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Induction by Interleukin-15 of Human Killer Cell Activity against Lung Cancer Cell Lines and Its Regulatory Mechanisms

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Interleukin (IL)-15 is a novel cytokine with IL-2-like activity. In the present study, we examined IL-15-mediated induction of killer activity of peripheral blood mononuclear cells (MNC) against lung cancer cell lines, and the regulatory mechanisms of this induction by IL-15. Cytotoxic activity was measured by ^{51}Cr release assay. IL-15 at concentrations of more than 10 ng/ml induced significant killer activity of blood MNC against a small cell lung cancer cell line (SBC-3), as well as Daudi cells, and 50 ng/ml was considered its optimal concentration. A time course study revealed that an incubation period of 4–6 days was optimal for induction of killer activity. MNC cultured with IL-15 also exhibited killer activity against other lung cancer cell lines (H-69, N-291 and PC-9 cells). IL-15 and IL-12 had additive effects on induction of killer activity against SBC-3 cells. On the other hand, IL-15 had no synergistic or additive effect on induction of killer activity by IL-2. Fresh human monocytes isolated by centrifugal elutriation augmented the development of killer activity of lymphocytes stimulated by IL-15. As a humoral regulatory factor, IL-4 had a suppressive effect on induction of killer activity by IL-15. IFN-γ, IL-1β, TNF-α, IL-6 or IL-10 had no effect on induction of killer activity by IL-15 at the optimal concentration. These results suggest that IL-15 has potential for the immunotherapy of lung cancer.

Key words: Interleukin-15 — Mononuclear cell — Tumor cytotoxicity — Lymphokine-activated killer cell

Human lung cancer is one of the most frequent causes of death and has a poor prognosis,[1,2] due to difficulty in its therapy. As conventional therapies, including chemotherapy, radiation therapy and surgical resection, are not usually effective to eradicate lung cancer, various new methods such as immunotherapy have been tested both *in vitro* and *in vivo*.[3–6]

Interleukin (IL)-15 is a novel M_r 15,000 cytokine, first identified in the supernatant of monkey epithelial cell line CV-1/EBNA as a factor that enhanced the antitumor response. IL-15 induces T-cell proliferation,[7–9] enhances natural killer (NK) cell cytotoxicity, upregulates production of NK cell-derived cytokines, including interferon-γ (IFN-γ), granulocyte/macrophage-colony-stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α),[10] and stimulates proliferation and differentiation of B cells activated with anti-immunoglobulin M (anti-IgM).[11] In addition, IL-15 stimulates locomotion and chemotaxis of normal T cells.[12] Recently, attention has been focused on clinical application of IL-15 for cancer immunotherapy.[13]

In this work we studied the ability of IL-15 to induce killer activity of blood mononuclear cells (MNC) against lung cancer cell lines, and the cellular and humoral mechanisms regulating its effect.

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MATERIALS AND METHODS

Cell lines Cell lines of human Burkitt lymphoma (Daudi) and human lung small cell carcinoma (N-291) were obtained from the American Type Culture Collection (Rockville, MD). A human lung small cell cancer line (SBC-3) was kindly provided by Dr. Hiraki (Okayama University, Okayama).[14] A human lung adenocarcinoma cell line (PC-9) and human lung small cell carcinoma cell line (H-69) were gifts from Dr. Y. Hayata (Tokyo Medical College, Tokyo) and Dr. Shimosato (National Cancer Center Research Institute, Tokyo), respectively. Cell lines were maintained in culture in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY) and gentamicin (Schering-Plough, Osaka), designated as complete RPMI-1640 (CRPMI) medium, at 37°C in a humidified atmosphere containing 5% CO_2. For cytotoxicity assays, cultured target cells were used in the exponential growth phase.

Isolation and culture of human peripheral blood monocytes Leukocyte concentrates were prepared from peripheral blood (200 ml) of healthy donors in an RS-6600 rotor of a Kubota KR-400 centrifuge, and MNC were separated from the leukocyte concentrates in lymphocyte separation medium (Litton Bionetics). Then monocytes were separated from the mononuclear cell samples by

centrifugal elutriation in a Hitachi SRR6Y elutriation rotor.[15] A fraction containing more than 95% of the total monocyte population was obtained at 2000 rpm and a 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 5×10^5 MNC per ml. These cells were plated for 1 h in 96-well Microtest III plates (Falcon, Oxnard, CA).

Reagents Recombinant human IL-15 was obtained from PeproTech, Inc. (Rocky Hill, NJ). According to the company manual, the ED_{50} as determined by dose-dependent stimulation of the proliferation of CTLL cells was 1.0 ng/ml. Recombinant human IL-12 (specific activity: 5.26×10^6 U/mg protein) was supplied by the Genetics Institute, Inc. (Cambridge, MA), and recombinant human IL-2 (specific activity: 1.14×10^7 U/mg protein as assayed on IL-2-dependent murine NKC3 cells)[16] was a gift from Takeda Pharmaceutical Co. (Osaka). Recombinant human IFN-γ (specific activity: 5.36×10^6 U/mg) was a gift from Nippon Roche (Tokyo). Natural (n)TNF-α (lot 906045, specific activity 3.25×10^5 JRU/mg protein) was a gift from Hayashibara Institute (Okayama). Recombinant human IL-4 (specific activity, 1×10^6 U/mg protein) was a gift from Ono Pharmaceutical Co. (Osaka). Human recombinant IL-1β was supplied by Otsuka Pharmaceutical Co. (Tokushima). Recombinant human IL-6 was supplied by Ajinomoto (Tokyo). Recombinant human IL-10 (specific activity: 1×10^7 U/mg protein) was a gift from DNAX Research Institute (Palo Alto, CA). None of these materials contained endotoxins, as judged by *Limulus* amebocyte assay (sensitivity limit, 0.1 ng/ml, Seikagaku Kogyo Co., Tokyo).

Cytotoxicity assay Cell-mediated cytotoxicity was assayed by measuring ^{51}Cr release in a 4-h test as described previously.[15] Briefly, peripheral blood MNC (10^5 per well) were incubated in CRPMI-1640 with the indicated concentrations of IL-15, IL-12 and/or IL-2 in 96-well Microtest III plates (Falcon) for 4 days. In some experiments the supernatants of cultured cells were collected and frozen for measurement of cytokines. Remaining cells were washed and used as effectors, and various cells labeled with ^{51}Cr were used as targets. The effector-to-target (E : T) ratios were 10 : 1 and 20 : 1, and experiments were performed in triplicate. After incubation for 4 h, the radioactivities of the supernatant fluids were determined. Results are expressed as percent cytotoxicity, calculated by use of the following formula: $(E-S/M-S)\times100$ (where E = experimental cpm, S = spontaneous cpm and M = maximum cpm). The spontaneous releases observed with different target cells ranged from 5 to 15% of that on total lysis.

Quantitative measurements of cytokines Enzyme immunoassays (EIAs) for human GM-CSF, TNF-α and IFN-γ were performed essentially as described in detail previously,[17] the sensitivity limits of all these EIAs being 20 pg/ml.

Statistical analysis The statistical significance of differences between groups were analyzed by means of Student's t test.

RESULTS

Effect of IL-15 on induction of killer activity of blood mononuclear cells We first examined the effect of IL-15 on induction of killer activity peripheral blood MNC.

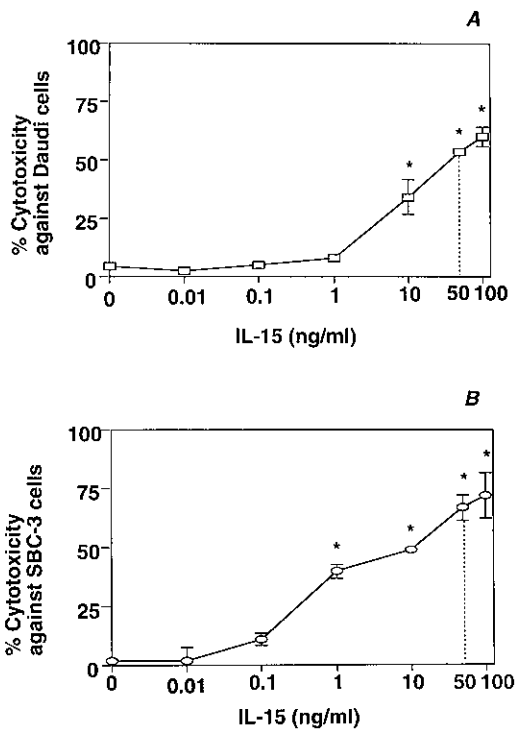

Fig. 1. Dose response relation for induction by IL-15 of killer activity mediated by blood MNC. MNC (10^5/well) were incubated for 4 days in medium containing the indicated amounts of IL-15 before cytotoxicity assay. Cytotoxic activity was assayed on Daudi cells (A) or SBC-3 cells (B) at an E/T ratio of 10 : 1, as described in "Materials and Methods." Bars show SDs of means for triplicate cultures. Asterisks indicate significant differences from values in medium alone ($P<0.01$). Dotted lines indicate optimal concentrations of IL-15. Results are representative of three independent experiments that gave similar results.

MNC were incubated with various concentrations of IL-15 for 4 days and then their killer activities against Daudi cells and a lung cancer cell line (SBC-3) were measured at E/T ratios of 10. Representative results are shown in Fig. 1. MNC (1×10^5/well) cultured in medium alone exhibited only marginal cytotoxicity against Daudi cells and SBC-3 cells, but MNC cultured with IL-15 at doses of more than 10 ng/ml developed significant killer activity against both SBC-3 cells and Daudi cells. We used 50 ng/ml of IL-15 as the optimal concentration to induce killer activity in the following experiments. Experiments with 10 different donors showed that the mean values (± SD) of cytotoxicity at an E/T ratio of 10 of MNC cultured with or without IL-15 were 36.0 (±4.0) and 2.9 (±1.9) for Daudi cells, and 45.0 (±5.9) and 2.3 (±1.4) for SBC-3 cells, respectively.

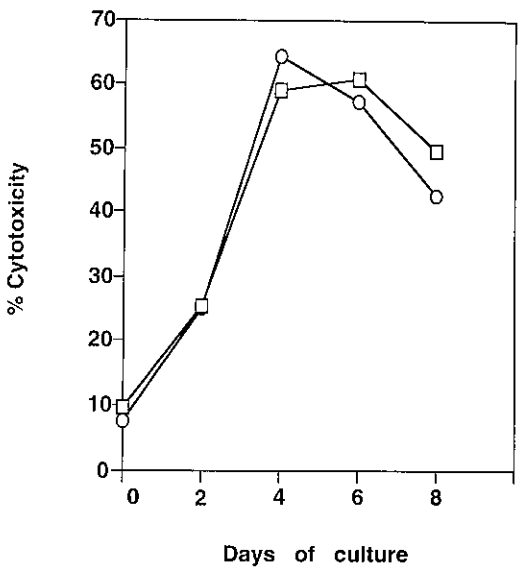

Fig. 2. Time course of induction by IL-15 of killer activity mediated by blood MNC. MNC (10^5/well) were incubated for various times with 50 ng/ml of IL-15. Cytotoxic activity was assayed on Daudi cells (□) or SBC-3 cells (○) at an E/T ratio of 10 : 1, as described in "Materials and Methods." The SDs were consistently less than 10% of the means. Results are representative of three independent experiments that gave similar results.

Time course of induction of killer activity by IL-15 We examined the time course of induction of MNC-mediated killer activity by IL-15. For this, MNC (1×10^5/well) were cultured in medium with or without 50 ng/ml of IL-15 for various periods, and then killer activities against Daudi and SBC-3 cells were measured. As shown in Fig. 2, an incubation period of at least 4 days was required for full expression of the killer activity.

Induction by IL-15 of killer activity against various lung cancer cell lines Next, we examined the killer activities of MNC cultured with IL-15 against various lung cancer cell lines. Blood MNC (1×10^5/well or 2×10^5/well) were incubated with or without IL-2 (500 U/ml), IL-12 (100 U/ml) and IL-15 (50 ng/ml) for 4 days and then killer activities against various lung cancer cell lines were measured. The concentrations of IL-2 and IL-12 were optimal for inducing killer activity.[18] As shown in Table I, MNC cultured with IL-15, as with IL-2, developed significant killer activities against all the lung cancer cell lines (SBC-3, H-69, N-291 and PC-9 cells) examined at E/T ratios of 10 : 1 and 20 : 1, as well as against Daudi cells.

Additive effects of IL-12 and IL-15 on induction of killer activity of blood MNC We examined the effect of IL-15 in combination with IL-2 or IL-12, because these cytokines have been reported to induce killer activity of blood MNC. For this, blood MNC (1×10^5/well) were incubated in medium with or without 20 ng/ml of IL-15 in the presence or absence of suboptimal or optimal concentrations of IL-2 or IL-12 for 4 days and then their killer activities against SBC-3 cells were measured at an E/T ratio of 10 : 1. As shown in Fig. 3, IL-12 had an additive effect with IL-15 (20 ng/ml) on induction of killer activity against SBC-3 cells. On the other hand, IL-2 had no synergistic or additive effect with IL-15 on induction of killer activity. Moreover, IL-12 had no additive effect

Table I. Spectrum of Killer Activity of IL-15-activated MNC

Target cells	Medium only		+IL-2 (500 U/ml)		+IL-12 (100 U/ml)		+IL-15 (50 ng/ml)	
	E/T=10	20	10	20	10	20	10	20
Daudi	0.7±0.1[a)]	6.0±1.3	31.6±4.0[b)]	61.6±3.0[b)]	3.2±0.9[b)]	22.0±3.6[b)]	20.6±0.5[b)]	58.6±2.2[b)]
SBC-3	1.8±1.2	14.1±3.0	41.3±6.2[b)]	86.8±7.0[b)]	6.7±1.9[b)]	62.3±10.7[b)]	76.3±4.4[b)]	92.1±5.2[b)]
H 69	7.1±2.2	23.4±0.9	47.2±6.4[b)]	44.6±4.1[b)]	10.1±1.6[b)]	29.7±4[b)]	32.2±4.3[b)]	44.8±5.3[b)]
N 291	6.3±2.7	20.1±3.3	49.9±4.2[b)]	67.9±1.4[b)]	16.5±1.2[b)]	37.8±5.6[b)]	56.2±9.9[b)]	83.1±0.2[b)]
PC-9	0±0.9	1.6±0.7	9.7±3.4[b)]	41.8±3.9[b)]	9.2±1.0[b)]	11.1±5.0[b)]	6.2±2.0[b)]	27.9±2.0[b)]

a) Mean±SD for triplicate cultures.
b) Significantly different from the value without cytokines ($P<0.05$).

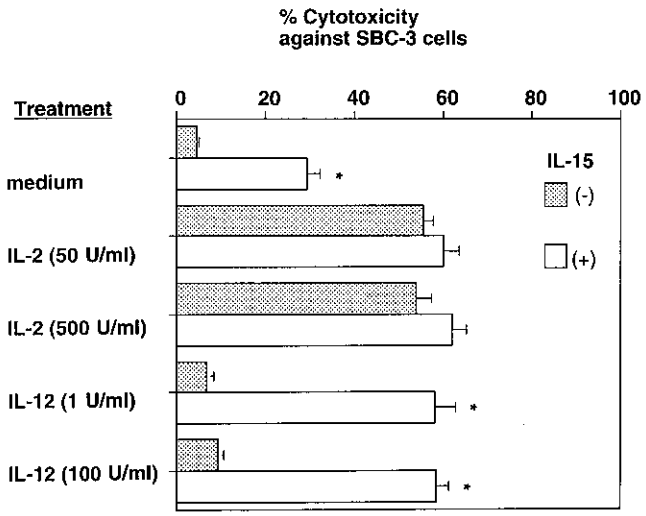

Fig. 3. Effects of IL-15 on IL-2- and IL-12-induced induction of killer activity mediated by blood MNC. MNC (10^5/well) were incubated for 4 days with or without IL-15 (20 ng/ml) in the presence or absence of IL-2 (50 U/ml, 500 U/ml) or IL-12 (1 U/ml, 100 U/ml). Cytotoxic activity was assayed on SBC-3 cells at an E/T ratio of 10 : 1, as described in "Materials and Methods." Bars show SDs of means for triplicate cultures. Asterisks indicate significant differences from values without IL-15 ($P<0.005$). Results are representative of three independent experiments that gave similar results.

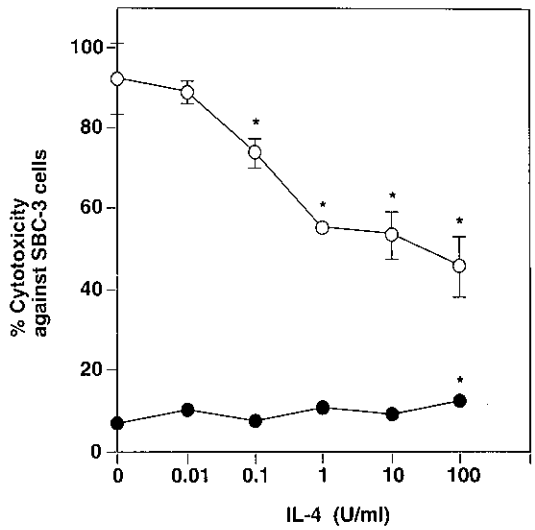

Fig. 4. Effect of IL-4 on induction by IL-15 of killer activity mediated by blood MNC. MNC (10^5/well) were incubated for 4 days with various concentrations of IL-4 in the presence (○) or absence (●) of IL-15 (50 ng/ml). Cytotoxic activity was assayed on SBC-3 cells at an E/T ratio of 10 : 1, as described in "Materials and Methods." Bars show SDs of means for triplicate cultures. Asterisks indicate significant differences from values without IL-4 ($P<0.05$). Results are representative of two independent experiments that gave similar results.

Table II. Effect of Various Cytokines on IL-15-induced Killer Activity[a]

Cytokine (100 U/ml)	% Cytotoxicity against SBC-3 cells		
	Medium	+IL-2 (500 U/ml)	+IL-15 (50 ng/ml)
Medium	5.3±0.7[b]	73.4±5.9	62.2±7.4
IFN-γ	10.4±2.0	56.8±5.1	52.8±6.0
IL-1β	3.8±2.5	64.4±8.4	50.3±5.3
TNF-α	0±1.8	63.1±9.5	50.5±6.7
IL-6	2.0±1.9	67.0±2.8	56.1±3.6
IL-4	3.6±1.0	48.2±4.0[c]	31.6±1.9[c]
IL-10	6.6±1.1	70.5±6.8	55.0±3.3

a) MNC (10^5/well) were incubated in medium with or without IL-2 or IL-15.
b) Mean±SD for triplicate cultures.
c) Significantly different from the value without IL-4 ($P<0.005$).

with the optimal dose of IL-15 (50 ng/ml) on induction of killer activity (data not shown).

Effects of various cytokines on induction of killer activity by IL-15 There are reports that induction of killer activity by IL-2 is regulated by various cytokines, such as IFN and IL-4.[19–21] We, therefore, examined the regulatory effects of various cytokines on the induction by IL-15. Blood MNC (1×10^5/well) were incubated in medium with or without 50 ng/ml of IL-15 in the presence or absence of various concentrations of cytokines for 4 days and then killer activity against SBC-3 cells was measured at an E/T ratio of 10 : 1. As shown in Table II, of the various cytokines examined in the present study, only IL-4 inhibited killer cell induction by IL-15. Moreover, we examined the effect of various concentrations of IL-4 on induction of killer activity by IL-15. As shown in Fig. 4, IL-4 at concentrations of more than 0.1 U/ml significantly inhibited the induction of killer activity of blood MNC by IL-15.

IL-15 was previously reported to induce GM-CSF, TNF-α and IFN-γ production by NK cells.[10] To examine further the effect of IL-4 on the IL-15-mediated response of lymphocytes, we examined whether IL-4 could affect cytokine production by MNC in the presence of IL-15. MNC with or without various concentrations of IL-4 were incubated for 4 days in medium with or without IL-15 (50 ng/ml), and then the supernatants were harvested for quantitative measurements of GM-CSF, TNF-α, IFN-γ. TNF-α and IFN-γ were produced by MNC stimulated with IL-15, but their production was suppressed dose-dependently by IL-4 (data not shown).

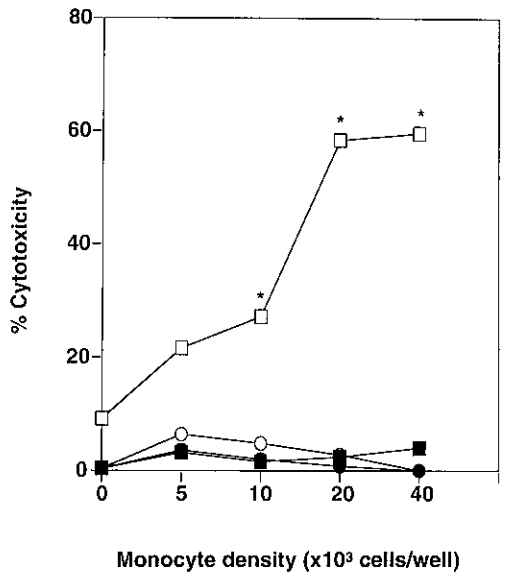

Fig. 5. Effect of number of added monocytes on induction by IL-15 of killer activity against SBC-3 cells. Lymphocytes at concentrations of 1×10^5/well were incubated for 4 days with the indicated numbers of monocytes with (□) or without (■) IL-15 (50 ng/ml). The indicated numbers of monocytes alone were also incubated for 4 days with (○) or without (●) IL-15 (50 ng/ml). Cytotoxic activity was assayed on SBC-3 cells, as described in "Materials and Methods." The SDs were consistently less than 10% of the means. Asterisks indicate significant differences from values without monocytes ($P<0.05$). Results are representative of two independent experiments that gave similar results.

Killer cell induction by IL-15 in the presence or absence of blood monocytes We have reported that cells of monocyte-macrophage lineage regulate killer cell induction by IL-2 and IL-12, depending on their functional states.[18, 22–24] We examined the effect of blood monocytes on the induction of killer cell activity by IL-15. For this, highly purified lymphocytes were incubated in medium with or without 50 ng/ml of IL-15 in the presence or absence of various amounts of blood monocytes for 4 days, and killer cell activity against SBC-3 cells was measured. Representative results are shown in Fig. 5. Lymphocytes incubated in medium with or without monocytes showed no killer activity. Lymphocytes incubated with IL-15 in the absence of monocytes showed low levels of cytotoxicity. Full induction of killer cell activity by IL-15 was dependent on the density of the monocytes added.

We also examined whether IL-15 could induce cytokine production by blood lymphocytes in the presence or absence of blood monocytes. Lymphocytes (1×10^5 cells/well) with or without autologous monocytes at various densities from 5×10^3 cells/well to 4×10^4 cells/well were incubated for 4 days in medium with or without IL-15 (50 ng/ml), and then the supernatants were harvested for quantitative measurements of GM-CSF, TNF-α and IFN-γ. TNF-α and IFN-γ were produced by lymphocytes (1×10^5 cells/well) stimulated with IL-15 only when 4×10^4 monocytes were added. In the absence of IL-15, addition of blood monocytes to a culture of lymphocytes resulted in no detectable production of either cytokine (data not shown).

DISCUSSION

Our study showed that recombinant human IL-15 induces cytotoxic activities of human blood MNC against lung cancer cell lines, and that the generation of cytotoxic lymphocytes by IL-15 is significantly up-regulated by cocultivation with monocytes. We further showed that IL-15-inducible killer activity is augmented by IL-12, but suppressed by IL-4.

For therapeutic purposes, much attention has been paid to cytokines capable of inducing cytotoxic activity of blood lymphocytes. IL-15 is a cytokine that induces non-MHC restricted cytotoxicity of normal blood MNC against various allogeneic cells of lung tumors (Table I). This observation confirms and extends a recent report of IL-15-activated induction of killer cells against human malignant cells such as colon adenocarcinoma (COLO 205), leukemia (K562), lymphoma (Daudi), and melanoma (FMEX).[13]

Monocyte-macrophages play important roles in regulating killer cell induction by IL-2 and IL-12. For example, human monocytes isolated by centrifugal elutriation augmented the development of LAK activity stimulated by recombinant IL-2[18, 22, 23] or by IL-12.[18] This was confirmed in the present study using recombinant human IL-15; addition of freshly isolated monocytes to cultures of autologous lymphocytes with IL-15 resulted in density-dependent augmentation of cytotoxic killer cell induction (Fig. 5). Monocytes incubated alone for 4 days in medium with IL-15 did not kill SBC-3 target cells, so the present findings indicate that monocytes cause up-regulation of killer cell induction from blood lymphocytes. However, the mechanism of monocyte-mediated augmentation of IL-15-activated killer cell activity is unknown.

IL-15 seems to be the only cytokine other than IL-2 that is able to utilize the IL-2Rβ chain.[7, 10, 25] Since monocytes are involved in switching expression from the low-affinity IL-2R to high-affinity receptors,[26, 27] one possible mechanism by which fresh monocytes may up-regulate killer cell induction by IL-15 would be the augmentation of expression of the IL-2Rβ chain of killer cell precursors.

The effects of various cytokines, such as IFN-γ, IL-1β, TNF-α and IL-6 on IL-2-induced LAK activity have been investigated.[19, 28–30] Little is known, however, about the cytokine-mediated regulatory mechanism of killer cell induction by IL-15. The IL-15-mediated induction was not affected by addition of exogenous IL-10 (Table II). This finding is consistent with the finding of the absence of its effect on IL-2-mediated killer cell induction.[31, 32] The present findings also showed that other cytokines such as IL-1β, TNF-α and IL-6 had no effect on killer cell induction by an optimal dose of IL-15. Similarly, no synergistic or even additive effect on cytotoxic activity was observed on incubation with IL-15 in the presence of an optimal or suboptimal concentration of IL-2.

Interestingly, we found that IL-4 significantly inhibited induction of killer cell activity by IL-15 in a dose-dependent manner (Table II, and Fig. 4). Moreover IL-4 significantly reduced the production of TNF-α and IFN-γ by MNC in a dose-dependent manner (data not shown). IL-4 was previously found to suppress killer cell induction by IL-2,[20, 21] but not by IL-12.[17] There is suggestive evidence that IL-4 acts directly on killer precursors (NK cells and T cells), and inhibits IL-2 receptor expression,[33] and some steps in IL-2 activation pathways mediated by the IL-2Rβ-chain.[34–37] Since IL-4 and IL-2 share the IL-2Rγ chain,[38] which is an indispensable component of functional IL-2R,[39] sharing of the IL-2Rγ chains between IL-2 and IL-4 may be another reason why IL-4 suppresses killer induction by IL-2. The IL-2Rγ chain also plays an important role in IL-15-mediated signaling.[40] Thus, sharing of IL-2Rγ chain by IL-4 is one possible mechanism of IL-4-mediated suppression of killer cell induction by IL-15.

IL-15 was previously found to augment the effect of IL-12 on IFN-γ production and induction of killer activity of highly purified CD56^{dim} NK cells against NK-resistant human colon cancer cells.[10] This observation is extended by the present finding that IL-15 had an additive effect with IL-12 on induction of killer activity of blood MNC against SBC-3 cells (Fig. 3). Moreover, addition of IL-12 to cultures of MNC with IL-15 resulted in augmented production of IFN-γ as compared to that with IL-12 alone (data not shown). In the present study, augmented IFN-γ production might not be responsible for the observed additive effect on killer cell induction of the combination of IL-12 and IL-15, because addition of exogenous IFN-γ to cultures of MNC with IL-15 did not cause any increase in killer cell induction (Table II). It would be interesting to know how these two cytokines induce killer activity of human lymphocytes.

In contrast to IL-2, IL-15 appears to be abundantly expressed in various tissues and cell types, including activated monocytes/macrophages.[7] Our findings support the idea that IL-15 is another cytokine that can induce cytotoxicity of peripheral blood MNC against lung cancer cells and that IL-15 and IL-12 have additive effects in induction of killer cell activity. Thus, IL-15 may be useful clinically in the immunotherapy of lung cancer.

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