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ORIGINAL ARTICLE

Koji Tamada · Mamoru Harada · Tadao Okamoto
Mitsuhiro Takenoyama · Osamu Ito · Goro Matsuzaki
Kikuo Nomoto

Specific antitumor activity of tumor-infiltrating lymphocytes expanded first in a culture with both anti-CD3 monoclonal antibody and activated B cells and then in a culture with interleukin-2

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Abstract In order to expand tumor-infiltrating lymphocytes (TIL) efficiently and in order to use them for immunotherapy, we utilized lipopolysaccharide-activated B cells (LPS blasts) as costimulatory-signal-providing cells in an in vitro culture system. TIL, prepared from subcutaneously inoculated B16 melanoma, failed to expand when cultured with anti-CD3 monoclonal antibody (mAb) alone followed by a low dose of interleukin(IL)-2. In contrast, such TIL did expand efficiently in culture with both anti-CD3 mAb and LPS blasts followed by culture with IL-2. These findings suggest that the presence of LPS blasts in the initial culture was essential for the cell expansion. The expansion of TIL was partially blocked by the addition of CTLA4 Ig, which is an inhibitor of costimulatory molecules such as CD80 and CD86, and was almost blocked by the addition of anti-(Fc receptor γII)mAb. These findings thus indicate that such molecules, in conjunction with the receptor on the LPS blasts, participate in the efficient expansion of TIL. The B16-derived TIL, which expanded in our culture system, were predominantly CD8+ T cells and showed a higher level of cytolytic activity against B16 melanoma than either lymphokine-activated killer cells or TIL cultured with a high dose of IL-2. In addition, the in vitro expanded B16-derived TIL produced interferon γ, but not IL-4, in response to B16 melanoma. What is more important, the adoptive transfer of such TIL had a significant antitumor effect against pulmonary metastasis in B16 melanoma, even without the concurrent administration of IL-2. Collectively, our results thus indicate the therapeutic efficacy of the protocol presented here for antitumor immunotherapy with TIL.

Key words TIL · Adoptive immunotherapy · Activated B cells · Costimulation signal

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K. Tamada · T. Okamoto · M. Takenoyama · O. Ito · G. Matsuzaki · K. Kikuo
Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan

M. Harada (✉)
Department of Virology, Medical Institute of Bioregulation, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-82, Japan
(Fax: 092 641 1315)

Introduction

As a cell source for adoptive immunotherapy against cancer, peripheral blood lymphocytes, tumor-draining lymph node cells and tumor-infiltrating lymphocytes (TIL) have been proposed. Among them, TIL are considered to contain the highest frequency of either tumor-specific cytotoxic T lymphocytes (CTL) or their precursors [26]. However, the number of TIL available is generally small and therefore they inevitably require a lengthy culture time to grown to a sufficiently large cell number to be used for adoptive immunotherapy. Considering that the number of effector cells to be transferred is critical in regard to the therapeutic effect [9], an in vitro culture system, by which TIL are able to expand efficiently within a short period, would be therapeutically useful.

Recently, it has been revealed that T cells essentially require two different signals through both T cell receptor and CD28 molecules in order to attain optimal activation [10, 13, 18]. Activated B cells, macrophages and dendritic cells are known to express costimulatory molecules such as CD80 and CD86 [1, 6, 7, 8, 20], and such molecules provide costimulatory signals to T cells through CD28. In particular, activated B cells are easy to prepare from tumor-bearing hosts in comparison to other populations. On the basis of such evidence, we previously reported that activated B cells are able to provide costimulatory signals optimally in vivo [11]. In addition, we also reported that the tumor-draining lymph node cells can be efficiently expanded in the culture first by stimulation with both anti-CD3 monoclonal antibody (mAb) and activated B cells and then by further stimulation with interleukin

(IL)-2 [25]. In addition, the lymph node cells expanded in vitro show a specific antitumor effect against metastatic melanoma [25].

In the present study, we tried to extend our culture protocol to TIL. We attempted to demonstrate that a culture with both anti-CD3 mAb and activated B cells, followed by a culture with IL-2 can efficiently expand TIL, and that such expanded TIL also exhibit both cytolytic activity and interferon (IFN) γ production with tumor specificity. What is more important, such TIL also show a specific antitumor effect against pulmonary metastasis of melanoma. The implications of these findings are discussed.

Materials and methods

Mice

Female C57BL/6 (B6) mice, 7 weeks of age, were purchased from Japan SLC (Shizuoka, Japan). All mice were bred under specific-pathogen-free conditions, and were later used for the experiments at 8 weeks of age.

Tumors

The B16, 3LL and EL-4 cell lines originated from melanoma, Lewis lung carcinoma, and T cell lymphoma, respectively. All tumor cell lines were of B6 origin and were maintained in vitro.

IL-2

Highly purified human recombinant IL-2 was kindly provided by Takeda Chemical Industries Ltd., Osaka, Japan. The specific activity of IL-2 was 1.4×10^7 Japan reference units/mg protein; when the Biological Response Modifiers Program standard was used, this corresponded to 1.2×10^7 units/mg protein. In this report, the unit of IL-2 is the Japanese reference unit.

Anit-CD3 mAb

Anti-CD3 mAb was purified from the supernatants of anti-CD3-mAb-producing hybridoma 145-2C11 (kindly provided by Dr. J. A. Bluestone, Department of Pathology, University of Chicago, Chicago, Ill.). The antibody was obtained by collecting supernatants of the hybridoma cells growing in a serum-free medium (101; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

Preparation of TIL

B6 mice were inoculated subcutaneously (s. c.) with either 1×10^6 B16 or 1×10^6 3LL into the bilateral abdomen. On day 10 or 14, the tumors were harvested aseptically and teased into suspension in a complete culture medium. RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah) 50 μM 2-mercaptoethanol, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 30 μg/ml gentamicin (Schering Corporation, Kenilworth, N.J.), and 0.02% sodium bicarbonate was used as the complete medium. The suspensions of TIL were resuspended in 5 ml 45% Percoll (Sigma Chemical Co., St. Louis, Mo.), and layered on 5 ml 67.5% Percoll solution. The gradient was centrifuged at 2500 rpm at 20 °C for 25 min. The lymphocytes at the interface were harvested, and washed with the complete culture medium. This procedure was not able to remove the tumor cells and, on the basis of a microscopic examination, the percentage of lymphocytes in the prepared TIL was determined to only be approximately 10% in any experiment (data not shown).

Preparation of LPS blasts

Spleen cell suspensions (4×10^6 cells/ml) obtained from naive B6 mice were cultured with 5 μg/ml lipopolysaccharide (LPS) (*Escherichia coli* 0111, Difco Laboratories, Detroit, Mich.) for 2 days in the complete culture medium. The LPS blasts were enriched by Percoll gradient centrifugation. The Percoll gradient was prepared by layering 40% Percoll containing LPS-stimulated spleen cells over 50% Percoll and centrifuging them at 3000 rpm for 13 min at 4 °C. The cells at the interface were collected and washed with the complete culture medium. The enriched cells were $94 \pm 3\%$ positive for $B220^+$ and negative for Mac-1 on the basis of a flow-cytometric analysis.

In vitro culture with anti-CD3 mAb and/or LPS blasts and subsequently with IL-2

TIL (1×10^5 cells/ml) were cultured with anti-CD3 mAb (1 μg/ml) and/or LPS blasts (5×10^5 cells/ml) at 37 °C in 5% CO_2 for 2 days. Tumor-draining lymph node cells (1×10^6 cells/ml) were also cultured with anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^6 cells/ml) under the same conditions. The cultured cells were harvested and the viable lymphocytes were enriched by using Lympholyte-M (Cedalane Laboratories, Ontario, Canada). Thereafter, TIL (1×10^5 cells/ml) and tumor-draining lymph node cells (1×10^6 cells/ml) were cultured in the complete culture medium containing 50 U/ml IL-2 for 5 days.

To examine the participation of the costimulatory signals and Fc receptor (FcR) γII in T cell proliferation, either CTLA4 Ig (kindly provided by Dr. P. S. Linsley, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Wash.) or the supernatant of anti-FcRγII-mAb-producing hybridoma (2.4G2, rat IgG2b) was added to each well at the initial culture. For a control, the supernatant of anti-Mac-1α-mAb-producing hybridoma (M1/70.15.11.5, rat IgG2b) was added to each well. In some experiments, TIL (1×10^5 cells/ml) were cultured with 1000 U/ml IL-2 for 7 days. To prepare the lymphokine-activated killer (LAK) cells, naive spleen cells (4×10^6 cells/ml) were cultured with 1000 U/ml IL-2 for 4 days.

Flow-cytometric analysis

For flow cytometry, a cell suspension was incubated with the following mAb and then analyzed by fluorescence-activated cell sorting (FACScan, Becton Dickinson, Mountain View, Calif.). Biotin-conjugated anti-(mouse IL-2 receptor) (IL-2R) α chain was purchased from PharMingen (San Diego, Calif.). Phycoerythrin-conjugated streptavidin was purchased from Gibco BRL (Gaithersburg, Md.). To exclude any dead cells, the prepared cells were stained with 2 μg/ml propidium iodide (Sigma Chemical Co.).

Assay of cytotoxicity

The in vitro cytolytic activity of the effector cells was examined by the standard 4-h ^{51}Cr-release assay. The specific release was calculated according to the following formula:

$$\text{specific } ^{51}\text{Cr release } (\%) = \frac{\text{test release (cpm)} - \text{spontaneous release (cpm)}}{\text{total release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Spontaneous release was determined by the supernatant of the sample that was incubated with no effector cells, and the total release was determined by the supernatant of the sample that was incubated with 10% Triton X (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Table 1 The efficient expansion of tumor-infiltrating lymphocytes (TIL) in the culture of anti-CD3 plus lipopolysaccharide (LPS) blasts/ interleukin-2 (IL-2). TIL (1×10^5 cells/ml), from B6 mice bearing B16 melanoma for 14 days, were first cultured with anti-CD3 mAb (1 mg/ ml) and/or LPS blasts (5×10^5 cells/ml) for 2 days, then with IL-2 for 5 days. In experiment 3, the B16-derived TIL were cultured with 1000 U/ml IL-2 for 7 days. The expansion(-fold) represents the increase between the initial cell number and that observed on the indicated day. *ND* not done

Expt.	Group	Culture system		Expansion(-fold) on day:		
		Initial culture with	Subsequent culture with IL-2 (U/ml)	2	7	10
1	1	Anti-CD3	50	0.3	1	ND
	2	Anti-CD3+LPS blasts	50	3.0	140	ND
2	1	Anti-CD3	50	1.4	0	0
	2	Anti-CD3+LPS blasts	50	2.8	36	167
3	1	Anti-CD3	50	0.1	0	0
	2	Anti-CD3+LPS blasts	50	2.6	62	98
	3	IL-2 1000 U/ml	1000	2.4	10	28

In vitro culture of TIL with the tumor cells

The expanded TIL (1×10^6 cells/ml) were cultured with mitomycin-C-treated tumor cells (5×10^4 cells/ml) in a 24-well plate (Coster, Cambridge, Mass.). After 3 days, the supernatants were collected and assayed for IFN-γ and IL-4. Mitomycin-C-treated tumor cells were prepared by culture with 100 μg/ml mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) at 37 °C for 60 min.

Cytokine assay

The detection of mouse IFNγ and IL-4 was performed by a sandwich enzyme-linked immunosorbent assay (ELISA), modifying the technique previously reported by Kitamura et al. [15]. Briefly, 96-well ELISA plates (Greiner, Frickenhausen, Maybachstraße, Germany) were coated overnight at 4 °C and 50 μl/well with either anti-(mouse IFNγ) mAb (PharMingen) or anti-(mouse IL-4) mAb (PharMingen) at a final concentration of 2 μg/ml. The plates were washed with 0.05% Tween/phosphate-buffered saline (PBS) and unbound sites were saturated by incubation with 100 μl PBS containing 0.1% bovine serum albumin (fraction V, Sigma Chemical Co.) for 1 h at room temperature. After the plates had been washed, a 50-μl aliquot of each sample was added to each well. The samples were incubated at room temperature for 1 h, and standard dilutions for recombinant IFNγ (PharMingen) and recombinant IL-4 (PharMingen) were also evaluated. The plates were then washed and 50 μl 2 μg/ml biotinylated anti-(mouse IFNγ)mAb (PharMingen) or anti-(mouse IL-4)mAb (PharMingen) was added to each well and incubated for 1 h at room temperature. The plates were then washed and 50 μl streptavidin-β-galactosidase (Gibco BRL) at a dilution of 1/1000 was added to each well and incubated for 45 min. After the plates had been washed, 100 μl substrate solution containing 0.2 mM 4-methylumbelliferyl-β-D-galactopyranoside (Wako Pure Chemical Industries Ltd.) was added to the wells, which were left for 45–60 min while being protected from any direct light. Finally, after addition of 100 μl 0.1 M glycine, NaOH, absorbance was measured and monitored with a fluorescence microplate reader (MTP-32, Corona Co. Ltd., Ibaragi, Japan) calibrated for excitation at 360 nm and emission at 460 nm. The values vor IFNγ and IL-4 were calculated from a standard curve of recombinant mouse IFNγ and IL-4.

Adoptive immunotherapy model

To establish a model of metastasis, 2×10^5 B16 melanoma cells at a volume of 0.5 ml were injected intravenously (i. v.) into B6 mice through the tail vein. On day 3, when micrometastases had become established in the lungs, the cultured cells were then transferred i. v. into these mice. On day 21, the treated B6 mice were sacrificed and the numbers of pulmonary colonies were counted. In some mice, 30000 U IL-2 in a volume of 0.5 ml was injected intraperitoneally (i. p.), twice daily from day 3 to day 6.

Statistics

The statistical significance of the data was determined using Student's *t*-test. A *P* value of less than 0.05 was considered to be statistically significant.

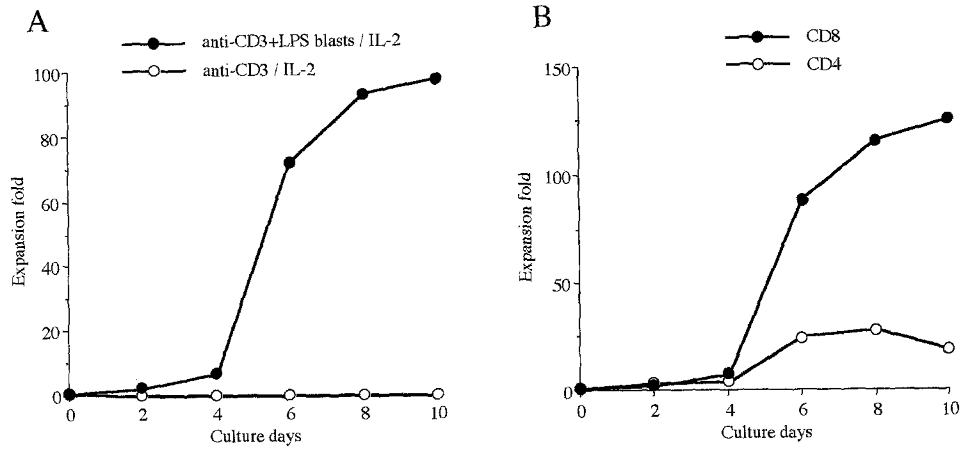
Results

Efficient expansion of TIL in the culture of anti-CD3 plus LPS blasts/IL-2

We have previously reported that tumor-draining lymph node cells are able to expand efficiently in culture with both anti-CD3 mAb and LPS blasts followed by culture with a low dose (50 U/ml) of IL-2 (anti-CD3 plus LPS blasts/IL-2) [25], probably because of the optimal delivery of the costimulatory signals from LPS-activated B cell blasts [11]. In this study, we tried to extend this culture system to TIL in order to utilize it for immunotherapy. As shown in Table 1, TIL, prepared from s. c. inoculated B16 melanoma, efficiently expanded in this culture system. On the other hand, a culture with anti-CD3 mAb alone, followed by a culture with IL-2 was not able to exhibit any proliferation of TIL. In addition, the degree of expansion of TIL in the culture with 1000 U/ml IL-2 was significantly smaller than that of our culture system. We also examined the kinetic change of phenotypes of TIL in the culture of anti-CD3 plus LPS blasts/IL-2. As shown in Fig. 1, $CD8^+$ T cells expanded more efficiently than $CD4^+$ T cells, and the percentage of $CD8^+$ T cells in the expanded TIL on day 7 of the culture was approximately 95% in all experiments. In addition, the LPS blasts added at the beginning of the culture disappeared after 2 or 3 days of culture (data not shown).

Costimulatory molecules and FcRγII on LPS blasts participate in the efficient expansion of TIL in the culture protocol

Since activated B cells express costimulatory molecules and FcRγII [11], we tried to determine whether or not such



molecules and the receptor on LPS blasts participated in the efficient expansion of TIL in our culture system. Figure 2 shows that the addition of CTLA4 Ig, as an inhibitor of costimulatory signals, into the initial culture partially inhibited the cell expansion of TIL. This finding suggests that the efficient expansion of TIL in the culture protocol could, in part, be attributed to the delivery of costimulatory signals from CD80 and CD86 on LPS blasts. We also examined the possibility that FcRγII on LPS blasts participated in the culture protocol, and found that the efficient expansion of TIL was almost completely inhibited by the addition of anti-FcRγII mAb, but not by anti-Mac-1α mAb as an isotype-matched control mAb. These results suggest that both the costimulatory molecules and the FcRγII on LPS blasts are necessary for the efficient expansion of TIL in the culture of anti-CD3 plus LPS blasts/IL-2.

Fig. 1 A, B The efficient expansion of tumor-infiltrating lymphocytes (TIL) in the culture of anti-CD3 plus lipopolysaccharide (LPS) blasts/interleukin-2 (IL-2). **A** TIL (1×10^5 cells/ml), from B6 mice bearing B16 for 14 days, were cultured with anti-CD3 mAb (1 μg/ml) alone or with both anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^5 cells/ml) for 2 days, and thereafter with 50 U/ml IL-2 for 8 days. After the indicated number of days, the cultured cells were harvested and counted. **B** The cultured cells were analyzed for their surface phenotypes by flow cytometry. The numbers of $CD4^+$ or $CD8^+$ T cells were calculated by multiplying the total number of the cultured cells by the percentage of either $CD4^+$ or $CD8^+$ T cells. *Vertical scale* the expansion (-fold) in comparison with the initial cell number. The same results were obtained in four independent experiments

Group	Initial 2 days		Subsequent 5 days	Cell expansion fold on day 7 (0, 40, 80, 120)
	Cultured with	Added with		
1	anti-CD3	-	IL-2 50 U/ml	
2	anti-CD3/LPS blasts	-	IL-2 50 U/ml	
3	anti-CD3/LPS blasts	CTLA4 Ig	IL-2 50 U/ml	
4	anti-CD3/LPS blasts	anti-FcRγ II	IL-2 50 U/ml	
5	anti-CD3/LPS blasts	anti-Mac-1α	IL-2 50 U/ml	

Fig. 2 Both the costimulation molecules and Fc receptor(FcR)γII on LPS blasts are necessary for the optimal expansion of TIL in the culture of anti-CD3 plus LPS blasts/IL-2. TIL (1×10^5 cells/ml), from B6 mice bearing B16 for 14 days, were cultured with either anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^5 cells/ml) or anti-CD3 mAb (1 μg/ml) alone for an initial 2 days and then were cultured with 50 U/ml IL-2 for 5 days. CTLA4 Ig was added to each well at a dose of 10 μg/ml in the initial culture. The supernatant of either anti-FcRγII mAb (rat IgG2b) or control anti-Mac-1α mAb (rat IgG2b) was added to each well at a dose of 25% in the initial culture. The cell expansion (-fold) was then calculated by dividing the cell number on day 7 by the initial cell number. The same results were obtained in three independent experiments

Expression of IL-2Rα on TIL expanded in the culture protocol

To investigate the mechanism by which the culture protocol induced the efficient cell expansion of TIL, we examined

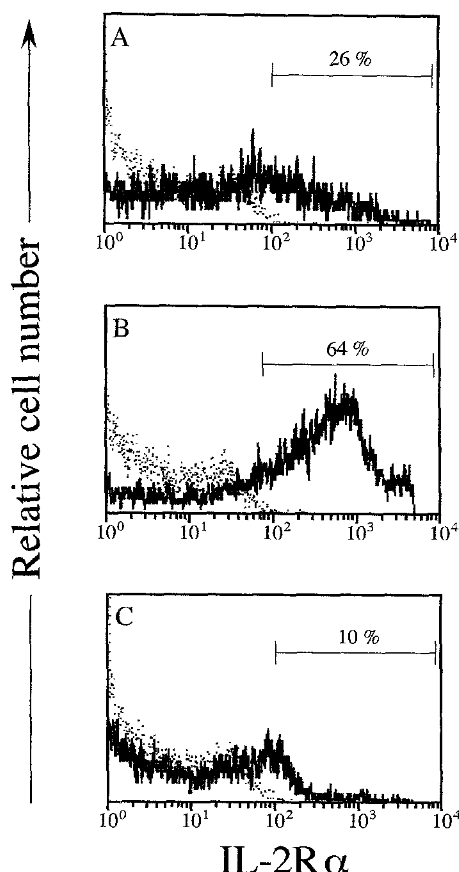


Fig. 3A–C Expression of interleukin-2 receptor (IL-2R) α chain on the in vitro cultured TIL. TIL (1×10^5 cells/ml), from B6 mice bearing B16 for 14 days, were cultured with either anti-CD3 mAb (1 μg/ml) alone (**A**), anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^5 cells/ml) (**B**) or 1000 U/ml IL-2 (**C**) for 2 days and were stained first with the biotin-conjugated anti-(IL-2Rα chain) and then with streptavidin-phycoerythrin (*solid line*). *Dotted line* the background stained with streptavidin-phycoerythrin alone. The numbers indicate the percentage of IL-2Rα-chain-positive cells. The fluorescence histograms are plotted on a logarithmic scale

the expression of IL-2 receptor (IL-2R) on the TIL cultured in vitro. As shown in Fig. 3, TIL cultured with both anti-CD3 mAb and LPS blasts for 2 days expressed a higher level of IL-2Rα chain than those with either anti-CD3 mAb alone or 1000 U/ml IL-2. We also examined the expression of the IL-2Rβ chain, but no difference was observed for any group (data not shown). Considering that the expression of IL-2Rα is regarded as an activation marker [24], these findings suggest that the addition of LPS blasts was essential for the optimal activation of TIL in the presence of anti-CD3 mAb. In addition, these findings also suggest that such T cells, which highly expressed IL-2Rα, expanded even in the subsequent culture with a low dose of IL-2.

Specific CTL activity of TIL expanded in the culture protocol

We next examined the CTL activity of the TIL expanded in vitro. Figure 4 shows that B16-derived TIL, which expanded in the proposed culture system, significantly exhibited specific CTL activity against B16 melanoma. TIL, prepared from 3LL and which expanded in vitro in the culture protocol, showed a higher level of CTL activity against 3LL, but it was not significant. These expanded TIL also exhibited an approximately 25%–30% cytotoxic activity against P815 at an effector/target ratio of 25/1 (data not shown). These results, however, suggest that TIL, after expanding in the culture protocol, have a specific amount of CTL activity against the tumor from which they were prepared.

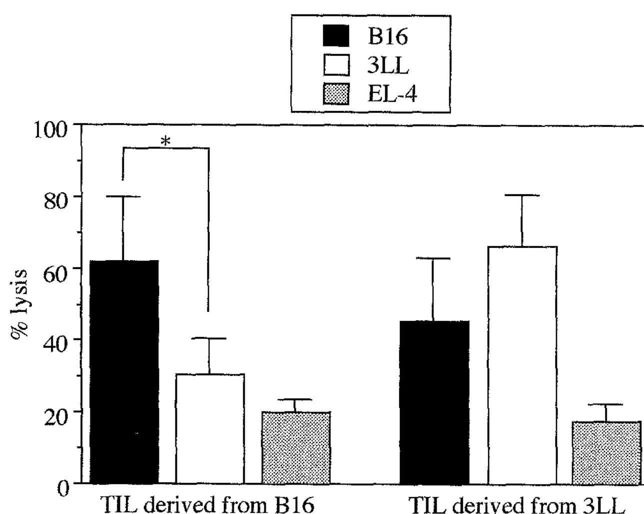


Fig. 4 The cytotoxic T lymphocyte (CTL) activity of TIL expanded in the culture of anti-CD3 plus LPS blasts/IL-2. TIL (1×10^5 cells/ml), from B6 mice bearing either B16 melanoma or 3LL carcinoma for 14 days, were cultured first with both anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^5 cells/ml) for 2 days and then with 50 U/ml for 5 days. These cultured cells were analyzed for their cytolytic activities against three tumor cell lines by a 4-h ^{51}Cr-release assay at an E/T ratio of 50/1. The same results were obtained in three independent experiments. * $P < 0.05$, significant

We next compared the CTL activity of B16-derived and in vitro expanded TIL with those cultured with a high dose of IL-2. As shown in Fig. 5, the TIL that expanded in the culture protocol showed a higher level of specific CTL activity than those cultured with 1000 U/ml IL-2. In addition, their CTL activity was higher than that of spleen cells cultured with 1000 U/ml IL-2 for 4 days, the so-called LAK activity. In contrast, no difference was observed in the CTL activity against 3LL in any group.

Cytokine production of the TIL expanded in vitro in response to the tumor cells

We further examined whether or not TIL expanded in the culture protocol could produce cytokines with specificity. As shown in Table 2, the B16-derived and in vitro expanded TIL produced a marginal level of IFNγ when cultured either with 3LL or without any tumor line. In contrast, such TIL produced a higher level of IFNγ when cultured with B16. The production of IL-4 was not detected in any group.

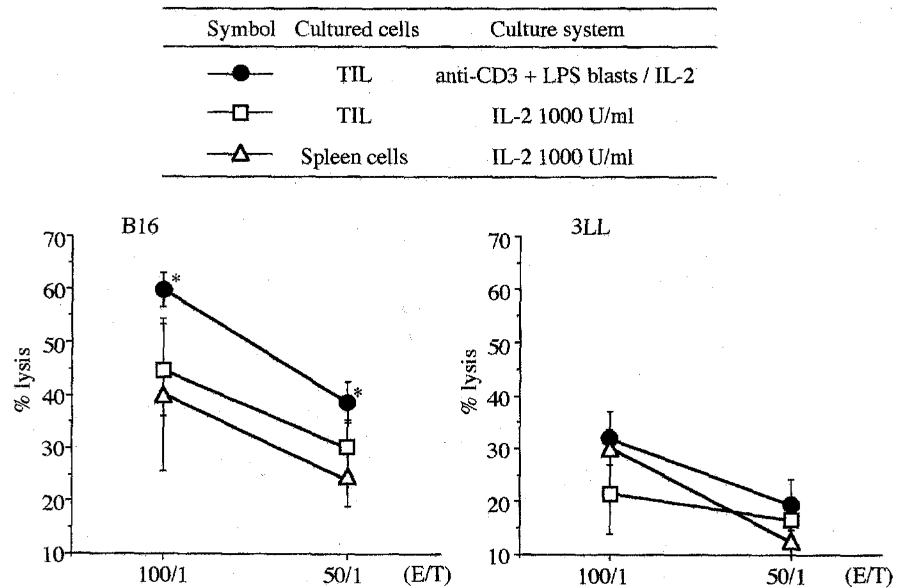


Fig. 5 The CTL activity of the in vitro expanded TIL and lymphokine-activated killer cells. TIL (1×10^5 cells/ml), from B6 mice bearing B16 for 14 days, were cultured first with both anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^5 cells/ml) for 2 days and then with 50 U/ml IL-2 for 5 days, or were cultured with 1000 U/ml IL-2 for 7 days. The naive spleen cells (4×10^6 cells/ml) were cultured with 1000 U/ml IL-2 for 4 days. These cultured cells were analyzed for their cytolytic activities against B16 melanoma and 3LL carcinoma by a ^{51}Cr-release assay. The same results were obtained in three independent experiments. * $P < 0.05$, significant compared with the other groups

Table 2 Specific cytokine production of B16-derived and in vitro expanded TIL in response to B16 melanoma. TIL (1×10^5 cells/ml), from B6 mice bearing B16 for 14 days, were first cultured with both anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^5 cells/ml) for 2 days and then with 50 U/ml IL-2 for 5 days. These cultured cells were used as responder cells. Either mitomycin-C-treated B16 or mitomycin-C-treated 3LL cells were used as stimulator cells. Responder cells (1×10^6 cells/ml) were cultured either with or without the stimulator cells (5×10^4 cells/ml) in a 24-well plate for 3 days and then the supernatants were harvested and assayed to determine the cytokine levels by enzyme-linked immunosorbent assay. *IFN* interferon

Expt.	Responder	Stimulator	Cytokine production: IFNγ (U/ml)	Cytokine production: IL-4 (U/ml)
1	B16 TIL	B16	16.0	<1
	B16 TIL	3LL	3.8	<1
2	–	B16	<1	<1
	–	3LL	<1	<1
	B16 TIL	–	2.9	<1
	B16 TIL	B16	11.2	<1
	B16 TIL	3LL	3.4	<1

Table 3 The antitumor effect of TIL expanded in vitro in the culture of anti-CD3 plus LPS blasts/IL-2. TIL (1×10^5 cells/ml) were first cultured with both anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^5 cells/ml) for 2 days, and then with 50 U/ml IL-2 for 5 days. The tumor-draining lymph node cells (1×10^6 cells/ml) were also first cultured with both anti-CD3 mAb (1 μg/ml) and LPS blasts (5×10^6 cells/ml) for 2 days, and then with 50 U/ml IL-2 for 5 days. Both types of effector cells were prepared from B6 mice bearing B16 for 10 days. The injection of IL-2 was started on the day the cultured cells were transferred. IL-2 was i. p. injected twice daily for 4 days. On day 0, 2×10^5 B16 melanoma cells were injected i. v. into B6 mice and, on day 3, the cultured cells were i. v. transferred. On day 21, the mice were sacrificed and the numbers of pulmonary metastatic colonies were counted. Each group consisted of six mice

Group	Effector cells: Cell source	Effector cells: Number of cells transferred	IL-2 injected (U)	Mean number of pulmonary metastases ± SD
1	–	–	0	100 ± 11
2	–	–	30000	74 ± 20*
3	TIL	1×10^7	0	11 ± 3.8**
4	TIL	1×10^7	30000	5 ± 3.9**
5	Tumor-draining lymph node cells	1×10^7	30000	4 ± 2.8**

* $P < 0.05$ compared with group 1
** $P < 0.01$ compared with group 1

These results thus suggest that TIL, expanded in our culture system, were able to produce the Th1-type cytokines with specificity.

Adoptive immunotherapy with TIL expanded in the culture of anti-CD3 plus LPS blasts/IL-2

Thus far we have demonstrated the efficacy of the culture protocol of anti-CD3 plus LPS blasts/IL-2 in regard to cell growth, CTL activity and cytokine production of TIL. We finally determined whether or not the TIL expanded in vitro could show any antitumor effect in vivo. As shown in Table 3, B16-derived and in vitro expanded TIL showed a significant antitumor effect against the pulmonary metastasis of B16 melanoma either with or without i. p. injections of IL-2 (groups 3 and 4). These findings are in contrast to the result that the in vivo antitumor effect of the tumor-draining lymph node cells, expanded in our culture system, needs the concurrent i. p. injections of IL-2 [25]. The in vivo antitumor effect of the in vitro expanded TIL was almost equivalent to that of the in vitro expanded tumor-draining lymph node cells (group 5). We also determined whether or not the 3LL-derived and in vitro expanded TIL could show an antitumor effect against metastatic B16 melanoma and found that no such effect was induced, even when the treatment included i. p. injections of IL-2 (data not shown).

Discussion

It has been reported that TIL are the most effective lymphocytes for antitumor immunotherapy, and their efficacy has been demonstrated in various systems in both mice [26, 31] and humans [14, 16, 17, 28]. However, it has also been reported that TIL are functionally impaired [5, 21–23], probably because of their proximity to the tumor cells, and therefore an in vitro culture system is needed to restore their functions. On the other hand, although the availability of recombinant IL-2 enables us to expand T cells, both the proliferative response of TIL to IL-2 and their antitumor cytotoxicity have been reported to decline as the length of the culture period increases [4]. In order to overcome these difficulties in expanding TIL, we proposed an in vitro culture system, by combining anti-CD3 mAb with activated B cells as costimulatory-signal-providing cells, to expand TIL efficiently within a short period of time and with antitumor specificity.

Recent reports have revealed that several kinds of costimulatory signals are needed for optimal T cell activation [10, 13, 18]. B7-1 (CD80), B7-2 (CD86), intracellular adhesion molecule-1 and heat-stable antigen are reported to be able to deliver costimulatory signals to T cells [1, 3, 8, 12, 19, 20]. In addition, it has also been reported that activated B cells strongly express such costimulatory molecules on their cell surfaces [1, 6, 8, 19, 20]. The costimulatory signals provided by these molecules play an important role in the antitumor T cell response and several researchers have reported therapeutic models utilizing CD80 in vivo [2, 32]. On the other hand, we previously reported that activated B cells are useful as costimulatory-signal-providing cells in an in vitro culture system [25]. That is, culture of the tumor-draining lymph node cells with both activated B cells and anti-CD3 mAb followed by a low dose of IL-2 (anti-CD3 plus LPS blasts/IL-2) resulted in an efficient expansion, and such expanded lymph node cells show an antitumor effect in vivo. In the present study, we applied the same culture protocol to expand TIL. In contrast to the inability to expand TIL in the culture with anti-CD3 mAb followed by IL-2 (anti-CD3/IL-2), the TIL expanded efficiently in our culture system (Table 1, Fig. 1 A). We observed that, in the culture of anti-CD3/IL-2, the initially contaminating tumor cells expanded and thus consequently overcame the expansion of TIL. In contrast, in our culture system, the contaminating tumor cells had disappeared within 4 or 5 days of culture and thereafter the TIL expanded vigorously. These findings indicate that the culture protocol presented here can indeed expand TIL even with a substantial contamination of the tumor cells. Regarding the difficulty in completely excluding the tumor cells from prepared TIL, these results are considered to be immunotherapeutically useful.

Concerning the mechanisms by which TIL efficiently expanded in our culture system, we propose the following. The cross-linking with anti-CD3 mAb, which bound to the FcRγII on LPS blasts, provided the first signals and then the costimulatory molecules on the LPS blasts simultaneously provided the secondary signals to TIL. Such an optimal stimulation induced the increased expression of IL-2R and, as a result, TIL could expand in the subsequent culture with a low dose of IL-2. This assumption was also supported by the findings that demonstrated that both the costimulatory molecules and the FcRγII participated in the efficient expansion of TIL (Fig. 2), while the expression of the IL-2Rα chain on TIL, cultured with both anti-CD3 mAb and LPS blasts for 2 days, was higher than that following culture with anti-CD3 mAb alone (Fig. 3). Nevertheless, further investigations are needed to elucidate the precise mechanism fully.

It has been reported that LAK cells show a high level of cytolytic activity against many kinds of tumors, but the in vivo antitumor effect has been reported to be insufficient [27]. Several researchers therefore devised an in vitro restimulation system to induce the tumor-specific CTL [29, 30]. As demonstrated in this study, our culture system was able to induce the tumor-specific T cells in vitro against not only immunogenic B16 melanoma but also poorly immunogenic 3LL carcinoma, although some degree of cross-reactivity was observed between B16 melanoma and 3LL (Fig. 4). This cross-reactivity seemed to be due to the LAK activity contained in the expanded TIL. Moreover, the tumor-specific CTL activity of the TIL, which expanded in the culture protocol presented in this study, was higher than that of the LAK cells and TIL cultured with a high dose of IL-2 (Fig. 5). We also confirmed that the TIL cultured in vitro produced IFNγ but not IL-4, in response to restimula-

tion with the tumor cells (Table 2). It is thus likely that TIL were restimulated by contaminating tumor cells during the in vitro culture. The high and specific CTL activity of the in vitro expanded TIL and their Th1-type cytokine production may thus reflect the in vivo antitumor effect of TIL.

Although some investigators have revealed that the tumor-draining lymph node cells, which were cultured in vitro, show antitumor activity in vivo, such activity depends on the concomitant administration of IL-2 [25, 33], probably because the transferred cells need IL-2 for survival and maturation in vivo [34]. In contrast, TIL expanded in the culture with anti-CD3 plus LPS blasts/IL-2 showed a significant antitumor effect against the pulmonary metastasis of melanoma even without the concurrent administration of IL-2 (Table 3). This difference between the tumor-draining lymph node cells and TIL might be due to the presence of tumor cells during the in vitro culture of TIL. In this regard, Shu et al. reported that the antitumor effect of tumor-draining lymph node cells expanded in the presence of irradiated tumor cells was dependent on the concurrent administration of IL-2 [29, 30]. Although the in vitro culture systems are different from each other, these lines of evidence may indirectly suggest that the antitumor effect of TIL in vivo without the simultaneous administration of IL-2 was not entirely dependent on the presence of tumor cells during the culture in vitro. Although one possible explanation of this finding is that the TIL had already matured in the in vitro culture and showed the antitumor effect soon after the i. v. transfer, further examination is needed to elucidate the precise mechanism.

In conclusion, we have demonstrated that the culture system, utilizing activated B cells as costimulatory-signal-providing cells, was able to expand TIL efficiently within a short time and that such expanded TIL demonstrate specific antitumor activity both in vitro and in vivo. The culture protocol described in this study may thus eventually be utilized as a potent strategy for adoptive immunotherapy with TIL.

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