Mechanism of Immune Dysfunction in Cancer Mediated by Immature Gr-1+ Myeloid Cells

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*J Immunol* 2001; 166:5398-5406; doi: 10.4049/jimmunol.166.9.5398

http://www.jimmunol.org/content/166/9/5398
Mechanism of Immune Dysfunction in Cancer Mediated by Immature Gr-1⁺ Myeloid Cells

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The mechanism of tumor-associated T cell dysfunction remains an unresolved problem of tumor immunology. Development of T cell defects in tumor-bearing hosts are often associated with increased production of immature myeloid cells. In tumor-bearing mice, these immature myeloid cells are represented by a population of Gr-1⁺ cells. In this study we investigated an effect of these cells on T cell function. Gr-1⁺ cells were isolated from MethA sarcoma or C3 tumor-bearing mice using cell sorting. These Gr-1⁺ cells expressed myeloid cell marker CD11b and MHC class I molecules, but they lacked expression of MHC class II molecules. Tumor-induced Gr-1⁺ cells did not affect T cell responses to Con A and to a peptide presented by MHC class II. In sharp contrast, Gr-1⁺ cells completely blocked T cell response to a peptide presented by MHC class I in vitro and in vivo. Block of the specific MHC class I molecules on the surface of Gr-1⁺ cells completely abrogated the observed effects of these cells. Thus, immature myeloid cells specifically inhibited CD8-mediated Ag-specific T cell response, but not CD4-mediated T cell response. Differentiation of Gr-1⁺ cells in the presence of growth factors and all-trans retinoic acid completely eliminated inhibitory potential of these cells. This may suggest a new approach to cancer treatment. The Journal of Immunology, 2001, 166: 5398–5406.

Failure of T cells from tumor-bearing hosts to effectively recognize and eliminate tumor cells is one of the major factors of tumor escape from immune system control. Success of cancer immune therapy depends on the ability of the treatment to activate T cells against the tumor. Therefore, elucidation of the mechanisms of T cell nonresponsiveness in cancer is critical for the design of an effective cancer immunotherapy. Inhibition of various T cell functions in tumor-bearing mice and cancer patients has been described in numerous reports. This is attributed to inhibition of signal transduction in these cells (1, 2). However, it is not clear what may cause these defects. One of the possible mechanisms is an effect of several tumor-derived factors like TGF-β and IL-10 (3). Another possible mechanism is an effect induced by accumulation of immature myeloid cells in tumor-bearing hosts. Several groups have reported an increased production of these cells capable of inhibiting T cell functions in cancer patients and tumor-bearing mice (4–8). In mice, recently these immature myeloid cells were more precisely characterized as Gr-1⁺ cells. Increased presence of these cells has been described in bone marrow and spleens of tumor-bearing mice (9–11). These cells express the Mac-1 (CD11b) marker of myeloid cells and are able to cause a significant decrease in CD3ζ molecule expression in T cells, which is important for signal transduction (9). It is possible that Gr-1⁺CD11b⁺ cells play an important role in T cell deficiency in cancer. However, it is not clear how these cells could affect T cell function. In particular, it is not known whether Gr-1⁺ cell-mediated inhibition of T cells is Ag-specific. In this study we have investigated the mechanism of Gr-1⁺ cell-mediated inhibition of T cell functions. We demonstrate that Gr-1⁺ cells inhibit Ag-specific CD8⁻, but not CD4-mediated T cell responses. This inhibition is dependent on MHC class I expression on the Gr-1⁺ cells and could be reversed by differentiation of these cells in the presence of growth factors and differentiation agents.

Materials and Methods

Animals

Female BALB/c and C57BL/6 mice (6–8 wk old) were purchased from Harlan (Indianapolis, IN) and were housed in specific pathogen-free units of the Division of Comparative Medicine at Loyola University Medical Center. TCR-transgenic mice expressing an αβ TCR specific for aa 110–120 from influenza hemagglutinin (HA) presented by I-Ek were a generous gift from Harald von Boehmer (Basel Institute for Immunology, Basel, Switzerland) (12). These mice were crossed to a BALB/c background for >10 generations. Transgenic mice used in these experiments were heterozygous for the transgene.

Tumor models

MethA sarcoma is a transplantable 3-methylcholanthrene-induced sarcoma of BALB/c origin passaged as an ascitic tumor (13). MethA sarcoma is a relatively immunogenic tumor that carries a carcinogen-induced mutant endogenous p53 gene. MHC class I-restricted peptide (KVICNSSCM) derived from mutant p53 is specific for this model (13, 14). Immunization of mice with this peptide results in tumor protection and partial regression of established tumor. The wild-type p53 counterpart (KYMCSNCCSM) does not induce antitumor response and has been used as a control peptide. C3 tumor cell line was made by transfection of C57BL/6 B6 mouse embryonic cells with EJ-ras and plasmid containing the human papillomavirus (HPV) type 16 (15). This is a poorly immunogenic tumor. MHC class I-restricted HPV-16-derived peptide RAHYNIVTF expressed by this tumor was shown to elicit potent anti-tumor immune response (16).

Immunization protocol

The Helios gene gun system (Bio-Rad, Hercules, CA) was used for intradermal gene delivery. A DNA construct containing the H2Dβ restricted dominant HPV16E7 peptide RAHYNIVTF was used in this study (35). Bullets containing 2 μg DNA per shot were generated according to the

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Received for publication November 1, 2000. Accepted for publication February 21, 2001.

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† This work was supported by National Institutes of Health Grants CA84488 (to D.I.G.) and CA78,999 (to W.M.K.). M.P.V. is a fellow of the Cancer Research Institute.

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Abbreviations used in this paper: HA, hemagglutinin; HPV, human papillomavirus; ATRA, all-trans retinoic acid; ELISpot, enzyme-linked immunospot; LLMA, N°-monomethyl-l-arginine.

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0022-1767/01/$02.00
overnight at 4°C with a different clone of biotinylated anti-IFN-γ pulsed with [3 H]thymidine (1 Ci/well; Amersham, Arlington Heights, IL) and cultured for 3 days. Eighteen hours before harvesting, cells were presence of Con A or synthetic HA peptide (aa 110–120, SFERFEIFPKE) (BCIP/NBT) substrate. The number of spots per 106 splenocytes, which (PharMingen). Reactions were visualized using avidin-alkaline phosphatase; and purified anti-Gr-1, anti-TER-119, FITC- or PE-conjugated anti-MHC class II (I-Aq, TIB-120). Mouse GM-CSF, IL-4, and TNF-α were obtained from Research Diagnostics (Flanders, NJ); Con A, all-trans retinoic acid (ATRA), and polyclonal anti-mouse Ig were obtained from Sigma; and purified anti-Gr-1, anti-TER-119, FITC- or PE-conjugated anti-Gr-1, CD11c, CD11b, CD86 (B7-2), I-Aq, and IAq Abs were purchased from PharMingen (San Diego, CA). Isotype-matched FITC- and PE-conjugated IgG was used as a control of nonspecific binding. Low-Tox-M complement and Lympholyte-M were obtained from Cedarlane Laboratories, (Hornby, Ontario, Canada). Cell culture inserts with a pore size of 0.2 μm were obtained from NalgeNunc. Complete medium EHS contained RPMI 1640 supplemented with 10% FCS, antibiotics, and 5 × 10−5 2-ME.

Cell separation and analysis of cell surface receptors

A single cell suspension was prepared from spleens and inguinal, axillary, and brachial lymph nodes, and red cells were removed by hypotonic shock using ACK lysis buffer. For analysis of cell surface receptors, cells were washed in PBS supplemented with 0.1% FCS and labeled with appropriate Abs for 30 min at 4°C. Cells were then washed and analyzed on FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Cell sorting was performed on a FACStar flow cytometer (Becton Dickinson). Macrophages were isolated from spleens of control mice. Briefly, splenocytes were cultured overnight in complete medium, monadherent cells were removed, and adherent cells were dislodged using a cell scraper. Cells were washed and then used in experiments. An enriched population of T cells was obtained from lymph nodes by incubating cells on ice for 30 min with anti-MHC class II mAb (TIB-120) and polyclonal rabbit anti-mouse Ig. Cells were then washed and incubated with Low-Tox-M complement for 60 min at 37°C. Dead cells were removed by gradient centrifugation on Lympholyte-M.

Ag-specific proliferation

Splenocytes or lymph node cells (10^7/well) from transgenic mice were mixed with Gr-1+ cells from MethA sarcoma-bearing BALB/c mice in the presence of Con A or synthetic HA peptide (aa 110–120, SFRSEFIPKE) and cultured for 3 days. Eighteen hours before harvesting, cells were pulsed with [3 H]thymidine (1 μCi/well; Amersham, Arlington Heights, IL). [3 H]Thymidine uptake was counted using a liquid scintillation counter and expressed as cpm.

Enzyme-linked immunospot (ELISPOT) assay

The number of IFN-γ-producing cells was measured using an ELISPOT assay. Briefly, Millipore MultiScreen-HA plates were coated with anti-mouse IFN-γ Ab (PharMingen). Splenocytes (2 × 10^6 cells/well) were cultured for 24 h at 37°C in 5% CO2 incubator in the complete medium alone or in the presence of the specific or control peptides at a concentration of 10 μM. After that time, wells were washed and then incubated overnight at 4°C with the presence of a common biotinylated anti-IFN-γ Ab (PharMingen). Reactions were visualized using avidin-alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate. The number of spots per 10^6 splenocytes, which represented the number of IFN-γ-producing cells, was calculated blindly by two investigators.

IL-2 ELISA

ELISA was performed using Abs and protocol developed by PharMingen. The sensitivity of the assay was 6 pg/ml.

Statistical methods

Statistical analysis was performed using parametric methods and JMP statistical software (SAS Institute, Cary, NC).

Results

As was previously reported production of Gr-1+ cells is dramatically increased in tumor-bearing mice. To test the levels of induction in our two experimental models BALB/c mice were inoculated s.c. with 3 × 10^7 MethA sarcoma cells, and C57BL/6 mice were inoculated s.c. with 5 × 10^6 C3 tumor cells. Mice were sacrificed 4–5 wk later when tumor size reached 1.5 cm in diameter. Splenocytes were collected, and the presence of Gr-1+ cells was evaluated by flow cytometry. Control BALB/c and C57BL/6 mice had a similar proportion of Gr-1+ cells in spleens (2.7 ± 0.4 and 3.1 ± 0.5%, respectively). The proportion of Gr-1+ in MethA sarcoma-bearing BALB/c mice was increased >5-fold (14.5 ± 1.3%, p < 0.05), whereas the presence of these cells in C3 tumor-bearing C57BL/6 mice increased almost 10-fold (24.6 ± 4.2%, p < 0.05). To clarify the phenotype of Gr-1+ in control and tumor-bearing mice, splenocytes were double-labeled with FITC-conjugated anti-Gr-1 Ab and PE-conjugated Abs against different markers. Almost all Gr-1+ cells from control (93.7 ± 5.6%) as well as from the tumor-bearing mice (95.8 ± 4.1%) were positive for CD11b marker specific for myeloid cells of the macrophage lineage (Fig. 1). Most of Gr-1+ cells (86.2 ± 8.7%) from control mice expressed MHC class I molecules (H2Dq), and 36.7 ± 5.8% of these cells were also MHC class II positive (IAq) (Fig. 1). A slightly lower proportion of Gr-1+ cells from tumor-bearing mice was MHC class I positive (67.3 ± 6.8%, p > 0.05), and only a minor part (11.9 ± 3.5%) of Gr-1+ cells from tumor-bearing mice expressed MHC class II molecules (Fig. 1). These cells did not express markers of progenitor or stem cells (CD34 and Sca-1; data not shown). Similar results were obtained from mice bearing C3 tumors (data not shown). Thus, production of Gr-1+ cells was significantly increased in tumor-bearing mice. These cells, similarly to Gr-1+ cells from control mice, were CD11b and MHC class I positive. However, most of these cells were MHC class II negative.
three performed experiments are shown. Similar results were obtained with sarcoma-bearing mice using cell sorting with FITC-conjugated anti-Gr-1 Ab. Splenocytes were obtained from control C57BL/6 mice and incubated (5 x 10^5 cells per ml) for 72 h in triplicate in U-bottom 96-well plates with different numbers of Gr-1^+ cells as indicated. Cells were stimulated with 1 mg/ml Con A. SI, Stimulation index calculated as T cell proliferation in the presence of Con A/spontaneous T cell proliferation.

**GR-1^+ CELLS MEDIATED IMMUNE DYSFUNCTION**

**Tumor-induced Gr-1^+ cells do not affect CD4-mediated T cell responses**

Gr-1^+ cells were isolated from spleens of tumor-bearing mice using cell sorting. First, we measured the effect of these cells on Con A-induced T cell proliferation. Gr-1^+ cells were incubated with syngenic control splenocytes and Con A. Gr-1^+ cells alone did not proliferate either spontaneously or in response to Con A (Fig. 2A). In both experimental tumor models, the presence of Gr-1^+ cells in cell culture at a Gr-1^+ cells:splenocytes ratio as high as 1:1 did not affect response of control T cells to Con A (Fig. 2, A and B). Effect of Gr-1^+ cells on T cells might depend on time of exposure. To test this possibility, splenocytes from control C57BL/6 mice were cultured overnight with Gr-1^+ cells obtained from C3 tumor-bearing mice at a 1:1 ratio. After that time cells were stimulated with Con A and proliferative response was measured using [3H]thymidine uptake. This extended incubation of splenocytes with Gr-1^+ cells did not affect T cell proliferation (data not shown). To verify the effect of Gr-1^+ cells on T cell function, we measured IL-2 production by T cells in response to Con A. Splenocytes isolated from control C57BL/6 mice were cultured at concentration 5 x 10^6 cells/ml with different numbers of Gr-1^+ cells in the presence of 1 μg/ml Con A. After 48-h incubation IL-2 was measured in supernatants using ELISA. Gr-1^+ cells did not inhibit Con A-inducible IL-2 production by T cells. At the highest ratio (1:1) Gr-1^+ cells stimulated Con A inducible, but not spontaneous IL-2 production by T cells (Fig. 2C). Gr-1^+ cells alone did not produce a detectable amount of IL-2 with or without Con A (data not shown).

To elucidate the possible impact of Gr-1^+ cells on Ag-specific CD4^+ T cell response we used T cells from TCR-transgenic mice. These mice express the γδ TCR specific for aa 110–120 from influenza HA presented by MHC class II (I-E^d^). T cells from these mice demonstrated a high level of a proliferative response to the specific HA peptide in vitro (17, 18). Lymph node cells isolated from these transgenic mice were cultured with Gr-1^+ cells and specific peptide. Gr-1^+ cells at ratio as high as 1:1 did not affect peptide-specific T cell proliferation (Fig. 3, A and B). No significant effect of Gr-1^+ cells on peptide-specific IL-2 production was found at ratios from 1:10 to 1:2. However, Gr-1^+ cells stimulated IL-2 production in response to the specific peptide at ratio 1:1 (Fig. 3C).

These results indicate that Gr-1^+ cells even at concentrations higher than those observed in tumor-bearing mice did not affect a CD4-mediated T cell response.

**Tumor-induced Gr-1^+ cells inhibit CD8-mediated T cell response**

Next, we asked whether Gr-1^+ cells were able to affect Ag-specific CD8-mediated response. BALB/c mice were immunized s.c. twice within a 2-wk interval with mutant p53 peptide in incomplete

catened (from 5 x 10^7 to 5 x 10^8). Cells were stimulated with 1 μg/ml Con A. [3H]Thymidine (1 μCi/well) was added 18 h before the end of the cultures. Cells were harvested and [3H]thymidine uptake was analyzed using a liquid scintillation counter. Cumulative results (average ± SD) of three performed experiments are shown. Similar results were obtained with 5 x 10^5 and 10^6 Gr-1^+ cells (data not shown). B, Gr-1^+ cells were obtained from spleens of C3 tumor-bearing mice using cell sorting with FITC-conjugated anti-Gr-1 Ab. Splenocytes were obtained from control C57BL/6 mice and incubated (5 x 10^6 cells per well) for 72 h in triplicate in U-bottom 96-well plates with different numbers of Gr-1^+ cells as indicated (from 5 x 10^7 to 5 x 10^8). Cells were stimulated with 1 μg/ml Con A. [3H]Thymidine (1 μCi/well) was added 18 h before the end of the cultures. Cells were harvested and [3H]thymidine uptake was analyzed using a liquid scintillation counter. Two experiments with the same results were performed. SI, Stimulation index calculated as T cell proliferation in presence of Con A/spontaneous T cell proliferation. C, Gr-1^+ cells were obtained from spleens of C3 tumor-bearing mice. Splenocytes were obtained from control C57BL/6 mice and incubated (5 x 10^6 cells per ml) for 48 h in triplicate in U-bottom 96-well plates with different numbers of Gr-1^+ cells as indicated. Cells were stimulated with 1 μg/ml Con A. Supernatants were collected and IL-2 was measured using ELISA as described in Materials and Methods. Average ± SD is shown. * Statistically significant differences from control (no Gr-1^+ cells) level (p < 0.05).
The number of Gr-1+ cells isolated from MethA sarcoma-bearing mice were cultured in triplicate with different concentrations of the specific HA peptide were used. [3H]Thymidine uptake was measured as T cell proliferation in the presence of peptide/spontaneous T cell proliferation. Typical results of two performed experiments are shown. C, Gr-1+ cells were obtained from splenocytes of MethA sarcoma-bearing mice. Lymph node cells were obtained from HA TCR-transgenic mice and incubated (5 x 10^6 cells per well) for 48 h in triplicate in U-bottom 96-well plates with different numbers of Gr-1+ cells as indicated. Cells were stimulated with 12.5 μg/ml of HA specific peptide. Supernatants were collected, and IL-2 was measured using ELISA as described in Materials and Methods. *, Statistically significant differences from control (no Gr-1+ cells) level (p < 0.05).

Mechanism of Gr-1+ cells mediated inhibition of immune response

We asked whether the described effects of Gr-1+ cells might be mediated by soluble factors. To test this possibility Gr-1+ cells were cultured for 24 h at 37°C in a concentration 10 times higher than that used in previous experiments (10^6 cells/ml). Conditioned media were collected and added to splenocytes from immunized control mice. Presence of Gr-1+ cell-conditioned media at a concentration as high as 20% did not affect T cell response to the specific peptide (Fig. 5A). Similar results were observed when conditioned media were obtained from Gr-1+ cells stimulated with 10 μM specific peptide (data not shown). Because many soluble factors able to affect CD8+ T cells are short-lived, another set of experiments has been performed. Gr-1+ cells (2 x 10^6 cells per well) were placed on top of semi-permeable membrane (pore size 0.2 μm). Splenocytes from immunized mice (10^5 cells per well) were placed in the bottom chamber of a 96-well plate. Cells were cocultured in the presence of the specific peptides for 24 h, and an ELISPOT assay was performed as described in Materials and Methods.
Methods. No inhibition of IFN-γ production was detected under these experimental conditions (Fig. 5B).

Gr-1+ cells are comprised of immature macrophages and myeloid cells known to produce NO. NO is a well-described factor that inhibits T cell function (19). To investigate a possible role of NO in the observed effects we used a competitive inhibitor of NO synthase N(G)-monomethyl-L-arginine (LMMA) (20). LMMA at a concentration of 0.5 mM significantly decreased the inhibitory effect of Gr-1+ cells on splenocytes (Fig. 5C). The effect of LMMA was more prominent with increased numbers of Gr-1+ cells. This suggests that NO production is involved in Gr-1+ cell-mediated inhibition of T cell responses. As we demonstrated above, a substantial proportion of tumor-induced Gr-1+ cells expressed MHC class I molecules, but were negative for MHC class II. To investigate possible involvement of MHC class I in the described inhibition of T cell response, Gr-1+ cells from tumor-bearing C57BL/6 mice (H2Db) were incubated on ice for 30 min with 3 μg of either specific anti-H2Db Ab or control anti-H2Dd Ab. After that time cells were washed and incubated at 37°C for 1 h in complete culture medium to allow for internalization of the specific molecules. Cells were then washed again and added to splenocytes obtained from immunized C57BL/6 mice and stimulated with the specific peptide as described above. Pretreatment of Gr-1+ cells with anti-H2Db, but not with H2Dd Ab completely abrogated the inhibitory effect of these cells on T cells (Fig. 5D). These data indicate that MHC class I molecules are closely involved in Gr-1+ cell-mediated T cell inhibition.

Differentiation of Gr-1+ cells abrogates negative effect on T cell response

Next we investigated whether differentiation of Gr-1+ cells might eliminate their inhibition of T cells. Gr-1+ cell were isolated from

*FIGURE 4. Gr-1+ cells inhibit CD8-mediated T cell response. A, Splenocytes were obtained from immunized BALB/c mice as described in Materials and Methods. Red cells were removed by osmotic lysis, and 2 × 10^5 splenocytes were cultured in triplicate with 2 × 10^4 Gr-1+ cells isolated from MethA sarcoma-bearing mice. The number of IFN-γ-producing cells in response to stimulation with specific and control peptides was analyzed in triplicate in an ELISPOT assay as described in Materials and Methods. The number of IFN-γ-producing cells was recalculated per 10^6 splenocytes. Averages ± SD of two performed experiments is shown. *, Statistically significant differences from control peptide level (p < 0.05). B, Splenocytes were obtained from immunized C57BL/6 mice as described in Materials and Methods. RBC were removed by osmotic lysis, and 2 × 10^5 splenocytes were cultured in triplicate with different numbers of Gr-1+ cells isolated from C3 tumor-bearing mice. The number of IFN-γ-producing cells in response to stimulation with specific and control peptides was analyzed in an ELISPOT assay as described in Materials and Methods. The number of IFN-γ-producing cells was recalculated per 10^6 splenocytes. Averages ± SD of one typical experiment is shown. Five experiments with the same results were performed. *, Statistically significant differences from control peptide level (p < 0.05). C, Gr-1+ cells (3 × 10^5) obtained from C3 tumor-bearing mice were pulsed for 1 h with 10 μM of control or specific peptide, washed, and injected i.v. into the tail vein of immunized C57BL/6 mice. Injection was repeated 24 h later. Twenty-four hours after the second injection, mice were sacrificed and an ELISPOT assay was performed with splenocytes stimulated with control or specific peptides. The number of IFN-γ-producing cells was recalculated per 10^6 splenocytes. Cumulative results (average ± SD) of four performed experiments are shown. *, Statistically significant differences from control peptide level (p < 0.05). D, Splenocytes were obtained from mice after in vivo treatment with Gr-1+ cells as described above. Cells were then stimulated with Con A for 3 days, and [3H]thymidine uptake was measured as described in Fig. 2A. Cumulative results of three performed experiments are shown.
A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

FIGURE 5. Mechanism of Gr-1+ effects on T cell responses. Splenocytes were isolated from immunized C57BL/6 mice, and Gr-1+ cells were isolated from C3 tumor-bearing mice as described above. A, Gr-1+ cell-conditioned medium was obtained after 24-h incubation of Gr-1+ cells in complete culture medium at a concentration of 10^6 cells/ml. Supernatants were collected and added immediately in triplicate to 2 × 10^5 splenocytes at indicated concentrations. The number of IFN-γ-producing cells was analyzed using the ELISPOT assay as described above. Cumulative results (average ± SD) of three performed experiments are shown. *, Statistically significant differences from control peptide level (p < 0.05). B, Splenocytes from immunized C57BL/6 mice were placed in triplicate (10^5 cells per well) into 96-well Millipore MultiScreen-HA plates precoated with anti-IFN-γ Ab. C, 60% of the cells treated with GM-CSF or GM-CSF and IL-4, respectively, showed similar results. Phenotypic analysis of these cells by flow cytometry revealed that all cells retained CD11b and MHC class I. More than half of the cells lost Gr-1 expression after incubation with GM-CSF alone and >80% after incubation with GM-CSF and IL-4. A combination of GM-CSF and ATRA decreased the presence of Gr-1+ cells even further (Fig. 6A). The proportion of the cells with surface expression of MHC class II increased >2-fold compared with freshly isolated Gr-1+ cells. Almost 10% of the cells treated with GM-CSF or GM-CSF and IL-4.
but not with ATRA, expressed both MHC class II and B7-2 (Fig. 6A). No such cells were detected among freshly isolated Gr-1+ cells (data not shown). To investigate whether these cultured cells retained their ability to inhibit peptide-specific T cell response, splenocytes from immunized mice were stimulated with peptides in the presence of freshly isolated Gr-1+ cells or Gr-1+ cells cultured for 5–6 days with GM-CSF alone or with a combination of GM-CSF and IL-4 or ATRA. Freshly isolated Gr-1+ cells decreased the number of peptide-specific IFN-γ-producing cells >5-fold (Fig. 6B). This inhibitory potential was completely lost after a 5- to 6-day culture with all tested substances. Control levels of IFN-γ-producing cells were seen in the presence of as much as 10% of these cells (Fig. 6B).

Discussion
In this study we have demonstrated that Gr-1+ immature myeloid cells produced in large numbers in tumor-bearing mice inhibit Ag-specific CD8-, but not CD4-mediated T cell responses. Presentation of the specific Ags by MHC class I molecules apparently plays a critical role in this inhibition.

Several groups have previously reported increased accumulation of immature myeloid cells and Mac-1+ (CD11b) macrophages in tumor-bearing hosts (2, 5, 6, 24). More recently, increased production of a more defined population of Gr-1+ CD11b+ immature myeloid cells has been described in several mouse tumor models (10, 11). In this study we have also observed a 5- to 10-fold increase in the presence of these cells in two different tumor models. Thus it appears that increased production of immature myeloid cells in cancer is a widespread phenomenon. Increased production of these cells might be triggered by different soluble tumor-derived factors. Treatment of mice with one of these factors, vascular endothelial growth factor (VEGF), resulted in dramatic accumulation of Gr-1+ cells in peripheral lymphoid organs (25). These cells may play an important role in the inability of the immune system to recognize and eliminate tumor cells.

In this study we sought to clarify the effect of these cells on specific T cell functions. Previous studies have demonstrated that macrophages and myeloid cell-enriched population of splenocytes from tumor-bearing mice inhibited T cell proliferation in response to CD3 ligation or various mitogens (4, 11, 26). To our surprise, in both tested models Gr-1+ cells isolated by cell sorting from tumor-bearing mice did not affect Con A-inducible proliferation or IL-2 production by T cells isolated from syngeneic control animals. To test the effect of Gr-1+ cells on T cell proliferation induced via TCR complex we used Ag-specific T cells from transgenic mice. These cells have αβ TCR specific for HA peptide presented by MHC class II. Stimulation of these T cells with the specific peptide resulted in strong proliferative response and IL-2 production, and Gr-1+ myeloid cells did not affect T cell response to this peptide. Thus, Gr-1+ cells did not impair CD4-mediated T cell response. These data are somewhat in contrast to previously published observations (9, 11). It can be explained by the fact that in this study we used highly purified Gr-1+ cells. In all previous studies enriched populations of APCs, macrophages, or myeloid cells were used. It is likely that the presence of mature or immature macrophages in those cell fractions may dramatically affect the result of the experiments. Mature and immature macrophages are known to produce a variety of different factors that nonspecifically inhibit T cell function. Indeed, blockade of TGF-β and inhibition of NO production by these cells completely abrogated the observed inhibitory effect of myeloid cells in previous studies (11, 26).

We have investigated whether the same number of Gr-1+ cells might affect CD8-mediated T cell response. Two models with defined MHC class I restricted peptides were used. These peptides are presented by MHC class I and specifically activate CD8+ CTL (14, 16, 27). To test T cell responses we measured peptide-specific IFN-γ production by T cells in an ELISPOT assay. Specific peptides induced a 3- to 5-fold increase in the number of IFN-γ-producing cells in immunized mice. In seven independently performed experiments using two different experimental models, Gr-1+ cells at a ratio as low as 1:20 almost completely abrogated this increase. This suggests that the presence of small numbers of Gr-1+ myeloid cells may be sufficient to inhibit a CD8-mediated T cell response. These findings were confirmed by in vivo experiments. T cells from mice treated with specific peptide-loaded
Gr-1$^+$ cells lost their ability to respond to this peptide although they reacted normally to stimulation with Con A. This supports the hypothesis that relatively small numbers of Gr-1$^+$ cells induce Ag-specific, but not a general immune suppression.

What could be a mechanism of this inhibition? It appears that direct cell-to-cell contact is required for Gr-1$^+$ cell-mediated inhibition of T cell response, because conditioned medium from Gr-1$^+$ cells did not affect T cell response to the peptide. Experiments with semi-permeable membranes confirmed these conclusions. Our data suggest that NO may be involved in described Gr-1$^+$ cell-mediated effects. It appears that the role of NO increases with increased number of Gr-1$^+$ cells. If high concentration of Gr-1$^+$ cells is used (as was done in previous studies), NO production plays a critical role in nonspecific T cell inhibition by Gr-1$^+$ cells. If a relatively low proportion of Gr-1$^+$ cells is used (2.5–10%), the NO role is not so prominent, although still important. It is possible that NO production by Gr-1$^+$ cells is increased after their contact with other cells. The mechanism of NO involvement is under investigation at this time.

However, these data could not explain a dichotomy in Gr-1$^+$ cell effects on CD4$^+$ and CD8$^+$-mediated T cell responses. The explanation may lie in the phenotype of these cells. In contrast to Gr-1$^+$ cells present in control mice, Gr-1$^+$ cells from tumor-bearing animals expressed little or no MHC class II molecules, whereas they retained relatively high level MHC class I molecules. We hypothesized that these cells were unable to present Ags via MHC class II and, therefore, could not affect CD4$^+$-mediated T cell response. Conversely, immature Gr-1$^+$ myeloid cells were able to present Ags in the context of MHC class I. Therefore, these cells can specifically block MHC class I-restricted T cell response. To test this hypothesis we blocked MHC class I expression on the surface of Gr-1$^+$ cells using mAb and then tested their ability to inhibit T cell response to the specific peptide. mAb against MHC class I completely abrogated Gr-1$^+$ cell-mediated inhibition of T cell response to the peptide. This indicates that observed inhibition is mediated by MHC class I presentation of the Ag. The exact mechanism of this inhibition is under investigation at this time.

We also investigated whether differentiation of Gr-1$^+$ cells may decrease or eliminate their inhibitory potential. A previous study has suggested that differentiation of immature cells may reduce their inhibitory activity (5). In this study we have shown that Gr-1$^+$ cells were not able to survive 5- to 6-day culture without presence of growth factors. This supports the hypothesis that these cells are not a subset of macrophages, but truly immature myeloid cells. In vitro culture of Gr-1$^+$ cells with GM-CSF alone or with GM-CSF and IL-4 down-regulated Gr-1$^+$ expression and increased the proportion of cells expressing MHC class II molecules. These cells did not block a peptide-specific T cell response. A naturally occurring isomer of retinoic acid, ATRA is a well-known naturally occurring isomer of retinoic acid, ATRA is a well-known

These cells did not block a peptide-specific T cell response. ATRA may also affect the growth of normal hemo-cell line HL-60 and freshly isolated acute promyelocytic leukemia

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