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Gr-1\(^+\) Myeloid Cells Derived from Tumor-Bearing Mice Inhibit Primary T Cell Activation Induced Through CD3/CD28 Costimulation\(^1\)

Sergei A. Kusmartsev, Yu Li, and Shu-Hsia Chen\(^2\)

Activation of T cells is a necessary step in the development of a specific antitumor immune response. In the present study, we evaluated the ability of Gr-1\(^+\) myeloid cells, derived from the bone marrow or spleen of tumor-bearing mice, to inhibit CD3/CD28-mediated T cell activation. Using flow cytometry, we found that growth of a murine colon carcinoma (MCA-26) induces a significant increase in the number of Gr-1\(^+\) and Gr-1\(^+\)/Mac-1\(^+\) myeloid cells in both bone marrow and spleen of the tumor host. The proliferative response of T cells was dramatically decreased when naive T cells were activated by anti-CD3 and anti-CD28 Abs in the presence of a myeloid-enriched cell fraction derived from spleen or bone marrow of tumor-bearing mice vs the bone marrow of naive mice. Reversal of the inhibitory effect could be achieved by adding a combination of MnTBAP (manganese [III] tetrakis [4-benzoic acid]) porphyrin and \(\text{L-NMMA} (\text{N}^6\text{-monomethyl-L-arginine})\), a superoxide dismutase mimetic and inducible NO synthase inhibitor, respectively, or by depletion of the Gr-1-positive cells. IFN-\(\gamma\), which is endogenously produced by CD3/CD28-stimulated naive T cells, is involved in induction of the inhibitory activity of myeloid cells. Importantly, when T cells pre-activated with anti-CD3 Abs were used as responder cells, the bone marrow- or spleen-derived Gr-1\(^+\) myeloid cells were unable to suppress CD3/CD28-induced T cell proliferation. Our findings suggest that one mechanism by which an increased number of immune suppressive Gr-1\(^+\) cells can induce T cell unresponsiveness or immune tolerance in tumor hosts could be through peroxynitrite production upon primary T cell activation. *The Journal of Immunology, 2000, 165: 779–785.*

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\(^3\) Abbreviations used in this paper: BM, bone marrow; MCA-26, murine colon carcinoma; SC, spleen cell; iNOS, inducible NO synthase; \(\text{L-NMMA}\), \(\text{N}^6\text{-monomethyl-L-arginine}\); MnTBAP, manganese [III] tetrakis [4-benzoic acid] porphyrin; SOD, superoxide dismutase; Fr., fraction.

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Materials and Methods

**Mice**

Female BALB/c mice (Taconic Farms, Germantown, NY) between 8 and 12 wk of age were used in the reported experiments. Syngeneic metastatic colon carcinoma was induced in the liver by intraperitoneal implantation of $1 \times 10^6$ MCA-26 tumor cells, as described previously (18).

**Media and reagents**

RPMI 1640 medium was supplemented with 10% FCS, 20 mM HEPES, 200 U/ml penicillin, 50 $\mu$g/ml streptomycin, 0.05 mM 2-ME, and 2 mM glutamine (all from Sigma, St. Louis, MO). t-NMMA ($N^\beta$-monomethyl-L-arginine) and MnTcBAD (manganese [III] tetrakis [4-aminobenzic acid] porphyrin) were obtained from Calbiochem (San Diego, CA). Sulfanilamide (N-1-naphthyl)-ethylenediamine, phosphoric acid, sodium nitrite and Percoll were purchased from Sigma. Methyl-$[3H]$thymidine (5 mCi/mmol) from New Life Science Products (Boston, MA).

**Monoclonal Abs**

Purified CD3ε (clone 145-2C11), CD28 (37.51), Gr-1 (RB6-8C5), anti-rat IgG2b (Rg7/11.1) mAbs, anti-mouse IFN-γ, anti-mouse GM-CSF, anti-mouse/rat TNF-α neutralizing Abs, as well as purified rat IgG1 and IgG2a isotype control Igs were purchased from Pharmingen (San Diego, CA).

**Cell preparation**

Mice were sacrificed by cervical dislocation, and their spleens, tibias, and femurs were harvested under sterile conditions. BM cells were obtained by flushing the cavities of the mouse femora and tibia with cold PBS using a syringe and a 26-gauge needle in the standard way. Spleen cell (SC) suspensions were prepared by teasing the spleen. Isolated BM and SC were centrifuged for 5 min at 200 x g and resuspended in complete culture medium.

To obtain plastic nonadherent cells, 20 ml of BM or SC (3–4 x 10^6/ml) were placed in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) and incubated overnight at 37°C in 5% CO₂. The next day, the cells were gently washed with medium pre-warmed to 37°C to recover the plastic nonadherent cells, which were then washed and counted.

To separate the isolated plastic nonadherent cells according to their density characteristics, BM or SC were fractionated by centrifugation on a Percoll density gradient as described by Angulo et al. (4). Briefly, 0.5–1 x 10^8 plastic nonadherent cells were resuspended in 2 ml of 100% Percoll solution. Two milliliters each of 70, 60, 50, and 40% Percoll and 1 ml of HBSS were carefully layered over the cell suspension. After centrifugation at 1800 x g for 30 min, cells were collected from the gradient interfaces. Cells banding between 40 and 50% ($[3H]$thymidine for the final 18 h of incubation. Cells were harvested onto 10^8 plastic nonadherent cells were resuspended in 2 ml of 100% Percoll and 1 ml of HBSS were carefully layered over the cell suspension. After centrifugation at 1800 x g for 30 min, cells were collected from the gradient interfaces.

**Flow cytometry**

BM or SC (10^6/sample), which were kept on ice throughout the procedure, were incubated for 30 min with optimal concentrations of Abs. Controls included unstained cells (cell control), samples in which the first step was either omitted (conjugate control), or replaced by an isotype-matched control mAb (mAb control). The cells were analyzed using a FACSscan (Becton Dickinson, Mountain View, CA).

**NO production**

Equal volumes of culture supernatants (100 μl) were mixed with Griess reagent (21). After a 10-min incubation at room temperature, the absorbance at 550 nm was measured on a Spectramax plate reader (Molecular Devices, Sunnyvale, CA). Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.125 mM sodium nitrite.

**Statistical analysis**

The statistical significance between values was determined by the Student’s t test. All data were expressed as the mean ± SD of triplicates. Probability values >0.05 were considered nonsignificant.

**Results**

**Tumor growth induces the accumulation of Gr-1^- cells in BM and spleen**

Flow cytometric analysis was used to study the frequency of Gr-1- and Mac-1-positive cells in the spleen or BM of mice bearing MCA-26 tumors. While there is a very low number of Gr-1^-/Mac-1^- cells in naive spleen (2.8 ± 0.3%), the results in Table I indicate that 43.8 ± 2.5% of the SC from tumor-bearing animals express both Gr-1 and Mac-1 Ags. BM was also examined for cells expressing myeloid markers. The percentages of double positive, as well as single stained, myeloid cells in the bone marrow of MCA-26 tumor-bearing mice are twice as high as the percentages seen in cells from naive BM.

Although low density BM and SC, derived from tumor-bearers, can inhibit the CD3-induced proliferative response of naive splenocytes, they become more suppressive in the presence of CD3/CD28-activated T cells

To enrich myeloid cell populations according to their density characteristics, splenic or BM cell suspensions were depleted of plastic

### Table I. Increase of Gr-1^- and Mac-1^- cell population in BM and spleen of MCA-26 tumor-bearing mice, as detected by flow cytometry

<table>
<thead>
<tr>
<th>Marker</th>
<th>SC naive</th>
<th>SC TB</th>
<th>BM naive</th>
<th>BM TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gr-1^-</td>
<td>13.2 ± 1.2</td>
<td>48.3 ± 2.4</td>
<td>41.7 ± 6.3</td>
<td>80.9 ± 5.3</td>
</tr>
<tr>
<td>Mac-1^-</td>
<td>6.6 ± 0.8</td>
<td>45.8 ± 2.6</td>
<td>48.1 ± 11.7</td>
<td>90.1 ± 1.9</td>
</tr>
<tr>
<td>Gr-1^-/Mac-1^-</td>
<td>2.8 ± 0.3</td>
<td>43.8 ± 2.5</td>
<td>34.5 ± 3.8</td>
<td>79.3 ± 1.3</td>
</tr>
</tbody>
</table>

* Fresh-derived spleen or BM cells after erythrocyte depletion were used for staining. The results shown are representative of three separate experiments. TB, tumor-bearing cells.
adherent cells and fractionated using Percoll gradient. Functional analysis of the fractionated cell populations was conducted by evaluating their ability to inhibit CD3 or CD3/CD28-induced activation of naive T cells (Fig. 1). When naive splenocytes were stimulated by adding anti-CD3 and anti-CD28 agonistic mAbs, the proliferative response was found to be inhibited by >90% in the presence of the low density cell Fr. II derived from BM of tumor-bearing mice (Fig. 1B) as compared with the response in splenocytes alone. Similarly, Fr. II cells derived from the spleen of tumor-bearing mice (Fig. 1C) also strongly inhibited the CD3/CD28-induced proliferation of naive T cells.

Fig. 2 shows that the immune suppressive activity of Fr. II cells, derived from BM of MCA-26-bearing mice, was markedly higher in comparison to the activity of the same cells derived from naive BM.

In addition to the CD2/CD28 costimulation assay, we have performed the MLR assay in the presence of immune-suppressive myeloid cells derived from BM of MCA-26 tumor-bearing mice (Fig. 3). When purified naive T cells were stimulated by adding allogeneic irradiated splenocytes, the proliferative response was found to be inhibited in the presence of the low density cell fraction (Fr. II) derived from BM of tumor-bearing mice. Cell Fr. III from the same BM did not display significant inhibitory activity.

To assess the contribution of Gr-1\(^+\) cells to immune suppression, Fr. II was depleted of cells expressing Gr-1 Ag using a panning technique. The depleted nonadherent (postpanning) cell population was tested for its ability to inhibit CD3/CD28-stimulated T cell proliferation. Our results demonstrate (Fig. 4) that the T cell response can be restored by depletion of Gr-1-positive cells. Thus, it appears that Gr-1\(^+\) cells are responsible for inhibition of CD3/CD28-induced responses.

**FIGURE 1.** Cell Fr. II derived from BM or spleen of MCA-26 tumor-bearing mice inhibits the CD3/CD28-induced T cell proliferative response. Cells from Fr. II or Fr. III, obtained by Percoll fractionation from BM of naive mice (A) or BM or spleen of tumor-bearers (B and C), were added (2 \times 10^5/well) to naive splenocytes (2 \times 10^5/well) activated with CD3 (1 \mu g/ml) and CD28 (5 \mu g/ml) mAbs. Cells were cocultured for 72 h, and incorporation of \[^{3}H\]thymidine was measured. The results shown are the average of triplicates and are representative of four separate experiments. □, Naive splenocytes only; ■, naive splenocytes plus cell Fr. II; ▪, naive splenocytes plus cell Fr. III.

**FIGURE 2.** Comparative inhibitory activity of cell fraction II, derived from BM of naive and tumor-bearing (TB) mice. Freshly isolated BM cells were fractionated on a Percoll gradient. A graded number of Fr. II cells (0.5–2 \times 10^5/well) was added to naive splenocytes (2 \times 10^5/well) activated with CD3 (1 \mu g/ml) and CD28 (5 \mu g/ml) mAbs. Cells were cocultured for 72 h, and incorporation of \[^{3}H\]thymidine was measured. Results presented are the average of triplicates and are representative of three such experiments.

**FIGURE 3.** Myeloid immune suppressive cells can inhibit the proliferative response of naive T cells to alloantigens in MLR. BM cells were derived from MCA-26 tumor-bearing mice and fractionated on a Percoll gradient. A graded number of Fr. II or Fr. III cells (0.5–2 \times 10^5/well) was added as a third part to MLR as described in *Materials and Methods*. Cells were cocultured for 96 h, and incorporation of \[^{3}H\]thymidine was measured. In the figure, × is responder cells alone (purified T cells, BALB/c origin); ◊ is stimulator cells alone (irradiated C57BL/6 splenocytes); ▲ is responder plus stimulator cells; ♦ is responder plus stimulator cells plus cell Fr. III from TB BM; and □ is responder plus stimulator cells plus cell Fr. II from tumor-bearing BM. Results presented are the average of triplicates and are representative of two separate experiments.
CD28-stimulated T cell proliferation in the presence of the immune suppressive cell fraction derived from BM or spleen of MCA-26 tumor-bearers.

**Immune suppression mediated by Gr-1<sup>+</sup> cells may be reversed in vitro by using a combination of peroxynitrite and NO inhibitors**

Because NO is known as the major mediator of natural suppressor activity (4), we decided to measure the level of nitrates in cocultures of fractionated tumor-bearer-derived BM (or spleen) cells and naive SC stimulated with CD3 (1 μg/ml) and CD28 (5 μg/ml) mAbs. Cells were cocultured for 72 h, and incorporation of [3H]thymidine was measured. Results presented are the average of triplicates and are representative of two separate experiments.

In other experiments, no more than 20–25% of the suppression was reversed when L-NMMA was used alone (data not shown). Fig. 5B shows that immune suppression, mediated by Gr-1<sup>+</sup> cells, L-NMMA, a competitive inhibitor of inducible NO synthase (iNOS), was added to the cultures. However, the presence of L-NMMA did not markedly block the inhibitory effect of Fr. II cells. In other experiments, no more than 20–25% of the suppression was reversed when L-NMMA was used alone (data not shown).

Fig. 5B shows that immune suppression, mediated by Gr-1<sup>+</sup> cells, can be almost completely reversed in vitro using a combination of L-NMMA and MnTBAP, an iNOS inhibitor and superoxide dismutase (SOD) mimetic, respectively. However, the inhibitory activity of Fr. II cells derived from BM or spleen of MCA-26 tumor-bearing mice was unaffected when the proliferation assays were performed in the presence of either L-NMMA or MnTBAP alone (data not shown). The presence of MnTBAP in concentrations exceeding 10 μM led to the impairment of CD3/
We demonstrated a quantitative increase in the number of Gr-1+ activation. Several findings can be drawn from our experiments. In a representative experiment, immune suppression mediated by spleen- or BM-derived myeloid cells was almost completely abolished in the presence of neutralizing Abs against IFN-γ (Fig. 6A), but not in the presence of iso-type control Abs or neutralizing Abs against GM-CSF or TNF-α (Fig. 6C). NO production by myeloid cells was also strongly inhibited when anti-IFN-γ mAbs were added (Fig. 6B). Thus, it appears that the inhibition of CD3/CD28-mediated T cell proliferation by Fr. II cells is strictly dependent on endogenously produced IFN-γ.

T cells pre-activated with anti-CD3 mAbs are not sensitive to the inhibitory effect of immune suppressive cells derived from BM or spleen of tumor-bearers

It is important to know whether pre-activated T cells instead of the primary T cell activation can be inhibited by the immune suppressive cells derived from MCA-26 tumor-bearing mice. When SC preincubated with anti-CD3 agonist Ab for 72 h were used as responder cells (Fig. 7A) in cocultures with Fr. II cells derived from BM or spleen of tumor-bearers, no inhibition was observed. In a control experiment (Fig. 7B); however, BM or SC derived from the same MCA-26-bearing mice were able to inhibit CD3/CD28-induced primary activation of naive T cells.

Discussion

Previous observations in cancer patients and tumor-bearing animals suggested that myeloid lineage cells were involved in tumor-induced immunosuppression. Expansion of immature myeloid cells or macrophage/monocytes with characteristics of immune suppressive cells has been described in patients with head and neck cancer (6), gastric cancer (7), in mice bearing most carcinomas: Lewis lung carcinoma (8), Ehrlich carcinoma (9, 22), mammary adenocarcinoma (23), chemically induced fibrosarcoma (24), and colon carcinoma (13–15). This expansion was associated with a decline in T cell responses; however, little is known regarding the mechanism by which immune-suppressive cells inhibit primary T cell activation.

In the present study, we investigated how Gr-1+ myeloid cells, isolated from MCA-26 tumor-bearing mice and enriched by fractionation, contribute to inhibition of CD3/CD28-induced T cell activation. Several findings can be drawn from our experiments. We demonstrated a quantitative increase in the number of Gr-1+ cells in both BM and spleen from mice bearing MCA-26 tumors. These myeloid cells are able to strongly inhibit the proliferation of naive T cells that have been triggered with anti-CD3 and anti-CD28 agonistic mAbs. However, the same suppressive cell population failed to inhibit CD3/CD28-induced proliferation when the responder T cells were pre-activated with anti-CD3 mAbs. IFN-γ, which released from T cells activated by CD3/CD28, is involved in Gr-1+ cell-mediated immune suppressive activity. Indeed, neutralization of IFN-γ, but not TNF-α or GM-CSF, with specific mAbs almost completely abolished the immune suppressive activity of Gr-1+ cells. Finally, inhibition of T cell proliferation mediated by myeloid cells could be prevented by adding a combination of l-NMMA plus MnTBAP, an iNOS inhibitor and SOD mimetic, respectively, to the cocultures. These inhibitors could only prevent immune suppression when used in combination, whereas using...
tumor-bearing mice, were added to pre-activated splenocytes in the presence of TCR signaling induces full T cell activation. However, our data clearly indicate that when CD3/CD28-induced T cell activation occurs in the presence of Gr-1\(^+\) natural suppressor cells, T cells become deactivated or tolerated. This deactivation of T cells, at least in vitro, can be achieved upon CD3/CD28 triggering, as Gr-1-positive cells in murine BM and spleen of tumor-bearers can be differentiated into fully functional APC by T cells. Our findings point toward a mechanism for negative control of primary T cell activation if our model for T cell activation, which uses anti-CD3 and anti-CD28 Abs, is relevant to naturally occurring APC-mediated, T cell activation (17). In part, the physiological relevance of these myeloid immune suppressive cells could be reflected our data obtained using MLR (Fig. 3).

Regarding the nature of the described inhibitory activity of Gr-1\(^+\) myeloid cells under the conditions used in our experiments, it could be caused by peroxynitrite (ONOO\(^-\)), the reaction product of NO and superoxide (O\(_2\)). It has been previously demonstrated that a significant portion of the toxic effects attributed to NO is due to the generation of ONOO\(^-\) (30). Peroxynitrite is a powerful oxidant that can inhibit T cell activation and proliferation by impairment of tyrosine phosphorylation and apoptotic death (31). Our results suggest that production of reactive nitrogen and oxygen intermediates by myeloid cells could play a major role in the impairment of T cell activation upon CD28 costimulation. It is not surprising that immature myeloid cells can generate reactive oxygen species (ROS) and nitrogen species, because ROS is involved in signal transduction of hematopoietic growth factors (32), whereas NO is involved in mechanisms of differentiation and maturation of myeloid cells in BM (33, 34).

It is known that costimulation through B7-CD28 interaction in the presence of TCR signaling induces full T cell activation. However, our data clearly indicate that when CD3/CD28-induced T cell activation occurs in the presence of Gr-1\(^+\) natural suppressor cells, T cells become deactivated or tolerated. This deactivation of T cells, at least in vitro, can be achieved upon CD3/CD28 triggering, and IFN-\(\gamma\) plays a critical role in this deactivation. We suggest that the presence of immune suppressive myeloid cells in secondary lymphoid organs, such as spleen, can down modulate, or even prevent, development of an adaptive T cell antitumor immune response. This type of feedback mechanism could also play an important role in the development of tolerance, one of the means by which a growing tumor may escape the T cell immune response.

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**References**


