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# Enhancement by Monocytes of Perforin Production and Its Gene Expression by Human CD8+ T Cells Stimulated with Interleukin-2

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**Pore-forming protein (PFP) is an important effector molecule for cytotoxicity mediated by cytotoxic T cells and NK cells. In the present study, the effect of monocytes on PFP production by interleukin-2 (IL-2)-stimulated T lymphocytes was examined. Highly purified lymphocytes (>99%) and monocytes (>90%) were isolated by centrifugal elutriation from peripheral blood of healthy donors, and, CD4+ and CD8+ cells were isolated from the purified lymphocytes by using antibody-bound magnetic beads. PFP production was quantitated with a universal microspectrophotometer in combination with immunostaining using anti-PFP antibody. Monocytes did not produce any PFP. High levels of PFP production were observed in CD8+ cells, but not CD4+ cells after incubation for 4 days with IL-2. Addition of monocytes to cultures of CD8+ cells resulted in significant augmentation of PFP production after 3 days' stimulation with IL-2. Monokines (TNF$\alpha$ and IL-6) caused a significant increase in PFP production by IL-2-stimulated CD8+ cells. Northern blot analysis revealed that the PFP mRNA level was enhanced by stimulation with IL-2, and that addition of monocytes to cultures of CD8+ cells plus IL-2 augmented their PFP mRNA expression. These observations suggest that monocytes are important in *in situ* regulation of the CD8+ T cell-mediated cytotoxic response through production of PFP.**

Key words: Perforin — CD8+ T cell — Monocyte — Interleukin-2

Non-specific cytotoxic effector cells against autologous and allogeneic solid tumors can be induced without antigenic stimulation by culturing unprimed lymphocytes for 4–6 days with interleukin 2 (IL-2). These lymphokine (IL-2)-activated killer cells are called LAK cells,[1,2] and their precursors are known to be T cells and NK cells.[3,4] Accumulating evidence indicates that induction of LAK activity by IL-2 can be up- and down-regulated by various humoral and cellular factors.[5–9] We have shown that freshly isolated blood monocytes from normal donors up-regulate LAK induction by IL-2, and that activated monocytes down-regulate LAK induction through production of a new monokine.[10–12] Moreover, induction of LAK activity in T cells has been found to be largely regulated by blood monocytes.[13] These findings raise the problem of what effector mechanism responsible for tumor cell killing by IL-2-activated killer cells is regulated by blood monocytes.

Cytotoxic CD8+ T lymphocytes (CTL) constitute one of the major effector cell populations of the immune system involved in the control of tumor cells and virally infected cells, most probably by lysis of infected and transformed cells.[14] Much attention has been paid to the effector mechanism by which CTL kill tumor cells. The cytoplasmic granules of CTL and NK cells have been found to contain a cytolytic, calcium-dependent, pore-forming protein (PFP, perforin), which is released into the extracellular space upon antigen stimulation.[15,16] The formation of pores by perforin in target cell membranes is thought to be responsible for death of these cells.[16–18] Recently, IL-2 was shown to induce PFP at mRNA and protein levels in human blood CD8+ cells which had been enriched by adherence of CD8+ cells to $F(ab)_2$ anti-mouse Ig-coated petri dishes.[19] Since the activation of T lymphocytes by antigen or IL-2 requires the accessory function of monocytes,[10,20,21] these findings raise the question of whether PFP production by IL-2-activated lymphocytes is regulated by blood monocytes. In this study we found that freshly isolated monocytes up-regulated production of PFP and its mRNA expression in blood CD8+ cells.

## MATERIALS AND METHODS

**Subjects** Cells were obtained from healthy male volunteers (21–28 years old), who had no evidence by history or physical examination of infectious disease, and who were not taking medication. These subjects all gave informed consent to participate in the experiments.

**Reagents** Fetal bovine serum (FBS) was purchased from Gibco Lab., Grand Island, NY. Recombinant human IL-2 was kindly provided from Takeda Pharmaceutical

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Co. (Osaka), and had a specific activity of $3.5\times10^4$ U/mg as assayed on IL-2-dependent murine NKC3 cells.[22] One unit obtained by this method was equivalent to approximately 400 Japanese Reference Units (JRU). Mouse anti-PFP monoclonal antibody ($IgG_{2a}$) was produced as described previously.[23] Human recombinant IL-6 (lot 735-3; specific activity, $6\times10^6$ U/mg of protein) was a gift from Ajinomoto Co., Kawasaki. Human natural tumor necrosis factor $\alpha$ (TNF$\alpha$: specific activity, $4.0\times10^5$ JRU/mg of protein) was a gift from Hayashibara Institute, Okayama. Human recombinant IL-1$\alpha$ was kindly supplied through Y. Hirai (Otsuka Pharmaceutical Co., Tokushima). None of these materials contained endotoxins, as judged by *Limulus* amebocyte assay (sensitivity limit, 0.1 ng/ml) (Seikagaku Kogyo Co., Tokyo).

**Isolation and culture of human peripheral blood monocytes** Leukocyte concentrates were collected from peripheral blood (200 ml) of healthy donors in an RS-6600 rotor of a Kubota KR-400 centrifuge, and mononuclear cells were separated from the leukocyte concentrates on lymphocyte separation medium (Litton Bionetics). Then monocytes were separated from the mononuclear cell samples by centrifugal elutriation in a Hitachi SRR6Y elutriation rotor.[10,11] A fraction containing more than 95% of the total monocyte population was obtained at a speed of 2000 rpm and flow rate of 20 ml/min. More than 90% of these cells were monocytes as determined by nonspecific esterase staining and morphological examination, and more than 97% were viable, as judged by the trypan blue dye exclusion test. This fraction was washed twice with phosphate-buffered saline, and resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated FBS and gentamycin, designated as CRPMI 1640, at a concentration of $5\times10^5$ monocytes per ml. These cells were then plated in 96-well Microtest III plates (Falcon, Oxnard, CA), and after 1 h, nonadherent cells were removed by two washes with warm RPMI 1640 medium. At this point the purity of the monocytes was >99% as judged by their morphology and nonspecific esterase staining.

**Isolations of CD4+ and CD8+ cells with antibody (Ab)-coated immunomagnetic bead** Anti-CD4 Ab and anti-CD8 Ab adsorbed on uncoated Dynabeads M-450 were obtained from Dynal Inc., Great Neck, NY. Sterile Ab-coated immunomagnetic beads were washed twice with ice-cold CPMRI 1640, using a magnet to pellet the beads. The pellet of beads ($3\times10^7$/ml) was suspended in 5 ml of cell mixture ($1\times10^7$ cells/ml) and incubated on ice with gentle agitation every 5 min for 30 min. The tube was then inverted to resuspend the pellet and placed in a magnetic holder for 2 min. The supernatant was pipetted off and the pellet was resuspended in 25 ml of cold RPMI 1640 and replaced in the magnet. This washing procedure was repeated once more, then the pellet was suspended in 50 ml of CRPMI 1640 at 37°C for 16 h. The beads were removed from the cultures by vigorous agitation of the plates with a Pasteur pipette and transfer of the mixture to a magnetic holder. After 2 min, the supernatant was pipetted off and saved for replating. By this separation method we could isolate more than 99% of the CD4+ or CD8+ cells, as judged by FACScan analysis (data not shown). CD4+ or CD8+ cells with or without monocytes were plated at $10^5$ cells/well in 24-well plates (Costar, Cambridge, MA) and incubated for 4 days in medium with or without IL-2 at 37°C.

**PFP staining method** Blood lymphocytes with or without monocytes were incubated for 4 days in medium with or without IL-2 at various concentrations, and then the cells were cytocentrifuged on fluorescence-free glass slides, and stained with anti-human perforin antibody using an ABC kit (Vector Laboratories, Inc., Burlingame, CA), as described previously.[23] Briefly, the cytocentrifuged preparations were fixed with cold acetone, rehydrated in PBS, and incubated sequentially with anti-PFP monoclonal antibody P1-8, biotinylated sheep anti-rat Ig, and avidin-biotinylated peroxidase complexes, and then developed with 3,3′-diaminobenzidine.

**PFP measurement** The point absorption of the stained preparations was measured in a universal microspectrophotometer (UMSP-1: Carl Zeiss) with a spot diameter of 10 $\mu$m and a ×40 objective, as described in detail previously.[24] A preliminary experiment showed that there was an approximately linear relationship between the absorbance at 500 nm and the staining of IL-2-stimulated CD8+ cells by the ABC method with anti-PFP antibody, and that the straight line passed through the origin (data not shown). Under the same conditions, there was no PFP staining by the ABC method with mouse $IgG_{2a}$ serum as a negative control. Assuming that the concentration of reactive groups in the measured area is constant, we concluded that Beer-Lambert's law held for ABC staining and that the absorbance was proportional to the amount of reactive groups. Thus, we used this method for quantitative measurement of the amount of intracellular PFP per cell.

**Isolation of RNA and Northern blot hybridization** CD8+ cells with or without monocytes at a CD8+ cell/monocyte ratio of 10:1 were cultured in polypropylene tubes (Falcon) for 6 h in RPMI 1640 medium with or without IL-2 (1 U/ml). Total cellular RNA was isolated by the guanidium isothiocyanate method.[25] Electrophoresis and RNA blotting were carried out as described previously.[26] Equal amounts of RNA (10 $\mu$g) were denatured in a solution of 20 m*M* 3-(N-morpholino)propanesulfonic acid buffer (pH 7.0) containing 16% formaldehyde and 50% formamide at 65°C for 5 min and then subjected to electrophoresis in 1.2% agarose gel containing 16% formaldehyde. The separated RNA was transferred to

a Hybond-$N^+$ nylon membrane (Amersham) and hybridized with a $^{32}P$-labeled human PFP cDNA probe[27] or with a cDNA probe of $\beta$-actin (Wako Pure Chemical Industries, Osaka). Before hybridization, the nylon membrane was prehybridized with 50% formamide, 5×SSPE (1×SSPE = 0.15 *M* NaCl, 10 m*M* $NaH_2PO_4$, 1 m*M* EDTA), 200 $\mu$g/ml sonicated salmon sperm DNA, 0.1% SDS, and 5×Denhardt's solution (1×Denhardt's= 0.02% each of Ficoll, polyvinylpyrrolidone and BSA) at 42°C for 6 h. Hybridization was performed at 42°C for 14 h in the same solution containing labeled cDNA ($10^6$ cpm/ml) with a specific activity of about $10^9$ cpm/$\mu$g. After hybridization, the nylon membrane was washed four times with 2×SSPE containing 0.1% SDS for 15 min each time at 42°C and then with 1×SSPE containing 0.1% SDS for 30 min at 42°C. The membrane was autoradiographed with Kodak XAR-5 film at −70°C with an intensifying screen. Film images were scanned with a transmittance/reflectance scanning densitometer (Bio-Rad model 1650; Bio-Rad Laboratories, Richmond, CA) for quantitative determination of variations in RNA.

**Statistical analysis** The statistical significance of differences between groups was analyzed by using Student's *t* test (two-tailed).

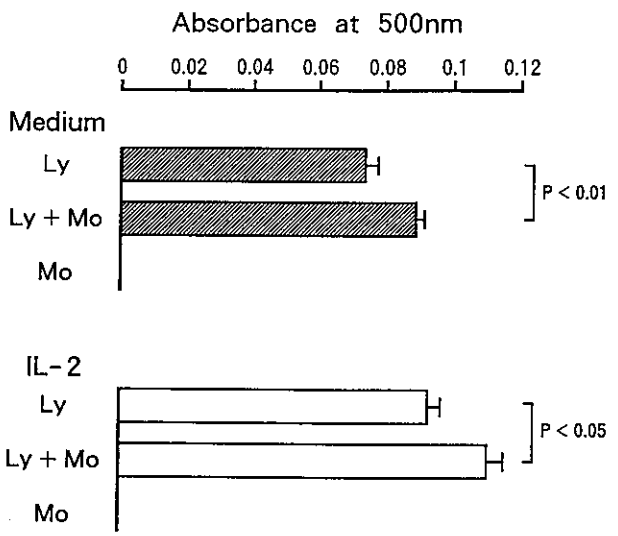


Fig. 1. Effect of monocyte addition on PFP production by blood lymphocytes with or without IL-2. Lymphocytes ($10^5$) and monocytes ($2\times10^4$) were incubated for 4 days in medium with or without 1 U/ml of IL-2. Then, they were washed and stained by the ABC method with anti-PFP antibody. PFP production was measured as described in "Materrials and Methods." Data are representative of two separate experiments from different donors. Columns and bars show ±SE for 60 lymphocytes.

RESULTS

**PFP production by CD8+, but not CD4+ cells or monocytes** LAK induction by IL-2 from human blood lymphocytes was previously found to be up-regulated by freshly isolated monocytes.[10-13] First, we examined whether monocytes increased the production of PFP by blood lymphocytes with or without IL-2 treatment. As shown in Fig. 1, blood lymphocytes produced relatively high levels of PFP spontaneously, without IL-2 stimulation, and addition of monocytes resulted in a significant increase in their PFP production. Under the same experimental conditions, IL-2 caused a significant increase in PFP production in blood lymphocytes, and this PFP production in IL-2-stimulated lymphocytes was augmented by monocytes. Monocytes treated with or without IL-2 in the presence or absence of lymphocytes did not produce PFP at all.

Next, we examined whether the up-regulation by monocytes of PFP production occurred in CD4+ or CD8+ cells or both. For this, we purified CD4+ and CD8+ cells from human peripheral blood lymphocytes. Flow cytometry showed that the resulting populations contained >98% CD4+ cells and >99% CD8+ cells, respectively. The effect of IL-2 on PFP production in these subsets was examined with a UMSP. As shown in Fig. 2, induction of PFP production occurred in CD8+ cells that had been cultured for 4 days with IL-2 (1 U/ml), but little effect was seen in CD4+ cells, irrespective of

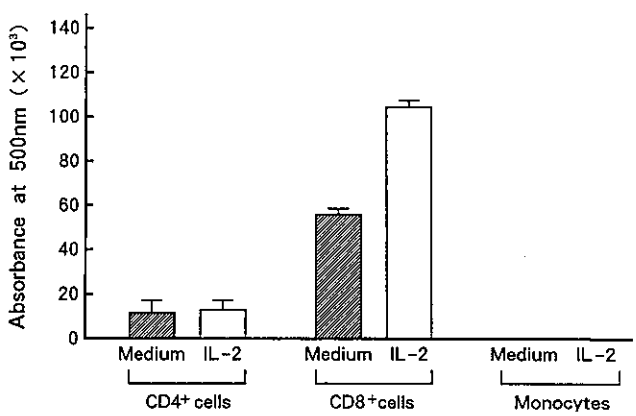


Fig. 2. IL-2-induced augmentation of PFP production by CD8+ cells. CD4+ cells, CD8+ cells and monocytes at a lymphocyte/monocyte ratio of 10:1 were incubated for 4 days in medium with or without 1 U/ml of IL-2, and then washed and stained by the ABC method with anti-PFP antibody. PFP production was measured as described in "Materials and Methods." Data are representative of two separate experiments from different donors. Columns and bars show ±SE for 60 lymphocytes.

Table I. Effects of Monocytes on PFP Productions by CD8+ Cells Stimulated with IL-2

| Cells | IL-2 | Absorbance at 500 nm ($\times 10^3$) | |
|---|---|---|---|
| | | Medium | Monocytes |
| CD4+ | − | 10.2±2.1[a] | 11.2±1.9 |
| | + | 9.9±2.7 | 9.8±2.6 |
| CD8+ | − | 57.3±3.4 | 49.5±3.0 |
| | + | 105.9±4.2 | 132.6±7.2[b] |

*a*) Mean±SE for 60 lymphocytes.
*b*) $P<0.05$ versus value for CD8+ cells incubated with IL-2 alone without monocytes.

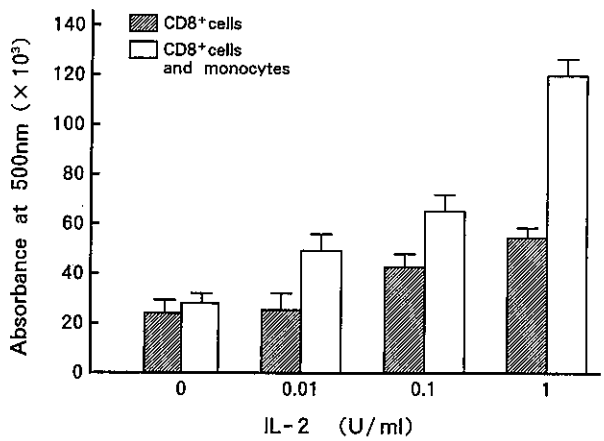


Fig. 3. Dose-response effect of IL-2 on PFP production by CD8+ cells with or without monocytes. CD8+ cells ($10^5$) were incubated for 4 days in medium with various concentrations of IL-2 in the presence or absence of $10^4$ monocytes. The cells were washed, and then stained by the ABC method with anti-PFP antibody. PFP production per cell was measured as described in "Materials and Methods." Columns and bars show ±SE for 60 lymphocytes.

IL-2 treatment. Monocytes with or without IL-2 treatment produced no appreciable PFP. Under the same experimental conditions, we examined whether monocytes up-regulated PFP production by CD8+ cells induced by IL-2. CD4+ and CD8+ cells separated with immunomagnetic beads were tested for ability to produce PFP. For this, CD4+ and CD8+ T cells with or without monocytes at a lymphocyte/monocyte ratio of 10:1 were incubated for 4 days in medium with or without IL-2 (1 U/ml), and then their PFP production was examined with a UMSP. As shown in Table I, addition of monocytes to cultures with or without IL-2 had no effect on PFP production by CD4+ cells, but up-regulated PFP production by IL-2-stimulated CD8+ T cells.

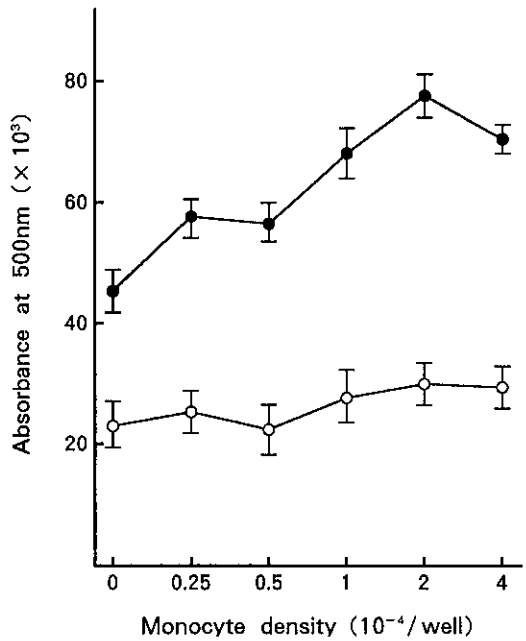


Fig. 4. Effects of monocyte density on PFP production by CD8+ cells stimulated with IL-2. CD8+ cells ($10^5$) were incubated for 4 days in medium with (closed circles) or without (open circles) IL-2 (1 U/ml) in the presence or absence of various numbers of monocytes, and then stained for measurement of PFP as described in "Materials and Methods." Data are representative of two separate experiments. Bars show ±SE for 60 lymphocytes.

We examined the effect of IL-2 concentration on PFP production by CD8+ cells and its regulation by monocytes. As shown in Fig. 3, PFP production by CD8+ cells was enhanced does-dependently by IL-2, and addition of monocytes up-regulated PFP production by CD8+ cells induced by IL-2 at concentrations of more than 0.01 U/ml. The effect of the number of monocytes added on PFP production by CD8+ T cells was also examined. As shown in Fig. 4, PFP production by IL-2-activated CD8+ T cells increased with increase in numbers of monocytes added, being maximal at a lymphocyte/monocyte ratio of 10:2.

**Kinetics of up-regulation by monocytes of PFP production by CD8+ cells** For examination of the kinetics of PFP production by CD8+ T cells and its up-regulation by monocytes, CD8+ T cells with or without monocytes were incubated for up to 4 days in medium with or without IL-2 (1 U/ml), and then their PFP activity was measured as described in "Materials and Methods." The results in Fig. 5, showed that PFP production by CD8+ T cells incubated for up to 4 days in medium alone was not increased by addition of monocytes.

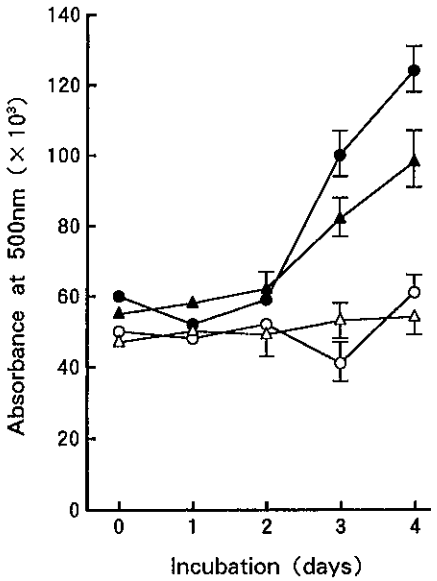


Fig. 5. Time course of PFP production by CD8+ cells with or without monocytes. CD8+ cells ($10^5$) were incubated for up to 4 days in medium with (closed symbols) or without (open symbols) IL-2 (1 U/ml) in the presence (circles) or absence (triangles) of $10^4$ monocytes. At the indicated times, the cells were harvested, and stained for measurement of PFP as described in "Materials and Methods." Bars show ±SE for 60 lymphocytes.

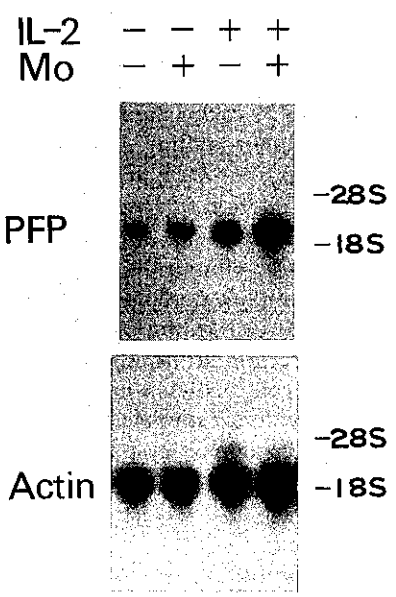


Fig. 6. Effects of monocytes on IL-2-stimulated expression of PFP mRNA in CD8+ cells. CD8+ cells were incubated for 6 h in medium with or without IL-2 in the presence or absence of monocytes (Mo) at a CD8+ cell/monocyte ratio of 10:1. Total RNA was isolated and analyzed as described in "Materials and Methods."

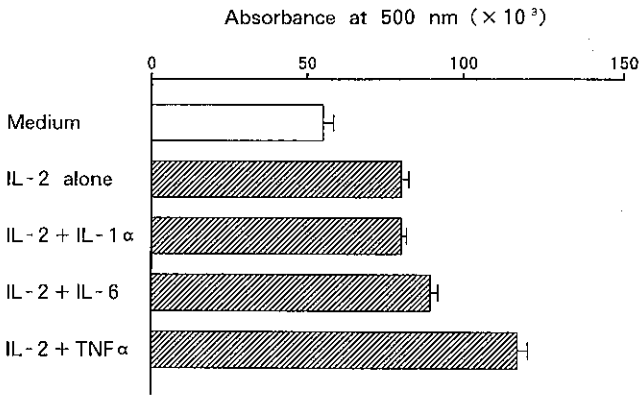


Fig. 7. Effects of monokines on PFP production by CD8+ cells. CD8+ cells were incubated for 3 days in medium with or without IL-2 in the presence or absence of IL-1$\alpha$ (100 U/ml), IL-6 (0.1 ng/ml) or TNF$\alpha$ (1000 U/ml), and then stained for measurement of PFP as described in "Materials and Methods." Columns and bars are means ±SE for 60 lymphocytes in three different experiments.

However, IL-2 induced a significant increase in PFP production by CD8+ cells 3 days after its addition, and this increase was significantly enhanced by addition of monocytes (Fig. 5).

**Up-regulation by monocytes of PFP mRNA expression in CD8+ cells** Preliminary experiments showed that the level of PFP mRNA in CD8+ cells was maximal 6 h after stimulation with the optimal concentration of IL-2 (1 U/ml), but disappeared almost completely by 24 h. For this reason, total RNAs were isolated from CD8+ T cells that had been incubated for 6 h in medium with or without IL-2 (1 U/ml) in the presence or absence of monocytes, and were examined by Northern blot analysis with a cDNA probe specific for the PFP gene, as described in "Materials and Methods." The results are shown in Fig. 6. Indices of the PFP gene expression (ratio of the scanning unit for PFP to that for $\beta$-actin) were 0.26 for CD8+ cells alone, 0.33 for CD8+ cells and monocytes alone, 0.55 for CD8+ cells plus IL-2, and 0.78 for CD8+ cells and monocytes plus IL-2. Thus, on incubation in medium alone without monocytes, CD8+ T cells showed slight spontaneous expression of PFP mRNA. This expression was increased in medium with IL-2, and the addition of monocytes to cultures of CD8+ cells with IL-2 resulted in a further 42% increase in their expression of PFP mRNA. No expression of PFP mRNA was seen in monocytes treated with or without IL-2 (data not shown).

**Effect of monokines on PFP production by CD8+ cells stimulated with IL-2** We examined whether monokines (IL-1$\alpha$, IL-6 and TNF$\alpha$) affected the production of PFP

in CD8+ cells by incubating the cells in medium with or without these monokines for 4 days before quantitation of PFP. Monokine alone did not affect the production of PFP in CD8+ cells in the absence of IL-2 (data not shown). As can be seen in Fig. 7, addition of IL-6 or TNF$\alpha$ to cultures of CD8+ cells with IL-2 significantly increased PFP production, whereas addition of IL-1$\alpha$ did not. TNF$\alpha$ was the most effective in augmenting PFP production by CD8+ cells activated with an optimal dose of IL-2.

## DISCUSSION

The present study showed that PFP production by CD8+ cells was up-regulated at the protein and mRNA levels by blood monocytes, and that, of the three monokines tested, TNF$\alpha$ and IL-6 significantly augmented PFP production by IL-2-stimulated CD8+ cells, whereas IL-1$\alpha$ did not.

PFP, which is responsible for lysis of tumor target cells, is known to be present in granules of LAK cells.[28] CD8+ cells and NK cells are precursors of killer cells induced by IL-2.[3,4,13] IL-2 was previously found to induce PFP mRNA expression in CD8+ cells, but not in CD4+ cells.[19] The present studies by a microphotospectrometry also showed that intracellular PFP was detectable in CD8+, and that its level in CD8+ cells was significantly augmented by *in vitro* treatment with IL-2 alone (Fig. 2). Interestingly, a small amount of intracellular PFP was detected in CD4+ cells, compared to that in monocytes, but this level was not enhanced by IL-2. The reason for this phenomenon is, however, not clear.

IL-2-activated killer cell induction from T cells was previously found to be up-regulated by freshly isolated human blood monocytes.[13] We confirmed and extended this finding by showing that human blood monocytes significantly augmented PFP production in IL-2-stimulated CD8+ T cells, which are responsible for tumor cytotoxicity. Interestingly, blood monocytes induced PFP production by CD8+ T cells even in the presence of a low concentration of IL-2 (Fig. 3). Moreover, a kinetic study showed that significant augmentation by monocytes of PFP production in CD8+ cells occurred at least 3 days after stimulation with IL-2. Blood monocytes also augmented PFP mRNA expression (Fig. 6), suggesting that monocytes induce up-regulation of PFP production in IL-2-stimulated CD8+ cells by enhancing its transcription.

Various monokines such as IL-1, TNF$\alpha$ and IL-6, which are products of activated monocyte-macrophages, have been found to augment IL-2-activated killer induction.[29-32] Recently, IL-6 was found to act synergistically with a low concentration of IL-2 in augmenting PFP gene expression in human blood T cells.[33] This finding was confirmed and extended by our demonstration that TNF$\alpha$ as well as IL-6 acted with IL-2 to enhance significantly PFP production by CD8+ cells. With an optimal concentration of IL-2 to induce PFP production in CD8+ cells, TNF$\alpha$ was the most effective in augmenting PFP production by CD8+ T cells (Fig. 7). This finding suggests that the enhancing effect of monocytes observed in the present study might be due to monokine production. This, however, seems unlikely for the following reasons. First, freshly isolated blood monocytes from normal donors were previously found not to produce monokines extracellularly (IL-1, TNF$\alpha$ and IL-6).[34,35] In contrast, fully activated monocytes were recently found to produce a new monokine (MW, 68 kDa) responsible for suppression of LAK induction.[12] Second, our preliminary experiments showed that these monokines could not be detected in the supernatants obtained from 3-day cultures of CD8+ cells and monocytes with IL-2 (data not shown). Third, augmentation of LAK induction by freshly isolated monocytes was previously found not to be mediated by the supernatants of lymphocyte/macrophage cultures,[10] suggesting the requirement of cell-to-cell contact. Nevertheless, it is still possible that PFP production by CD8+ cells may be augmented by expression of a membrane-bound monokine such as TNF$\alpha$. Thus, the mechanism by which monocytes up-regulate PFP production in CD8+ T cells remains to be explored.

The mechanism by which cytotoxic CD8+ cells kill tumor cells may involve various processes such as target cell recognition, cellular binding, a cytolytic potential and triggering events, but PFP has been strongly implicated in the lytic function of IL-2-activated CD8+ T cells.[28,36,37] Thus, the present and previous[13] studies suggest that human blood monocytes may play an important role in *in vivo* amplification of the PFP production responsible for tumor cell killing by CD8+ cells in response to IL-2. Therefore, information on up-regulation of human CD8+ T cell-mediated tumor cytotoxicity by monocytes may provide a rationale for *in situ* enhancement of the antitumor defense system in humans.

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