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The Role of T Cells in Allografted Tumor Rejection: IFN-γ Released from T Cells Is Essential for Induction of Effector Macrophages in the Rejection Site

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Allografted Meth A tumor rejection is T cell dependent, but T cells are inactive toward the allograft; rather, the main effector cells are allograft-induced macrophages (AIM) with MHC haplotype specificity. Here, we examined the role of T cells in the induction of AIM in the rejection site. On day 4.5 after i.p. transplantation of Meth A fibrosarcoma cells to C57BL/6 (B6) mice, we obtained a kind of precursor of AIM (pro-AIM) from the transplantation site by an enrichment technique involving adherence to serum-coated dishes. The noncytotoxic pro-AIM-rich population put into a diffusion chamber became cytotoxic against Meth A cells after 2 days in the peritoneal cavity of an untreated B6 mouse. Similar activation of the chambered B6 pro-AIM-rich population occurred in IFN-γ −/− B6 mice, whereas there was no activation when chambers containing an IFN-γ −/− mouse-derived pro-AIM-rich population were placed in normal or IFN-γ −/− mice, suggesting that IFN-γ is involved in the activation. RT-PCR experiments demonstrated that among bulk infiltrates T cells were the major producer of IFN-γ; and most of the cells in a T cell-eliminated pro-AIM population in a diffusion chamber kept for 2 days in a B6 mouse did not become AIM. Furthermore, IFN-γ −/− B6 mice could not reject allografted Meth A tumor cells, whereas the grafts were rejected by i.p. injections of IFN-γ into the mutant mice. These results indicate that IFN-γ released from allograft-induced T cells is essential for both the activation of a kind of pro-AIM to AIM in the transplantation site and the rejection of an allografted tumor. The Journal of Immunology, 1998, 160: 6012–6017.

Tumor allografts have been generally used as one of the in vivo indicators of alloreactivity in transplantation immunology. Some investigators have reported that the rejection of tumor allografts was mediated mainly by the class I-restricted CD8+ T cells that are responsible for CTL activity (1–3). In 1988, however, we established a model of tumor allograft rejection in which Meth A (H-2b) fibrosarcoma cells were transplanted into the peritoneal cavity of C57BL/6 (B6) (H-2b) mice (4) and reported that allograft-induced T cells (5, 6) or MLR-induced CTLs (6) were inactive toward allografted Meth A tumor cells and that there was no infiltration of NK cells in the rejection site (6). Rather, allograft-induced macrophages (AIM)4 were found to be cytotoxic against allografted Meth A tumor cells and to be the major effector cells responsible for the rejection (5–7), whereas both CD4+ T cells and CD8+ T cells were required for the induction of effector macrophages (Mφ) in the rejection site (8). Consistently, the cytotoxic activity of AIM against the allografted tumor cells was Ca2+ dependent, but Fas/Fas ligand or perforin—both of which are the mechanisms of killing by CTLs (9)—independent (10). Also, in the case of skin or organ allograft models, Zijlstra et al. (11), Dalloul et al. (12), Van Buskirk et al. (13), and Krieger et al. (14) reported recently that CD8+ T cells are not essential for the rejection using β2-microglobulin, CD8, or CD4 knockout mice and indicated that the main effector cells appeared to be those other than T cells or NK cells. More recently, we revealed that the major effector cells mediating allografted skin rejection were AIM, and not T or NK cells (15). Taken together, it is conceivable that AIM, a type of activated Mφ (16), mediate direct lysis of allografts and that their growth, differentiation, or activation is dependent upon cytokine production by T cells.

The Mφ, a widely distributed mononuclear phagocyte, plays a critical role in host defense against microbial infections and tumor cells (17–22). It is generally assumed that for the host defense, Mφ must be activated before exhibiting antimicrobial activity or tumoricidal activity and that one of the critical activators for Mφ is IFN-γ (16, 21–24).

Here, we obtained a kind of precursor of AIM (pro-AIM) from the transplantation site on day 4.5 after i.p. transplantation of Meth A tumor cells by employing an enrichment procedure based on adherence of the progenitors to a serum-coated dish. Using IFN-γ −/− mice and diffusion chambers containing the noncytotoxic pro-AIM, we investigated the role of T cells in the induction of AIM in the rejection site. The results demonstrated that IFN-γ was produced mainly by T cells and that IFN-γ was essential for both the activation of a kind of pro-AIM to AIM in the transplantation site and the rejection of allografted Meth A tumor cells.

Materials and Methods

Animals

Specific pathogen-free male B6 and BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan), and were used at 7 to 10 wk of age. IFN-γ

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Labeled anti-Thy-1.2 Ab, and their surface Ag was analyzed by FCM.

Tumor cells
Meth A cells, a fibrosarcoma cell line of BALB/c (H-2b) origin, were donated by Dr. S. Muramatsu (Department of Zoology, Faculty of Science, Kyoto University, Kyoto, Japan) and maintained by i.p. passage of 3 $\times$ 10^6 cells into BALB/c mice. Meth A cells were cultured for 24 h in RPMI 1640 medium (Nikken, Kyoto, Japan) supplemented with 10% FCS (ICN Biomedicals, Costa Mesa, CA) and antibiotics (100 U/ml penicillin and 100 $\mu$g/ml streptomycin) and were used for cytotoxicity assay.

Antibodies
FITC-labeled rabbit anti-rat IgM Ab was purchased from Zymed (South San Francisco, CA). Monoclonal anti-mouse CD4 and CD8 Abs were obtained from Pharmingen (San Diego, CA).

Enrichment of peritoneal exudate cells (PEC) and preparation of adherent cells
On day 4.5 after an i.p. transplantation of Meth A cells (3 $\times$ 10^6 cells/mouse), a leukocyte-rich fraction of host peritoneal cells was prepared as described (4, 5). The leukocyte-rich fraction was suspended in fresh medium (4 $\times$ 10^6 cells/ml), and the cells (2 $\times$ 10^6 cells/dish) were incubated on a serum-coated dish (catalogue no. 3003; Falcon, Lincoln Park, NJ) for 20 min at 37°C. Nonadherent cells were aspirated off, and the dish was washed three times with 5 ml of HBSS (Life Technologies, Grand Island, NY) warmed to 37°C. The dish containing adherent cells was incubated with 5 ml of ice-cold PBS containing 2.5 mM EDTA for 40 min at 4°C, and the adherent cells were then recovered by pipetting.

Placement of diffusion chamber containing pro-AIM into the peritoneal cavity of mice
The procedure used for the preparation of the diffusion chamber was essentially the same as described previously (4, 27, 28). The diffusion chambers (Millipore, Bedford, MA) were covered at each end with a polycarbonate membrane filter of 0.2 $\mu$m porosity (Nuclepore; Costar, Cambridge, MA). The membrane was used to prevent adhesion of the enclosed cells to the membrane surfaces. These chambers were sterilized by ethylene oxide gas before use. A pro-AIM-rich population of cells (7 $\times$ 10^6 cells/160 $\mu$l of PBS) prepared as described above was injected into a chamber through a filling hole with a 1-ml syringe fitted with a 26-gauge needle. The filling hole was plugged with melted paraffin, and the chamber was placed into the peritoneal cavity of a mouse. Thereafter, the peritoneum was sutured, and the skin was clipped.

Harvesting of cells enclosed in diffusion chambers
The diffusion chambers were harvested 2 days after having been put into the peritoneal cavity of B6 mice. The paraffin on the filling hole was removed with forceps. After the chamber fluid had been recovered with a 1-ml syringe attached to a 26-gauge needle, the inside of the diffusion chamber was filled with medium containing 10% FCS and 0.5% pronase (Calbiochem, San Diego, CA) to lyse the fibrin clot; then the filling holes were plugged again. After a 10-min incubation at room temperature, the contents were removed and added to the chamber fluid described above. The cell number, which was determined by trypan blue exclusion test after the cells had been washed twice with fresh ice-cold medium, was 45 to 50% of the number of cells that had been enclosed in the chamber 2 days before.

Complement-dependent cell lysis
Apparently homogeneous pro-AIM were obtained after T cell elimination from an adherent pro-AIM-rich population (prepared as described above) by complement-dependent cell lysis with anti-Thy-1.2 Ab. The cells (2 $\times$ 10^6) in the pro-AIM-rich population were suspended in 500 $\mu$l of fresh medium, and then 16 $\mu$l of anti-Thy-1.2 Ab and 160 $\mu$l of diluted Low-Tox-M rabbit complement (Cedarlane Labs, Hornby, Ontario, Canada) were added to the suspension. The complement was reconstituted with 1 ml of cold distilled water and was diluted (3$x$3) with medium before use. The mixture was incubated for 45 min at 37°C. To confirm that T cells had been eliminated specifically from an adherent pro-AIM-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 was analyzed by FCM.

FACS analysis
Allograft-induced peritoneal cells were analyzed and the granulocyte-, lymphocyte-, or AIM-rich population was isolated by FACS (FACStar; Becton Dickinson, Mountain View, CA), as described previously (5). For direct immunofluorescence labeling, cells (10^6 cells/50 $\mu$l of PBS containing 2% FCS and 0.1% NaN3) were incubated with 10% rat serum before the addition of primary Ab to inhibit nonspecific binding. For indirect labeling, cells were incubated with appropriate first Ab, washed twice with PBS containing 10% FCS, and incubated with second Ab that had been adsorbed with 5% rat serum. The labeled cells were washed twice and resuspended in PBS containing 2% FCS and 0.1% NaNO3 for FACS analysis.

Cytotoxicity assay
Meth A tumor cells were used as target cells. The tumor cells were labeled with $^{51}$Cr ($Na_2^{51}$CrO_4; Dupont New England Nuclear, Boston, MA), and the cytotoxic activity against the target cells was determined by the method of Ascher et al. (29). The cytotoxicity assay was performed with an E:T ratio of 50 and an 18-h incubation in round bottom 96-well microplates (Costar), as described previously (5). The amount of released $^{51}$Cr in an aliquot (0.1 ml) of supernatant was measured by a gamma counter (Cobra 5010; Packard, Zurich, Switzerland). Percentage of specific lysis was calculated by the following formula: % specific lysis $= (cpm_{experiment} - cpm_{spontaneous})/(cpm_{max} - cpm_{spontaneous}) \times 100$.

RT-PCR
Total RNA was purified with a total RNA purification kit (RNasey; Qiagen, Hilden, Germany) and the total RNA was reverse transcribed using a pre-amplification system (SuperScript; Life Technologies) according to the manufacturer’s instructions. About 10% of the single-stranded cDNA was subjected to PCR amplification. A mouse IFN-γ primer set (Stratagene, La Jolla, CA) was used to amplify a 405-bp fragment by PCR conducted by a 5-min denaturation at 94°C and a 5-min annealing at 65°C, followed by 30 cycles of 1 min at 72°C, 1 min at 94°C, and 30 s at 65°C, with a final extension of 5 min at 72°C in a PCR Thermal Cycler MP (TP-3100; Takara, Otsu, Japan). As a control, mouse β-actin, IL-2, IL-4, and TNF-α primer sets (Stratagene) were used to amplify 514-, 451-, 279-, and 276-bp fragments, respectively, by PCR involving a 5-min denaturation at 94°C and a 5-min annealing at 57°C, followed by 30 cycles of 1 min at 72°C, 1 min at 94°C, and 1 min at 57°C, with a final extension of 5 min at 72°C. The PCR products were electrophoresed on 3% NuSieve 3:1 agarose gels (Takara) and analyzed after ethidium bromide staining.

Results
Enrichment of a kind of pro-AIM and their activation to AIM
On day 4.5 after the i.p. transplantation of Meth A fibrosarcoma cells to B6 mice, we obtained morphologically AIM-like cells possessing no cytotoxic activity against Meth A tumor cells from the transplantation site by an enrichment technique involving adherence to serum-coated dishes, suggesting that the adherent cells may contain a kind of pro-AIM. Although the cytotoxic activity of AIM recovered on day 8 after transplantation against Meth A cells was essentially the same as that of AIM recovered on day 7, AIM that had been harvested on day 7 became inactive toward Meth A cells after a 1-day culture period in a 96-well plate (data not shown). Therefore, to elucidate the mechanism(s) of AIM induction in the rejection site and to prevent the negative effect, possibly due to adherence to the culture plate, on AIM induction, we cultured the pro-AIM in a diffusion chamber in the peritoneal cavity of another untreated B6 mouse. After a 2-day incubation of the diffusion chamber containing a B6 pro-AIM-rich population in the peritoneal cavity of a B6 mouse, the cells in the pro-AIM-rich population were found to have become activated to AIM: apparently homogeneous cells in the diffusion chamber exhibited a cytotoxic activity being essentially the same as that of AIM. As expected, however, the same cells in the pro-AIM-rich population did not become cytotoxic against Meth A tumor cells after a 2-day in vitro culture period on the plastic culture plate (data not shown).
FIGURE 1. Cytotoxic activities of AIM, pro-AIM activated in a diffusion chamber, and freshly isolated pro-AIM against Meth A tumor cells. AIM were prepared from PEC on day 7 after an i.p. transplantation of Meth A tumor cells to B6 mice. Activated pro-AIM were obtained from a diffusion chamber containing pro-AIM that had been kept in the peritoneal cavity of an untreated B6 mouse for 2 days. Each value represents the mean ± SD of 20 cultures from five different experiments. The difference is significant (*p<0.01) by Student’s t test.

Similar activation of the pro-AIM to AIM in the diffusion chamber was observed when the chamber containing a B6 pro-AIM-rich population was placed in IFN-γ −/− B6 mice, whereas there was no activation when the chamber contained a pro-AIM-rich population from IFN-γ −/− mice and was kept for 2 days in the peritoneal cavity of normal or IFN-γ −/− mice (Fig. 2), suggesting that IFN-γ may be essential for the activation of a kind of pro-AIM to AIM in the diffusion chamber.

IFN-γ released from T cells is essential for activation of a kind of pro-AIM to AIM

To identify which type of cells in the transplantation site releases IFN-γ, we prepared cDNAs from various types of cells infiltrating into the transplantation site and conducted RT-PCR experiments to detect the message for the expression of IFN-γ. As shown in Figure 3, among the bulk infiltrates T cells were found to be the major producers of IFN-γ; and CD8+ T cells as well as CD4+ T cells seemed to be potent producers. Consistently, when T cells were eliminated from the population, the expression of IFN-γ mRNA in the whole PEC was very low. Furthermore, no message was detected in allografted Meth A tumor cells.

Since FACS data indicated that the pro-AIM-rich population contained a small but significant number of adherent Thy-1.2+ T cells (=1.2% of the total cell number in the pro-AIM-rich population), we prepared a T cell-free pro-AIM population by complement-dependent cell lysis with anti-Thy-1.2 Ab. When the apparently homogeneous pro-AIM were used in the diffusion chamber experiments, the activation of this population to AIM was significantly impaired (Fig. 4). Furthermore, the numbers of Thy-1.2+ cells infiltrating into the rejection site was almost equal to the sum of the numbers of CD4+ and CD8+ T cells (data not shown), suggesting that there are few non-T cells that express Thy-1.2 Ag. These results taken together indicate that IFN-γ released mainly from allograft-induced T cells is essential for the activation of a kind of pro-AIM to AIM.

Expression of other cytokine mRNAs in IFN-γ −/− mice

Because there is a possibility that other cytokine production is also dramatically compromised in IFN-γ −/− mice, we studied cytokine mRNA expression in IFN-γ +/+ and IFN-γ −/− mice (Fig. 5).
IFN-γ mRNA was not detected at all in the Con A blasts from IFN-γ−/− mice, whereas IL-2, IL-4, and TNF-α mRNAs were present in all Con A blasts from IFN-γ−/− mice; and the level of expression was similar to that in IFN-γ+/+ mice. Furthermore, the expression of MHC class I molecules on Meth A cells, which were recovered from the rejection site of IFN-γ−/− mice, was essentially the same as that in control mice (data not shown).

Role of IFN-γ in allografted tumor rejection

To know the in vivo role of IFN-γ in the rejection of allografted Meth A tumor cells, we treated IFN-γ−/− and normal B6 mice with allogeneic Meth A tumor cells. Figure 6 shows the time-dependent changes in the growth of allografted Meth A tumor cells in the transplantation site (peritoneal cavity). As described previously (5), the number of tumor cells in the Meth A cell-treated normal B6 mice increased within 4 days after transplantation, reached a peak on days 6–8, and gradually decreased thereafter. Around day 14, the recipient mice rejected the allografts. In IFN-γ−/− B6 mice, however, the allografted tumor cells continued to grow; and all the mice died on day 17 or later.

To ascertain whether the lack of IFN-γ is directly responsible for the inability to reject allografts, we injected IFN-γ−/− mice (Table I). On day 13, when PBS-injected mutant mice had a large number of allografted Meth A tumor cells in the transplantation site, IFN-γ−/−injected mutant mice had already rejected the allografts, as observed with normal B6 mice (Fig. 6).

These results taken together indicate that IFN-γ is essential not only for the activation of a kind of pro-AIM to AIM in a diffusion chamber but also for the rejection of an allografted tumor.

**Table I. Effect of i.p. injections of PBS or IFN-γ on the growth of i.p. allografted Meth A tumor cells in IFN-γ−/− B6 mice**

<table>
<thead>
<tr>
<th>Injection</th>
<th>Meth A Cells×10^5 (cells/mouse)</th>
<th>PBS</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5.76, 5.12, 3.86, 1.78</td>
<td>4.13 ± 1.75</td>
</tr>
</tbody>
</table>

|           |                                 | 0, 0, 0, 0 | 0 |

On days 4, 6, and 8 after i.p. transplantation of Meth A cells (3 × 10⁶ cells/mouse) into IFN-γ+/+ mice, IFN-γ (Life Technologies; 10 μg = 10⁵ U) in 0.1 ml of PBS (0.1 ml/mouse) was i.p. injected. On day 13 after transplantation, the number of Meth A cells in the peritoneal cavity was assessed.

**Discussion**

In our tumor allograft model, we transplanted allogeneic Meth A tumor cells i.p. to B6 mice to recover all infiltrates from the transplantation site by lavage of the peritoneal cavity (5), thus overcoming the technical difficulty (30–35) of harvesting host cells that infiltrate allografts. With this model system, we found two distinct populations of unique cytotoxic cells, AIM and CTLs, in the rejection site, whereas there was no infiltration of NK cells in the transplantation site (6). The AIM were cytotoxic against allografted Meth A tumor cells but not against donor-type lymphoblasts, whereas the allograft-induced T cells were cytotoxic against donor-type lymphoblasts but not against the allografts. Since Meth A cells, and not donor-type lymphoblasts, were transplanted, the cytotoxic activity of T cells against donor-type lymphoblasts may be an in vitro artifact. Moreover, we reported earlier that the specific in vivo elimination of MΦs, including AIM, by dichloromethylene diphenylphosphonate-containing liposome treatment resulted in the failure of allografted tumor rejection by B6 mice (7), and that specific lysis of allogeneic skin components was also mediated by AIM and not by T cells (15), suggesting that AIM are the major population of effector cells responsible for allograft rejection.

It is well known that not only released cytokines but also surface Ags are changeable depending on their functions during the differentiation of MΦs (36–38). Also in our cytotoxicity assay, adherence of MΦs to plastic culture dish altered their functions: fully activated AIM cultured on a plastic plate for 24 h showed a total loss of cytotoxic activity against Meth A cells; and the cells in a pro-AIM-rich population cultured for 2 days on a plastic plate were ineffective in displaying cytotoxicity against Meth A cells.
IFN-γ is suggested by the fact that IFN-γ/ −/− B6 mice failed to reject tumor allografts (Fig. 6 and Table I). As one of the essential in vitro roles of IFN-γ in allografted tumor rejection, here we have shown impairment in the activation of a kind of pro-AIM from IFN-γ/ −/− mice to AIM. In addition, IFN-γ is able to induce a tryptophan-degrading enzyme (indoleamine 2,3-dioxygenase) in Meth A tumor cells undergoing rejection (4, 41); and IFN-γ and IL-1α/β, both of which are released in the rejection site, synergistically inhibit the growth of allografted Meth A tumor cells in a cytostatic manner (24). Therefore, these biologic functions of IFN-γ, at least in part, may also play important roles in the growth inhibition of allografted Meth A tumor cells.

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References


