

Direct Activation of Human CD8⁺ Cytotoxic T Lymphocytes by Interleukin-18

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Direct activation of human cytotoxic T lymphocytes (CTL) by interleukin (IL)-18 was observed in a system in which CTL effective against autologous tumor cells were generated. Peripheral blood mononuclear cells (PBMC) from tumor-bearing patients, after removal of natural killer (NK) cells, were cultured in a medium containing IL-1, -2, -4, and -6, with or without IL-18, and stimulated with autologous tumor cells. IL-18 increased the activity of the CTL and the proportion of autologous CD8⁺ T cells present after 28 days in the induction culture. When purified CD8⁺ T cells were cultured in the presence of IL-18 and IL-2 for 7 days, the CTL showed enhanced cytotoxic activity against autologous tumor cells. Moreover, a purified CD8⁺ T cell population, which did not exhibit any apparent cytotoxic activity against autologous tumor cells, displayed cytotoxic activity after 7-day incubation with IL-18. These results suggest that IL-18 may be useful to generate autologous CTL in humans and may thereby contribute to adoptive immunotherapy for tumors.

Key words: IL-18 — Cytotoxic T lymphocytes — Adoptive immunotherapy

For efficient generation of autologous cytotoxic T cells (CTL) in humans, as desired in adoptive immunotherapy for tumors,^{1,2} an understanding of the cytokine requirements is critical. Many investigators have tried to induce CTL using only interleukin (IL)-12,³⁻⁵ but there have been few studies in which the combined use of IL-2 and other cytokines was examined. Nakashima *et al.*⁶ reported that CTL were expanded from the lymphocytes from tumor-associated lymph nodes in the presence of IL-1, -2, -4, and -6. We also have reported that, by using this IL-cocktail, the serum/plasma-free culture of allogeneic human CTL is possible for more than 1 month without loss of target specificity and cytotoxicity,⁷ and that autologous human CTL are inducible from peripheral blood mononuclear cells (PBMC) of tumor-bearing patients.^{8,9} If there are any other cytokines that contribute to activation and/or induction of CTL, such cytokines should be taken into consideration to fortify the IL-cocktail.

IL-18, formerly designated interferon (IFN)- γ inducing factor, is a recently cloned cytokine with a molecular weight of 18 kDa, synthesized in Kupffer cells and activated macrophages.^{10,11} The activities associated with IL-18 are induction of IFN- γ production in Th1 cells and natural killer (NK) cells, especially in the presence of IL-12,¹² and enhancement of the cytotoxic activity of these cells through the Fas ligand-mediated mechanism.^{13,14} The effect of IL-18 on human CD8⁺ T cell responses has not been established. We report here the possible involvement of IL-18 in human CD8⁺ T cell responses, in particular, the cytotoxic activity against autologous tumor cells.

MATERIALS AND METHODS

Cytokines Recombinant human IL-1 β , IL-2, IL-4, IL-6, and IL-18 were kindly provided by Ohtsuka Pharmaceuticals Co. (Tokushima), Shionogi & Co., Ltd. (Osaka), Ono Pharmaceuticals Ltd. (Osaka), Ajinomoto Inc. (Tokyo), and Hayashibara Biochemical Laboratories, Inc. (Okayama), respectively. IL-12 was purchased from Genzyme Co. (Cambridge, MA).

Cell purification and induction of CTL After informed consent had been obtained from each patient, human PBMC were separated from the peripheral blood of tumor-bearing patients by the conventional Ficoll/Hypaque method (Lymphosepal, Immuno-Biological Laboratories Inc., Gunma). For preparation of a CD56-negative population of PBMC, 10⁷ PBMC were suspended in 100 μ l of phosphate-buffered saline (PBS) supplemented with 2 mM EDTA and 0.5% plasma protein fraction (Baxter Co., Tokyo) and incubated at 4°C for 15 min after the addition of 10 ml of anti-CD56 MicroBeads (Miltenyi Biotec, Germany). Then the CD56⁺ cells were removed by means of a LS+ Separation Column (Miltenyi Biotec) according to the manufacturer's protocol. Induction of CTL was performed as previously described.^{8,9} The CTL population generated was maintained in RHAM α medium^{7,15} containing 5% autologous plasma or, when indicated, 5% plasma protein fraction and an IL-cocktail consisting of IL-1 (167 U/ml), IL-2 (67 U/ml), IL-4 (67 U/ml), and IL-6 (134 U/ml). CTL were restimulated every 2 weeks with X-ray-irradiated autologous tumor cells. IL-18 and IL-12 were used at 10 ng/ml and 50 pg/ml, respectively. CD8⁺ T cells were purified from T lymphocyte

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populations by the immunomagnetic separation method. Briefly, 10^7 T lymphocytes were incubated with FITC-labeled anti-CD8 monoclonal antibody (mAb) (Nichirei Co., Tokyo), appropriately diluted in 100 μ l of PBS supplemented with 2 mM EDTA, and 0.5% plasma protein fraction for 20 min. These cells were washed, exposed to anti-FITC MicroBeads (Miltenyi Biotec), and incubated at 4°C for 15 min. Then the CD8⁺ T cells attached to the MicroBeads were separated by means of the LS+ Separation Column.

Tumor cells Tumor cell lines TKB9 from glioblastoma multiforme, GT3TKB from ascites of a gastric adenocarcinoma patient, and TUHR13TKB from renal carcinoma were established in our laboratory. These lines were maintained in RHAM α medium containing 10% fetal bovine serum (FBS).

Cytotoxic activity Cytotoxic activity of CTL was determined by coculturing lymphocytes and the target tumor cells for 24 h. The number of target cells adhering to the bottom of wells was measured as the number of surviving cells by means of the crystal violet staining (CV) assay.¹⁶⁾ This CV assay used in the present study is as sensitive for assessment of the killing activity of CD8⁺ T cells towards adherent target cells as the standard ⁵¹Cr-release cytotoxicity assay at effector-to-target (E/T) ratios of 10 or lower.¹⁶⁾ Briefly, the target cells, 1×10^4 cells/well in 200 μ l of medium containing 10% FBS, were seeded in each well of 96-well plates and were precultured for 12 h. The target cells were washed once with medium, then the PBMC or CD8⁺ T cells suspended in 200 μ l of RHAM α containing 5% autologous plasma were added as effector cells to each well at the indicated E/T ratio. These cells were cocultured for 24 h. The wells were washed once gently with an appropriate amount of PBS. Adherent target cells were fixed for 1 h with 10% (v/v) formalin (200 μ l/well), then stained with crystal violet solution (0.4% in water, 100 μ l/well) for 30 min at room temperature. The plate was washed with tap water and dried at room temperature. To each well, 200 μ l of 80% methanol was added and the A_{570} of each well was determined. As the 100% control, we determined the A_{570} of the target cells in a separate plate just before the addition of the effector cells. Percentage of surviving target cells was defined as follows:

$$\text{Surviving target cell (\%)} = (A - C) / (B - C) \times 100$$

where A is the A_{570} of the well containing the target cells and the CTL, B is the A_{570} of the 100% control well of the target cells, and C is A_{570} of the well containing medium alone. In the present experiments, the wells containing the CTL alone showed almost the same A_{570} as C , or even slightly higher A_{570} , even in the wells corresponding to E/T of 8. Therefore, we thought that remaining lymphocytes in the wells after the 24 h coculture were

sufficiently washed out. A marginal amount of the lymphocytes may have remained attached on the target cell layer, but they were included as surviving target cells. Therefore, the possibility of slight underestimation of the killing activity of the CTL should be considered in this assay. Note that the tumor cells cultured at E/T=0 grew well during 24 h incubation and, therefore, a value of more than 120% surviving target cells was obtained. Inhibition of the cytotoxic activity of the CTL by anti-CD8 mAb (Nichirei Co.) was examined as described previously.¹⁶⁾

Immunophenotypic analysis For direct single- or two-color analysis in flow cytometry, the lymphocytes (5×10^5) were incubated for 30 min at 4°C with PE- and FITC-conjugated mAb (DAKO Japan Inc., Kyoto), or murine control antibodies for each subclass of immunoglobulin (FITC- or PE-conjugated immunoglobulin (Ig) G2a or IgG2b, DAKO Japan Inc.). The stained cells were examined with a FACScan (Becton Dickinson, Co., Mountain View, CA).

Enzyme-linked immunosorbent assay (ELISA) of IFN- γ and tumor necrosis factor (TNF)- α For determination of cytokine production, the purified T cells (8×10^4 /well) were added to wells of 96-well plates with 200 μ l of medium per well. The culture supernatant was collected 24 h later. The quantity of IFN- γ or TNF- α in the culture supernatants was determined by two-site ELISA according to the manufacturer's protocol (PharMingen, San Diego, CA).

RESULTS

Effect of IL-18 on the induction of autologous CTL from PBMC Initial experiments were performed to examine whether addition of IL-18 influences the development of human autologous CD8⁺ CTL from the PBMC of a tumor-bearing patient. Since IL-18 is known to activate mouse and human NK cells,^{11, 14, 17)} CD56⁺ cells including NK cells were removed from the PBMC of patient A (glioblastoma multiforme) before the induction culture. This population of PBMC was stimulated with X-ray-irradiated autologous target tumor cells, TKB9, to induce the development of autologous CTL. The lymphocytes were restimulated with the irradiated target cells every 2 weeks. After 14 days in the induction culture, over 95% of the lymphocytes appeared to be CD3⁺ cells (T cells) (data not shown). These T cells were collected after 28 days, and the cytotoxic activity against autologous TKB9 cells was examined by means of the CV assay. As shown in Fig. 1, the T cells cultured in medium containing the IL-cocktail (IL-1, -2, -4, and -6) together with IL-18 exhibited target killing activity in a dose-responsive manner. The addition of IL-18 resulted in remarkable enhancement of the cytotoxic activity of the

CTL at low E/T ratios of 2–8. However, the T cells cultured with IL-2, or IL-2 plus IL-18, did not exhibit apparent cytotoxic activity. The cytotoxic activity of the CTL was blocked by pretreatment of the cells with anti-CD8 or anti-MHC class I monoclonal antibody before the killing assay (Fig. 2), suggesting that the cytotoxic activity was dependent on CD8⁺ CTL in the T cell population and this cytotoxic activity was MHC class I restricted.

The phenotype of these lymphocytes was examined by flow cytometry. In spite of the different combinations of cytokines which were added to the culture medium, over 90% of the cells in the population became CD3⁺ T cells within the first 14 days, and CD56⁺ cells were not detectable (Table I). The proportion of CD8⁺ T cells remained at 25% or less in the population of PBMC cultured with

IL-2, but not in the presence of the IL-cocktail. The effect of added IL-18 was observed in the case of cells cultured with the IL-cocktail and IL-18 after 28 days. In this population, the proportion of CD8⁺ T cells reached 90% after 35 days in the induction culture, while 54% of the cells induced with the IL-cocktail in the absence of IL-18 were CD8⁺ T cells.

Direct activation of the purified CD8⁺ CTL To elucidate the nature of the augmentation of CD8⁺ CTL activity by IL-18, CD8⁺ T cells were purified by the method previously reported^{8,9} from the CTL population derived from the PBMC of patient A by induction with the IL-cocktail. Cytofluorometric analysis showed that over 95% of the cells in this population of PBMC became CD3⁺ T cells within the first 21 days, including both CD8⁺ T cells and

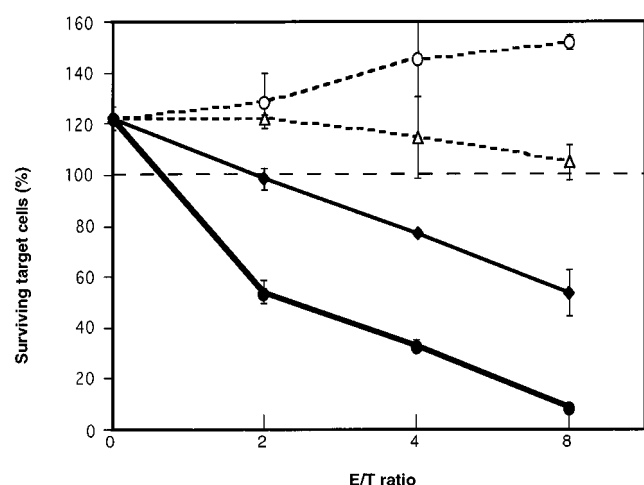


Fig. 1. Killing activity of the CTL induced from PBMC. PBMC from patient A were stimulated with autologous tumor cells (TKB9) in medium containing IL-2 (Δ), IL-2 plus IL-18 (○), an IL-cocktail consisting of IL-1, -2, -4, and -6 (◆), or the IL-cocktail plus IL-18 (●), for 28 days. Cytotoxic activity of the CTL against TKB9 cells was determined by means of the crystal violet staining assay (see “Materials and Methods”). Each point shown is the mean±SD of triplicate determinations.

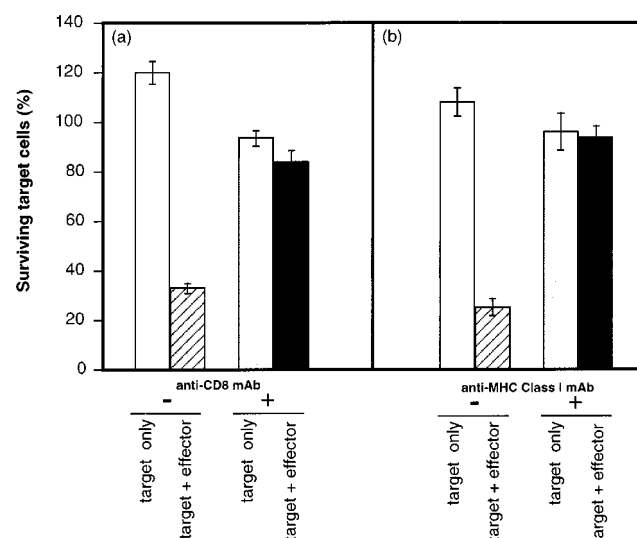


Fig. 2. Inhibition of the cytotoxic activity of CTL from patient A by anti-CD8 mAb (a) and anti-MHC class I mAb (b). Effector cells were pretreated with anti-CD8 mAb at 4°C for 1 h. Then these cells were incubated with autologous target tumor cells (TKB9) at an E/T ratio of 4 at 37°C for 24 h. Each bar shown is the mean±SD of triplicate determinations.

Table I. Phenotype of Lymphocytes Induced from PBMC

Culture conditions	Days	Proportion of											
		CD3 ⁺ (%)				CD56 ⁺ (%)				CD8 ⁺ (%)			
		0	14	28	35	0	14	28	35	0	14	28	35
IL-2		75	96	ND	ND	<1	<1	ND	ND	23	14	25	12
IL-2+IL-18		75	91	ND	ND	<1	<1	ND	ND	23	13	23	16
IL-2+IL-4+IL-6+IL-1		75	97	ND	ND	<1	<1	ND	ND	23	32	30	54
IL-2+IL-4+IL-6+IL-1+IL-18		75	98	ND	ND	<1	<1	ND	ND	23	24	71	90

ND: not determined.

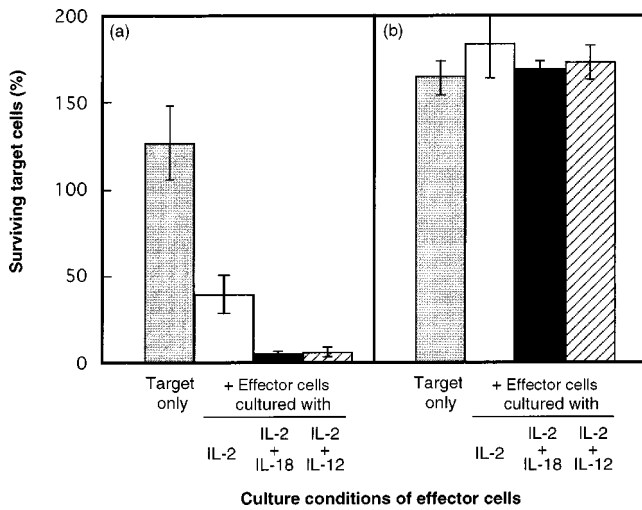


Fig. 3. Direct activation of purified CD8⁺ CTL by IL-18. CD8⁺ T cells were purified from the IL-cocktail-induced CTL population derived from patient A, and then cultured in medium containing IL-2 alone or IL-2 in combination with IL-18 and IL-12 for 7 days. The killing assay was performed at an E/T ratio of 4 (a) with autologous TKB9 tumor cells and (b) with allogeneic GT3TKB tumor cells, both of which carry the same MHC class I molecule, HLA-A2402. Each bar shown is the mean±SD of triplicate determinations.

Table II. IFN- γ and TNF- α Production by CD8⁺ T Cells in Response to IL-18

Cytokine	IFN- γ (pg/ml)	TNF- α (pg/ml)
IL-2	79	248
IL-2+IL-18	156	>1250
IL-2+IL-12	157	1230

CD4⁺ T cells (data not shown). The CD8⁺ T cells were then positively selected by means of the immunomagnetic beads. Since the purified lymphocytes were able to survive, though scarcely proliferated, in culture medium containing IL-2 but lacking IL-1, -4, and -6 (data not shown), these cells were cultured with IL-2 in the absence or presence of IL-18 or IL-12 for 7 days. We adopted IL-12 as the positive control since it is known to enhance the cytotoxic activity of human CTL.^{18,19} The results showed that IL-18, like IL-12, augmented the cytotoxic activity of CD8⁺ CTL against the autologous tumor cells, TKB9 (Fig. 3a). Moreover, the CD8⁺ CTL exhibited no cytotoxic activity against an allogeneic tumor cell line, GT3TKB cells (gastric adenocarcinoma), which carry the same MHC class I molecule, HLA-A2402 (Fig. 3b).

We also tested whether IL-18 is able to induce cytotoxic activity in a population of purified CD8⁺ T cells that

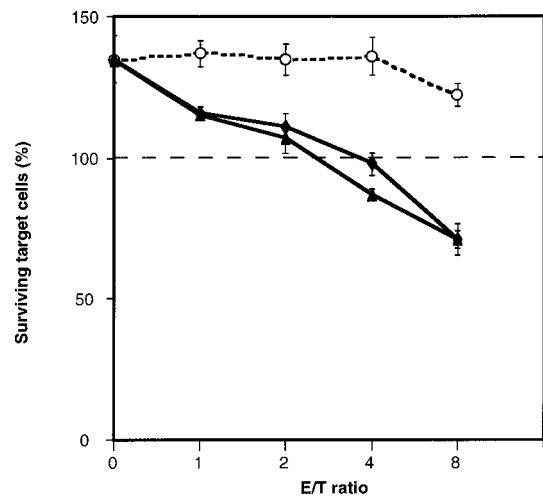


Fig. 4. Induction of cytotoxic activity in inert CD8⁺ T cells against TUHR13TKB autologous tumor cells. CD8⁺ T cells derived from patient B were purified from an inert T cell population which had been cultured in induction medium containing the IL-cocktail. The CD8⁺ T cells were cultured with IL-2 alone (○) or IL-2 in combination with IL-18 (◆) or IL-12 (▲) for 7 days. Each point shown is the mean±SD of triplicate determinations.

failed to display any apparent cytotoxic activity against autologous tumor cells. The PBMC from patient B (renal carcinoma) were cocultured with autologous tumor cells, TUHR13TKB, for 21 days in culture medium containing the IL-cocktail. However, the resulting T cell population showed no killing activity against TUHR13TKB cells, although the population contained over 95% CD3⁺ T cells, of which 90% were CD8⁺ T cells (data not shown). The CD8⁺ T cells were purified from this population and cultured with or without IL-18 in the presence of IL-2 for 7 days, and then their cytotoxic activity against the autologous TUHR13TKB cells was examined. IL-18 induced cytotoxic activity comparable to that obtained with the positive control cytokine, IL-12 (Fig. 4).

Since IL-18 was initially identified as an IFN- γ -inducing factor (IGIF),^{10,11} we confirmed its stimulatory effect on IFN- γ and TNF- α production by purified CD8⁺ T cells derived from patient B. When the CD8⁺ T cells were cultured in the presence of IL-2 and either IL-18 or IL-12 for 2 days, both IFN- γ and TNF- α production, especially the latter, were apparently enhanced (Table II).

DISCUSSION

The present results suggest that IL-18 directly activated autologous tumor-specific CD8⁺ CTL *in vitro*, just like IL-12^{18,19} (Fig. 3a and Fig. 4). In a preliminary study, when PBMC from patient A were cultured with the IL-

cocktail (IL-1, -2, -4, and -6), the PBMC could be expanded for nearly 1 month. However, the major population in the PBMC was NK cells and CD8⁺ T cells were hardly detected (data not shown). When the PBMC of patient A were cultured with allogeneic tumor cells (GT3TKB), CD8⁺ T cells became the major population within 2 weeks. They killed the allogeneic tumor cells almost completely at a very low ratio (E/T=2), but did not show cytotoxicity to autologous tumor cells (data not shown). This type of allogeneic tumor-oriented, but not autologous tumor-oriented, CTL induction were easily performed even when the PBMC were cultured with the target tumor cells and with IL-2 as the sole cytokine. In the present study, NK cells were negatively selected at the beginning of the CTL induction by the immunomagnetic separation method. Therefore, the resulting population of CTL scarcely contained NK cells (Table I), which are known to respond to IL-18¹⁴⁾ and IL-12.^{20, 21)}

Generation of autologous tumor-specific CD8⁺ CTL is a complex process, and cytokines are important factors in determining the differentiation to autologous tumor-specific CD8⁺ CTL. Previous studies have shown that IL-2 is an essential factor for the generation of CD8⁺ CTL.³⁻⁵⁾ Our laboratory and others showed that the combination of IL-1, -2, -4, and -6 works efficiently for the induction of autologous tumor-specific CD8⁺ CTL *in vitro*.^{6, 7)} In addition to these cytokines, IL-18 probably has an important role in induction of autologous tumor-specific CTL through promoting an increase in the CD8⁺ T cell population. In the culture in which CTL were induced from the PBMC of patient A, IL-18 functioned cooperatively with the IL-cocktail, but not in the culture supplemented with only IL-2 (Fig. 1, Table I).

At present, we have no evidence to explain the mechanism by which IL-1, -4, and -6 act together with IL-18.

The IL-cocktail might be necessary for the expression of human IL-18 receptor (IL-18 R) molecules²²⁾ on the CD8⁺ T cells and, therefore, once the CD8⁺ CTL were generated, IL-18 might be able directly to activate their killing activity without the cooperative function of IL-1, -4, and -6 (Figs. 3 and 4).

The direct activation of CD8⁺ CTL may be dependent on an increase in levels of IFN- γ and TNF- α production (Table II), since IFN- γ and TNF- α are known to augment CD8⁺ T cell activity.²³⁻²⁵⁾ Unfortunately, in the present study, the amounts of purified CD8⁺ T cells and autologous tumor cells from both patient A and patient B were insufficient to test this possibility in further killing assays. Successful preparation of pairs of established tumor cell lines and autologous, easily expandable CTL in the case of every tumor-bearing patient is still difficult and further improvement of the induction culture system is needed.

We could not establish normal autologous cells such as fibroblasts from the patients in the present study. However, as shown in our previous studies,^{8, 9)} CTL generated on autologous tumor cells in the medium containing the IL-cocktail did not kill autologous normal cells⁸⁾ or normal fibroblasts.⁹⁾ Therefore we assume that the CD8⁺ T cells induced with IL-18 would not kill autologous normal cells. The present results have important implications for the induction and activation of CD8⁺ CTL for cancer immunotherapy.

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