Clonal and functional analysis for the augmentation of tumour-infiltrating lymphocytes by interleukin 4

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Summary In the adoptive immunotherapy for cancer, the amounts of induced effector cells play a major role in improving therapeutic efficacy. We have already demonstrated that interleukin 4 (IL-4) augments proliferation of tumour-infiltrating lymphocytes (TILs) without altering the cytotoxic activity against autologous tumour cells. The present study is designed to investigate how IL-4 augments TILs by using established TIL clones in terms of IL-2/IL-2 receptor system. $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ (double positive) TIL clones were established from cancer patients. At clonal level, IL-4 augmented the proliferation of IL-2-activated TIL clones irrespective of phenotypes. In order to clarify the mechanism of IL-4 at clonal level, the blocking assay by anti-IL-2 receptor α and β chain and binding assay of IL-2 on the cell surface and the measurement of the internalisation of IL-2 in the cell were performed. It was clarified that IL-4 up-regulated the IL-2 receptor and then augmented the action of IL-2 molecule on the cell surface stimulated by IL-4. Furthermore, binding IL-2 internalised rapidly into the cells. Thus, it is suggested that signal transduction is augmented and proliferation of TILs is enhanced by IL-4 via the action of IL-2/IL-2 receptor system.

Keywords: interleukin 4; tumour-infiltrating lymphocyte; interleukin-2 receptor; scathard analysis

Adoptive immunotherapy (AIT) using tumour-infiltrating lymphocytes (TILs) has been carried out by various investigators (Rosenberg et al., 1986; Topalian et al., 1988; Yamaue et al., 1990). The number of transferred TILs is a critical factor for obtaining a therapeutic effect. Interleukin 4 (IL-4) is a pleiotrophic cytokine that acts on various cell types (Paul and O'Hara, 1987; Swain et al., 1988; Spits et al., 1987; Horohov et al., 1988; Mitchell et al., 1989), and is produced by helper T cell type 2 (Mosmann et al., 1986). It has been reported that IL-4 mainly acts on B cells (BCGF, B-cell growth factor) (Howard et al., 1982), and that it can augment the cytotoxic activity of lymphokine-activated killer (LAK) cells in a murine model (Mule et al., 1987). Recently, it has been demonstrated that IL-4 inhibited LAK activity in humans (Nagler et al., 1988). However, it has been reported that IL-4 enhanced the proliferation of TILs obtained from different types of human tumours (Kawakami et al., 1988, 1993). We have previously demonstrated that IL-4 augments the proliferation of interleukin 2 (IL-2)-activated TILs without inhibiting their cytotoxic activity against autologous tumour cells. Thus, the combination of IL-2 with IL-4 may lead to improved therapeutic efficacy of AIT using TILs (Tsunoda et al., 1992a). However, the mechanism of IL-4 on the augmentation of proliferation about TILs has not been clarified yet. In the present study, we demonstrated that the functional mechanism of IL-4 was clarified under clonal level.

Materials and methods

Culture media and tumour cell lines

Recombinant human IL-2 (Shionogi Pharmaceutical Co., Japan) and recombinant human IL-4 (Ono Pharmaceutical Co., Japan) were used for cell stimulation. RPMI-1640 medium (Gibco, Grand Island, NY, USA) was supplemented with 10% heat-inactivated human AB serum obtained from healthy subjects, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} amphotericin B, 2 mmol l^{-1} glutamine and 25 mmol l^{-1} Hepes (complete medium).

Separation of TILs and autologous tumour cells

TILs were separated as previously described (Yamaue et al., 1990; Tsunoda et al., 1992b). In brief, malignant fluids were obtained from cancer patients and centrifuged. The cell pellets were washed twice and resuspended with complete medium. Cells were then layered onto Ficoll–Hypaque gradients and centrifuged at 400 g for 30 min at 20°C. The interface fraction was collected, washed and resuspended in complete medium, and layered onto discontinuous gradients of 70% and 100% Ficoll–Hypaque. After centrifugation at 400 g for 30 min, the TILs were concentrated at the 100% interface, and the tumour cells were concentrated at the 70% interface. The purity of lymphocytes in the TIL-rich fraction was 60–85%, and this fraction was used as the source of TILs. The tumour cell-rich fraction was contaminated by mesothelial cells and mononuclear cells. To eliminate these cells, the fraction was layered onto discontinuous gradients composed of 4 ml each of 20%, 15% and 10% Percoll (Pharmacia) in complete medium in 15 ml plastic tubes, and then centrifuged at 25 g for 7 min at 20°C. Tumour cells depleted of lymphocytes were collected from the bottom of each tube, washed and resuspended in complete medium. The purity of the tumour cells was usually more than 90% after this extra centrifugation procedure. Freshly isolated tumour cells were over 95% viable according to the trypan blue dye exclusion test, and only cell fractions having less than 10% contamination by non-malignant cells were accepted for use.

Cloning method

TIL clones were established by limiting dilution method (Iwahashi et al., 1993). Briefly, maintained phase TILs were seeded in 96-well U-bottom plates at 0.7 cells per well in 0.2 ml of complete medium containing 10^5 allogenic peripheral blood mononuclear feeder cells which were treated with 50 μg ml^{-1} Mitomycin C (Kyowa Hakko, Japan) for 30 min at 37°C. IL-2 (1500 U ml^{-1}) and optimal IL-4 (250 pg ml^{-1} or 2.5 ng ml^{-1}) were used for stimulation

from our previous data (Tsunoda *et al.*, 1992). Every 4 days, microcultures were supplemented with IL-2 and IL-4. After 16–24 days of culture, microcultures were scored microscopically for growth. Grown microcultures were considered as clones. Clonal microcultures were then split into several microwells, expanded and analysed for surface marker expression and functional properties.

DNA synthesis

TIL clones were cultured in triplicate in round-bottomed microtitre plates with 1500 IU ml^{-1} IL-2 and/or IL-4 (250 μg ml^{-1} or 2.5 ng ml^{-1}) at 37°C. After 80 h, 1 μCi of [^{3}H]thymidine [^{3}H]TdR, New England Nuclear, Boston, USA) was added to each well and culture was continued for an additional 16 h. Samples were harvested using a cell harvester (Cambridge Technology, Cambridge, MA, USA) and the amount of incorporated radioactivity was measured using a liquid-scintillation counter.

IL-2 receptor blocking assay

Anti-CD25 antibody (anti-IL-2 receptor α chain, 10 μg ml^{-1}, CosmoBio, Tokyo, Japan) and TU27 (anti-IL-2 receptor β chain, 50 μg ml^{-1}, kindly provided by Dr Sugamura, Tohoku University, Japan) were used for IL-2 receptor blocking assay (Yagita *et al.*, 1989). The concentrations used have been sufficiently proved for inhibition in the proliferation of T cells by preliminary studies. Inhibition of DNA synthesis was used to measure receptor blocking.

IL-2 binding assay and Scatchard analysis

IL-2 binding assay was performed by modified Robb's method as previously described (Robb *et al.*, 1984). In brief, cells were washed three times in complete medium and incubated for 6 h at 37°C to remove IL-2. After an additional wash, the cells were resuspended in the same medium. Binding of [^{125}I]IL-2 (Amersham, 600 Ci mmol^{-1}) was performed in triplicate in 100 μl containing 4–6 × 10^6 cells. The incubation was performed for 90 min at 4°C. For every time point non-specific binding was determined with a 100-fold excess of cold IL-2. The specific binding represents the difference between the total and non-specific binding.

Determination of the internalisation of IL-2

Cells (2 × 10^6) were incubated with 200 pM [^{125}I]IL-2 in RPMI–Hepes at 4°C on ice for 30 min. They were then washed three times, resuspendend in 1.0 ml RPMI–Hepes, and incubated at 37°C for the indicated times. The suspension was centrifuged, and the cell pellet was then

treated for 10 min at 4°C with chilled 0.2 M glycin–HCl buffer (pH 2.8). The radioactivity of the non-acid-eluted fraction was then counted with a gamma counter and taken to represent the labelled IL-2 internalised by clones (Fujii *et al.*, 1986; Yoshimoto *et al.*, 1990).

Results

Characteristics of TIL clones

Thirty-five TIL clones incubated with IL-2 (1500 IU ml^{-1}) and IL-4 (250 pg ml^{-1}) were established from seven cancer patients. All TIL clones were CD3$^+$ and TCRα/β^+. Of these, 19 were CD4$^+$ (54.3%), six were CD8$^+$ (17.1%) and ten were CD4$^+$CD8$^+$ (double positive) (Figure 1).

IL-4 augmented the proliferation of TIL clones

As shown in Table I, IL-4 alone augmented DNA synthesis of TIL clones, and moreover, IL-4 enhanced the proliferation of IL-2-activated TIL clones irrespective of phenotype. Furthermore, these data demonstrate that the effect of IL-4 was synergistic, not additive.

Blocking assay by IL-2 receptor

In order to clarify the mechanism of augmentation of proliferation by IL-4, blocking assays using anti-IL-2 receptor α chain and β chain antibody were performed. Data of representative TIL clones are shown in Table II. Proliferation of the 1F7, CD4$^+$ clone, was increased from 15.3 × 10^3 c.p.m. to 32.1 × 10^3 c.p.m. by IL-4. Neither the anti-IL-2 receptor α chain antibody or the β chain antibody alone inhibited proliferation. However, the simultaneous addition of the α chain and β chain antibody inhibited the proliferation to the level of background (which was 3344 ± 1850 c.p.m.), induced by IL-2 alone as well as IL-4 plus IL-2. Interestingly, the baseline level of inhibition produced by the IL-2 receptor antibody was similar between IL-2 alone and IL-4 combined with IL-2. The same results were obtained with the TIL clone, 2F4 and 2D10. These data suggest that IL-4 augmentation of IL-2-induced proliferation operates through an IL-2 pathway.

IL-2 binding assay and Scatchard analysis

In order to reveal the mechanism of IL-4 in terms of the IL-2/IL-2 receptor system, IL-2 binding assays and Scatchard analysis were performed on the representative TIL clone, 2D10 (Figure 2). The K_d of the high- and low-affinity IL-2 receptor of this clone was 200 pM and 2.8 nM respectively. IL-4 increased the amount of IL-2 bound to the high-affinity
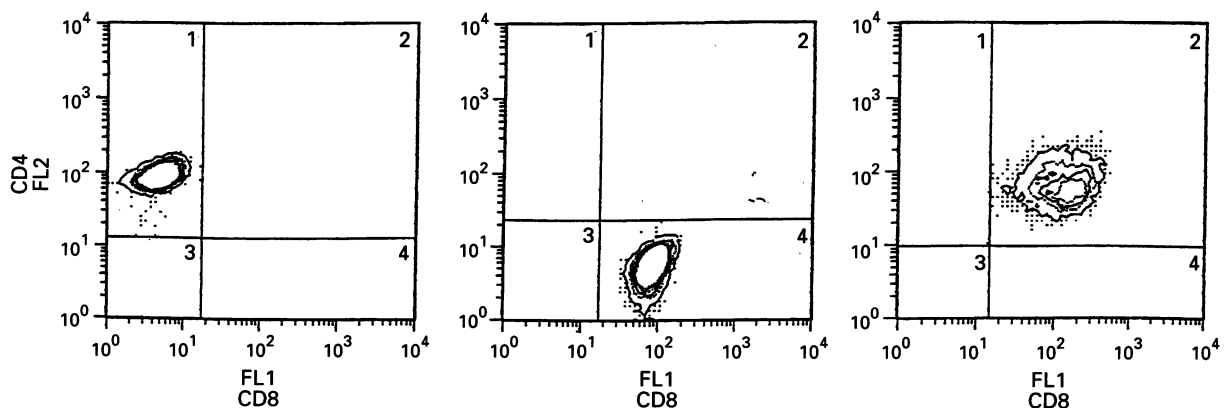

Figure 1 TIL clone established by IL-2 + IL-4. CD4$^+$, CD8$^+$ and CD4$^+$CD8$^+$ (double-positive) clones were obtained. All TIL clones were CD3$^+$, TCRα/β, and CD16$^-$.

Table I Effect of IL-4 on the proliferation of TIL clones

| Phenotype | Clone | Medium | $[^3H]$-TdR incorporation $(c.p.m. \times 10^3)$ | | | |
			IL-4	IL-2	IL-2+IL-4
CD4	1E5	0.3	0.7	2.1	7.3
	1F7	0.3	2.6	15.3	32.1
	2F4	0.5	2.4	10.0	27.8
	3B8	0.2	5.9	1.0	8.4
CD8	1E4	0.7	1.3	34.5	43.3
	2F7	0.7	0.9	7.1	13.4
	2F8	0.4	0.9	5.1	8.2
	3B9	NT	NT	34.3	40.5
CD4.CD8	2D10	6.2	9.3	40.5	81.0
	2E9	0.4	2.0	70.1	80.5
	2F5	0.2	0.4	50.0	70.5

At clonal level, IL-4 augmented the proliferation of every phenotype of clones. Representative data are shown.

Table II Blocking assay by anti-IL-2 receptor Ab

TIL clone	In vitro treatment	Control Ab	Blocking site α chain	β chain	($\times 10^3$, c.p.m.) α chain + β chain
1F7	IL-2	15.3	15.0	14.8	5.2
	IL-2+IL-4	32.1	31.8	31.5	7.4
2F4	IL-2	10.0	9.8	9.7	3.5
	IL-2+IL-4	27.8	26.2	25.8	9.0
2D10	IL-2	40.5	36.3	36.6	14.0
	IL-2+IL-4	81.0	80.3	79.9	19.5

Blocking assay by IL-2 receptor α- and/or β-chain Ab was performed. Simultaneous use of both Abs inhibited the proliferation of clones stimulated with IL-2 and IL-4. Data of the representative TIL clone are shown.

IL-2 receptor from 12 500 to 15 500 molecules per cell. Similarly, IL-4 increased IL-2 binding to the low-affinity IL-2 receptor from 49 000 to 67 000 IL-2 molecules per cell. However, IL-4 did not change the slope of the high- or low-affinity IL-2 receptor binding curves.

Internalisation of IL-2

To analyse the binding of IL-2 to the TIL clones further, internalisation of IL-2 was studied. Data of representative TIL clones are shown in Table III. At early time points (10 and 30 min) following incubation with IL-4, the internalisation of IL-2 was enhanced as compared with incubation with IL-2 alone. However, at later time points (60 and 120 min) of incubation, there was no difference between IL-2 alone and IL-2 plus IL-4 in the amount of IL-2 internalised. Moreover, after incubation with IL-4 for only 10 min, over 75% of the IL-2 was internalised. This demonstrates that IL-4 augments the internalisation of IL-2 at an early phase.

Discussion

It has been reported that IL-4 augments the specific cytotoxic activity against autologous melanoma cells and other types of tumour cells (Kawakami et al., 1988, 1993). We have already demonstrated that IL-4 accelerates the proliferation of IL-2-activated TILs without altering the cytotoxic activity against autologous adenocarcinoma cells, and IL-4 does not alter the phenotypes of TILs (Tsunoda et al., 1992). However, the mechanism of IL-4 has remained unclear. In order to clarify the mechanism of IL-4, we first established clones of TILs. CD4+, CD8+ and CD4+CD8+ (double positive) T-cell clones were established from freshly isolated TILs. About 3% of T cells in peripheral blood are double-positive T cells (Blue et al., 1985). Double-positive T cells are thought to be an intermediate phenotype between immature double-negative T cells and mature single-positive (CD4 or CD8) T cells during the differentiation of T cells in the thymus (McPhee et al., 1979; Penit, 1986). Our studies (Yamaue et al., 1990) and those of others (Whiteside et al., 1986; Ebert et al., 1989;
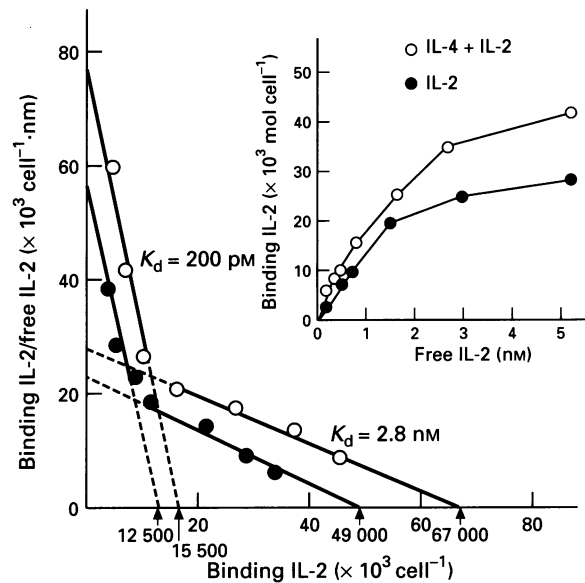
Figure 2 Binding assay and Scatchard analysis of TIL clone stimulated with IL-4. IL-4 up-regulated the low- and high-affinity IL-2 receptors without altering the slope of either receptor. Results were obtained using the representative TIL clone, 2D10.

Shimizu et al., 1990; Whitford et al., 1990; Viale et al., 1990) have not observed a subpopulation of double-positive T cells in TILs. In our system, IL-4 was used to establish TIL clones and it was thought that IL-4 augments the expression of the CD8 molecule in CD4+ T cells (Paliard et al., 1988). This might be the reason why we could establish double-positive T-cell clones.

The cytokine production of these TIL clones was measured. IL-1, IFN-γ and TNFα were produced from these TIL clones (data not shown). It may be indicated that IL-4 augments the cytokine network system (Lorre et al., 1990).

Next, by using three different T-cell clones, proliferation

Table III Internalisation of IL-2 molecule in TIL clones incubated with IL-4

TIL clone	In vitro treatment	Time course (min) of $[^{125}I]IL$-2 ($\times 10^3$ c.p.m.)				
		0	10	30	60	120
IF7	IL-2	2.7	4.1	4.5	7.2	8.1
	IL-2 + IL-4	2.8	6.9	7.1	7.7	9.3
2F4	IL-2	2.5	3.4	3.3	5.2	6.1
	IL-2 + IL-4	2.5	6.4	6.8	5.6	7.2
2D10	IL-2	4.2	5.0	6.0	9.5	11.2
	IL-2 + IL-4	4.2	8.1	9.3	9.9	12.0

IL-4 augmented the internalisation of IL-2 during the early phase (10 and 30 min). Over 75% of internalisation was acheived by 30 min. Representative data were shown. The value of time 0 means the background proliferation.

induced by IL-4 and/or IL-2 was measured. IL-4 alone augmented proliferation of TIL clones irrespective of phenotype, and furthermore, IL-4 combined with IL-2 enhanced the proliferation of every TIL clone in comparison with IL-2 alone. These results indicate two important points. First, IL-4 augments the proliferation of TILs at the clonal level independent of their phenotype. Second, the augmentation of IL-4 combined with IL-2 is synergistic. In the present report, we focused on the mechanism by which IL-4 enhanced the proliferation induced with IL-2. In this mechanism, IL-2 and IL-2 receptor system were thought to be crucial (Fernandez-Botran et al., 1989; Kawakami et al., 1989; Ishikawa et al., 1991). In our studies, a blocking assay using anti-IL-2 receptor α-chain antibody (CD25) and β-chain antibody (TU27) was performed on representative clones. IL-2 receptor α chain alone (low affinity) and β chain alone (intermediate) did not inhibit proliferation in comparison with control antibody. However, simultaneous addition of both IL-2 receptor antibodies inhibited proliferation. Interestingly, the inhibition was similar in clones cultured with IL-2 alone or with IL-2 plus IL-4. It is suggested that the augmentation of IL-4 is related to high-affinity IL-2 receptor. However, cell surface expression of IL-2 receptor α-chain, β-chain and IL-4 receptor by these clones was analysed by flow cytometry. There was no difference in receptor expression between cells stimulated with IL-2 alone or IL-2 plus IL-4 (data not shown).

Furthermore, the IL-2 binding assay and Scatchard analysis at clonal level showed that IL-4 did not change the value of K_d of the high-affinity IL-2 receptor (200 pM) and the low-affinity IL-2 receptor (2.8 nM) in comparison with IL-2 alone. These data suggest that IL-4 does not change the functional characteristics of the IL-2 receptor. On the other hand, IL-4 increased the binding amount of IL-2 molecule on the cell surface at both the IL-2 receptors. It is especially important that IL-4 increases the binding of IL-2 on the high-affinity IL-2 receptor, since this receptor is thought to be related to signal transduction (Yagita et al., 1989). This may be the mechanism for the synergistic effect of IL-4 on proliferation.

The increase of binding IL-2 molecule on TILs exposed to IL-4 prompted us to examine the internalisation of IL-2, which depends on the latter receptor (Fujii et al., 1986; Siegel et al., 1987; Robb et al., 1987; Yoshimoto et al., 1990). It was found that internalisation of IL-2 was augmented by IL-4 at the early incubation time points (10 and 30 min). Thus, the enhanced internalisation of IL-2 induced by IL-4 may accelerate delivery signals of transduction that is followed by the heightened proliferation of TILs. The combined effects that IL-4 has on up-regulation of the high-affinity IL-2 receptor, increasing the amount of IL-2 bound per TIL, and enhancement of internalisation of IL-2 together lead to augmentation of TIL proliferation.

Recently, IL-2 receptor γ chain has been sequenced and analysed in detail (Takeshita et al., 1992). High-affinity IL-2 receptor was clarified to consist of α-, β- and γ-chain, and the γ-chain was shown to be crucial to signal transduction (Nakamura et al., 1993). Moreover, the IL-2 receptor γ-chain has high homology to the IL-4 receptor (Kondo et al., 1993). IL-2 receptor γ-chain may play an important role in the augmentation of TIL proliferation caused by IL-4. We have shown at the clonal level that IL-4 augmented the proliferation of TIL clones via the IL-2 receptor. At present, we are now attempting to analyse the involvement of the IL-2 receptor γ-chain in the stimulation of proliferation produced by IL-4.

The expression of IL-4 receptor was analysed by radio-immunoassay. There was no significant difference by stimulation with IL-4 (data not shown). However, it is possible that the phenomena reported reflect some inside-out modulation of surface IL-2 receptor mediated via the IL-4/IL-4 receptor system.

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