contribution of $CD14^+$ cells in the different timing of culture process and by the different proportion.

Modification of IL-2 Supplementation Schedule

In our original protocol established by Mikami and Harada, we added IL-2 to the cell culture medium every 3 days to maintain its biologic activity. However, in this study, we modified the schedule of IL-2 administration to determine the suitable culture conditions for $V\alpha24^+$ NKT expansion as follows: addition of IL-2 i) only on day 0, ii) days 0 & 3, iii) days 0, 3 & 6, and iv) days 0, 3, 6 & 9. Each supplementation of IL-2 was oriented to 100 IU/ml as a final concentration. The cell numbers and their phenotypes were analyzed on day 12. α-GalCer was also supplemented at final concentration 100 ng/ml.

Statistical Analysis

Student's t test was used to compare 2 groups and P values of < 0.05 were considered statistically significant. Correlation was estimated by the ordinary least squares method. Correlation coefficients are shown as squared values (r^2).

RESULTS

Efficient Expansion of $V\alpha24^+$ NKT Cells Derived from G-CSF-Mobilized PBSCT of Normal Healthy Donors

We compared the expansion-fold of $V\alpha24^+$ NKT cells in PBSCT before and after G-CSF mobilization in 20 healthy donors. The expansion fold of percentage and absolute number of $V\alpha24^+$ NKT cells increased, respectively, 18(SD ± 23)- and 182(± 158)-fold in PBMC before G-CSF mobilization, whereas these were 333(± 347)- and 669(± 925)-fold in G-CSF-mobilized PBMC. Apheresis products from collection bags showed more efficient expansion capacities, from 1384(± 1434)- to 7091(± 2160)-fold (Figure 1A,B). Thus, G-CSF mobilization significantly increased the capacity for $V\alpha24^+$ NKT cell expansion.

Relationship Between the Concentration of $CD34^+$, $V\alpha24^+$ and $CD14^+$ Cells on V α 24^+ NKT Expansion

To analyze the contribution of $CD34^+$, $V\alpha24^+$ and $CD14^+$ cells on the proliferation of $V\alpha24^+$ NKT cells in apheresis product, we compared the percentage of $CD34^+$, $V\alpha24^+$ and $CD14^+$ cells on day 0 and $V\alpha24^+$ NKT expansion efficacy on day 12. The results suggested only $CD14^+$ cells showed the correlation with the expansion of $V\alpha24^+$ NKT cells. (Figure 2A).

Contribution of $CD14^+$ Cells to the Ex Vivo Expansion of $V\alpha24^+$ NKT Cells

It has been reported that $CD14^+$ cells, dendritic cells and monocytes play a critical role in the initiation of proliferation of $V\alpha24^+$ NKT cells.[9] In PB after G-CSF treatment, the absolute number of $CD14^+$ cells significantly increased (from 350 ± 81 to 2353 ± 1220/μL),

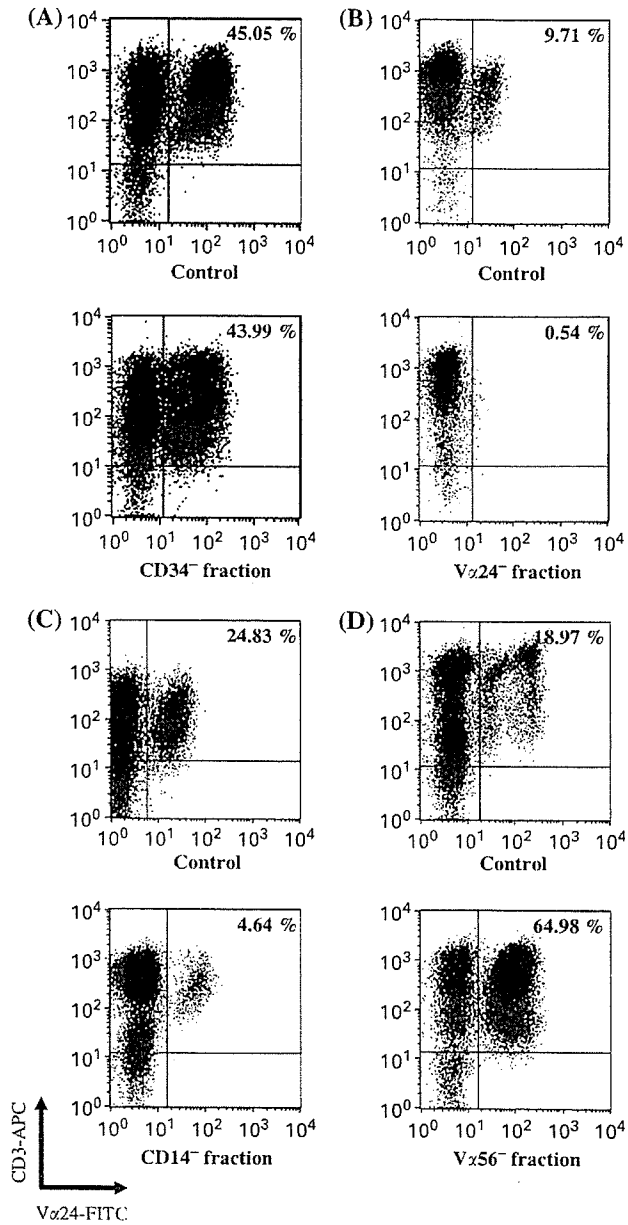

FIGURE 3. Effects of $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$ and $CD56^+$ NK cell depletion on the expansion of $V\alpha24^+$ NKT cells $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$, and $CD56^+$ NK cells were depleted using a MACS sorting system. (A) When $CD34^+$ cells were depleted, $V\alpha24^+$ NKT cells proliferated the same as in culture without $CD34^+$ cell-depletion. When (B) $V\alpha24^+$ NKT cells or (C) $CD14^+$ cells were depleted, $V\alpha24^+$ NKT cells did not expand. (D) When $CD56^+$ NK cells were depleted, the expansion efficiency of $V\alpha24^+$ NKT cells improved. These are each representative results from four experiments. The control in this experiment means the result by using apheresis product without target cell depletion.

although their percentage in PB did not change (from 7.24 ± 5.07 to 5.53 ± 2.10%) due to an overwhelming increase in granulocytes. In apheresis products, the proportion of $CD14^+$ cells in nuclear cells also increased 5.7- to 38-fold compared with before G-CSF mobilization, because the apheresis products included low granulocyte contaminations, less than 20%. We obtained $CD14^+$ cells using the MACS system with a purity of > 95%, and made a $CD14^+$ cell gradation (0%, 20%, 40%, 60%, 80% and 100%) under a fixed total cell count of 2.0×10^5 cells/mL/well. The efficacy of $V\alpha24^+$ NKT expansion was related to the initial proportion of $CD14^+$ cells, and the percentage of $V\alpha24^+$ NKT after expansion was increased in $CD14^+$ cell dose dependent manner (Figure 2B).

Effect of Depletion of Cells, Including $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$ and $CD56^+$ Cells, on $V\alpha24^+$ NKT Cell Expansion

To determine the origin of $V\alpha24^+$ NKT cells and the contribution of each cell population on $V\alpha24^+$ NKT cell expansion, we tested the following cell culture conditions with apheresis products: 1) $CD34^+$ cell-depleted, 2) $V\alpha24^+$ NKT cell-depleted, 3) $CD14^+$ cell-depleted, and 4) $CD56^+$ cell-depleted culture. When $CD34^+$ cells were depleted, $V\alpha24^+$ NKT cells proliferated the same as in non-depleted culture (Figure 3A). However, the depletion of $V\alpha24^+$ NKT cells completely abrogated the expansion of $V\alpha24^+$ NKT cells (Figure 3B). Depletion of $CD14^+$ cells also abrogated $V\alpha24^+$ NKT cell expansion to result in the complete disappearance of $V\alpha24^+$ NKT cells on day 12 (Figure 3C). Interestingly, when $CD56^+$ NK cells were depleted, a remarkable improvement in V $\alpha24^+$ NKT cell proliferation was observed (Figure 3D). In experiments with $CD56^+$ NK cells separated from $CD56^-$ fraction using a 3.0 μm-pore membrane, the proliferation of $V\alpha24^+$ NKT cells was maintained in $CD56^-$ fractions. The mixed culture of $CD56^+$ NK cells with $CD56^-$ fraction in the same wells resulted in the suppressed proliferation of $V\alpha24^+$ NKT cells, even though there were 1.0×10^5 $CD14^+$ cells (data not shown).

Add-Back of Cells, Including $CD14^+$ Cells, to $V\alpha24^+$ NKT Cell Cultures

The analysis of cell kinetics during culture suggested that $CD14^+$ cells gradually decreased in the early phase (days 0–3), whereas $V\alpha24^+$ NKT cells gradually increased in the latter phase of culture (days 9–12). With regard to $CD56^+$ NK cell kinetics, cell numbers continued to increase during culture in good responders (Figure 4A), whereas they peaked on day 9 in poor responders (Figure 4B). To evaluate the effects of $CD14^+$ NK cells in the early phase and late phase of $V\alpha24^+$ NKT cell expansion, we depleted and added back $CD14^+$ cells to the $CD14^-$ cell population, which included $V\alpha24^+$ NKT cells, on days 0, 3, 6 and 9, respectively (Figure 4C). Figure 4D shows that add-back of $CD14^+$ cells on day 0 induced the highest expansion of $V\alpha24^+$ NKT cells, whereas the

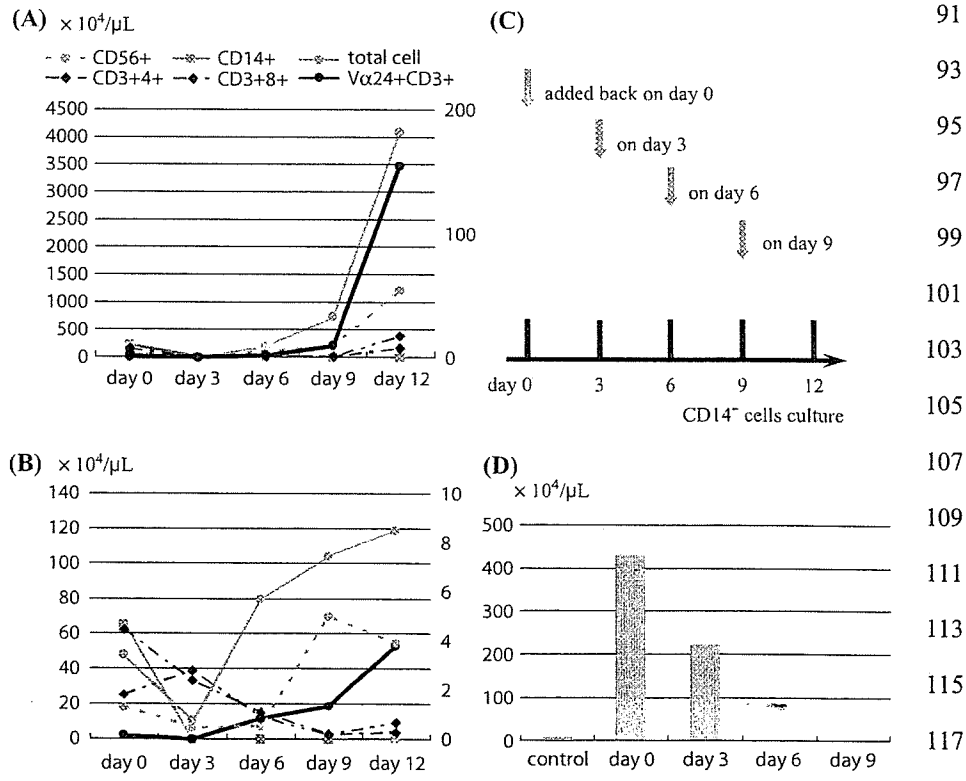

FIGURE 4. Cell kinetics of $V\alpha24^+$ NKT cells and $CD56^+$ NK cells in good and poor expanders (A) In a good-expanding donor, both $CD56^+$ NK cells and $V\alpha24^+$ NKT cells continued to proliferate without decline (representative results from four experiments). The right hand y-axis is used for the cell number of $V\alpha24^+$ NKT cells. (B) In a poor-expanding donor, $CD56^+$ NK cells proliferated more efficiently than $V\alpha24^+$ NKT cells, with a peak on day 9, concomitant with a suppression of $V\alpha24^+$ NKT cell proliferation on day 12 (representative results from four experiments). The right hand y-axis is used for the cell number of $V\alpha24^+$ NKT cells. (C) We added-back $CD14^+$ cells to $CD14^-$ cells on days 0, 3, 6, and 9. (D) Add-back of $CD14^+$ cells before day 3 enhanced the proliferation of $V\alpha24^+$ NKT cells. These are representative results from four independent experiments. All of four experiments were comparable and had a same tendency.

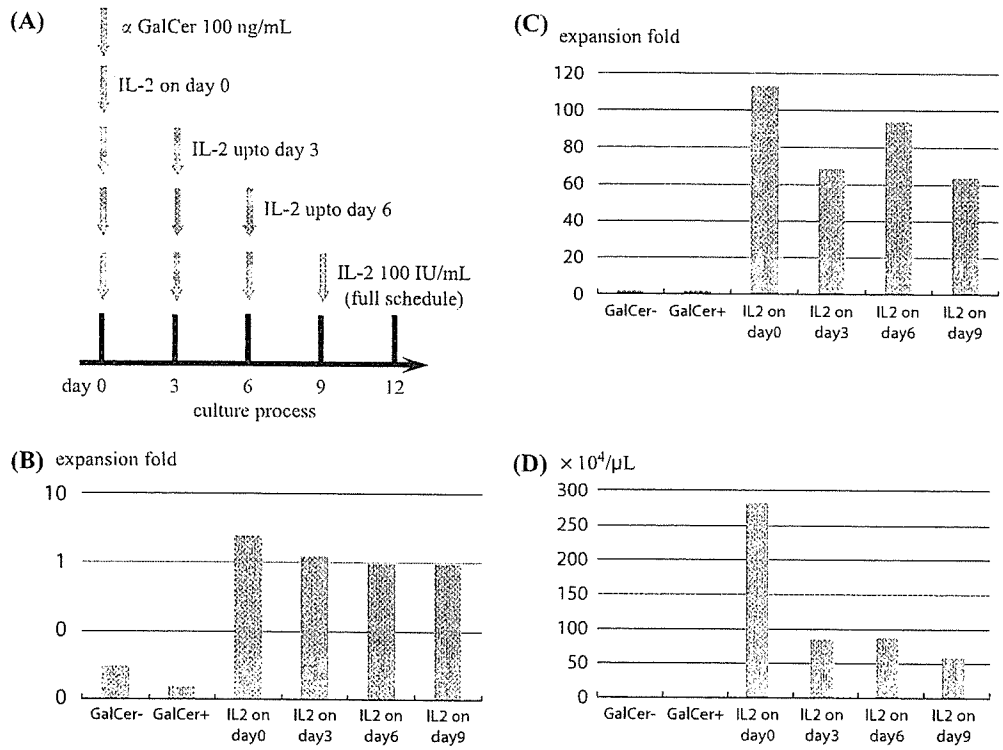

FIGURE 5. Effects of treatment with IL-2 on the expansion of Vα24$^+$ NKT cells (A) We tested different schedules for the administration of IL-2, as follows: on day 0 only, on days 0 & 3, on days 0, 3 & 6, and on days 0, 3, 6 & 9. We found that (B) the expansion-fold of whole cells, and the expansion-fold of the proportion (C) and absolute number (D) of Vα24$^+$ NKT cells were higher when IL-2 was supplemented on day 0 only (representative results from four experiments). All of four experiments were comparable and had a same tendency. In this experiments, α-GalCer was also supplemented at the concentration of 100 ng/mL without non-α-GalCer supplemented control.

addition of CD14$^+$ cells in the late phase did not show any remarkable benefit.

Effect of IL-2 Supplementation on the Expansion of CD56$^+$ NK Cells and Vα24$^+$ NKT Cells

We hypothesized that repeated IL-2 supplementation could result in the enhancement of CD56$^+$ NK activity to suppress the proliferation of Vα24$^+$ NKT cells.[10] In Figure 5, we tested four different schedules of IL-2 administration: on day 0 only, on days 0 & 3, on days 0, 3 & 6, and on days 0, 3, 6 & 9 (Figure 5A). We found that whole cells and Vα24$^+$ NKT cells expanded most effectively when IL-2 was added on day 0 only (Figure 5B,C,D).

DISCUSSION

The methods that have been used for the ex vivo expansion of human NKT cells can be divided into two categories: simple culture of PBMC with α-GalCer,[12] and a two-step culture method that uses α-GalCer-pulsed monocytes as feeder cells[15],. A single culture system has the benefit of simplicity and a low risk of contamination, and a major obstacle in a two-step culture system is the availability of a large number of feeder cells. Hence, in this study of the former type, we intended to improve and establish culture conditions for realistic clinical application. Previously, we used a single stimulation of α-GalCer on the initial day, and then administered IL-2 every 3 days to obtain satisfactory expansion of human Vα24$^+$ NKT cells.[7] We have also reported that the addition of 5% autologous plasma was also effective.[8] G-CSF mobilization increased the efficacy of Vα24$^+$ NKT cell expansion, and our data suggested that this was due to a change in cellular component including CD14$^+$ cells[16] and serous factors in the blood. In our present study, we found that CD14$^+$ cells, which are effectively mobilized together with CD34$^+$ cells by G-CSF,[16] are one of the candidates hat contribute to the effective ex vivo expansion of Vα24$^+$ NKT cells. Only the number of pre-cultured CD14$^+$ cells affected the magnitude of the expansion of Vα24$^+$ NKT cells, and this agreed with a previous report by van der Vliet et al that dendritic cells (DC) derived from monocytes including CD14$^+$ cells could efficiently mediate the expansion of Vα24$^+$ NKT cells[17](18). Additionally, we showed that 1) depletion of CD14$^+$ cells resulted in the loss of Vα24$^+$ NKT cell expansion, and 2) the expansion efficacy of Vα24$^+$ NKT

cells depended on the ratio of $CD14^+$ cells at the initiation of culture. Based on these observations, we speculated that the initial presence of $CD14^+$ cells plays an important role in the subsequent effective expansion of $V\alpha24^+$ NKT cells. We observed that the intensity of CD1d molecules on $CD14^+$ cells, which is critical for interaction with α-GalCer for the expansion of $V\alpha24^+$ NKT cells,[19] increased after G-CSF mobilization (data not shown). Hence, it is reasonable to speculate that more $CD14^+$ cells with a high intensity of CD1d molecules plays a key role in NKT cell expansion. The higher expansion efficiency in apheresis products compared with G-CSF-mobilized PB may be secondary to a higher concentration of $CD14^+$ cells.

The removal of $V\alpha24^+$ NKT cells before culture resulted in the loss of $V\alpha24^+$ NKT cell proliferation, and this supported previous reports that *ex vivo*-expanded $V\alpha24^+$ NKT cells were neither committed nor supported by $CD34^+$ cells, but were derived from peripheral circulating V α 24^+ NKT cells.[17] Whereas $CD34^+$ cells do not appear to be directly involved in the expansion of $V\alpha24^+$ NKT cells, they might make the circumstances suitable for $V\alpha24^+$ NKT cell expansion, through the secretion of unidentified soluble factors from bone marrow-derived stromal cells, as suggested by Johnston et al.[20] Although the presence of $V\alpha24^+$ NKT cells on day 0 is critical for the expansion of $V\alpha24^+$ NKT cells, no correlation was found between the proportion of $V\alpha24^+$ cells before culture and the proportion of $V\alpha24^+$ NKT cells at the end of culture. This suggests that some other factor(s) might regulate the expansion kinetics of $V\alpha24^+$ NKT cells. The inhibition of cell expansion by $CD56^+$ NK cells was restored when direct cell-to-cell contact was interrupted, which suggests that direct interaction between $V\alpha24^+$ NKT cell and $CD56^+$ NK cells plays a role. This hypothesis was indirectly supported by the phenomina that IL-2 supplementation in every 3 days suppressed expansion of $V\alpha24^+$ NKT cells. Indeed, NK cell-mediated interference of NKT cells is well known to be a primary immune regulatory mechanism.[21] Another possibility is indirect inhibition through the modulation of DC functions. It has been reported that NK cells could yield cytolytic activity against DC during their expansion.[22–24] NKT cells were also activated by DC, resulting in the suppression and killing of DC[25](26) in the same manner as NK cells.

In conclusion, for the efficient ex vivo expansion of $V\alpha24^+$ NKT cells, the presence of $V\alpha24^+$ cells and $CD14^+$ cells at the initiation of culture is critical. NK cells may interact with antigen presenting cells (APC) and interfere with the expansion of NKT cells by hindering the function of antigen presentation or providing direct cytotoxicity against APC. We believe that these findings may be useful for the development of an efficient system for the expansion of NKT cells for future adaptive immunotherapy.

ACKNOWLEDGMENTS

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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 ß 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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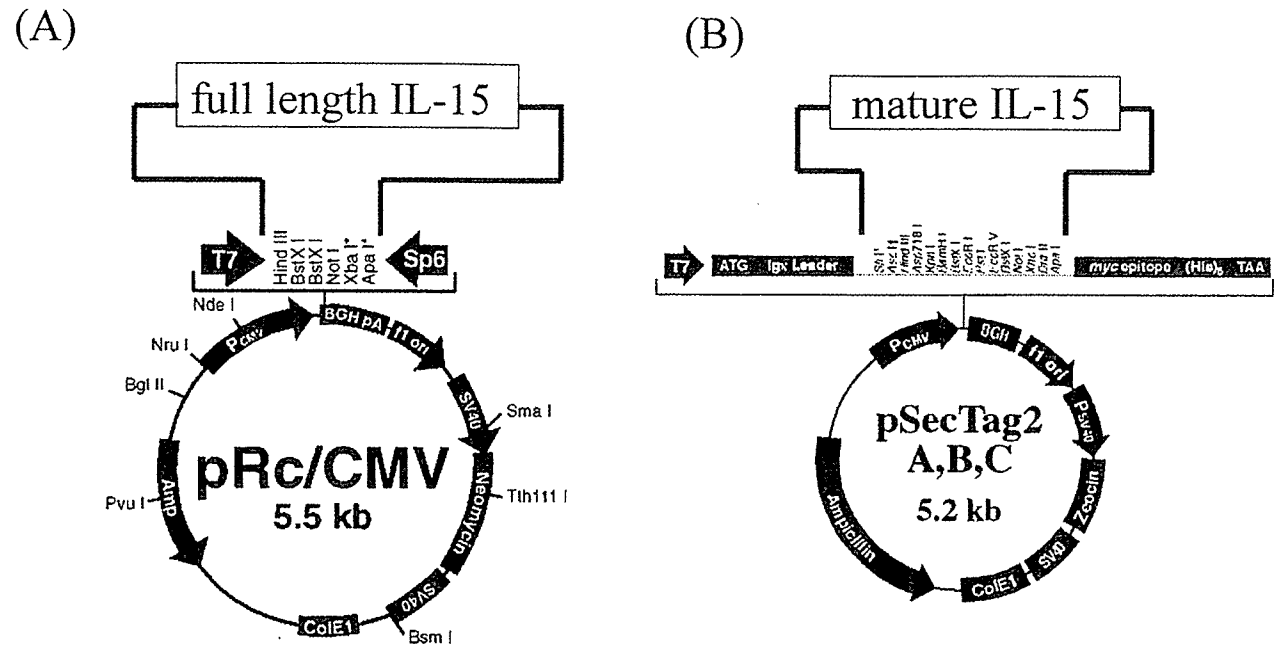

Figure 1. (A), Illustration of full-length IL-15 expression vector. (B), Illustration of mature-IL-15 expression vector.

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 ($1x10^6$ 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 $5x10^{-5}$ 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 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.

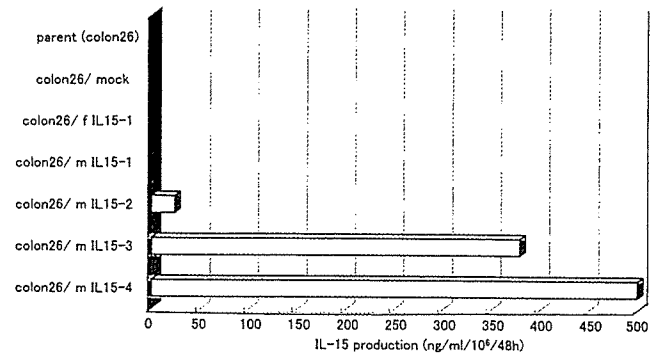

Figure 2. Production of IL-15 by transfectants was confirmed by CTLL-2 proliferation assay. Levels of IL-15 produced by mature-IL-15 transfectants (clones 1, 2, 3 and 4) were between 0 and 500 ng/10^6 cells/48 h, whereas full-length IL-15 transfectants and mock transfectants did not produce IL-15.

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.

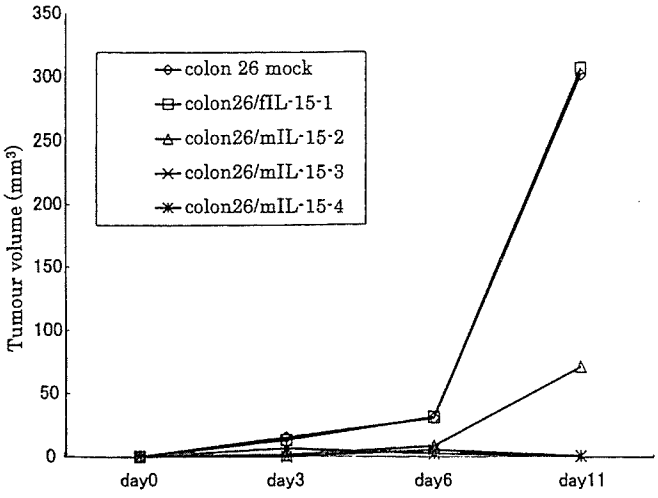

Figure 3. Tumorigenicity of full-length IL-15, mature-IL-15 (clones 1, 2, 3 and 4) and mock transfectants were examined by subcutaneous injection into BALB/c mice. Mean tumor volumes of clones 3 and 4 were significantly smaller on day 11 ($P<0.01$), whereas mock transfectants and clone 1 grew progressively.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

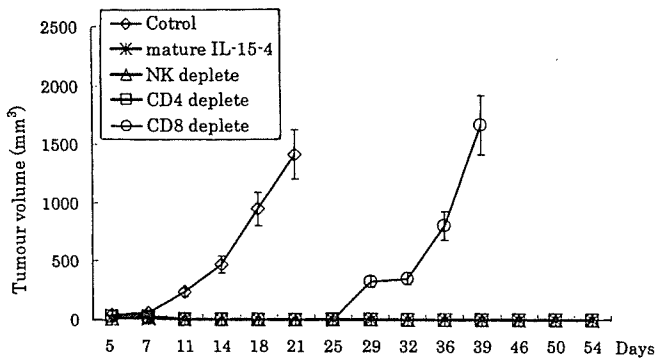

Figure 4. Depleted mice and control mice (HBSS only) were implanted with colon 26/mIL-15-4 cells. There was no difference in tumor growth between the NK-depleted mice, $CD4^+$-depleted mice, and control mice. Tumors in mice treated with anti-$CD8^+$ antibodies was initially rejected but grew rapidly 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 $5x10^5$ 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, $5x10^5$ 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).

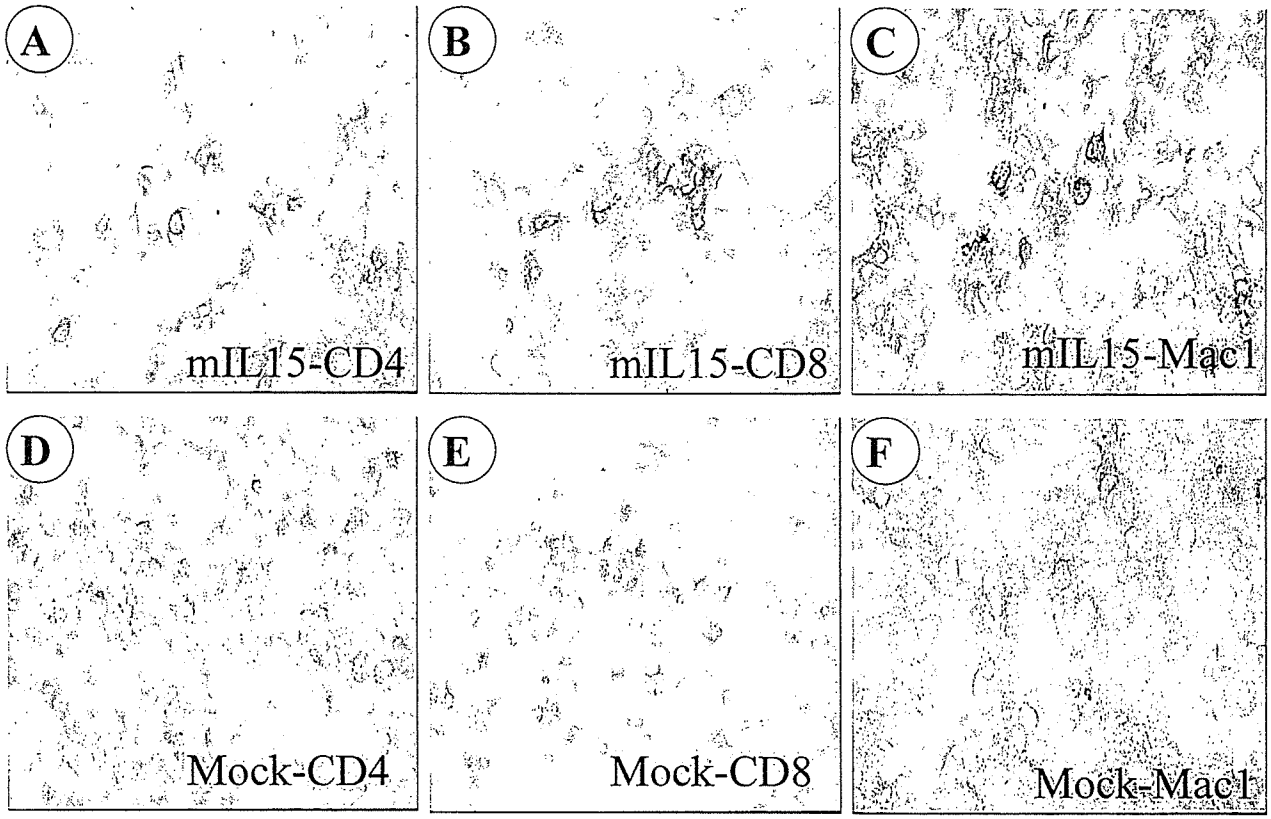

Figure 5. Immunohistochemical analysis of tumor implantation sites in BALB/c mice 14 days after subcutaneous injection of colon 26/mIL-15-4 cells (A-C) and mock transfectants (D-F). Staining with L3/T4 (CD4) (A), KT15 (CD8) (B) and Mac-1 (CD11b) (C) revealed infiltration of $CD4^+$ and $CD8^+$ lymphocytes and Mac-1-positive monocytes, respectively. Such infiltration was not observed in mice treated with mock transfectants (D-F).

Fourteen days after subcutaneous injection of colon 26/mIL-15-4 cells infiltration of $CD4^+$ and $CD8^+$ lymphocytes and Mac-1-positive monocytes was observed at the site of injection (Fig. 5). These findings were supported by previous reports that described the function of IL-15 in proliferation and functional activation of T, B and NK cells (3,4) and monocytes (29). To further clarity of anti-tumor mechanisms, we performed *in vivo* depletion of NK cells, $CD4^+$ and $CD8^+$ T cells. The anti-tumor effects of colon 26/mIL-15-4 cells were partially abrogated by treatment with anti-$CD8^+$ antibodies but not by depletion of NK cells or $CD4^+$ T cells (Fig. 4). In $CD8^+$ T cell-depleted mice, colon 26/mIL-15-4 cells disappeared temporarily, but re-grew 1 month after implantation. These results indicate that inoculation of IL-15-secreting tumor cells may mediate initial anti-tumor effects through $CD8^+$ T cells, $CD4^+$ T cells, NK cells and a variety of immunocompetent cells and that long-lasting specific immunity is mediated only through $CD8^+$ T cells. However, Meazza *et al* (30) reported that TS/A tumor cells, which secrete high levels of IL-15, reduced tumorigenicity, and that depletion of $CD8^+$ T cells or NK cells abrogated the efficacy of IL-15. This inconsistency may be due to differences in IL-15 between murine and human. Although murine and human IL-15 cross-react, there is only 73% amino acid identity between murine and human IL-15 (1). Therefore, it may be more appropriate to use murine IL-15 in murine studies. Indeed, Yajima *et al* (31) reported that murine IL-15 transgenic mice have anti-tumor activity against MHC class I-negative and -positive malignant melanoma through augmented NK activity and cytotoxic T-cell response, respectively. The present study is the first to show that murine tumor cells secreting high levels of murine IL-15 can mediate complete rejection of weakly immunogenic tumor cells and induce long-lasting specific anti-tumor immunity.

These results suggest that IL-15 is important in tumor immunity and that IL-15 may be an excellent candidate for a tumor-vaccine adjuvant for boosting $CD8^+$ memory T cells as therapy for weakly immunogenic human cancers.

Acknowledgements

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特集 コンセンサス 胆嚢癌の治療

CONTENTS

へるす出版

山口大学医学部消化器・腫瘍外科 硲 彰一／岡 正朗
Shoichi Hazama／Masaaki Oka

免疫逃避機構

tumor escape in immune responses

腫瘍細胞においては腫瘍特異抗原や腫瘍関連抗原が発現しているにもかかわらず，腫瘍組織は増殖し，宿主の生命を脅かす。これは腫瘍組織が宿主の免疫監視機構をかいくぐる免疫逃避機構をもっているからに他ならない。

HLA class I 抗原の発現低下

腫瘍細胞の免疫逃避機構として，腫瘍抗原の発現低下があげられる。

腫瘍細胞表面に腫瘍抗原が提示されるためには，まず腫瘍抗原蛋白が細胞内に存在し，イムノプロテアソームによりプロセシングを受けて蛋白が提示ペプチドとなり，transporter associated with antigen processing (TAP)により細胞内輸送され，HLA-A，B，Cならびにβ_2-microgloburin (β_2m)と結合して腫瘍細胞表面に提示されることが必要である。class I 発現を認めない分子機構として，まずβ_2m の不活化があげられる。この場合には抗原提示が不可能となるため，特異的腫瘍免疫誘導は不可能となる。腫瘍抗原のプロセシング機能低下の要因として，low molecular weight polypeptide proteasome subunit (LMP)，multicatalytic endopeptidase complex-like-1 (MECL-1)，PA28などのプロテアソームサブユニットの発現の低下ないしは欠失があげられる。TAPに代表される細胞内輸送機能の低下も知られている。限局癌が浸潤癌，転移癌へと進展する過程においてもHLAの発現は低下し，悪性度・免疫逃避機構が助長される。このように，HLA class I発現を認めない腫瘍に対してはMHCに依存しない免疫監視機構が重要となり，MHC非拘束性様式で腫瘍を認識・攻撃する効果細胞の増強が重要となる。

免疫抑制物質の産生

腫瘍局所において，腫瘍産生因子あるいは腫瘍間質に存在する免疫細胞や間質細胞から産生される免疫抑制物質(TGF-β，IL-6，IL-10，PGE_2)などの作用により，腫瘍局所の免疫監視機構や全身の免疫能が低下する。これが腫瘍の逃避機構の一因となっている。免疫系は大きくtype 1(細胞性免疫)ならびにtype 2(液性免疫)に分類することができるが，抗腫瘍免疫はtype 1(細胞性免疫)が担っている。腫瘍局所に浸潤したマクロファージ(M)は腫瘍局所環境によりtype 2 Mへと誘導される。Type 2 Mが産生するIL-6やIL-10はヘルパーTリンパ球(Th)のうちTh2を誘導し，細胞性免疫は抑制される結果となる。さらに，Thから産生されるIL-4，IL-10，IL-6などのいわゆるTh2系サイトカインはtype 2 Mを誘導するため，癌患者の抗腫瘍免疫はさらに抑制される結果となる。一方，癌免疫療法により強力な抗腫瘍エフェクターの誘導に成功しても，癌局所におけるTGF-βなどの免疫抑制物質により抗腫瘍活性が不活化され，十分な抗腫瘍効果が得られない。

腫瘍細胞におけるFas ligandの発現

Fas発現細胞はFas ligandとの結合刺激により，アポトーシスに陥るとされている。通常リンパ球はFas ligandを発現しており，腫瘍細胞に発現しているFasに結合してアポトーシスに陥らせると考えられている。これとは逆に，腫瘍細胞に発現したFas ligandがリンパ球上に発現したFasを刺激してリンパ球をアポトーシスに陥らせる機構が，腫瘍の免疫逃避機構の一つとして報告されている。

表1 腫瘍の免疫逃避機構

1. HLA class I 抗原の発現低下
   a. 腫瘍抗原蛋白分解酵素の発現低下
   b. 細胞内輸送機構の不活化
   c. β_2-microgloburin の不活化
2. 腫瘍細胞および浸潤免疫細胞からの免疫抑制物質の産生
   a. TGF-β
   b. IL-6
   c. IL-10
   d. PGE_2
3. 腫瘍細胞における Fas ligand の発現