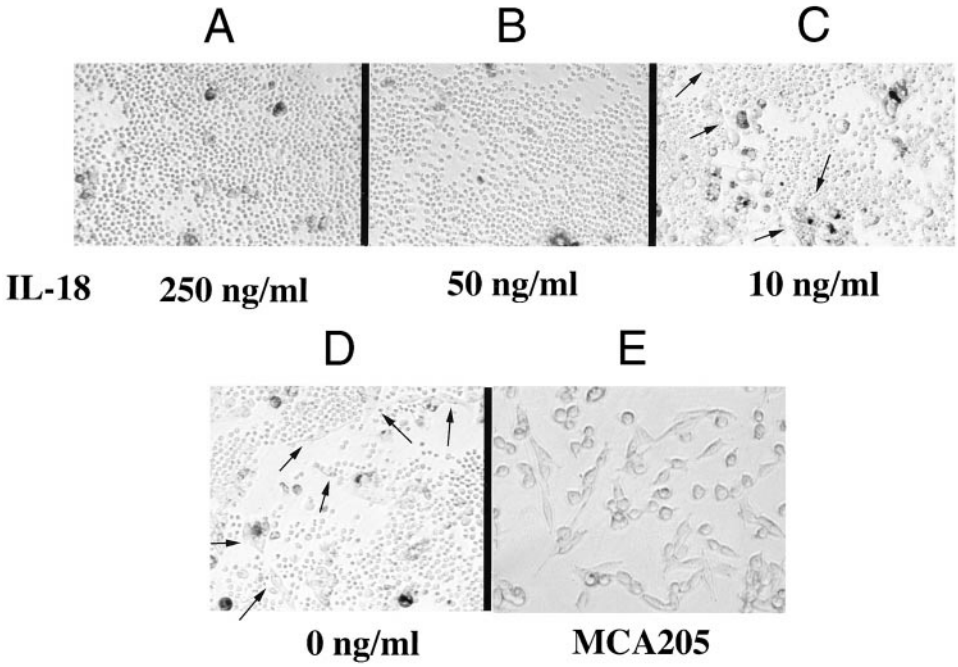

Fig. 2. IL-18 enhances NK cytolytic activity and eliminates tumor cells in an *in vitro* coculture system. Cocultures consist of tumor cells, NK cells, DCs, and T cells with various doses of rmIL-18. On day 3 of coculture, cells were examined and photographed microscopically. rmIL-18 [250 ng/ml (*A*), 50 ng/ml (*B*), 10 ng/ml (*C*), and 0 ng/ml (*D*)] was added to culture media. *Arrows* indicate viable tumor cells. *E*, growth of MCA205 cells alone on day 3 using the same number of cells of the coculture. The original magnification was ×200 of each sample.

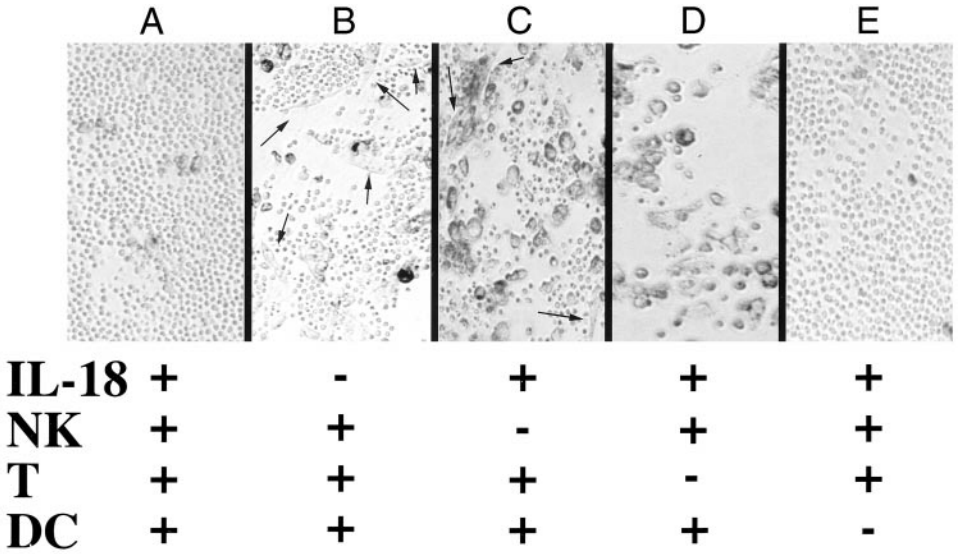

Fig. 3. Presence of DCs in tissue culture does not affect IL-18-induced NK activity. To examine which factors (rmIL-18, NK cells, DCs, or T cells) are essential for generation of significant NK activity, effector cells were cocultured under various conditions: *A*, IL-18, NK cells, DCs, and T cells; *B*, NK cells, DCs, and T cells, (no IL-18); *C*, IL-18, DCs, and T cells (no NK cells); *D*, IL-18, NK cells, and DCs (no T cells); and *E*, IL-18, NK cells, and T cells (no DCs). On day 3 of coculture, culture conditions were examined and photographed microscopically. *Arrows* indicate viable tumor cells. Original magnification, ×200.

To investigate the role of NK cells in the induction of CTLs by IL-18, we analyzed cytotoxic activity in a coculture system containing NK cells, T cells, bone marrow-derived DCs (cultured with GM-CSF and IL-4 for 6 days), and tumor cells. As shown in Figs. 2 and 3, tumor cell death was enhanced by the addition of rmIL-18 in a dose-dependent manner only when both NK cells and IL-18 were included. Thus, the immediate cytotoxic effect seems to be mediated by NK cells stimulated with rmIL-18, consistent with the results shown in Fig. 1, and was not significantly affected by the presence of T cells or DCs (Figs. 2 and 3).

The cytotoxicity of CTLs induced in culture containing live MCA205 cells and all of the individual immune cell components with IL-18 was similar to that obtained from the cultures containing UV-treated MCA205 cells and the immune cell components other than NK cells with rmIL-18. The cytolytic activity was generated against MCA205 cells, but not against YAC-1 cells (data not shown). Expression of IFN-γ in the culture was enhanced by rmIL-18 in a dose-dependent manner (4380 ± 68 pg/ml, 3880 ± 115 pg/ml, 2972 ± 312 pg/ml, and 1097 ± 287 pg/ml in the presence of rmIL-18 at 250 ng/ml, 50 ng/ml, 10 ng/ml and 0, respectively). Although the potent CTL activity against MCA205 was induced in this culture system by rmIL-18 in a dose-dependent fashion, no cytolytic activity was observed against NK-sensitive YAC-1 cells (Fig. 4*A*). These findings suggest that IL-18 enhances NK cytolytic activity, promotes tumor cell destruction, and stimulates IFN-γ production in the culture system used.

IL-18 Induces Tumor-specific Cytolytic T Cells in this Coculture More at a Greater Extent than Other IL-2 or IL-12. To compare the efficiency of IL-18 with other known T-cell growth factors (IL-2 and IL-12) in inducing cytolytic T cells *in vitro*, we tested the same culture systems but with IL-2 (1000 IU/ml), IL-12 (1000 pg/ml, 100 pg/ml, or 10 pg/ml), or IL-18 (250 ng/ml). The cytolytic activity of CTLs generated with IL-18 was the highest against MCA205 tumor when compared with that induced by IL-2 or IL-12. The cytolytic activity of CTLs generated with IL-2 was significantly enhanced against YAC-1 cells but only marginally against the MCA205 tumor. Use of high-dose IL-12 resulted in significant elevation of cytolytic activity against MCA205 cells. The level of cytolytic activity was substantially inferior to that induced by IL-18 (Fig. 4*B*). These results suggest that neither IL-2 nor IL-12 induces tumor-specific cytolytic T cells as efficiently as IL-18 does in this coculture system under these conditions.

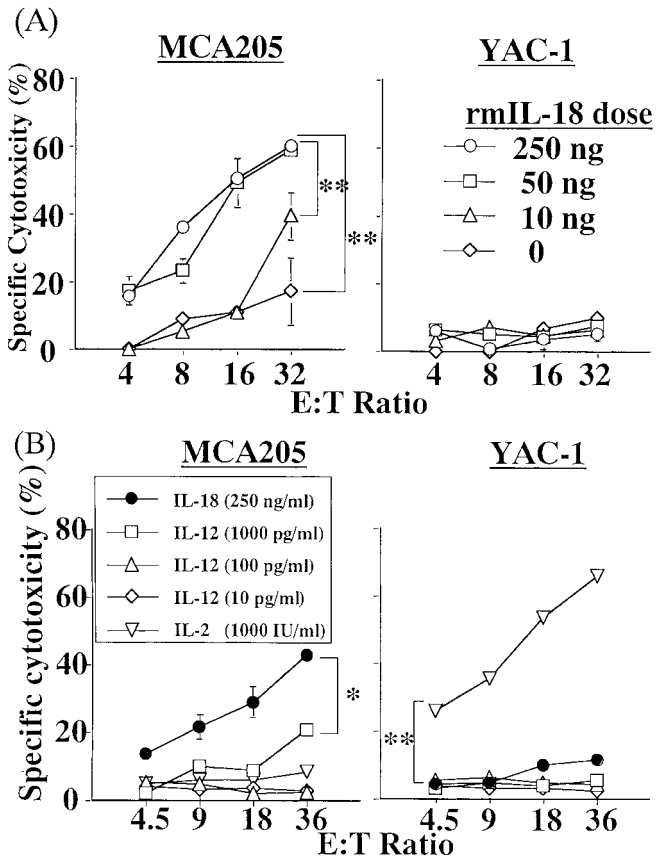

Fig. 4. NK cell killing and subsequent induction of tumor-specific CTL is augmented by IL-18 at a significant extent when compared with IL-2 or IL-12 *in vitro*. *A*, cytotoxic activity of effector cells from *in vitro* stimulation of cocultures of live tumor cells, NK cells, DCs, and T cells with various doses of rmIL-18, as shown in Fig. 2. On day 4 of coculture, effector cells were collected and cytolytic activity was assessed against MCA205 cells and YAC-1 cells at various E:T ratios. Data represent the mean ± SD of cytotoxicity. There is a statistically significant difference between a group of 250 ng of IL-18 dose *versus* that of 10 ng/ml and 0 ng/ml groups, respectively ($P < 0.01$). *B*, the cytolytic activity against MCA205 cells or YAC-1 cells was examined using effector cells obtained from the coculture with either IL-2 (1000 IU/ml), IL-12 (1000 pg/ml, 100 pg/ml, or 10 pg/ml), or IL-18 (250 ng/ml). Data represent the mean ± SD of cytotoxicity. Cytolytic activities of effector cells induced with IL-18 specifically against MCA205 and IL-2 nonspecifically against YAC-1 are significantly higher than those with other cytokines ($P < 0.05$ and $P < 0.01$, respectively). **, $P < 0.01$; *, $P < 0.05$.

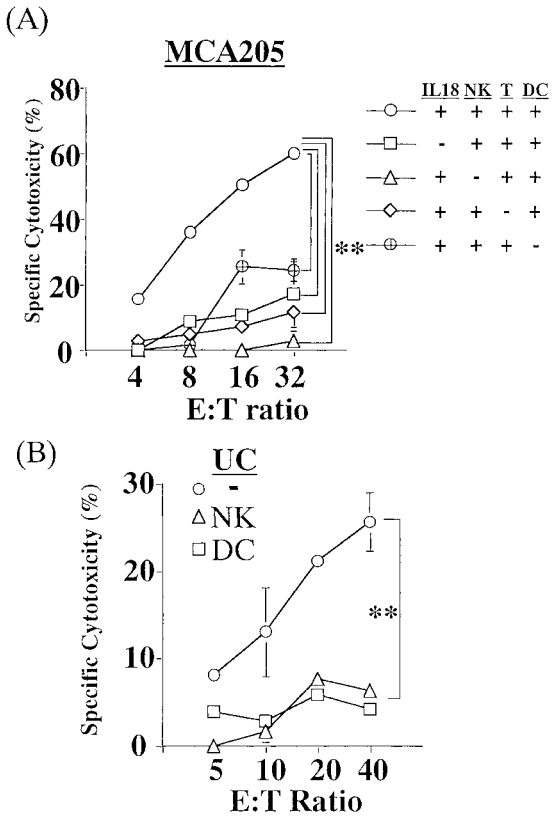

Fig. 5. Specific immunity is induced by this coculture system only when all components (NK cells, DCs, T cells, and IL-18) were present without a restriction of direct contact among the cell components. *A*, the cytotoxic activity of effector cells was examined from cocultures containing various components, including live tumor cells, NK cells, DCs, T cells, and IL-18. On day 4 of coculture, effector cells were collected and cytolytic activity was assessed against MCA205 cells at various E:T ratios. Data represent the mean ± SD of cytotoxicity. There is a statistically significant difference between the group that contained all components (○) *versus* all others lacking an individual element (**, $P < 0.01$ for all). *B*, effector cells were obtained from coculture separating either NK cells or DCs using a transwell system as described in "Materials and Methods." On day 4 of coculture, effector cells were collected and cytolytic activity was assessed against MCA205 cells and YAC-1 cells at various E:T ratios. *UC*, upper chamber separated cells. Data represent the mean ± SD of cytotoxicity. There is a statistically significant difference between the group with no separation and a group with either NK cells or DCs separated (**, $P < 0.01$).

Specific Immunity Is Induced Only When All Components (NK Cells, DCs, T Cells, and IL-18) Are Present without a Restriction of Direct Contact among Other Cell Components in this Coculture System. To examine the role of each individual cell type and cytokine in this culture system to generate potent CTLs, we examined the activity of CTLs induced in the culture system lacking an individual component. Significant CTL activity was induced ($P < 0.01$ for all others) against MCA205 cells only when all factors were included in the system (Fig. 5*A*). The cytolytic activity was not generated against YAC-1 cells (data not shown). Furthermore, the cytolytic activity was abrogated when either NK cells or DCs were separated using the transwell system, which allowed only the exchange of soluble factors (Fig. 5*B*). These data indicate that the MCA205-specific CTLs were induced only when all factors (NK cells, DCs, T cells, and IL-18) were present in the absence of barrier and in direct contact with each other.

Cytotoxic Activity Is Specific for MCA205 and MHC Class I following Coculture of NK Cells, DCs, and T Cells with IL-18. To analyze the specificity of effector cells generated in this system, the cytolytic activity of effector cells was analyzed against four different syngeneic cell lines (MCA205, D122, EL-4, and fibroblasts) and YAC-1 as a NK-sensitive cell line (Fig. 6*A*). Cytotoxicity of the effector cells was significantly enhanced only against MCA205 cells but not against any other cell lines ($P < 0.01$ for all). When the effector cells were incubated with anti-MHC class I antibody, the cytolytic activity generated was significantly inhibited (Fig. 6*B*). These results strongly suggest that cytolytic activity of the effector cells is MCA205 specific and the recognition of the target cells by the effector cells is restricted MHC class I manner in this system.

NK Cells, but not NKT Cells, Play the Major Role in Induction of CTL Activity in This Coculture System. To analyze the contribution of NK cells and NKT cells in this coculture system for generating CTLs, sorted NK cells and NKT cells were used. Using sorted NK cells, the cytolytic activity was greatly enhanced compared with that with NKT cells (Fig. 7). This suggests that NK cells, but not NKT cells, play the central and important role for induction of CTLs in this coculture system.

Potent and Specific CTLs Can Be Induced in the System Using Another Tumor Cell Line, MC38 Adenocarcinoma Cells. To analyze the efficiency of this coculture system using another cell line, we tested MC38 murine adenocarcinoma cells in this coculture system. As shown in Fig. 8, *A* and *B*, the cytolytic activity of CTLs obtained in this coculture was observed against MC38 cells, but not NK-sensitive YAC-1 cells. This cytolytic activity was enhanced by rmIL-18 in a dose-dependent manner. Furthermore, IL-12 or IL-2

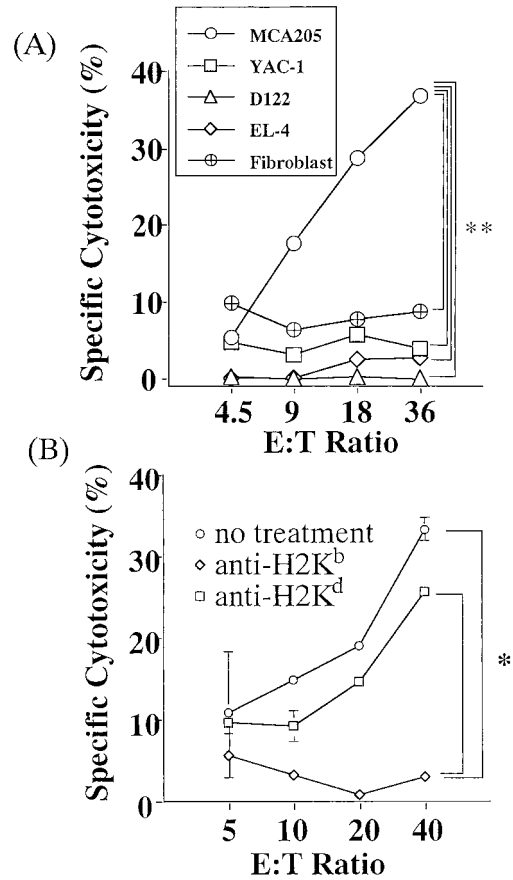

Fig. 6. Fine specificity of 4-day effector cells is obtained, and the cytolytic activity is MHC class I restricted in this CCS. *A*, cytolytic activity was assessed against MCA205 cells, D122 cells, EL-4 cells, and YAC-1 cells at various E:T ratios. Data represent the mean ± SD of cytotoxicity. There is a statistically significant difference between MCA205 and other targets ($P < 0.01$ for all). *B*, effector cells were collected from coculture on day 4, and the cytolytic activity was assessed against MCA205 cells in the presence of anti-H2K^b antibody or anti-H2K^d antibody, or without antibody at various E:T ratios. Data represent the mean ± SD of cytotoxicity. There is a statistically significant difference between the group following anti-H2K^b treatment and that of the nontreatment group or anti-H2K^d treatment group (*: $P < 0.05$).

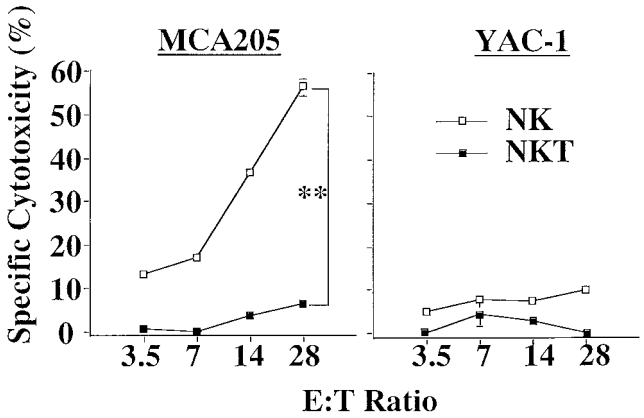

Fig. 7. NK cells, not NKT cells, play an important role in generating CTLs in this coculture system. T cells were cocultured with either NK cells or NKT cells with tumor cells and DCs in the presence of IL-18. After 4 days of coculture, effector cells were collected and specific cytotoxicity was assessed against MCA205 cells and YAC-1 cells at various E:T ratios. Data represent the mean ± SD of cytotoxicity. There is a statistically significant difference between cocultures containing NK cells and those containing NKT cells (**, $P < 0.01$).

failed to generate CTLs in this coculture at the level achieved with IL-18. These results support that this approach can be effective for inducing potent CTLs against weakly immunogenic tumors. Only modest cytolytic activity was induced for the B16 melanoma (data not shown).

DISCUSSION

Attempts to develop T-cell culture systems with outgrowth of specific T cells to tumor have been carried out for 20 years since the first identification of T-cell growth factor, now called IL-2 (16). Problems related to these attempts initially ascribed to contamination with mitogens or lack of nominal T-cell effectors (17) subsequently turned to those associated with use of concentrations of IL-2 that were sufficient to maintain nonspecific T cells with lymphokine-activated killer cell-like activity (18). Although the addition of IL-4 (19), IL-7 (20), IL-10 (21), or IL-12 (22) to cultures could either decrease lymphokine-activated killer cell-like activity or increase expansion, simple strategies did not allow rapid selection of specific T cells, and many groups turned to use of complex feeder systems, purposeful use of mitogens, or early cloning strategies. Here, we explored the use of DCs coupled with activated NK and näive T cells in conjunction with IL-18 to derive specific effector cells to tumor. This was developed based on the observation of potent and specific tumor responses induced by i.t. injection of adenoviral vector-expressing IL-18.

We have previously reported that i.t. injection of adenovirus vector-expressing IL-18 induces specific immunity but depletion of asialo GM1-positive cells, primarily NK cells, which substantially abrogate the IL-18-mediated antitumor effects. Considering these findings, we hypothesized that tumor-specific immunity could be efficiently promoted by first inducing tumor cell destruction by NK cells stimulated with IL-18. To examine the role of IL-18 in inducing specific immunity, we used an *in vitro* CCS containing viable tumor cells, NK cells, DCs, and T cells as well as cytokines, including IL-18, IL-2, or IL-12. The results of this CCS suggest that IL-18 enhances NK activity, inducing death of viable tumor cells that in turn seem to be acquired and processed by DCs to promote CTL generation from näive T cells. As shown in Figs. 1–3, tumor cells were destroyed by NK cells activated with IL-18, and tumor-specific CTLs were induced as an apparent consequence. This NK-mediated destruction of tumor cells was enhanced by the presence of IL-18 in a dose-dependent manner (Fig. 2, *A–E*). As a result, the specific cytolytic activity of CTLs against MCA205 fibrosarcoma cells was augmented by the presence of IL-18 in a dose-dependent manner (Fig. 4*A*). As shown in Fig. 1, NK cells after coculture of 96 h exhibited marginal cytolytic activity against tumor cells involved in the coculture. Thus, the effector cells that showed cytolytic effect against tumor cells after coculture were considered to be CTL effectors. Similar findings were also observed when MC38 adenocarcinoma cells were used in the system (Fig. 8*A*). These findings, indeed, suggest that tumor cell destruction by NK cells is an important initiating event for inducing tumor-specific CTLs.

To investigate the role of the individual components involved in this system for generating MCA205-specific CTLs, we examined a CCS lacking individual components. As shown in Fig. 3, *A–E*, MCA205 cells were completely killed only when both rmIL-18 and NK cells were present during culture. Presence of DCs in culture did not alter the extent of MCA205 death and relevant lymphocyte proliferation. However, the most potent and specific cytolytic activity was observed only when the culture contained all components, including IL-18, NK cells, DCs, and T cells. When any of the factors were missing in the culture, the cytolytic activity was significantly reduced ($P < 0.01$ for all; Fig. 5*A*). To analyze the role of the direct contact of NK cells with tumor cells, we performed the CCS using transwells, separating NK cells and their targets. As shown in Fig. 5*B*,

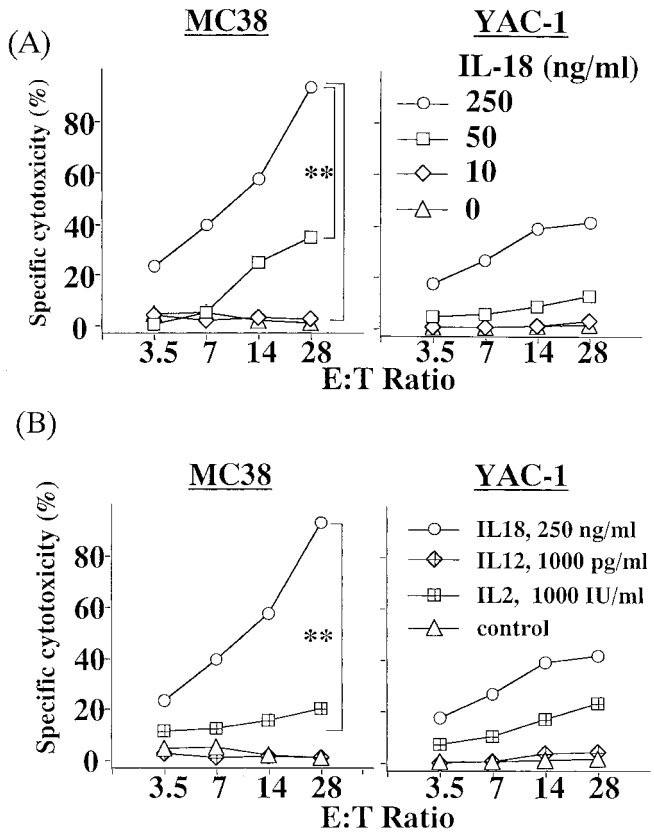

Fig. 8. Tumor-specific cytolytic activity is generated against another murine tumor (MC38 adenocarcinoma) in an IL-18 dose-dependent manner at a greater degree than IL-2 or IL-12. *A*, cells were cocultured for 4 days. When MC38 adenocarcinoma cells were involved in this coculture system with various doses of IL-18, the cytolytic activity was assessed against MC38 and YAC-1 cells at various E:T ratios. Data represent the mean ± SD of cytotoxicity. There is a statistically significant difference between the group using 250 ng of IL-18 *versus* those of the 50, 10, and 0 ng/ml groups (**, $P < 0.01$). *B*, the cytolytic activity of effector against MC38 cells or YAC-1 cells was examined using effector cells obtained from the coculture cells consisting of MC38 cells, NK cells, DCs, T cells, and 25 IU/ml IL-2 with either IL-2 (1000 IU/ml), IL-12 (1000 pg/), IL-18 (250 ng/ml), or no extra cytokine. Data represent the mean ± SD of cytotoxicity. Cytolytic activities of effector cells induced with IL-18 against MC38 is significantly higher than those with other cytokines ($P < 0.01$).

the cytolytic activity was greatly reduced when direct contact was inhibited between NK cells and other cells components. These results would indicate that the presence of NK cells play an important role in inducing specific immunity against tumor cells.

To determine the role of NK and NKT cells in CCS for efficient and rapid CTL induction, we sorted NK cells and NKT cells from liver MNCs. As shown in Fig. 7, potent cytolytic activity of the effector cells was induced when NK cells were included in the CCS, but not when NKT cells were used. Some reports have shown the results consistent with our data. Local production of IFN-γ by NK cells, but not NKT cells, was shown to be important for generating xenospecific CTLs (23). Leite-de-Moraes *et al.* (24) also demonstrated that NKT cells could kill the Fas+ cells following stimulation with IL-18 and IL-12, but cannot do with IL-18 alone. Thus, it seems that NK cells, but not NKT cells, play an important role in supplying antigen to DCs by mediating tumor cell death.

Furthermore, we examined the efficiency of other established and IFN-γ inducing T-cell growth factors, IL-2 and IL-12, and compared them with IL-18 in the induction of cytolytic T cells against MCA205 fibrosarcoma cells and MC38 adenocarcinoma cells, respectively. The expression of IFN-γ including either IL-12 or IL-18 in the coculture is 4869 pg/ml and 3315 pg/ml (when cocultured with MCA205) and 4844 pg/ml and 3751 pg/ml (when cocultured with MC38), respectively. These data showed that the difference of the effect of IL-12 and IL-18 for induction of IFN-γ was considered to be marginal. These results suggest that neither IL-2 nor IL-12 induces tumor-specific cytolytic T cells as IL-18 does in this CCS (Figs. 4*B* and 8*B*). Effective and rapid generation of such potent cytolytic activity against tumor cells, and potentially other targets, could be quite useful for development of adoptive immunotherapies as well as for generation of T cells important for antigen identification.

DCs play an important role as primary antigen-presenting cells to initiate and maintain T-cell responses. We and others have shown that DCs pulsed with tumor-associated antigens (25–27), tumor lysates (28), or RNA (29, 30) allow selection and/or activation of specific CTLs. DCs present exogeneous antigens on MHC class I molecules to induce priming or tolerance of CD8+ cells (31). As a source of the antigens, DCs phagocyte apoptotic cells to provide antigenic peptides and necrotic cells to be matured (32). In this study, we have demonstrated that DCs are an important cell population in generating effective CTLs against tumor in combination with IL-18 and NK cells in this CCS (Fig. 5). As shown in a previous study (33), we have demonstrated here that CTL induction against tumor cells was suppressed when DCs were absent (Fig. 5*A*) or separated (Fig. 5*B*) in the coculture well. At the beginning of the coculture, the day 6 DC showed 85% of MHC class II, 81% of CD80, 81% of CD86, and 68% of CD11c. Our data have demonstrated that CD11c, CD80, and CD86 expression of DCs after coculture with apoptotic cells or without coculture was not altered within 48 h from the coculture, thereafter increased when cocultured with apoptotic cells (34). Although the addition of rmIL-18 does not directly affect surface markers of DCs (data not shown), DC functions are affected by contact with tumor cells. We have recently identified dynamic changes in chemokine receptor expression (up-regulation of CCR7) on DCs following contact with apoptotic tumor (34). Also, we demonstrated in this study that effective CTLs were not generated when DCs were separated in the coculture using transwell, showing that direct contact with other elements in the coculture could be important for generating CTLs. Taken together, IL-18 seems to induce more frequent and effective tumor cell death through enhancing NK activity, enabling, in turn, DCs to serve as effective antigen-presenting cells, and then inducing potent and specific immunity. Thus, the implications are that IL-18/NK/DC plays a critical inductive and interactive role in promoting tumor-specific immunity. Furthermore, such T cells might be useful to be used in the clinic for adoptive immunotherapy and the research for identification of novel tumor associated antigens.

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