IFN-γ- and Cell-to-Cell Contact-Dependent Cytotoxicity of Allograft-Induced Macrophages Against Syngeneic Tumor Cells and Cell Lines: An Application of Allografting to Cancer Treatment[1]

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In allogeneic tumor or skin transplantation, the rejection process that destroys the allogeneic cells leaves syngeneic cells intact by discrimination between self and nonself. Here, we examined whether the cells infiltrating into the allografts could be cytotoxic against syngeneic immortal cells in vitro and in vivo. The leukocytes (i.e., macrophages (Mϕ; 55–65% of bulk infiltrates), granulocytes (20–25%), and lymphocytes (15–20%)) infiltrating into allografts, but not into autografts, in C57BL/6 mice were cytotoxic against syngeneic tumor cells and cell lines, whereas the cytotoxic activity was hardly induced in allografted, IFN-$\gamma^{-/-}$ C57BL/6 mice. Among the leukocytes, Mϕ were the major population of cytotoxic cells; and the cytotoxic activity appeared to be cell-to-cell contact dependent. When syngeneic tumor cells were s.c. injected into normal C57BL/6 mice simultaneously with the Mϕ-rich population or allogeneic, but not syngeneic, fibroblastic cells, tumor growth was suppressed in a cell number-dependent manner, and tumor cells were rejected either with a Mϕ:tumor ratio of about 30 or with an allograft:tumor ratio of ~200. In the case of IFN-$\gamma^{-/-}$ C57BL/6 mice, however, the s.c. injection of the allograft simultaneously with tumor cells had no effect on the tumor growth. These results suggest that allograft or allograft-induced Mϕ may be applicable for use in cancer treatment and that IFN-γ induction by the allograft may be crucial for the treatment. ***The Journal of Immunology*, 1999, 163: 148–154.**

One implication of the varied responses of tumor cells to chemotherapy and other treatment modalities is that the successful eradication of disseminated cancer cells will have to be highly selective and circumvent the problems of biologic heterogeneity of neoplasms. Among leukocytes, macrophages (Mϕ)[3] activated in vitro or in vivo to the tumoricidal state may fulfill these demanding tasks (1, 2). In fact, gene transfer of monocyte chemoattractant protein-1 to a rodent tumor cell line stimulated recruitment of monocytes to tumors and inhibited tumor growth (3). It has also been reported, however, that tumor-associated Mϕ either could promote the growth of tumor cells (4) or were positively correlated with tumor invasion and progression (5). Therefore, the successful or unsuccessful eradication of Mϕ in tumor growth may depend either on the kind of Mϕs infiltrating into tumors or on the stage of Mϕ activation.

In allografts, the rejection process that destroys the allogeneic cells is inactive toward syngeneic cells, indicating that the leukocytes infiltrating into allografts discriminate between self and nonself (6). In the present study we examined whether the cells infiltrating into allografts could be cytotoxic against syngeneic tumor cells and cell lines in vitro and in vivo. The results indicated that among leukocytes, Mϕ were the major population of cells cytotoxic against syngeneic tumor cells and cell lines in an IFN-γ- and cell-to-cell contact-dependent manner and that s.c. injected syngeneic tumor cells were rejected by a simultaneous inoculation of the Mϕ-rich population or of allograft cells.

Materials and Methods

Reagents

$Na_2{}^{51}CrO_4$ (10.5 GBq/mg) was purchased from New England Nuclear (Boston, MA). RPMI 1640 medium was obtained from Nissui Seiyaku (Tokyo, Japan). FCS was obtained from ICN Biomedicals (Costa Mesa, CA) and was used after heat inactivation. Casein sodium was purchased from Wako Pure Chemicals (Osaka, Japan). D-[α-*methyl*]Mannoside and Con A were obtained from Sigma (St. Louis, MO). LPS (*Escherichia coli* O55:B5) prepared by the Westphal method and Brewer thioglycolate medium were products of Difco (Detroit, MI). IFN-γ, penicillin, streptomycin, and L-glutamine were obtained from Life Technologies (Gaithersburg, MD). Anti-TNF-α Ab was a product of Genzyme (Cambridge, MA). Anti-Thy-1.2 (53-2.1) Ab, anti-NK-1.1 (PK136 or 3A4) Ab, biotin-labeled anti-IL-2R (7D4) Ab, and PE-conjugated streptavidin were purchased from PharMingen (San Diego, CA). N^G-monomethyl-L-arginine (*N*-MMA) was obtained from Calbiochem (La Jolla, CA). All other chemicals were of reagent grade.

Animals

Male specific pathogen-free C57BL/6 (B6) (H-2^b) mice, 7 wk old, were purchased from Japan SLC (Hamamatsu, Japan). IFN-$\gamma^{-/-}$ B6 mice were donated by Dr. Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan) (7). In Con A blasts from IFN-$\gamma^{-/-}$ B6 mice, IFN-γ mRNA was not detected by RT-PCR analyses, whereas IL-2, IL-4, and TNF-α mRNAs were present at levels of expression similar to those in normal (IFN-$\gamma^{+/+}$) B6 mice (8). After an s.c. inoculation of tumor cells into normal or IFN-$\gamma^{-/-}$ B6 mice, the mice were maintained for several

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[3] Abbreviations used in this paper: Mϕ, macrophage; *N*-MMA, N^G-monomethyl-L-arginine; Meth A, 3-methylcholanthrene-induced ascites-type fibrosarcoma; PEC, peritoneal exudate cells; AIM, allograft-induced macrophages.

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weeks in our animal facility under specific pathogen-free conditions in an air-conditioned room (25 ± 2°C; ~50% humidity).

Tumor cells and cell lines

3-Methylcholanthrene-induced ascites-type fibrosarcoma (Meth A; H-2^d) cells were provided by Dr. S. Muramatsu (Department of Zoology, Kyoto University Faculty of Science, Kyoto, Japan), and were maintained by routine i.p. injection of 3×10^6 cells into a syngeneic mouse strain (BALB/c; H-2^d). EL-4 (lymphoma; H-2^b), 3LL (lung carcinoma; H-2^b), B16 (melanoma; H-2^b), and CMT-93 (rectum carcinoma; H-2^b) tumor cells, and NOR10 (H-2^b), NCTC4093 (H-2^b), and BALB/3T3 (H-2^d) fibroblastic cell lines were purchased from American Type Culture Collection (Manassas, VA).

Cell preparations

Meth A cell-induced peritoneal exudate cells (PEC) were obtained by peritoneal lavage on day 7 (or at various intervals) after an i.p. transplantation of Meth A tumor cells (3×10^6 cells/mouse) to normal or IFN-$\gamma^{-/-}$ B6 mice as previously described (8–18). The Mϕ-rich (~95% purity and ~5% T lymphoblast contamination) population was isolated from PEC by FACS (FACStar, Becton Dickinson, Mountain View, CA) with the gate set in the forward scattering/side scattering mode, as described previously (9–18). Skin grafting and preparation of the cells infiltrating into the skin graft-graft bed border were performed as described previously (19). The grafts and the surrounding tissues of recipients were removed en bloc and cut into small blocks with scissors. The blocks were digested with 0.15% protease/0.075% collagenase/0.001% DNase. All digested cells were centrifuged, and a granulocyte-, lymphocyte-, or Mϕ-rich population was sorted with the gate set in the forward scattering/side scattering mode by FACS. The morphological characteristics of the cells in each fraction were assessed by May-Giemsa stain. The cell number in all the digested cells from skin autografts was approximately half that in the case of skin allografts on days 5–9 after transplantation. PEC including in vivo elicited Mϕs were collected by lavage of mice that had received i.p. injection of 1 ml of 6% sodium casein or 2 ml of Brewer thioglycolate medium (4 days before). In vitro activated Mϕ were prepared by a further 8-h incubation of elicited Mϕ monolayers with LPS (10 ng/ml) and IFN-γ (20 U/ml), and then the cells were washed twice before the cytotoxic assay. Con A blasts were prepared by stimulating spleen cells (10^8 cells) with 5 μg/ml of Con A for 48 h at 37°C in a 50-ml flask, washing them twice with culture medium containing 0.3 mM D-[α-*methyl*]mannoside to avoid Con A-dependent cytotoxicity, and washing them a third time with culture medium. The viable Con A blasts were isolated by density gradient centrifugation in sodium metrizoate/Ficoll (Otsuka Pharmaceutical, Tokyo, Japan).

Complement-dependent cell lysis

Homogeneous (>99% purity, microscopically no other type of cell in the Mϕ population (126 cells)) Mϕ were obtained after T cell elimination from a Mϕ-rich population (prepared as described above) by complement-dependent cell lysis with anti-Thy-1.2 Ab. The cells (2×10^7 cells) in the Mϕ-rich population were suspended in 500 μl of fresh medium, and then 16 μl of anti-Thy-1.2 Ab and 160 μl of diluted Low-Tox-M rabbit complement (Cederlane, Ontario, Canada) was added to the suspension. The complement was reconstituted with 1 ml of cold distilled water and was diluted (three times) with medium before use. The mixture was incubated for 45 min at 37°C. To confirm that T cells had been eliminated specifically from a Mϕ-rich population or bulk PEC, the Ab plus complement-treated cells were stained with fluorescein-labeled anti-Thy-1.2 Ab, and their surface Ag were analyzed by FCM. For the in vitro specific elimination of NK or NKT cells from PEC, PEC were incubated with anti-NK-1.1 Ab and complement.

Negative immunomagnetic selection of PEC

Sterile magnetic polymer beads (Dynabeads M-450, Dynal, Oslo, Norway) were supplied coated with covalently bound affinity-purified sheep anti-mouse IgG. Anti-NK-1.1 Ab-coated immunomagnetic beads were prepared as described previously (20). Washed mAb-coated beads (10^8 beads/2 ml) were added to bulk PEC (5×10^6 cells). The mixture of cells and beads was incubated 5 min at 4°C, and beads with any attached cells were removed magnetically. The supernatant was centrifuged, the pellet was resuspended in the culture medium, and then the cell number and the cytotoxic activity were determined.

FACS analysis

The expression of the IL-2R Ag on Mϕ with Con A blasts used as a positive control was examined by FACS.

Cell number and viability

The cell number in the suspensions was determined with a hemocytometer after dilution of the cells in Turk's solution. The viability of cells was determined by the trypan blue exclusion method.

Cytotoxicity assay

Target cells (10^6 cells) in 25 μl of culture medium were labeled with 25 μl (925 KBq) of $Na_2{}^{51}CrO_4$ at 37°C for 2 h, and effector cells (3×10^5 to 5×10^5 cells) in 200 μl of culture medium were mixed with 20 μl of ^{51}Cr-labeled targets (10^4 cells). After an 18-h incubation, an aliquot of supernatant was removed from each well and assayed for released ^{51}Cr in a Hewlett-Packard (Meriden, CT) gamma counter. Lysis of ^{51}Cr-labeled targets in each well was quantified as the percent specific lysis, as described previously (21).

In vivo measurement of tumor growth

EL-4 (H-2^b) or 3LL (H-2^b) tumor cells (10^4 cells/mouse) were s.c. injected simultaneously with an Mϕ (H-2^b)-rich population, or BALB/3T3 (H-2^d) or NOR10 (H-2^b) fibroblastic cells into normal or IFN-$\gamma^{-/-}$ B6 (H-2^b) mice. At various intervals after the treatment, the growth of these syngeneic tumor cells was determined by measuring two diameters (perpendicular to each other) of the tumor with Vernier calipers (22). The surface area of the tumor was previously shown to correlate precisely with the tumor weight (23).

Results

Cytotoxic activities of Mϕ against various kinds of syngeneic tumor cells and cell lines

Bulk cells (Mϕ, 55–65%; granulocytes, 20–25%; lymphocytes, 15–20%) (11) infiltrating into allografts of Meth A tumor cells in B6 mice were cytotoxic against various kinds of syngeneic tumor (e.g., 3LL, EL-4, B16, and CMT-93) cells and cell lines (e.g., NOR10 and NCTC4093) with a 45–65% cytotoxic activity in an 18-h incubation (Fig. 1). Most of the activity was retained after T cell elimination (Fig. 1*A*); and the cytotoxic activity was not suppressed (3LL (~103% of the cytotoxic activity with control serum), B16 (~133%), NCTC4093 (~103%), and NOR10 (~123%)) by the addition of anti-CD3 Ab to the culture medium. Furthermore, bulk allospecific CTLs that had been induced in mixed lymphocyte cultures (B6 anti-BALB/c) were virtually inactive toward syngeneic tumor cells (e.g., 0.5 ± 3.1 and 3.4 ± 2.1% (mean ± SD; $n = 4$) specific lysis for EL-4 cells and 3LL cells, respectively) and cell lines (e.g., 5.2 ± 3.0% (mean ± SD; $n = 4$) and 3.2 ± 2.0% (mean ± SD; $n = 4$) specific lysis for NCTC4093 and NOR10 cells, respectively), indicating that T cells are not involved in the cytotoxic activity of bulk PEC against syngeneic tumor cells and cell lines. The granulocyte-rich population had no cytotoxic activities against 3LL cells (0.2 ± 1.3% (mean ± SD; $n = 4$) specific lysis) and NCTC4093 cells (0.5 ± 2.1% (mean ± SD; $n = 4$) specific lysis). In addition, the cytotoxic cells were phenotypically IL-2R$^-$ (Fig. 2)/NK-1.1$^-$ (10)/Mac-1$^+$ (19) by FCM, and neither the cytotoxic activity nor the cell number changed significantly after magnetic removal of cells attached to anti-NK-1.1 Ab-coated beads from PEC or after the in vitro specific elimination of NK or NKT cells from PEC with anti-NK-1.1 Ab and complement (Table I). These results taken together suggest that the cytotoxic activities of PEC against syngeneic tumor cells and cell lines appeared to be ascribable mainly to Mϕ, but not to T cells, granulocytes, lymphokine-activated killer cells, or NK or NKT cells.

Similarly, the bulk leukocytes (Mϕ (~60%), granulocytes (~20%), and lymphocytes (~20%)) (19) infiltrating into the graft-graft bed border of allografted BALB/c skin onto B6 mice were cytotoxic against syngeneic tumor (e.g., 3LL) cells and cell lines (e.g., NCTC4093), and most of the activity was recovered in Mϕ and not T cells (Fig. 1*B*). By contrast, the Mϕ were inactive toward self Con A blasts (0.1 ± 1.3% (mean ± SD; $n = 8$) specific lysis)

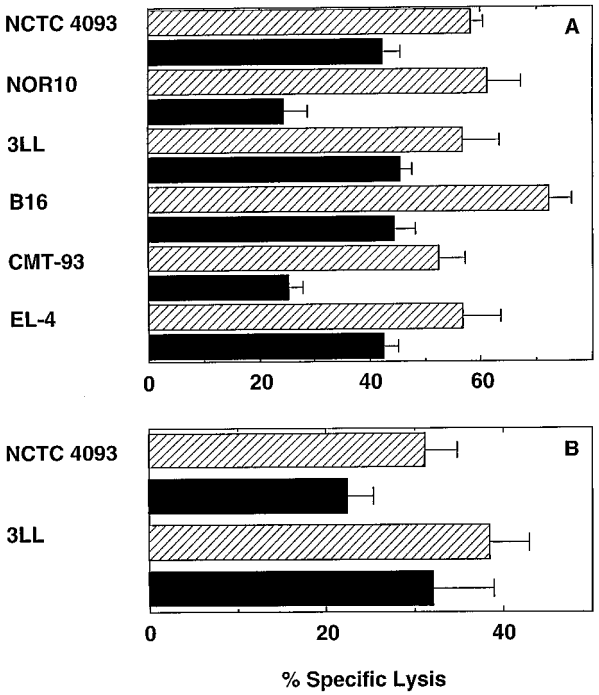

FIGURE 1. Effects of anti-Thy-1.2 Ab plus complement on cytotoxic activity either of PEC on day 7 after i.p. transplantation of Meth A tumor cells into B6 mice (*A*) or of bulk cells infiltrating into the graft-graft bed border on day 7 after BALB/c skin transplantation onto B6 mice (*B*) against various kinds of syngeneic tumor cells and cell lines. The cytotoxic activity was determined with an E:T ratio of 50 in an 18-h assay. Values represent the mean ± SD of eight cultures from two different experiments. ▨, −Ab, +complement; ■, +Ab, +complement.

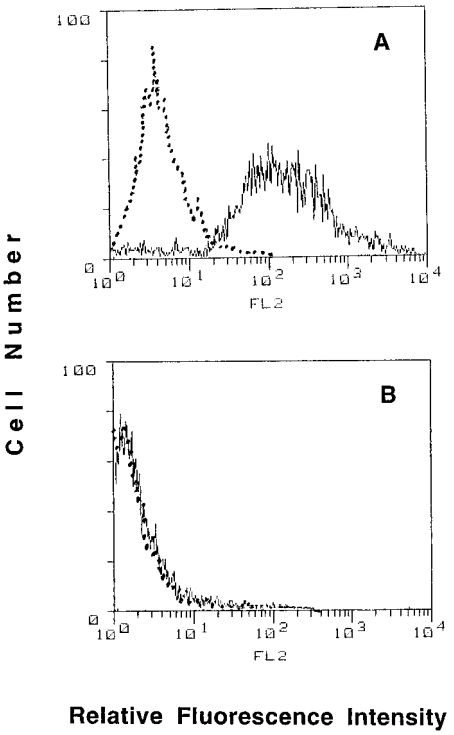

FIGURE 2. Phenotypic analyses of Mϕ. Bulk PEC were isolated on day 7 after Meth A tumor transplantation. Homogeneous (>99% purity) Mϕ were obtained after T cell elimination from a FACS-purified Mϕ-rich population by complement-dependent cell lysis with anti-Thy-1.2 Ab. B6 Con A blasts were used as a positive control. Cytofluorographic analyses were conducted with a FACS in the presence of biotin-labeled anti-IL-2R Ab and PE-conjugated streptavidin (——) or of PE-conjugated streptavidin (····). Vertical and horizontal axes represent the cell number and relative logarithmic fluorescence intensity, respectively. *A*, B6 Con A blasts; *B*, homogeneous Mϕ.

and autografts (2.0 ± 1.1% (mean ± SD; $n = 8$) specific lysis) as previously described (19).

Distribution of cytotoxic activities against syngeneic tumor cells and cell lines in i.p. Meth A cell-treated or BALB/c skin-grafted B6 mice

When the cytotoxic activities against 3LL cells of lymphoid cells in a variety of locations in i.p. Meth A cell-treated and untreated mice were quantified, the cytotoxic activities were associated exclusively with PEC of Meth A cell-treated mice and not with peritoneal cells of control mice (Fig. 3, *left*). Not only the cells in all lymphoid organs tested to date but also peripheral mononuclear leukocytes of Meth A cell-treated mice had little, if any, cytotoxic activity toward the targets. Furthermore, casein- or thioglycolate-elicited inflammatory Mϕ were totally inactive toward all these target cells (e.g., 0.2 ± 1.5 or 0.0 ± 1.2% (mean ± SD; $n = 8$) specific lysis for 3LL cells). Similarly, the cytotoxic activities against 3LL cells of cells in a variety of locations in BALB/c

Table I. *Cytotoxic activity of PEC against 3LL or NCTC4093 cells after removal of NK or NKT cells*

Target	Condition	Relative and Specific Lysis[a]	Relative Cell Number[b]
	Expt. 1: Complement-dependent cell lysis		
NCTC4093	Complement (C) alone	100 (50.9 ± 4.1)[c]	100 (10^6 cells)
	Anti-NK-1.1 (PK136) + C	92.9 ± 17.5	94.2 ± 9.6
	Anti-NK-1.1 (3A4) + C	87.2 ± 15.9	89.6 ± 13.5
3LL	Complement (C) alone	100 (29.2 ± 3.5)	
	Anti-NK-1.1 (PK136) + C	86.6 ± 16.9	
	Anti-NK-1.1 (3A4) + C	93.2 ± 19.2	
	Expt. 2: Anti-NK-1.1 Ab-coated beads		
NCTC4093	Bulk PEC	100 (44.2 ± 2.7)	100 (3×10^6 cells)
	Bulk PEC − NK-1.1$^+$ cells	91.9 ± 18.1	101.5 ± 25.3
3LL	Bulk PEC	100 (26.9 ± 2.3)	
	Bulk PEC − NK-1.1$^+$ cells	90.6 ± 16.7	

[a] Relative to the percent specific lysis of target cells exposed to control effector cells (E:T = 50).
[b] Relative to the cell number of control effector cells.
[c] Data in parentheses represent the mean ± SD of the observed percent specific lysis ($n = 8$) in an 18-h assay.

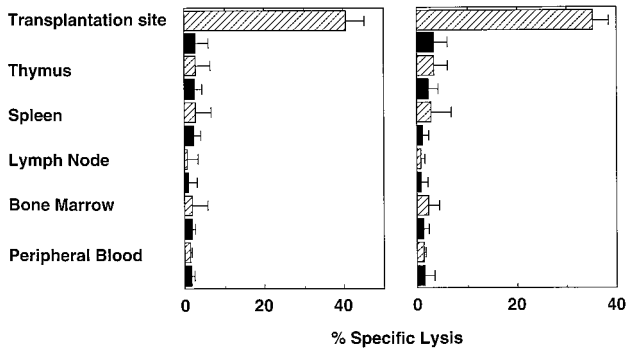

FIGURE 3. Distribution of cytotoxic activities against 3LL cells in (*left*) i.p. Meth A cell-treated and (*right*) BALB/c skin-grafted B6 mice. Bulk infiltrates into the transplantation site, all cells from spleen, lymph node, or bone marrow, or mononuclear leukocytes of peripheral blood were recovered from untreated (■) or allografted (▨) mice. The cytotoxic activity against ^{51}Cr-labeled 3LL cells with an E:T ratio of 50 was determined after an 18-h incubation. Each value represents the mean ± SE of six cultures from two different experiments.

skin-grafted B6 mice were distributed exclusively in the skin graft-graft bed border, but not in the thymus, spleen, lymph node, bone marrow, or peripheral blood (Fig. 3, *right*). By contrast, the bulk cells infiltrating into autografts had no cytotoxic activity against these tumor cells and cell lines at any time interval after transplantation (e.g., on day 6 after transplantation, 0.5 ± 1.1 and 0.8 ± 1.2% (mean ± SD; $n = 8$) specific lysis for 3LL and NCTC4093 cells, respectively).

Mechanisms of Mϕ-mediated cytotoxicity against syngeneic tumor cells and cell lines

When Mϕ isolated from tumor- or skin-allografted B6 mice were cocultured with 3LL or NCTC4093 cells, the Mϕ exhibited high cytotoxic activities against these targets, but they were totally inert against 3LL and NCTC4093 cells in a Transwell consisting of two (upper and lower) chambers with a cell-impermeable membrane (Table II). Although *N*-MMA, a NO synthase inhibitor, completely inhibited NO release from the Mϕ (58.3 ± 6.4 μM/18 h without inhibitor to 2.7 ± 1.3 μM/18 h with inhibitor), the inhibitor had no effect on the Mϕ-mediated cytotoxic activity against 3LL and NCTC4093 cells. By contrast, LPS/IFN-γ-activated casein Mϕ were highly cytotoxic (40.3 ± 4.2% specific lysis in an 18-h assay) against NO-sensitive P815 cells even in a Transwell, and the cytotoxic activity was completely suppressed by the addition of *N*-MMA (0.0 ± 2.3%). Furthermore, in contrast to LPS/IFN-γ-activated casein-Mϕ, the allograft-induced Mϕ (AIM) did not release TNF-α (16), and the addition of anti-TNF-α Ab did not affect the AIM-mediated cytotoxic activities against 3LL and NCTC4093 cells. LPS/IFN-γ-activated casein-Mϕ, however, were cytotoxic (38.5 ± 2.8% specific lysis in an 18-h assay) against TNF-α-sensitive L929 cells even in a Transwell, and anti-TNF-α Ab largely (~61%) inhibited the activity. These results indicated that in contrast to other activated Mϕ, AIM exhibited cytotoxic activity against syngeneic tumor cells and cell lines in a cell-to-cell contact-dependent, soluble factor-independent manner.

Table II. *Cell-to-cell contact-dependent cytotoxicity of PEC against 3LL or NCTC4093 cells*

Target	Culture Condition[a]	Inhibitor[a]	% Specific Lysis[b]
NCTC4093	Coculture		34.6 ± 3.0
		N-MMA	33.1 ± 4.2
		Anti-TNF-α	31.2 ± 5.1
	Transwell		0.0 ± 0.9
3LL	Coculture		26.6 ± 1.8
		N-MMA	28.4 ± 2.0
		Anti-TNF-α	23.7 ± 4.2
	Transwell		0.0 ± 0.8

[a] Allografted Meth A tumor cell-induced PEC in B6 mice were cocultured with target cells or cultured separately in a Transwell at an E:T ratio of 30 in the presence or absence of *N*-MMA (0.5 mM) or anti-TNF-α Ab (2 μg/ml).

[b] The cytotoxic activities were determined after an 18-h incubation. Each value represents the mean ± SE of six cultures from two different experiments.

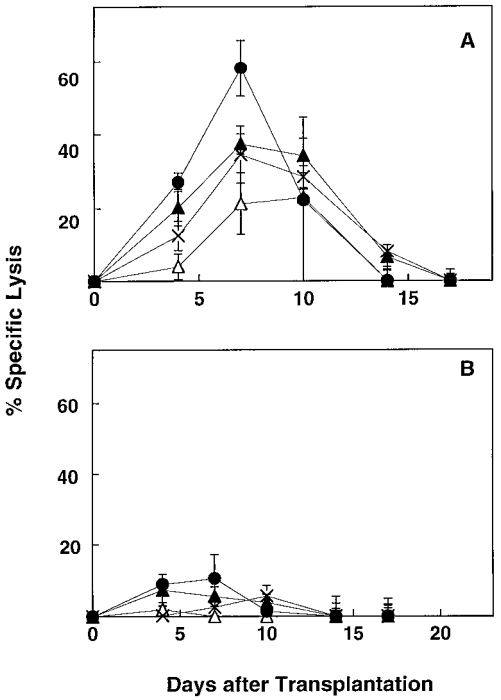

FIGURE 4. IFN-γ-dependent cytotoxicity of AIM against syngeneic tumor cells and cell lines. Meth A cells were i.p. injected into normal (IFN-$\gamma^{+/+}$; *A*) or IFN-$\gamma^{-/-}$ (*B*) B6 mice. At various intervals after transplantation, the cytotoxic activities of bulk PEC against syngeneic tumor cells and cell lines were determined at an E:T ratio of 50 in an 18-h assay. Each value represents the mean ± SE of six cultures from two different experiments. ×, EL-4; ●, 3LL; △, NOR10; ▲, NCTC4093.

The cytotoxic activities of PEC against various kinds of syngeneic tumor cells and cell lines were time dependently detected in the transplantation site of allogeneic Meth A tumor cells with a peak around day 8 after transplantation in normal (IFN-$\gamma^{+/+}$) B6 mice (Fig. 4*A*). Thereafter, the cytotoxic activities decreased, and almost no cytotoxicity remained against syngeneic tumor cells and cell lines on day 17. By contrast, very low cytotoxic activities of PEC against these syngeneic tumor cells and cell lines were induced in Meth A cell-treated IFN-$\gamma^{-/-}$ B6 mice (Fig. 4*B*), suggesting IFN-γ-dependent cytotoxicity.

Effects of AIM on growth of syngeneic tumor cells in the Winn assay

To examine the in vivo cytotoxic effects of AIM on syngeneic tumor cells, we performed the Winn assay with various AIM:tumor ratios (Table III). Subcutaneously injected 3LL lung cancer cells (2×10^5 cells/mouse) grew time dependently, and three of four animals with >30 mm diameter of tumor mass died ~1 mo

Table III. *Effects of AIM on growth of 3LL cells s.c. transplanted into B6 mice*

		+ AIM (cells/mouse)				
Days[a]	3LL Alone	10^5	2×10^5	5×10^5	8×10^5	2×10^6
14	10.3 ± 1.3[b]	9.8 ± 1.9	7.1 ± 4.8	3.5 ± 4.0	1.4 ± 2.8	0
19	15.9 ± 3.6	14.8 ± 2.2	11.3 ± 7.5	6.5 ± 7.7	2.6 ± 5.3	0
26	23.9 ± 2.6	23.4 ± 4.7	16.1 ± 10.9	10.4 ± 12.1	4.6 ± 9.3	0
29	28.0 ± 3.9	25.1 ± 4.9	19.3 ± 13.0	11.8 ± 3.1	6.0 ± 12.0	0
34	32[c]	29.0 ± 0.5	21.1 ± 14.3	12.4 ± 14.5	7.3 ± 14.5	0

[a] Days after s.c. injection of 3LL tumor cells (2×10^5 cells/mouse) into B6 mice.
[b] Diameter of tumor mass, mm; mean ± SD ($n = 4$).
[c] Value for one mouse only, as the other three mice died.

after tumor transplantation. However, the growth of 3LL cells was suppressed by a simultaneous inoculation of AIM in a cell number-dependent manner, and in the presence of 2×10^6 cells in an AIM-rich population the tumor cells were rejected without forming any tumor mass in the transplantation site. Table IV shows similar inhibitory effects of cells in the AIM-rich population on the growth of EL-4 lymphoma cells. The growth was completely abrogated by the simultaneous inoculation of AIM at an AIM:tumor ratio of 50.

Rejection of 3LL tumor cells by the simultaneous inoculation of allogeneic, but not syngeneic, fibroblastic cells

As it may sometimes be difficult to isolate AIM from tumor-bearing patients, we next tried to induce AIM in the transplantation site of syngeneic tumor cells (Fig. 5). 3LL cells (2×10^5 cells/mouse) s.c. injected into B6 mice grew time dependently, and the tumor mass reached 16 mm diameter on day 24 after transplantation. However, a simultaneous inoculation of allogeneic (BALB/3T3; H-2^d), but not syngeneic (NOR10; H-2^b), fibroblastic cells suppressed tumor growth in a cell-number dependent manner and resulted in rejection without forming any tumor mass in the transplantation site at an allograft:tumor ratio of 200. In contrast, sonicated BALB/3T3 fibroblastic cells totally lost their inhibitory effect; and the simultaneous inoculation of allogeneic BALB/3T3 fibroblastic cells with 3LL cells into IFN-$\gamma^{-/-}$ B6 mice had virtually no effect on tumor growth (Fig. 5).

Discussion

Mϕ recognize, phagocytose, and dispose of senescent RBC, damaged cells, and foreign invaders (24). These continuous functions are constitutive and therefore do not require external regulation. In contrast, sporadic functions, such as participation in host defense against chronic infections and cancer, require activation signals to initiate the recruitment and activation of Mϕ to perform a specific task. Once this task is completed, the cells can revert to the nonactivated state (25). AIM fulfill these demanding properties. 1) The cytotoxic activity against syngeneic tumor cells was found only in the rejection site of allografted tumor or skin (Fig. 3). 2) Resident (Fig. 3) and casein- or thioglycolate-elicited inflammatory (Table II) Mϕ were inactive toward syngeneic tumor cells or cell lines. 3) AIM are a type of activated Mϕ (9, 10, 16, 17). 4) When allografts were rejected, AIM reverted to the nonactivated state, and the number of AIM was rapidly reduced in the rejection site (9–12, 19) (Fig. 4).

Among bulk infiltrates in allografts, T cells were found to be the major producers of IFN-γ (8). Therefore, it is reasonable that, as was shown in Fig. 1, the IFN-γ-dependent cytotoxicity of cells in an AIM-rich population against syngeneic tumor cells and cell lines was significantly reduced after T cell elimination. Although IFN-γ was essential for the induction of AIM-mediated cytotoxicity against syngeneic tumor cells and cell lines (Figs. 4 and 5), IFN-γ alone was insufficient to induce significant Mϕ-mediated antitumor activity even in vitro, because resident or thioglycolate Mϕ activated by IFN-γ in vitro had very low cytotoxic activity against tumor cells (data not shown). Also in vivo, an s.c. injection of IFN-γ (0.05–5 μg in 0.2 ml of PBS) simultaneously with 3LL tumor cells was ineffective on the tumor growth (unpublished data). The nature of the additional factor(s) essential for the induction of AIM-mediated cytotoxic activity against syngeneic tumor cells and cell lines remains to be elucidated. Of particular interest, Hayashi (26) reported recently that the inducibility of IFN-γ in cancer patients is a good marker for the effectiveness of immunotherapy with bacillus Calmette-Guérin cell wall skeleton.

It has been generally believed that CTLs recognize allografts as nonself through the difference in the histocompatibility Ags and destroy the allografts (27). Recent studies using β_2-microglobulin, CD4, or CD8 knockout mice, however, revealed that the main

Table IV. *Effects of AIM on growth of EL-4 cells s.c. transplanted into B6 mice*

		+ AIM (cells/mouse)		
Days[a]	EL-4 Alone	10^6	7.5×10^6	10^7
13	16.5 ± 2.1[b]	5.0 ± 7.1	0.0 ± 0.0	0
17	20.3 ± 3.2	8.0 ± 11.3	4.5 ± 6.4	0
25	35.0 ± 1.4	16.5 ± 23.3	11.0 ± 15.6	0
34	—[c]	23.7 ± 16.8[d]	14.5 ± 20.5	0

[a] Days after s.c. injection of EL-4 tumor cells (2×10^5 cells/mouse) into B6 mice.
[b] Diameter of tumor mass, mm; mean ± SD ($n = 3$).
[c] All mice died.
[d] One mouse died.

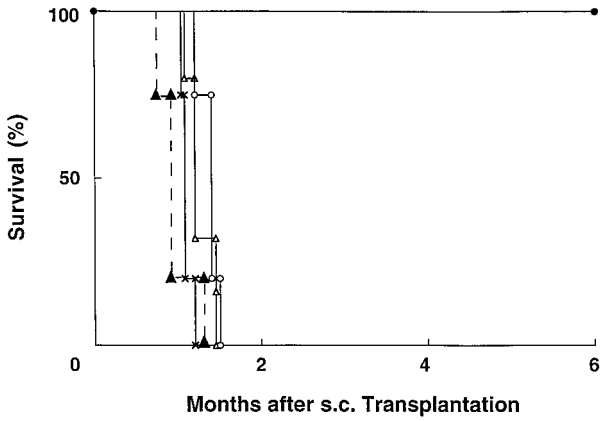

FIGURE 5. Effects of simultaneous inoculation of allografts on the growth of s.c. transplanted 3LL tumor cells in normal (IFN-$\gamma^{+/+}$) or IFN-$\gamma^{-/-}$ B6 mice. ○, 3LL cells (3×10^4 cells/mouse) into B6 mice; ●, 3LL cells plus BALB/3T3 fibroblastic cells (6×10^6 cells/mouse) into B6 mice; △, 3LL cells plus sonicated homogenate of BALB/3T3 cells (6×10^6 cells/mouse) into B6 mice; ×, 3LL cells plus NOR10 fibroblastic cells (6×10^6 cells/mouse) into B6 mice; ▲, 3LL cells plus BALB/3T3 fibroblastic cells (6×10^6 cells/mouse) into IFN-$\gamma^{-/-}$ B6 mice. The percent survival rates of the treated animals (12 mice each) were determined.

effector cells responsible for skin or cardiac allograft rejection appear to be cells other than T or NK cells (28–31). We also reported that a novel type of activated Mϕ was the major population of effector cells responsible for the rejection of either i.p. allografted Meth A (H-2^d) fibrosarcoma cells in B6 (H-2^b) mice (8–18) or of allografted BALB/c (H-2^d) skin on B6 mice (19). The Mϕ were cytotoxic against allografts in a cell-to-cell contact-dependent manner (11, 16). To determine whether the same population of Mϕ could react with syngeneic tumor cells or cell lines as well as with allografted Meth A tumor cells, we performed a series of cold target inhibition assays. The cytotoxic activity of Mϕ against ^{51}Cr-labeled allograft (Meth A cells) was inhibited by the addition of unlabeled H-2^d, but not H-2^k or H-2^b, Con A blasts (11), whereas the cytotoxic activities of Mϕ against ^{51}Cr-labeled 3LL and NCTC4093 cells were not affected by the addition of cold H-2^d, H-2^k, or H-2^b Con A blasts (our unpublished data).

The application of clinical allogeneic bone marrow transplantation has expanded as a treatment modality for hematologic malignancies (32), and it has been suggested that graft-vs-host disease is associated with an antileukemic effect (33, 34). Some patients without graft-vs-host disease, however, have remained free of leukemia 2–8 yr after bone marrow transplantation, and recurrent leukemia has developed in other patients with severe graft-vs-host disease (35). Furthermore, Champlin et al. (36) demonstrated that bone marrow transplantation using donor marrow depleted of CD8$^+$ lymphocytes decreased the incidence of a graft-vs-host disease without abrogating the graft-vs-leukemia effect. In the present study we revealed that host-derived AIM were cytotoxic not only against parenchymal tumor (e.g., 3LL, CMT-93, and B16) cells and cell lines (e.g., NCTC4093 and NOR10) but also against lymphoid tumor cells, including EL-4 lymphoma cells. Therefore, it is plausible that the cytotoxicity of recipient-derived Mϕ against the lymphoid tumor cells is a mechanism responsible for the antileukemic effect following allogeneic bone marrow transplantation and that it may be possible to make a therapeutic application of AIM (or the inducer) to leukemia.

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