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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 L-NMMA (N^G-monomethyl-L-arginine), a superoxide dismutase mimetic and inducible NO synthase inhibitor, respectively, or by depletion of the Gr-1-positive cells. IFN-γ, 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.

Tumor cells can escape from the attack by the immune system through various mechanisms, including immune evasion, immune suppression, or both. To achieve better therapeutic effect by immune modulating therapy, it is important to study the mechanisms of tumor-induced immune suppression. It has been demonstrated that immunoregulatory cells having myeloid origin and termed natural suppressor cells were found in normal adult bone marrow (BM)[3] of humans and animals (1–4) as well as in sites with intense hematopoiesis such as the spleen of newborn mice or in adult mice during graft-versus-host disease, or after cyclophosphamide injection or gamma-irradiation (4, 5). Tumor growth is accompanied by an increase in the number of immature myeloid cells having strong natural suppressive activity, in BM as well as peripheral lymphoid organs in cancer patients (6, 7) or tumor-bearing mice (8–10). It has been demonstrated that these immune suppressive cells are capable of inhibiting the T cell proliferative response induced by alloantigens (1), CD3 ligation (11), or various mitogens (3, 4), and can also inhibit IL-2 utilization (12) as well as NK cell activity (7). These studies indicate that pro-gressive tumor growth is associated with the down-regulation of T cell responses. It is also apparent that immature myeloid cells can be involved in negative immunoregulatory mechanisms of the tumor host. However, the mechanism by which these immune suppressive cells could affect the T cell response is still not clear.

It has been demonstrated previously that cells expressing Gr-1 or Mac-1 myeloid lineage cell markers are involved in T cell hyporesponsiveness in tumor-bearing mice. CTL activity and T lymphocyte proliferation in response to alloantigens can be significantly inhibited by Mac-1-positive cells derived from the spleen of mice bearing colon carcinoma (13). Direct interaction of T cells with Mac-1$^+$ cells, derived from mice with large tumors, can result in the lost of, or significant decrease in, CD3ξ expression (14). Depletion of Mac-1-positive cells from the cell suspension can result in the restoration of the CD3ξ molecule, which is important for signal transduction. Furthermore, the Gr-1$^+$/Mac-1$^+$ immune suppressive cell population was recently shown to be involved in inhibition of CD8$^+$ cell generation in the tumor host through apoptosis and a contact-dependent mechanism (15).

Considering the above information, it is essential to know whether immune suppressive myeloid cells can affect primary T cell activation. APC provide at least two signals that are required for T cell activation: a signal via the TCR/CD3 complex that is transmitted upon recognition of Ag and a additional signal(s) delivered through one or more costimulatory cell interactions, like B7-CD28 or LFA-ICAM (16, 17). Only in the presence of the appropriate costimulatory signal does the primed T cell become capable of a productive immune response characterized by proliferation and production of IL-2 (IL-2), differentiation, clonal expansion and effector function (16).

In the current study, we focused our investigation on immature Gr-1$^+$ myeloid cells and their involvement in the regulatory mechanisms of T cell activation and costimulation, as induced through CD3/CD28 ligation.

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 intrahepatic implantation of 1×10^5 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 μg/ml streptomycin, 0.05 mM 2-ME, and 2 mM glutamine (all from Sigma, St. Louis, MO). L-NMMA (N^G-monomethyl-L-arginine) and MnTBAP (manganese [III] tetrakis [4-benzoic acid] porphyrin) were obtained from Calbiochem (San Diego, CA). Sulfanilamide (N-1-naphtyl)-ethylenediamine, phosphoric acid, sodium nitrite and Percoll were purchased from Sigma. Methyl-[^{3}H]thymidine (5 mCi/mmol) from New Life Science Products (Boston, MA).

Monoclonal Abs

Purified CD3e (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 contents 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 $200 \times g$ and resuspended in complete culture medium.

To obtain plastic nonadherent cells, 20 ml of BM or SC ($3–4 \times 10^6$/ml) were placed in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) and incubated overnight at 37°C in 5% CO$_2$. 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, $0.5–1 \times 10^8$ 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 $1800 \times g$ for 30 min, cells were collected from the gradient interfaces. Cells banding between 40 and 50% (<1.063 g/ml) were labeled as fraction (Fr) I; between 50 and 60% (1.063–1.075 g/ml) as Fr. II; and between 60 and 70% (1.075–1.090 g/ml) as Fr. III. After washing, the cells were counted and adjusted to the appropriate concentrations in culture medium.

Myeloid cell-enriched Percoll fractions were depleted of Gr-1$^+$ cells by panning (19). Plastic petri dishes were each coated with 7 ml of secondary anti-rat IgG2b Abs (10 μg/ml; PharMingen). Fractionated BM or SC were incubated with primary Gr-1 Abs in PBS without Ca^{2+}/Mg^{2+} at a concentration 10 μg/10^7 cells After washing, the cells were plated onto the precoated petri dishes and incubated for 1 h at 4°C. Nonadherent cells were then collected by gently washing with PBS and used in the appropriate experiments.

In some experiments Fr. II cells, derived from the spleen of MCA-26 tumor-bearing mice were also depleted of T cells by means of complement dependent lysis using anti-CD3 mAbs, as described previously (20).

T cell activation and proliferation assay

For T cell activation experiments, freshly isolated naive splenocytes (2×10^5/well) were cultured in 96-well round-bottom plates in complete culture medium containing soluble, or plate-bound, anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml). In some experiments T lymphocytes (1×10^5/well), which were purified from naive spleens using T cell enrichment columns (MTCC-525; R&D Systems, Minneapolis, MN), were used as responder cells instead of bulk splenocytes.

BM or SC fractions, obtained by Percoll fractionation from MCA-26 tumor-bearing mice, were added to the naive splenocytes or purified T cells, activated with CD3 and CD28 Abs. These cell mixtures were cocultured for 72 h at 37°C in 5% CO$_2$. Each well was pulsed with 1 μCi [^{3}H]thymidine for the final 18 h of incubation. Cells were harvested onto filtermates (cat. no. 1450-421, Wallac, Turku, Finland) and radioactivity was measured in a 1450 Microbeta scintillation counter (Wallac). Data are expressed in cpm (mean ± SD of triplicate cultures) or percentage of

suppression calculated as follows: % suppression = [(100 − cpm test cells)/cpm control cells] × 100.

MLR

MLR was performed by using C57BL/6 splenocytes (H-2^b) as stimulator cells and purified BALB/c T cells (H-2^d) as responder cells. Irradiated stimulator cells (5×10^5/well) and responder cells (2×10^5/well) were cultured in 96-well round-bottom plates for 96 h, and [^{3}H]thymidine (1 μCi/well) was added for the final 18 h of incubation. To evaluate the inhibitory activity of the myeloid cell enriched population on MLR, BM or SC fractions were prepared by Percoll fractionation from MCA-26 tumor-bearers and applied in graded doses to MLR.

Flow cytometry

BM or SC (10^6/tube), 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 FACScan (Becton Dickinson, Mountain View, CA).

NO production

Equal volumes of culture supernatants (100 μl) were mixed with Greiss reagent (21). After a 10-min incubation at room temperature, the absorbance at 550 nm was measured on 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 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*

	% of Positive Cells			
Marker	SC naive^a	SC TB	BM naive	BM TB
Gr-1$^+$	13.2 ± 1.2	48.3 ± 2.4	41.7 ± 6.3	80.9 ± 5.3
Mac-1$^+$	6.6 ± 0.8	45.8 ± 2.6	48.1 ± 11.7	90.1 ± 1.9
Gr-1$^+$/Mac-1$^+$	2.8 ± 0.3	43.8 ± 2.5	34.5 ± 3.8	79.3 ± 1.3

^{a} 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.
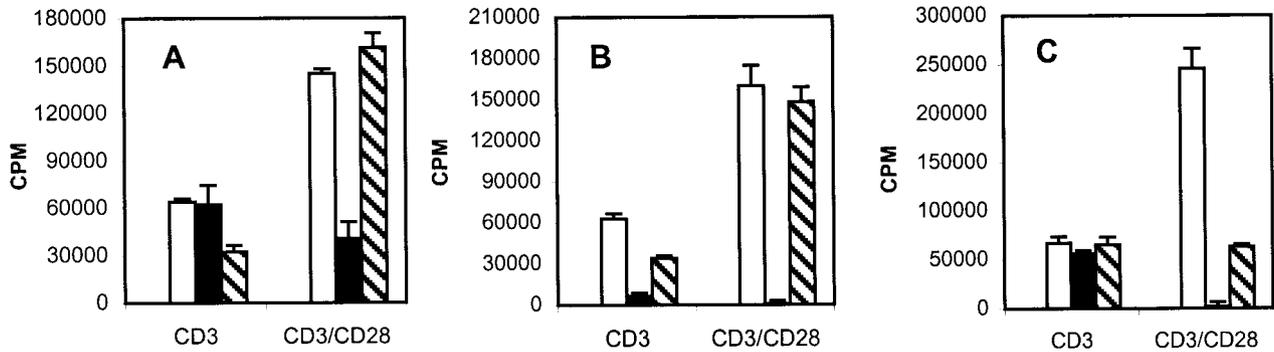
FIGURE 1. Cell Fr. II derived from BM or spleen of MCA-26 tumor-bearing mice inhibits the CD3/CD28-induced T cell proliferative response. Cells from Fr. II or Fr. III, obtained by Percoll fractionation from BM of naive mice (*A*) or BM or spleen of tumor-bearers (*B* and *C*), were added (2×10^5/well) to naive splenocytes (2×10^5/well) activated with CD3 (1 μg/ml) and CD28 (5 μg/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.

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. 1*B*) as compared with the response in splenocytes alone. Similarly, Fr. II cells derived from the spleen of tumor-bearing mice (Fig. 1*C*) 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/
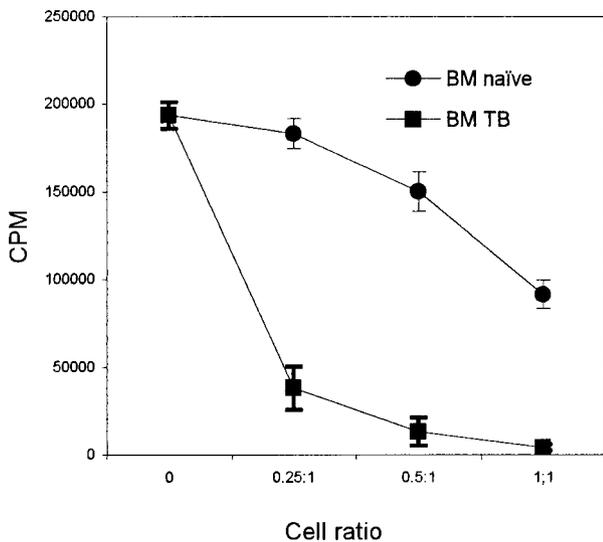
FIGURE 2. Comparative inhibitory activity of cell fraction II, derived from BM of naive and tumor-bearing (TB) mice. Freshly isolated BM cells were fractionated on a Percoll gradient. A graded number of Fr. II cells (0.5–2×10^5/well) was added to naive splenocytes (2×10^5/well) activated with CD3 (1 μg/ml) and CD28 (5 μg/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.
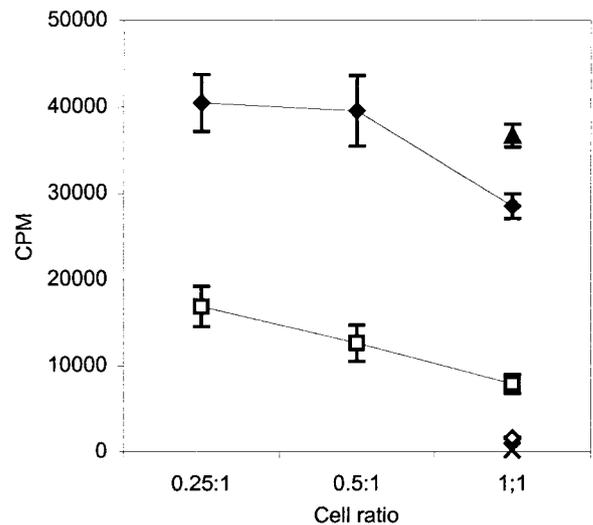
FIGURE 3. Myeloid immune suppressive cells can inhibit the proliferative response of naive T cells to alloantigens in MLR. BM cells were derived from MCA-26 tumor-bearing mice and fractionated on a Percoll gradient. A graded number of Fr. II or Fr. III cells (0.5–2×10^5/well) was added as a third part 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.
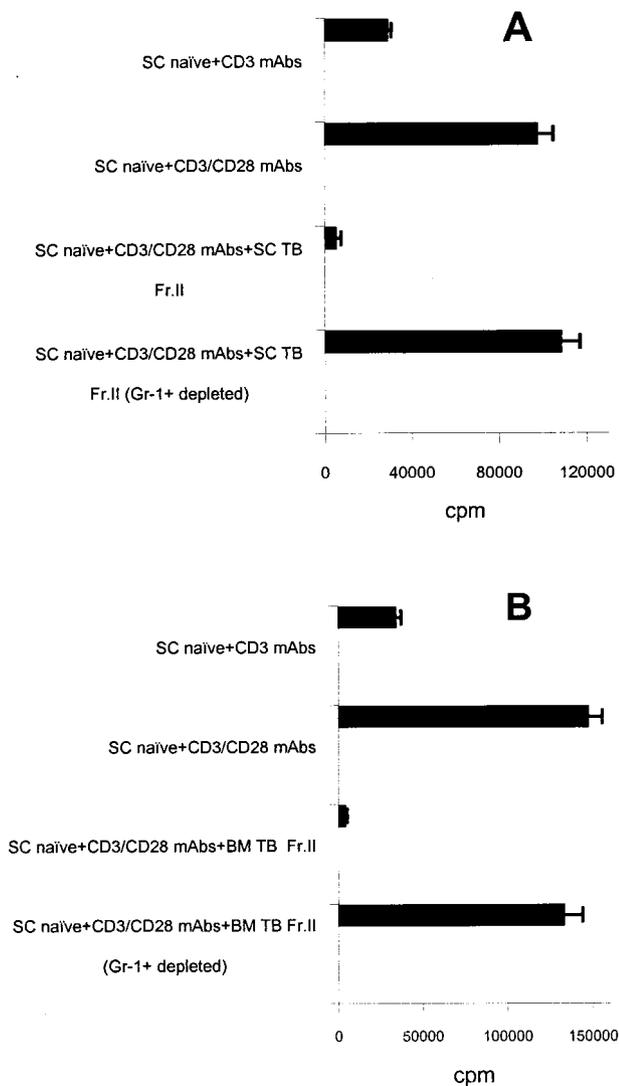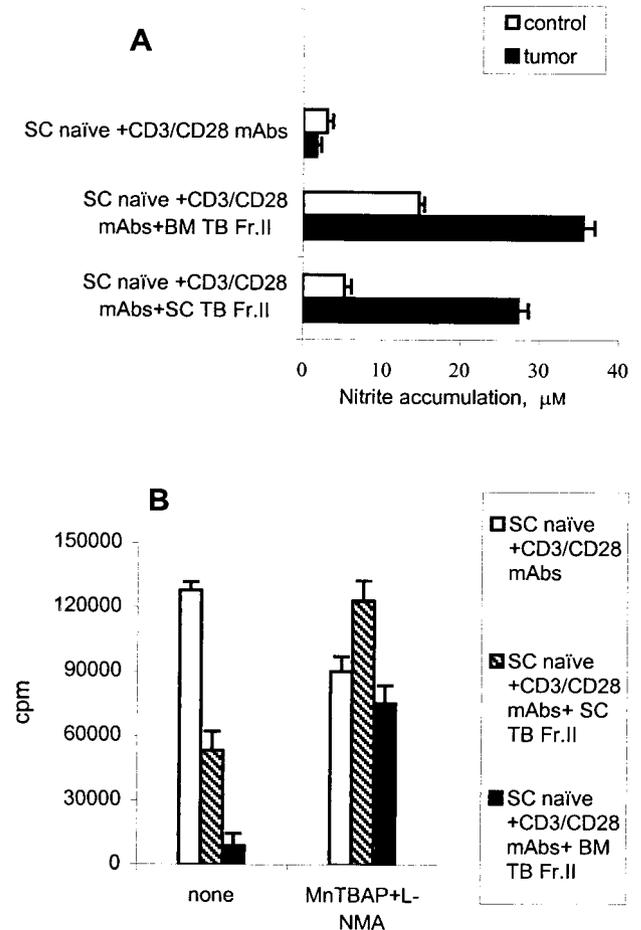
FIGURE 4. Depletion of Gr-1$^+$ cells from tumor-bearer SC Fr. II (*A*) or BM cell Fr. II (*B*) restores CD3/CD28 activated proliferation of naive T cells. Freshly isolated spleen or BM cells were fractionated on a Percoll gradient. Fr. II cells from BM or spleen were depleted of Gr-1-positive cells and added in a 1:1 ratio to naive splenocytes activated with CD3 (1 μg/ml) and CD28 (5 μg/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 two separate experiments.

FIGURE 5. Involvement of reactive nitrogen and oxygen species in mechanisms of immune suppression. *A,* Comparative levels of nitrites in cell culture supernatants. T cell activation assays were set up in the presence or absence of Fr. II cells derived from spleen or BM of naive or tumor-bearing mice, as described in *Materials and Methods.* Cells were cocultured for 72 h using a 1:1 cell ratio, and culture supernatants were collected. The amount of NO secreted into the culture supernatant was detected using Greiss reagent. Results presented are the average of triplicates and are representative of five separate experiments. *B,* Reversal of immune suppression in the presence of L-NMMA and MnTBAP. T cell activation assays were set up in the presence or absence of Fr. II cells derived from spleen or BM of tumor-bearing mice, as described in *Materials and Methods.* A combination of L-NMMA (0.5 mM) and MnTBAP (10 μM) was added to the cultures. Cells were cocultured using a 1:1 cell ratio for 72 h, and the incorporation of [^{3}H]thymidine was measured. 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$^+$ 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 nitrites in cocultures of fractionated tumor-bearer-derived BM (or spleen) cells and naive SC stimulated with anti-CD3 and anti-CD28 mAbs. In a representative experiment (Fig. 5*A*), high nitrite levels were detected in the culture supernatants of stimulated SC cocultured with Fr. II cells, i.e., cultures exhibiting a high degree of suppression. Fr. II cells cultured alone did not generate NO (data not shown). To test the involvement of NO in the suppression caused by Fr. II

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. 5*B* shows that immune suppression, mediated by Gr-1$^+$ 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/

CD28-induced T cell proliferation in control SC cultures (data not shown).

IFN-γ is responsible for induction of the immune suppressive activity of Gr-1+ cells

The results obtained indicate that to achieve inhibition of T cell proliferation by Fr. II cells, activation of some inducible enzymes in cocultured Gr-1+ myeloid cells is required suggesting a role for T cell-derived cytokine(s), at least in culture. To investigate the possible role of T cell-released cytokines in the induction of this inhibitory activity, cocultures were set up in the presence of various cytokine neutralizing Abs. 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 isotype 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 medi-
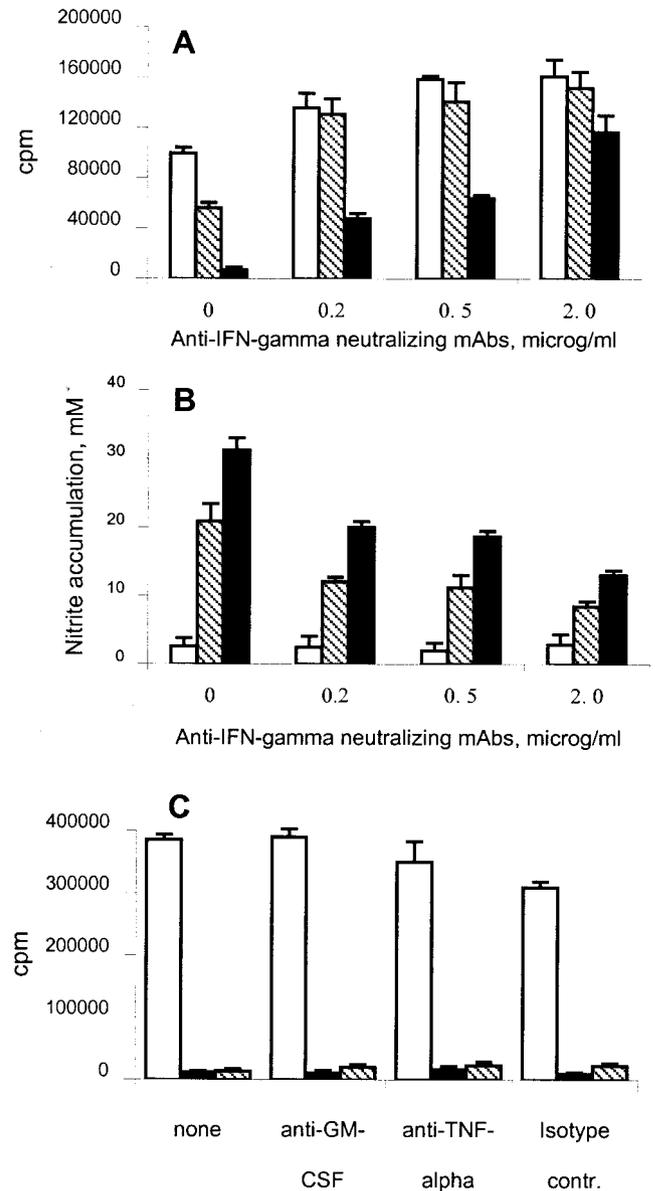
FIGURE 6. Involvement of IFN-γ in immune suppression. Neutralizing Abs to IFN-γ decrease the inhibitory activity of Gr-1+ cells (A) and NO production by Gr-1+ cells (B) in a dose-dependent manner. Fr. II cells from tumor-bearer BM or spleen were cocultured with naive splenocytes plus anti-CD3/CD28 mAbs in the presence or absence of neutralizing anti-IFN-γ mAbs. Cells were cultured for 72 h, and incorporation of [³H]thymidine was measured. The amount of NO secreted into the culture supernatants was detected using Greiss reagent. Results presented are the average of triplicates and are representative of two such experiments. C, Neutralization of GM-CSF or TNF-α has no influence on Gr-1+ cell-mediated immune suppression. The cells were cocultured in presence of anti-GM-CSF or anti-TNF-α mAbs, or isotype control Abs at high concentrations (20 μg/ml) were used instead of anti-IFN-γ. Myeloid-enriched cell Fr. II derived from spleen of tumor-bearers (SC TB II) was depleted additionally of T cells as described in *Materials and Methods*. □, Naive splenocytes plus CD3/CD28 mAbs; ■, naive splenocytes plus CD3/CD28 mAbs plus BM TB Fr. II; ▨, naive splenocytes plus CD3/CD28 mAbs plus SC tumor-bearing cell Fr. II.

ated 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
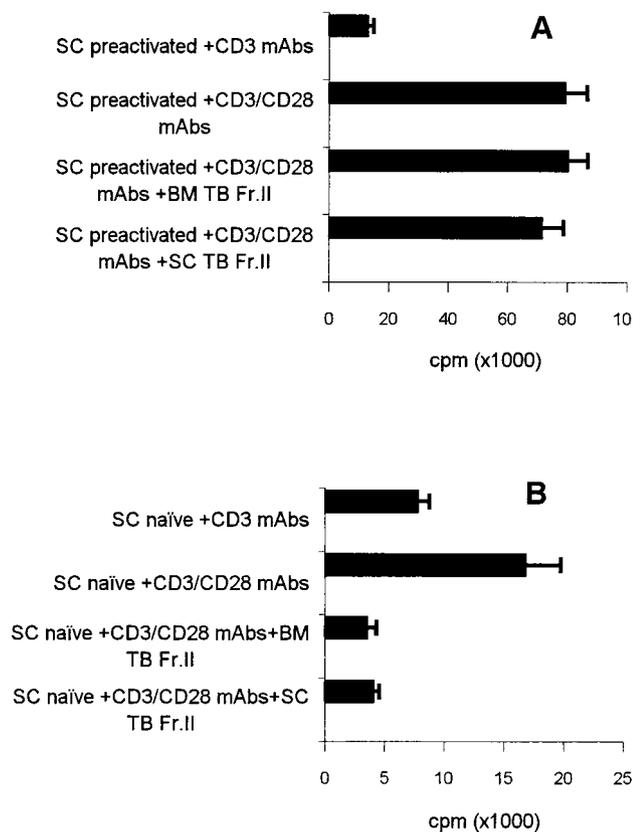
FIGURE 7. Myeloid immune suppressive cells do not inhibit CD3/CD28-induced proliferation of pre-activated T cells. *A,* Naive splenocytes were pre-activated by incubation with plate-bound anti-CD3 Abs (0.3 μg) for 72 h. After washing, they were ready for use in assays as indicated in *Materials and Methods.* Freshly isolated spleen or BM cells were fractionated on a Percoll gradient. Fr. II cells, derived from the BM or spleen of tumor-bearing mice, were added to pre-activated splenocytes in the presence of anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) mAbs. Results presented are the average of triplicates and are representative of two separate experiments. *B,* Control experiment. An aliquot of the same BM or SC Fr. II were added to naive splenocytes (2 $\times$ 10^5/well) activated with CD3 (1 μg/ml) and CD28 (5 μg/ml) mAbs using a 0.5:1 cell ratio. 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 separate experiments.

either one of them alone failed to restore CD3/CD28-induced T cell proliferation.

Gr-1 is a myeloid lineage differentiation Ag whose expression on hematopoietic cells can be regulated by certain cytokines, such as GM-CSF and IL-3 (25). These Gr-1-positive cells in murine BM represent a subset consisting mainly of precursors for both granulocytes and monocytes (26). Moreover, in the presence of GM-CSF plus IL-4, Gr-1$^+$/Mac-1$^+$ immune suppressive cells derived from tumor-bearers can be differentiated into fully functional APC in vitro (15). The significant increase in the number of immediate granulocyte-monocyte progenitors in mice bearing large MCA-26 tumors leads to BM of almost complete myeloid composition. As tumor growth progressed, splenomegaly and extramedullary hematopoiesis could be observed (data not shown). However, the exact mechanism by which the growing intrahepatic MCA-26 tumor induces myelopoiesis is not clear. Several cytokines produced by tumors, such as GM-CSF (11, 15), vascular endothelial growth factor (27, 28), and IL-10 (29), have been proposed as candidates for impairment of myelopoiesis in tumor hosts.

In the current study, BM or SC fractions, derived from MCA-26 tumor-bearing mice and enriched for immature myeloid cells, produced a significant amount of NO in the presence of naive T cells stimulated with anti-CD3 and anti-CD28 mAbs. This NO production was inducible and strictly dependent on the presence of endogenous IFN-γ, because addition of neutralizing anti-IFN-γ Abs to the cultures inhibited accumulation of nitrites in the culture supernatant in a dose-dependent fashion. However, NO was not primarily responsible for the observed immune suppression, because adding L-NMMA, a competitive iNOS inhibitor, to the cultures had no effect on the impaired T cell proliferation. The presence of MnTBAP, a SOD mimetic, alone in the culture, also did not restore the impaired T cell proliferative response to CD3/CD28 stimulation. Only a combination of these inhibitors was effective in blocking immune suppressive activity. When taken together, our results indicate that Gr-1$^+$ myeloid cells become immune suppressive only in the presence of IFN-γ due to CD3/28-triggered primary T cell activation induces releasing of IFN-γ 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 tolerized. This deactivation of T cells, at least in vitro, can be achieved upon CD3/CD28 triggering, and IFN-γ 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.

Acknowledgments

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