Expression of cytokine receptors on T cells and NKT cells after G-CSF mobilization

We evaluated the expression of cytokine receptors for IL-2, IL-3, IL-4, IL-7, G-CSF, and IFN-γ on CD3+ T cells and Vα24+ NKT cells in pre- and post-G-CSF PBMCs (n=5) (Fig. 4). The expression levels of IL-3, IL-7, and IL-4 receptor (which has IL-13 common receptor [19]) on CD3+ T cells and Vα24+ NKT cells were not effected by G-CSF mobilization, although the corresponding cytokine levels (IL-3, 7 and 13) were increased by G-CSF mobilization. The IL-7 receptor was expressed on most Vα24+ NKT cells, although some CD3+ T cells showed down regulation of the IL-7 receptor after G-CSF mobilization. There was no obvious tendency that G-CSF mobilization enhanced the expression level of the G-CSF receptor nor the α chain of the IL-2 receptor on both of CD3+ T cells and Vα24+ NKT cells. Interestingly, only the α chain of the IFN-γ receptor increased after G-CSF mobilization with a significant difference (P=0.009), and this increase occurred on Vα24+ NKT cells but not on CD3+ T cells.

Cell populations

Table 2 shows mean values and standard deviations for the cell kinetics of apheresis MNCs cultured with autologous apheresis plasma (n=11). The apheresis procedure did not affect the percentage of Vα24+ NKT cells. On day 0, Vα24+ NKT cells comprised only 0.10 (±0.06) % of apheresis MNCs, and the CD4+ to CD8+ T-cell ratio was >1.0. Monocytes accounted for approximately 30% of MNCs at day 0, which was substantially higher than the percentage of monocytes (2.7%-7.9%) in pre-G-CSF

PBMCs. When stimulated with α-GalCer, Vα24+ NKT cells propagated linearly until day 14. CD8+ T cells expanded to become the predominant T-cell population, changing the CD4+ to CD8+ T cell ratio to <1.0. B cells and monocytes almost completely disappeared by day 14 (2.33% and 0.16%, respectively). NK cells were also remarkably reduced after day 7, although they grew rapidly in the first 7 days of culture.

Cytokine production

We measured IFN-γ and IL-4 production in apheresis MNCs (n=10) that were cultured with or without α-GalCer for 14 days. Representative flow cytometry data are shown in Fig. 5. The percentage of IFN-γ-producing MNCs was 58.7±13.9% when cultured with α-GalCer and 44.8±15.6% when cultured without α-GalCer. The percentage of IL-4-producing MNCs was 8.6±8.5% when cultured with α-GalCer and 5.0±2.9% when cultured without α-GalCer. When cultured with α-GalCer, 75.7±12.2% of Vα24+ NKT cells produced IFN-γ and 16.2±10.5% produced IL-4. In the comparison of IFN-γ and IL-4 produced by Vα24+ NKT cells, IFN-γ was significantly dominant (P=0.023).

Cytotoxicity assays

Three tumor cell lines were used as target cells in the cytotoxic assay. CD1d expression on the target tumor cells was evaluated using CD1d mAb. CD1d was expressed on 87% of Jurkat cells and 13% of Daudi cells. K-562 did not express CD1d. Vα24+ NKT cells purified from mononuclear cells stimulated with α-GalCer mediated

strong cytotoxic effects against all of these hematological cell lines (Fig. 6). The cytotoxicities were unrelated to CD1d expression on the target cells.

Discussion

NKT cells help regulate a variety of immune responses, including the immune responses associated with autoimmune diseases [20] including inflammatory bowel diseases [21], graft-versus-host disease [22], and tumor rejection [23]. Two main strategies have been devised to use the specific ligand for NKT cells, α-GalCer, in therapeutic settings. These two strategies are the *in vivo* use of α-GalCer to enhance an immune response and the *ex vivo* use of α-GalCer to expand NKT cells for adoptive transfer. When the former approach was tested in patients with various solid tumors [24], there were short-term elevations in the IL-12 level, granulocyte macrophage–CSF level, and NK cell activity. Additionally, a slight elevation in serum IFN-γ and IL-4 occurred in some patients. Interestingly, the NKT cells disappeared from peripheral blood within 24 h of α-GalCer injection. Although no adverse events were associated with this approach, no therapeutic benefits were apparent. In murine models, high doses of α-GalCer showed significant liver toxicity [25].

The infusion α-GalCer-pulsed dendritic cells is an alternate approach that was studied by Nieda et al. They reported a transient decrease in the number of Vα24+ Vβ11+ NKT cells in the peripheral blood within 48 hours of the infusion. This transient decrease was followed by significant increases in Vα24+Vβ11+ NKT cells and the serum levels of IFN-γ and IL-12, in addition to the activation of NK cells and T cells [16]. No significant adverse events were reported in a clinical trial of this approach [26].

The clinical use of Vα24+ NKT cells requires the development of a highly effective expansion method for Vα24+ NKT cells *ex vivo*. Previous reports of *ex vivo* cell expansion for clinical applications have focused on T cells [27, 28], NK cells [29]

or dendritic cells [30], rather than NKT cells. A few reports have found that the expansion of human NKT cells from steady-state peripheral blood cells or cord blood cells can be mediated by α-GalCer and several cytokines [13-16]. However, the expansion ratios of these NKT cells were limited. Our previous study demonstrated that G-CSF mobilized peripheral blood cells, whether from normal donors or cancer patients, had a significantly higher expansion potential for Vα24+ NKT cells in a combination culture of α-GalCer and IL-2 [17]. These results provide realistic rationale for performing adoptive transfer of α-GalCer-expanded Vα24+ NKT cells in combination with high-dose chemotherapy and G-CSF treatment or in combination with autologous or allogeneic hematopoietic stem cell transplantation including G-CSF mobilization. Nevertheless, these approaches are seriously limited by the use of FBS, and the development of a non-FBS culture system is critical.

In the present study, we tested a culture system that uses autologous plasma for the expansion of Vα24+ NKT cells in the presence of α-GalCer and IL-2. We also evaluated the sustained usefulness of G-CSF-mobilized specimens. We found that autologous serum and autologous plasma had greater capacities to expand Vα24+ NKT cells than did FBS and rHSA. Indeed, there was no significant difference between Vα24+ NKT cells expansion in 5% or 10% autologous plasma or autologous serum. However, the percentage of Vα24+ cell in culture medium was the highest and 87%-95% of Vα24+ NKT cells reacted to the α-GalCer-CD1d tetramer after expansion in 5% autologous plasma. Additionally, plasma is easy to be obtained in the process of PBMCs preparation from the peripheral blood samples and in the process of apheresis. Then, we selected plasma as a medium supplement. We also found that G-CSF-

mobilized PBMCs and G-CSF-mobilized plasma, which were used instead of steady-state PBMCs and plasma, yielded the highest expansion ratio for Vα24+ NKT cells.

When we comparatively analyzed cells and plasma before and after G-CSF mobilization, we found that both G-CSF-mobilized PBMCs and G-CSF-mobilized plasma had the capability to support expansion of Vα24+ NKT cells (Fig. 2). In the clinical setting, we plan to use mobilized PBMCs and apheresis product derived from cancer patients as autologous setting, or derived from healthy donor as allogeneic setting. The clinical application of *ex vivo* expanded-NKT cells has a possibility of wide modification including combination therapy with stem cell transplantation.

Contrary to our expectations, our flow cytometry data revealed that the percentage of Vα24+ NKT cells *in vivo* decreased after G-CSF mobilization. As the absolute number of Vα24+ NKT cells did not change by G-CSF mobilization, decreased percentage of it was caused by the increment of other cell population after G-CSF mobilization. That means that the G-CSF does not mobilize Vα24+ NKT cells directly. And more, *ex vivo* supplementation of G-CSF did not enhance the expansion of Vα24+ NKT cells (data not shown), which means the indirect contribution of G-CSF in the expansion of NKT cells contrary to a previous report [31]. On the other hand, the post-G-CSF PBMCs (Table 2) and apheresis products contained a high percentage of monocytes, which include APCs capable of presenting α-GalCer. This observation indicates that the number of CD1d-expressing PBMCs also increased after G-CSF mobilization and might be one factor responsible for the significant expansion of Vα24+ NKT cells in post-G-CSF PBMCs. We previously reported the cell to cell contact with CD14+ cells was needed for the expansion of NKT cells [17].

The plasma collected after G-CSF mobilization also had an enhanced capacity for Vα24+ NKT cell expansion. IL-2, IL-7, IL-12, IL-15, IL-18, and IFN-γ directly induce proliferation and activation of NKT cells [13, 14, 32, 33]. However, none of these cytokines, with the exception of IL-7, was increased in the plasma of G-CSF-mobilized peripheral blood. When Vα24+ NKT cells were cultured with α-GalCer and increased levels of cytokines (IL-3, IL-7, IL-13, and G-CSF) in medium containing pre-G-CSF plasma, the expansion efficiency of Vα24+ NKT cells was not enhanced to the level achieved with post-G-CSF plasma (data not shown). These results suggest that IL-3, IL-7, IL-13, and G-CSF do not directly contribute to the proliferation of Vα24+ NKT cells. The identification of these unknown factors in post-G-CSF plasma, that promotes the proliferation of Vα24+ NKT cells, would help to further increase the effectiveness of Vα24+ NKT cell expansion.

To determine if the characteristics of cells were changed by G-CSF mobilization, we evaluated the expression of several cytokine receptors on CD3+ T cells and Vα24+ NKT cells isolated from peripheral blood before and after G-CSF mobilization. The significant increase was observed in the expression of the IFN-γ receptor α chain on Vα24+ NKT cells after G-CSF mobilization (P=0.009). This increased α chain expression may be partially responsible for the proliferative advantage of Vα24+ NKT cells after G-CSF mobilization. However, the variability of response between individuals is essential issue, especially in the evaluation of receptor intensity. This variation suggests that *ex vivo* expansion of NKT cells are controlled by mutual change, which exists in cellular and humoral factor.

We found that expanded Vα24+ NKT cells predominantly produced IFN-γ. The expanded Vα24+ NKT cells exhibited augmented cytotoxicity against CD1d-positive tumor cell lines (Daudi and Jurkat) as well as CD1d-negative tumor cell line (K562). In CD1d blocking experiments, we found that expanded Vα24+ NKT cells mediated cytotoxic activity against CD1d-blocked Jurkat cells that was comparable to the cytotoxic activity against CD1d-unblocked Jurkat cells (data not shown). Thus, the expanded Vα24+ NKT cells yielded lytic activity against tumor cells in a CD1d-independent manner. Although the mechanism of CD1d-related cytotoxicity mediated by Vα24+ NKT cells has not been clarified, other recent studies of NKT cells suggest that CD1d expression on the target tumor cells is not essential for cytotoxicity [34-36]. The Vα24+ NKT cells obtained in our culture system appear to be Th1-type NKT cells that have strong anti-tumor activity through direct and indirect mechanisms.

In the present study, we developed an effective method for Vα24+ NKT cell expansion through the use of G-CSF-mobilized peripheral blood. We also featured the possible clinical applications of Vα24+ NKT cells in adoptive immunotherapy both in autologous and allogeneous setting. Further research needed to achieve this goal is currently underway.

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Figure and Table Legends

Figure 1. Differences in Vα24+ NKT cell expansion according to the type of supplemented protein

PBMCs from normal healthy donors were cultured for 12-14 days with α-GalCer and IL-2 in the presence of 10% FBS, 10% rHSA, 5% or 10% autologous plasma, or 5% or 10% autologous serum. Vα24+ CD3+ cells were defined as Vα24+ NKT cells. Vα24+ CD1d-α-GalCer tetramer+ NKT cells were also stained, and the percentage of the gated population is shown. These flow cytometry results are representative of five independent experiments. (FBS, fetal bovine serum; rHSA, recombinant-human serum albumin)

Figure 2. Differences in Vα24+ NKT cell expansion influenced by a combination of PBMCs and plasma

The expansion of Vα24+ NKT cells was analyzed in several co-culture combinations of PBMCs and 5% plasma before and after G-CSF mobilization. Cells were cultured for 14 days in the presence of α-GalCer and IL-2. Values are the mean and standard deviation of the Vα24+ NKT cell expansion fold. Samples were obtained from the same donor (n=8), and the following co-culture conditions were examined: 1) pre-G-CSF PBMCs and pre-G-CSF plasma (pre-pre); 2) pre-G-CSF PBMCs and post-G-CSF plasma (pre-post); 3) post-G-CSF PBMCs and pre-G-CSF plasma (post-pre); and 4) post-G-CSF PBMCs and post-G-CSF plasma (post-post). (*, $p<0.05$. P values were determined using the Student's t test and Bonferoni adjustment.)

Figure 3. Cytokine levels in plasma

Cytokine levels in peripheral blood were measured by ELISA before G-CSF mobilization and in apheresis products from the same normal healthy donors (n=6). IL-2 levels are plotted in U/ml. All other cytokine levels are plotted in pg/ml. Results are shown as mean values with standard deviations. (*, $p<0.05$, compared with pre-G-CSF peripheral blood and apheresis product)

Figure 4. Cytokine receptor expression

Changes in the cytokine receptor expression of CD3+ T cells and Vα24+ NKT cells in peripheral blood before and after G-CSF mobilization are shown as five independent experiments. Peripheral blood before and after G-CSF-mobilization was obtained from the same healthy donors. (○, △, □, ◇, +; Figure symbols indicate individual donors.) (*, $p<0.05$)

Figure 5. Intracellular cytokines in cultured Vα24+ NKT cells

Intracellular IFN-γ and IL-4 were stained in whole cells after culture with or without α-GalCer. Cells were activated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours. Representative data from one of ten independent experiments are presented. (*, $p<0.05$, Difference between the production of IFN-γ and IL-4 were evaluated witn Student's t test.)

Figure 6. Cytotoxicity of purified Vα24+ NKT cells after culture

Vα24+ NKT cell-mediated cytotoxicity against tumor cells was measured with an effector to target ratio of 10:1, 3:1 and 1:1. Cell lines were classified to the following 4 groups on the expression level of CD1d: (-); 0-3%, (±); 3-10%, (+); 10-60% and (++); 60%-100%. Cytotoxicity was evaluated with ^{51}Cr release assays. The means and standard deviations of triplicate culture are shown in representative result of four independent experiments.

Table 1. Vα24+ NKT cell expansion

PBMCs were cultured for 12-14 days in medium containing 5% autologous plasma and α-GalCer. Pre-G-CSF cells and plasma were obtained from peripheral blood. Post-G-CSF cells and plasma were obtained from apheresis products. Results are mean values and standard deviation from the same healthy donors (n=18). (*; $p<0.05$, Differences between pre-G-CSF and post-G-CSF were evaluated with the Student's t test.)

Table 2. Changes in cell populations during the culture process

The kinetics of each cell population are shown. G-CSF mobilized cells from healthy donors were expanded in culture with α-GalCer and IL-2 for 14 days. Mononuclear cells and autologous plasma were obtained from apheresis products after G-CSF mobilization (n =11). Results are shown as mean values of percentage for each subpopulation and presented standard deviations.

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Tumor secreting high levels of IL-15 induces specific immunity to low immunogenic colon adenocarcinoma via CD8$^+$ T cells

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Abstract. Although interleukin (IL)-15 augments innate and acquired immunities, IL-15 expression is controlled at the levels of transcription, translation and intracellular trafficking. We constructed plasmid vectors encoding the murine mature-IL-15 cDNA linked to an Igκ leader sequence and full-length murine IL-15 cDNA to evaluate the efficacy of the mature-IL-15 vector. Weakly immunogenic colon 26 cells were transfected with the above-mentioned vectors or with empty vector (mock). Transfectants with mature-IL-15 produced significantly higher levels of IL-15 than did transfectants with full-length IL-15. When injected into syngeneic BALB/c mice, transfectants secreting high levels of IL-15 were rejected completely. Depletion of natural killer cells or CD4$^+$ T cells did not affect the growth of transfectants. In contrast, transfectants treated with anti-CD8 antibody re-grew 1 month later after implantation. These findings indicate that CD8$^+$ T cells are required for complete rejection of the tumor. Gene therapy with transfectants expressing mature-IL-15 containing the Igκ leader sequence may be useful as a tumor vaccine.

Introduction

Interleukin (IL)-15 is a 15-kDa cytokine (1,2) that uses the β and γ chains of the IL-2 receptor (R) for signal transduction and shares biologic activities with IL-2. In particular, IL-15 promotes proliferation and activities of T, B and natural killer (NK) cells (3,4) and is a potent inducer of lymphokine-activated cytotoxic activity against tumor cells (5-7).

However, IL-15R contains a unique α chain that has a higher affinity and broader tissue distribution than that of the IL-2Rα chain (8,9). Indeed, there are several significant differences in the molecular and cellular features of IL-2 and IL-15 (10). IL-15 shows stronger mediation of NK and NKT cell differentiation and survival *in vivo* in comparison with IL-2 (11,12). Moreover, IL-15 can selectively promote proliferation and long-lasting survival of memory CD8$^+$ T cells, in contrast to IL-2, which inhibits proliferation of CD8$^+$ memory T cells (13-15). In addition, doses of IL-15 required to induced severe hypotension and pulmonary vascular leak syndrome were six times higher that those of IL-2, resulting in a higher therapeutic index for IL-15 (16). Such findings have led to a renewed clinical interest in IL-15.

Though IL-15 mRNA is expressed in a broad normal organization and tumor cells (1), it has been difficult to detect IL-15 protein in supernatants of many cells that express IL-15 mRNA (17). IL-15 expression is controlled at the levels of transcription, translation, and intracellular trafficking (18-20). Although there was a 4- to 5-fold increase in translation of IL-15 mRNA with the alternative short signal peptide in comparison with that of the wild-type 48-aa signal peptide (17,21,22), only IL-15 containing the 48-aa signal peptide is secreted (21). We previously reported that highly immunogenic tumor (Meth-A) cells transfected with IL-15 containing the 48-aa signal peptide can elicit an anti-tumor immune response (23). However, in a preliminary study we found that these anti-tumor effects were not present in the weakly immunogenic colon 26 cells.

In the present study, we constructed plasmid vectors encoding the murine mature-IL-15 cDNA linked to a high efficiency Igκ leader sequence and evaluated the efficacy of high IL-15-producing tumor. We report that high IL-15 production caused complete rejection of weakly immunogenic colon 26 cells and induced long-lasting CD8$^+$ T cell-mediated specific anti-tumor immunity.

Materials and methods

Reverse transcription-PCR and primers. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) were carried out as described previously with some modifications (24). Briefly, cells (5x10^6) were lysed in 1 ml of TRIzol reagent (Life Technologies, Grand Island, NY), and total cellular RNA was isolated according to the manufacturer's instructions. One microliter of total RNA (1 μg) was added to 19 μl of RT-mixture (Takara, Ohtsu, Japan). After mixing, the samples were incubated at 30˚C for 10 min, 55˚C for 30 min, 95˚C

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Figure 1. (A), Illustration of full-length IL-15 expression vector. (B), Illustration of mature-IL-15 expression vector.

for 5 min, and 4°C for at least 5 min. PCR-mixture (80 µl) (Takara) containing 100 nM primers was added to each RT reaction. PCR amplification consisted of 34 cycles of denaturation at 94°C for 1 min, annealing at 58 or 68°C for 1 min, and extension at 72°C for 2 min. The annealing temparature was 68°C for vector construction and 58°C for mRNA detection.

The primer sequences for murine full-length IL-15 vector construction were sense, 5'-AGCTGCGGCCGCATGAAAA TTTTGAAACCATAT-3' and antisense, 3' primer 5'-AGCT AGTCTAGATCAGGACGTGTTGATGAACAT-3'. Those for murine mature-IL-15 vector construction were sense, 5'-CCTCGTGAATTCGCCAACTGGATAGATGTAAGA-3' and antisense, 5'-TACACACTCGAGTCAGGACGTGTTGA TGAACAT-3'. Primers for detection of murine IL-15 mRNA were sense, 5'-TAGATATAAGATATGACCTGGA-3' and antisense, 5'-TGTTGAACATTTGGACAAT-3', and those for detection of murine ß-actin mRNA were sense, 5'-TCGA CAACGGCTCCGGCATGT-3', and antisense, 5'-GCTGAT CCACATCTGCTGGAA-3'. The expected sizes of the PCR products were 513, 372, 330, and 1046 bp, respectively. The PCR products were separated by electrophoresis on 1% agarose gels and visualized by staining with ethidium bromide and UV transillumination.

Murine full-length-IL-15 expression vector (Fig. 1A). A murine IL-15 cDNA was isolated by RT-PCR from total RNA of lipopolysaccharide-stimulated murine spleen cells. The PCR fragment was cloned into the *NotI/XbaI* site of the pRC/CMV eukaryotic expression vector (Invitrogen, San Diego, CA). The nucleotide sequence was confirmed by sequence analysis with the T7 promoter.

Murine mature-IL-15 expression vector (Fig. 1B). A mature-IL-15 cDNA was isolated by PCR with the full-length IL-15 expression vector as a template. The PCR fragment was cloned into the *EcoRI/XhoI* site of the pSecTag2B eukaryotic expression vector (Invitrogen), which contains the Igκ leader sequence. The nucleotide sequence was confirmed by sequence analysis from the T7 promoter.

Tumor cells and transfection. Colon 26 cells (25), a murine colon adenocarcinoma cell line derived from BALB/c mice, were maintained in RPMI 1640 (Life Technologies) medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Sub-confluent cultures in 100-mm petri dishes were transfected with 5 µg of murine full-length or mature-IL-15 expression plasmid or vector alone with Lipofectamine reagent (Life Technologies) according to the manufacturer's instructions. G418 (100 µg/ml) (Life Technologies), which selects for the pRC/CMV vector, and Zeocin (100 µg/ml) (Invitrogen), which selects for the pSecTag2B vector, were added to the cells 48 h later. G418- or Zeocin-resistant clones were isolated and expanded in culture medium containing 100 µg/ml of G418 or Zeocin as appropriate.

Bioassay for IL-15 production by transfectants. Because an ELISA kit for murine IL-15 is not available, the activity of IL-15 in culture supernatants was quantified with the CTLL-2 bioassay as descrived previously (23). Briefly, each transfectant line (1×10^6 cells/ml) was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum without G418 or Zeosin for 48 h. Culture supernatants were collected by centrifugation for 5 min at 400 g and filtered (0.22 µm) prior to bioassay. IL-15 and IL-2 dependent CTLL-2 cells (4000 cells/well) in 96-well flat-bottom microtiter plates were incubated with culture medium supplemented with 5×10^{-5} M 2-mercaptoethanol in the presence of culture supernatant with or without anti-murine IL-15 monoclonal anti-

body (G277-3588, PharMingen, San Diego, CA). After 20-h incubation, 50 µg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) (Chemicon International, Temecula, CA) was added to each well, and the reaction was allowed to incubate for an additional 4 h at 37˚C. Isopropanol with 0.04 N HCl (100 µl) was then added to each well. Color development at a wavelength of 540 nm was monitored with an ELISA reader (SLT Labinstruments, Austria). Serial dilutions of murine recombinant IL-15 (BioSource, Camarillo, CA) were used as a standard.

Animal studies. Seven-week-old female BALB/c mice were purchased from Japan SCL (Hamamatsu, Japan). Mice were inoculated with freshly prepared suspensions of tumor cells at a concentration of $2x10^6$ cells/ml. Animals were inoculated with a total of $5x10^5$ cells; all were inoculated subcutaneously in the right lower abdominal quadrant with a 27-gauge needle. Tumor volumes were measured in mm^3 with a venire caliper and calculated according to the following formula: $a \times b^2/2$, where a is the larger and b is the smaller of the two dimensions. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Yamaguchi University School of Medicine.

Re-challenge with parental cells (colon 26) and Meth-A. Sixty days after disappearance of the initial implant of mature-IL-15/ colon 26 cells, 10 mice were injected with $5x10^5$ parental colon 26 cells in the previously uninjected side, left lower abdominal quadrant. Meth-A cells, which were derived from a methylcholanthrene-induced fibrosarcoma in BALB/c mice (26), were also injected into both nonimmunized (n=10) and immunized (n=10) mice.

In vivo depletion of NK, $CD4^+$ and $CD8^+$ T cells. Depletion of NK, $CD4^+$ and $CD8^+$ T cells was carried out as described previously (27). Briefly, to deplete NK cells, 200 µl of a 1:15 dilution of anti-asialo GM1 antibody (Wako Fine Chemicals, Osaka, Japan) in phosphate-buffered saline (PBS) or control rabbit serum (diluted 1:15 in PBS) was injected intraperitoneally into mice 2 days prior to tumor challenge and 5, 7 and 11 days after tumor challenge. Monoclonal antibodies against $CD4^+$ cells (GK1.5) and $CD8^+$ cells (2.43) (both purchased from American Type Culture Collection, Rockville, MD) or HBSS (Gibco-BRL) (control) were injected intraperitoneally (1.0 mg) into mice (n=6) to deplete subsets of immune cells 3 days before and once each week after the inoculation of tumor cells. Flow cytometric analysis was performed with an EPICS XL (Beckman Coulter, Fullerton, CA) to verify 95% depletion of specific cell subsets in the spleen after the administration of depleting antibodies. Tumor volume was recorded twice a week.

Histologic evaluation for immune cells infiltrating into tumor tissues. On days 7 and 14 after inoculation, tumors were dissected, fixed in 10% neutral buffered-formalin, and embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin. For immunohistochemical staining, tissues were embedded in OCT compound (Ames Division, Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -80˚C. Acetone-fixed 6-µm cryostat

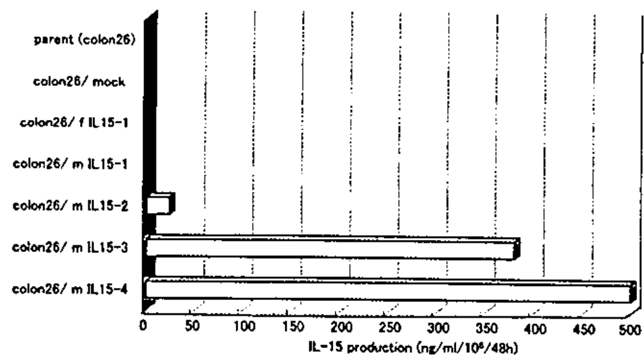

Figure 2. Production of IL-15 by transfectants was confirmed by CTLL-2 proliferation assay. Levels of IL-15 produced by mature-IL-15 transfectants (clones 1, 2, 3 and 4) were between 0 and 500 ng/10^6 cells/48 h, whereas full-length IL-15 transfectants and mock transfectants did not produce IL-15.

sections were blocked with goat serum and then immunostained with optimal dilutions of the following rat mAbs: L3/T4 (CD4, Becton Dickinson, Franklin Lakes, NJ) and KT15 (CD8, Serotec, Sapporo, Japan). Slides were then sequentially incubated with biotinylated goat anti-mouse IgG (Zymed laboratories, South San Francisco, CA) and ABComplex (Dako, Tokyo, Japan). Each incubation step lasted at least 30 min and was followed by a 10-min wash with PBS. Sections were then incubated with 0.03% H_2O_2 and 0.06% 3,3-diaminobenzidine for 2-5 min, rinsed with tap water, and counterstained with hematoxylin.

Statistical analysis. Statistically significant differences were evaluated with Student's t-test. A value of $P<0.05$ was considered statistically significant. Results are presented as mean ± SE.

Results

Expression of IL-15 mRNA by transfectants. Four independent G418-resistant full-length IL-15 clones (named colon 26/ fIL-15-1, 2, 3 and 4) and four independent Zeocin-resistant mature-IL-15 clones (named colon 26/mIL-15-1, 2, 3 and 4) were isolated and expanded. RNA was isolated, and RT-PCR was performed. Three of 4 (75%) clones (clones 1, 2 and 4) expressed full-length IL-15 mRNA, and 4 of 4 (100%) clones expressed mature-IL-15 mRNA. IL-15 mRNA was not detected in colon 26 cells transfected with empty vector or parental colon 26 cells (data not shown).

Bioassay for IL-15 produced by transfectants (Fig. 2). Production of IL-15 protein was confirmed by CTLL-2 proliferation assay. IL-15 levels in the supernatants of mature-IL-15 transfectants (10^6 cells/48 h) were 0 ng, 24 ng, 380 ng and 500 ng for clones 1, 2, 3 and 4, respectively. The bioactivity of each culture supernatant was neutralized completely by 10 µg/ml anti-IL-15 antibody. Colon 26 cells transfected with vector expressing full-length IL-15, mock transfected cells and parental colon 26 cells did not produce bioactive IL-15.

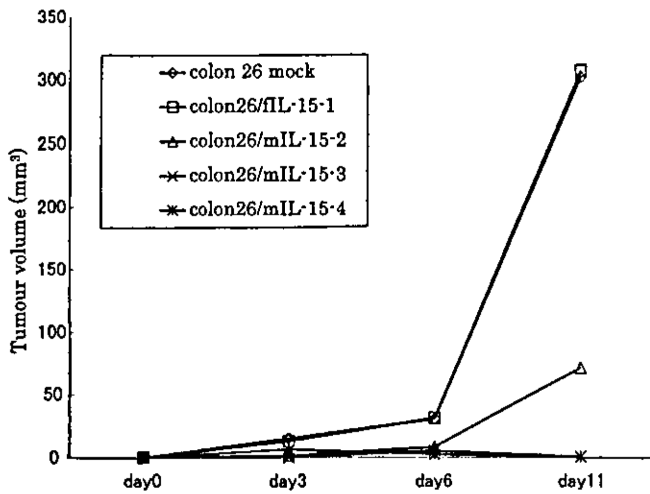

Figure 3. Tumorigenicity of full-length IL-15, mature-IL-15 (clones 1, 2, 3 and 4) and mock transfectants were examined by subcutaneous injection into BALB/c mice. Mean tumor volumes of clones 3 and 4 were significantly smaller on day 11 (P<0.01), whereas mock transfectants and clone 1 grew progressively. Each SEM was <10%.

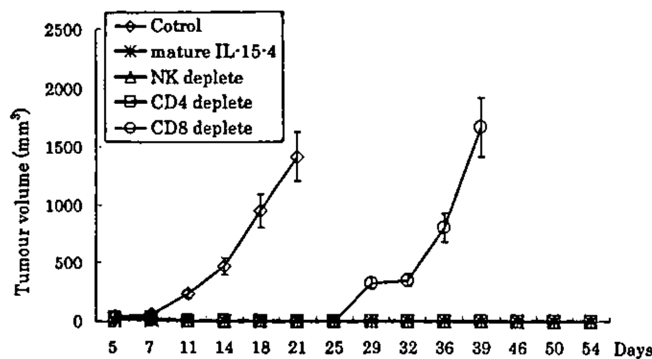

Figure 4. Depleted mice and control mice (HBSS only) were implanted with colon 26/mIL-15-4 cells. There was no difference in tumor growth between the NK-depleted mice, CD4⁺-depleted mice, and control mice. Tumors in mice treated with anti-CD8⁺ antibodies was initially rejected but grew rapidly approximately 1 month after transplantation.

Inhibition of tumor growth in vivo (Fig. 3). Transfection of colon 26 cells with full-length or mature-IL-15 expression vector did not alter the growth properties of the cells *in vitro* as assessed by doubling time or morphology (data not shown) in comparison with parental or mock transfected cells. The tumorigenicity of full-length IL-15, mature-IL-15, and mock transfected cultures were examined by subcutaneous injection into BALB/c mice. The mean tumor volumes of the mature-IL-15, -3, -4 were significantly reduced (n=7), whereas mock transfectants grew progressively (n=7). Full-length IL-15 transfectant showed growth similar to that of mock transfected cells.

Re-challenge with parental colon 26 cells and Meth-A. We next examined whether primary rejection of IL-15 transfectants led to protective immunity. Sixty days after the disappearance of the initial mature-IL-15/colon 26 implants, immunized mice (n=7) were injected with 5×10^5 parental colon 26 cells in the lower left abdomen. Seven non-immunized mice were injected in the same manner as controls. Tumor rejection was observed in all immunized mice, whereas colon 26 cells grew progressively in nonimmunized mice (data not shown). To confirm the specificity of the protective immunity, 5×10^5 Meth-A cells were injected into both nonimmunized and immunized mice. There was no significant difference in growth of implanted Meth-A cells between nonimmunized and immunized mice (data not shown).

Effects of anti-asialo GM1, anti-CD4 and anti-CD8 antibodies on growth of mature-IL-15 clones (Fig. 4). Depleted mice and control mice were inoculated with mature-IL-15-expressing cells. There was no difference in tumor growth among the NK-depleted group (n=6), $CD4^+$-depleted group (n=6) and the control group (n=7). Tumors in mice treated with anti-CD8 antibody were initially rejected completely but grew rapidly approximately 1 month after transplantation in comparison with those in mice treated with HBSS (control).

Histology at the site of tumor cell injection (Fig. 5). To characterize the host cellular responses augmented by IL-15 production, histological analysis of the injection site was performed 14 days following the injection of tumor cells. Immunohistochemical analysis of the site of injection of mature-IL-15 transfectants (clone 4) revealed infiltration of $CD4^+$ and $CD8^+$ lymphocytes and Mac-1-positive monocytes. This was not observed with mock transfectants.

Discussion

The anti-tumor effects of IL-15-transfected tumor cells have been described. Two areas have been the focus of recent studies, the low efficiency signal peptide of IL-15 and the induction of anti-tumor effector cells by IL-15.

Both murine and human IL-15 contain an unusually long 48-aa signal peptide, and an alternative short 21-aa signal peptide is also found in human (17,21) and an alternative 26-aa form is present in mice (22). We previously reported that highly immunogenic tumor (Meth-A) cells transfected with the human IL-15 containing the 48-aa signal peptide can elicit local and systemic T cell-dependent immunity (23). However, secretion of bioactive IL-15 was low, and these anti-tumor effects were not evident with weakly immunogenic colon 26 cells (Figs. 2 and 3). Although Kimura *et al* (28) reported the efficacy of Meth-A cells transfected with the alternative form of murine IL-15, which produces relatively large amounts of intracellular IL-15, they did not examine the efficacy with weakly immunogenic tumor cells.

We previously reported that replacement of the endogenous IL-18 leader sequence with the Igκ signal peptide caused efficient secretion of bioactive IL-18 protein (27). We constructed plasmid vectors containing the murine mature-IL-15 linked to the Igκ leader sequence and evaluated the efficacy of high IL-15-producing tumor. Tumorigenicity of mature-IL-15 transfectants of colon 26 cells was decreased in proportion to the level of IL-15 secretion. The clone with the highest production of IL-15 was rejected completely when injected subcutaneously (Figs. 2 and 3).