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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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.
B6 mice were donated by Dr. Y. Iwakura, Institute of Medical science, University of Tokyo, Tokyo, Japan (25, 26). The mice were kept in our animal facility under specific pathogen-free conditions in an air-conditioned room at 25 ± 2°C at ~50% humidity.

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 × 10⁶ 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 µg/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 × 10⁶ 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 × 10⁶ cells/ml), and the cells (2 × 10⁶ 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 µm 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 × 10⁶ cells/160 µl 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 holes 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 trypsin 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 × 10⁶) in the pro-AIM-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 (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) 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 (10⁶ cells/50 µl 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 dissolved in PBS containing 10% 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 ⁵¹Cr (Na₂⁵¹CrO₄; Du Pont 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 ⁵¹Cr 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 experimental - cpm spontaneous]/cpm spontaneous) × 100.

RT-PCR

Total RNA was purified with a total RNA purification kit (RNeasy; 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 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). 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 strong cytotoxic activity against Meth A tumor cells (Fig. 1), their 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).
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. 3).
Role of IFN-\(\gamma\) in allografted tumor rejection

To know the in vivo role of IFN-\(\gamma\) in the rejection of allografted Meth A tumor cells, we treated IFN-\(\gamma\) \(-/-\) 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-\(\gamma\) \(-/-\) 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-\(\gamma\) is directly responsible for the inability to reject allografts, we injected IFN-\(\gamma\) i.p. into IFN-\(\gamma\) \(-/-\) 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-\(\gamma\)-injected mutant mice had already rejected the allografts, as observed with normal B6 mice (Fig. 6).

These results taken together indicate that IFN-\(\gamma\) 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.

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 M\(\phi\)s, including AIM, by dichloromethylene diphosphonate-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 M\(\phi\)s (36–38). Also in our cytotoxicity assay, adherence of M\(\phi\)s 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.

Table I. Effect of i.p. injections of PBS or IFN-\(\gamma\) on the growth of i.p. allografted Meth A tumor cells in IFN-\(\gamma\) \(-/-\) B6 mice

<table>
<thead>
<tr>
<th>Injection</th>
<th>Meth A Cells(^a) ((\times 10^4) cells/mouse)</th>
<th>PBS</th>
<th>IFN-(\gamma)</th>
<th>PBS</th>
<th>IFN-(\gamma)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>5.76, 5.12, 3.86, 1.78</td>
<td>4.13 ± 1.75(^b)</td>
<td>0, 0, 0, 0</td>
<td>0</td>
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</table>

\(^a\) On days 4, 6, and 8 after i.p. transplantation of Meth A cells (3 \(\times 10^6\) cells/mouse) into IFN-\(\gamma\) \(-/-\) mice, IFN-\(\gamma\) (Life Technologies; 10 \(\mu\)g \((= 10^6\) U) in 0.1 ml of PBS/mouse) or 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.

\(^b\) Mean ± SD (n = 4).
It has been postulated that IFN-$\gamma$ promotes production of Th1-type cytokines (e.g., IFN-$\gamma$ and IL-2) while diminishing production of Th2-type cytokines (e.g., IL-4 and IL-10) (39). Saleem et al. (40) and our present data, however, revealed that IFN-$\gamma$ $\rightarrow$ mice did not preclude production of IL-2 mRNA and did not increase IL-4 mRNA expression, implying specific absence of IFN-$\gamma$ in the IFN-$\gamma$ $\rightarrow$ mice. In fact, i.p. injections of IFN-$\gamma$ into IFN-$\gamma$ $\rightarrow$ mice, in which there was no activation of a kind of pro-AIM to AIM after transplantation of allogeneic Meth A tumor cells and the allografts continued to grow, resulted in rejection. These results suggest that IFN-$\gamma$ may be directly responsible 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.

T cells infiltrating into the transplantation site of allogeneic Meth A cells recognize alloantigens processed by APC (6) but exhibit no cytotoxic activity against allografted Meth A tumor cells (6, 7). What is the biologic significance of allograft-induced T cells? Recently, we found that when Meth A cells were i.p. injected into adult-thymectomized, X-irradiated, bone marrow-reconstituted (ATXBM) B6 mice, the ATXBM mice could induce pro-AIM, but not AIM, and failed to reject the allografts; and that both CD4$^+$ T cells and CD8$^+$ T cells were required for the induction of AIM in the rejection site (8). In the present study, we demonstrated that both CD4$^+$ T cells and CD8$^+$ T cells produced IFN-$\gamma$ (Figs. 3 and 4), which was found to be the cytokine essential for both the activation of a kind of pro-AIM to AIM (Fig. 2) and for allografted tumor rejection (Fig. 6 and Table I). Also, in skin or organ allograft models, other investigators have recently reported that neither MHC class I-restricted CD8$^+$ T cells nor NK cells were essential to the rejection and that adoptively transferred noncytotoxic CD4$^+$ T cells from CD8$^+$-deficient mice to nude mice were sufficient to mediate the rejection (11–14).

Recent studies have demonstrated that IFN-$\gamma$ is essential for both the activation of a kind of pro-AIM to AIM in the rejection site and the rejection of allografted Meth A tumor cells. Our present study does not simply indicate that IFN-$\gamma$ alone is able to activate a kind of pro-AIM to AIM to reject the allografts, because the adoptive transfer of CD8$^+$ T cells, a main producer of IFN-$\gamma$ (Fig. 3), to ATXBM mice could hardly activate the precursors of AIM to AIM (8). Rather, the CD8$^+$ T cells could enhance the number of pro-AIM and/or AIM (8) in the rejection site. By contrast, the adoptive transfer of noncytotoxic CD4$^+$ T cells to the ATXBM mice could activate pro-AIM to AIM but was not sufficient for the allografted Meth A tumor rejection (8). Taken together, these results imply that in the case of nonproliferative allografts (e.g., heart), some cytokine(s), together with IFN-$\gamma$ from CD4$^+$ T cells, may be sufficient for both the induction of AIM in the rejection site and the allograft rejection and that both some cytokine from CD4$^+$ T cells and a high level of IFN-$\gamma$ from CD4$^+$ and CD8$^+$ T cells may be necessary to induce a large number of AIM and to reject proliferative allografts (e.g., Meth A fibrosarcoma cells).

The crucial role of IFN-$\gamma$ in allografted Meth A tumor rejection is suggested by the fact that IFN-$\gamma$ $\rightarrow$ B6 mice failed to reject tumor allografts (Fig. 6 and Table I). As one of the essential in vitro roles of IFN-$\gamma$ in allografted tumor rejection, here we have shown impairment in the activation of a kind of pro-AIM from IFN-$\gamma$ $\rightarrow$ mice to AIM. In addition, IFN-$\gamma$ is able to induce a tryptophan-degrading enzyme (indoleamine 2,3-dioxygenase) in Meth A tumor cells undergoing rejection (4, 41), and IFN-$\gamma$ and IL-1$\alpha$/B, 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-$\gamma$, at least in part, may also play important roles in the growth inhibition of allografted Meth A tumor cells.

Acknowledgments

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References


