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Mechanism of Immune Dysfunction in Cancer Mediated by Immature Gr-1⁺ Myeloid Cells¹

Dmitry I. Gabrilovich,^{2*} Markwin P. Velders,[†] Eduardo M. Sotomayor,^{*} and W. Martin Kast[†]

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 CD3 ζ 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 $\alpha\beta$ TCR specific for aa 110–120 from influenza hemagglutinin (HA)³ presented by I-E^d 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 (KYICNSSCM) 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 (KYMCNSSCM) 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 H2D^b restricted dominant HPV16E7 peptide RAHYNIVTF was used in this study (35). Bullets containing 2 μ g DNA per shot were generated according to the

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³ Abbreviations used in this paper: HA, hemagglutinin; HPV, human papillomavirus; ATRA, all-*trans* retinoic acid; ELISPOT, enzyme-linked immunospot; LMMA, N^ε-monomethyl-L-arginine.

manufacturer's protocols. Briefly, 100 μ g DNA was precipitated on 25 mg 1- μ m gold particles in the presence of 100 μ l 0.05 M spermidine (Sigma, St. Louis, MO) using 100 μ l 1 M CaCl₂. The particles were washed three times with 1 ml 100% ethanol and resuspended in 3 ml 0.1 mg/ml polyvinylpyrrolidone (PVP) in 100% ethanol. The gold was then loaded into the tubing using the tubing prep station (Bio-Rad), and the gold-loaded tubing was cut into 0.5-inch pieces, then loaded into the cartridges. C57BL/6 mice were anesthetized by i.p. injection of 2.4 mg ketamine (Abbott Laboratories, Abbott Park, IL) mixed in 80 μ l PBS with 0.48 mg xylazine (Sigma). The abdominal area was shaved and the DNA was delivered with the gene gun into the epidermis at a helium pressure of 450 psi. This procedure was repeated 2 wk after the first DNA delivery.

Reagents

The following Ab-producing hybridomas were obtained from American Type Culture Collection (ATCC, Manassas, VA) and used as culture supernatants: anti-CD4 (L3T4, TIB-207), anti-CD8 (Lyt-2.2, TIB-210), anti-MHC class II (I-A^d, 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-A^b, and I-A^d 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 μ m were obtained from Nalgen. Complete culture medium included RPMI 1640 supplemented with 10% FCS, antibiotics, and 5×10^{-6} 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 FACS-Calibur 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, nonadherent 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 (10^5 /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, SFERFEIFPKE) and cultured for 3 days. Eighteen hours before harvesting, cells were pulsed with [³H]thymidine (1 μ Ci/well; Amersham, Arlington Heights, IL). [³H]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 (2×10^5 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 μ M. After that time, wells were washed and then incubated overnight at 4°C with a different clone of 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 level of this induction in our two experimental models BALB/c mice were inoculated s.c. with 3×10^5 MethA sarcoma cells, and C57BL/6 mice were inoculated s.c. with 5×10^5 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 $3.1 \pm 0.5\%$, respectively). The proportion of Gr-1⁺ in MethA sarcoma-bearing BALB/c mice was increased >5-fold ($14.5 \pm 1.3\%$, $p < 0.05$), whereas the presence of these cells in C3 tumor-bearing C57BL/6 mice increased almost 10-fold ($24.6 \pm 4.2\%$, $p < 0.05$). 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 ($93.7 \pm 5.6\%$) as well as from the tumor-bearing mice ($95.8 \pm 4.1\%$) were positive for CD11b marker specific for myeloid cells of the macrophage lineage (Fig. 1). Most of Gr-1⁺ cells ($86.2 \pm 8.7\%$) from control mice expressed MHC class I molecules (H2D^d), and $36.7 \pm 5.8\%$ of these cells were also MHC class II positive (IA^d) (Fig. 1). A slightly lower proportion of Gr-1⁺ cells from tumor-bearing mice was MHC class I positive ($67.3 \pm 6.8\%$, $p > 0.05$), and only a minor part ($11.9 \pm 3.5\%$) 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.

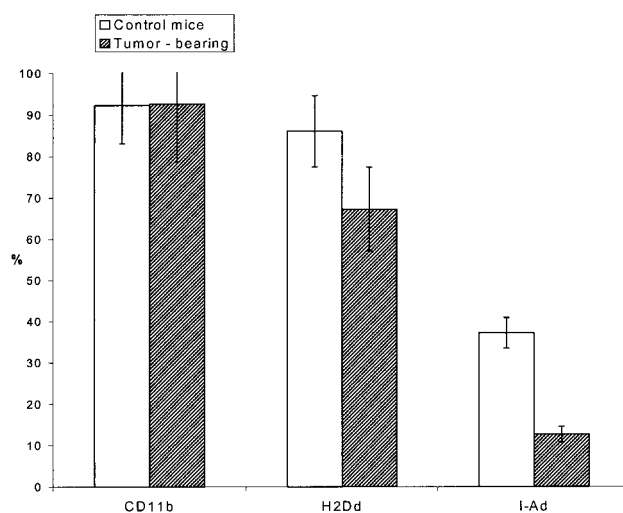


FIGURE 1. Tumor-induced Gr-1⁺ cells lack MHC class II molecules. Splenocytes obtained from control BALB/c and MethA sarcoma-bearing mice were labeled with FITC-conjugated anti-Gr-1 Ab and PE-conjugated anti-CD11b, IA^d, or H2D^d Abs. The proportion of PE-positive cells was calculated among FITC-positive Gr-1⁺ cells. Averages \pm SD of five performed experiments are shown. *, Statistically significant differences from control mice ($p < 0.05$).

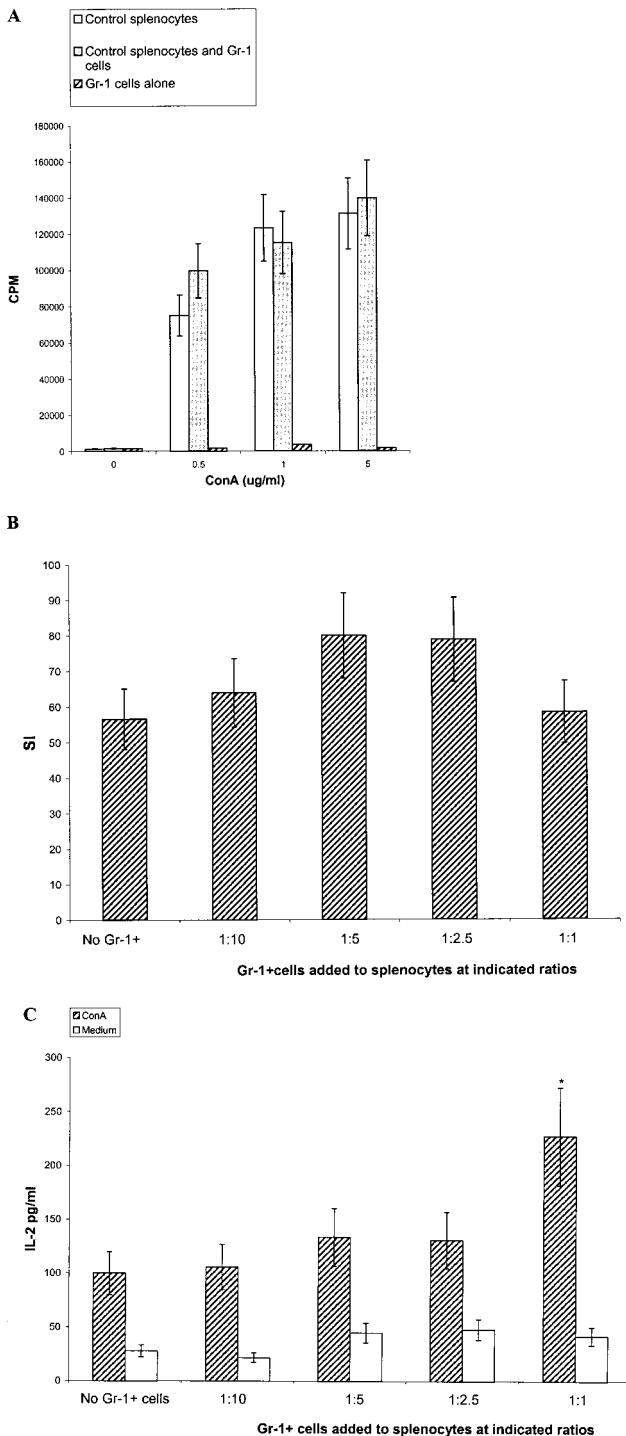


FIGURE 2. Tumor-induced Gr-1⁺ cells do not inhibit Con A-mediated T cell responses. **A**, Gr-1⁺ cells were obtained from spleens of MethA sarcoma-bearing mice using cell sorting with FITC-conjugated anti-Gr-1⁺ Ab. Splenocytes were obtained from control BALB/c mice and incubated (10^5 cells per well) for 72 h in triplicates in U-bottom 96-well plates with 10^4 Gr-1⁺ cells. For analysis of Gr-1⁺ cell proliferation, 5×10^4 of these cells were incubated alone. Different concentrations of Con A were used as indicated. [³H]Thymidine ($1 \mu\text{Ci}/\text{well}$) 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 \pm SD) of three performed experiments are shown. Similar results were obtained with 5×10^3 and 10^3 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 (5×10^5 cells per well) for 72 h in triplicate in U-bottom 96-well plates with different numbers of Gr-1⁺ cells as indi-

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 5×10^5 cells/ml with different numbers of Gr-1⁺ cells in the presence of $1 \mu\text{g}/\text{ml}$ 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 $\alpha\beta$ TCR specific for aa 110–120 from influenza HA presented by MHC class II (IE^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

ated (from 5×10^3 to 5×10^4). Cells were stimulated with $1 \mu\text{g}/\text{ml}$ Con A. [³H]Thymidine ($1 \mu\text{Ci}/\text{well}$) 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 (5×10^5 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 $1 \mu\text{g}/\text{ml}$ Con A. Supernatants were collected and IL-2 was measured using ELISA as described in *Materials and Methods*. Average \pm SD is shown. *, Statistically significant differences from control (no Gr-1⁺ cells) level ($p < 0.05$).

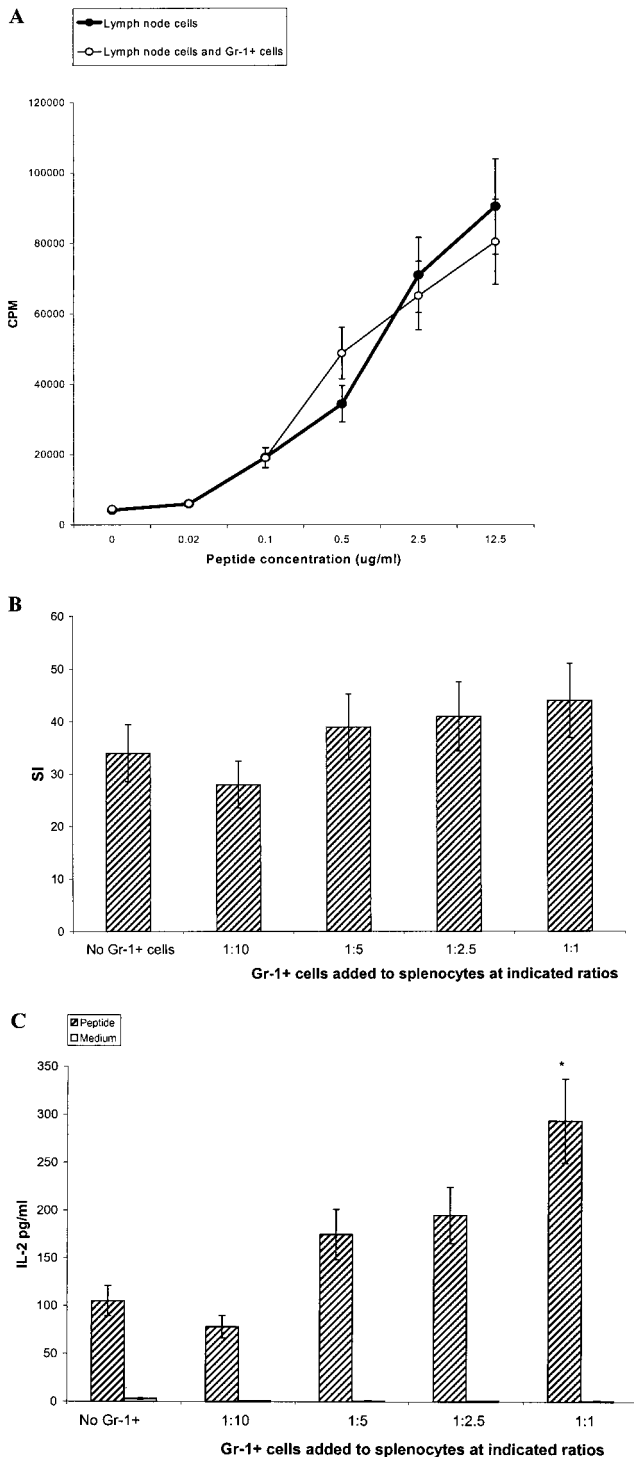


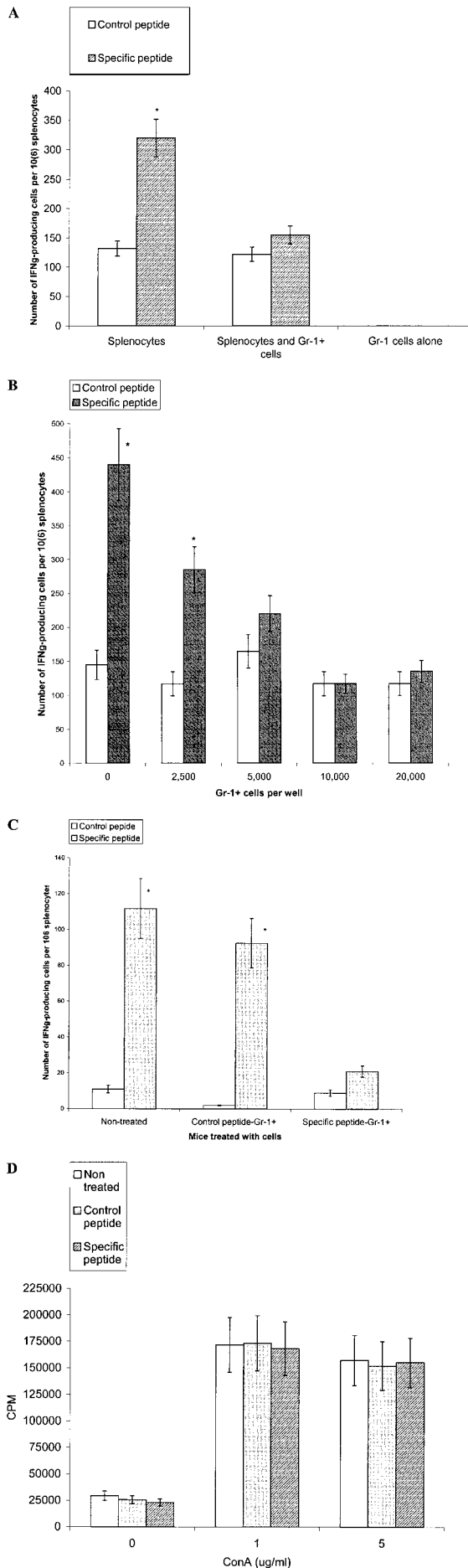
FIGURE 3. Tumor-induced Gr-1⁺ cells do not inhibit CD4-mediated T cell responses. *A*, Lymph node cells were obtained from HA TCR-transgenic mice and incubated in triplicate (10^5 cells per well) with 10^4 Gr-1⁺ cells from MethA sarcoma-bearing mice for 3 days. Similar results were obtained with 5×10^3 and 10^3 Gr-1⁺ cells (data not shown). Different concentrations of the specific HA peptide were used. [³H]Thymidine uptake was measured as described above. Typical results (average \pm SD) of three performed experiments are shown. Similar results were observed with splenocytes (data not shown). *B*, Lymph node cells (10^5 cells per well) from HA TCR-transgenic mice were cultured in triplicate with different numbers of Gr-1⁺ cells from MethA sarcoma-bearing mice in the presence of 12.5 μ g/ml of specific peptide. [³H]Thymidine uptake was measured 3 days later. SI, Stimulation index calculated as T cell proliferation in the presence of the peptide/spontaneous T cell proliferation. Typical results of two performed experiments are shown. *C*, Gr-1⁺ cells were obtained from

Freund's adjuvant. This peptide is presented by MHC class I. Ten days after the second injection mice were sacrificed and splenocytes were stimulated with control or specific peptides in the presence of Gr-1⁺ cells isolated from MethA sarcoma-bearing mice. The number of IFN- γ -producing cells was analyzed using an ELISPOT assay. An almost 3-fold increase in the number of these cells was detected in response to the specific peptide. However, the presence of Gr-1⁺ cells at a Gr-1⁺ cell:splenocyte ratio of 1:10 completely abrogated this increase (Fig. 4A). To confirm this effect in a different strain of mice and different tumor model, C57BL/6 mice were immunized with DNA encoding the H2D^b-restricted HPV16E7-specific peptide as described in *Materials and Methods*. Ten days after the last immunization the mice were sacrificed, and their splenocytes were collected and incubated with control or specific peptides in the presence of Gr-1⁺ cells isolated from syngeneic tumor-bearing mice. Incubation of splenocytes with the specific peptide significantly increased the number of IFN- γ -producing cells. The presence of Gr-1⁺ cells at a concentration as low as 2.5% significantly decreased that number and at a concentration of 5% completely eliminated peptide-specific response (Fig. 4B). To investigate whether the same effect can be observed in vivo, 3×10^5 Gr-1⁺ cells isolated from tumor-bearing mice were pulsed with control or specific peptide for 2 h, washed, and injected i.v. into immunized C57BL/6 mice. Injections were repeated the next day and 24 h later mice were sacrificed, and splenocytes were isolated and stimulated with control or specific peptides in an ELISPOT assay. Mice treated with Gr-1⁺ cells loaded with the specific, but not with control peptide almost completely blocked T cell response to the specific peptide as measured in the ELISPOT assay (Fig. 4C). To exclude the possibility that peptide nonresponsiveness was due to general suppression of T cell function, splenocytes obtained from the same treated mice were stimulated with Con A. Splenocytes from all mice responded equally well to Con A (Fig. 4D). These data indicate that Gr-1⁺ cells inhibited peptide-specific CD8-mediated immune responses in vitro and in vivo.

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 at 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 μ M 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 (2×10^4 cells per well) were placed on top of semi-permeable membrane (pore size 0.2 μ m). 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*

spleens of MethA sarcoma-bearing mice. Lymph node cells were obtained from HA TCR-transgenic mice and incubated (5×10^5 cells/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 12.5 μ g/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 ($p < 0.05$).



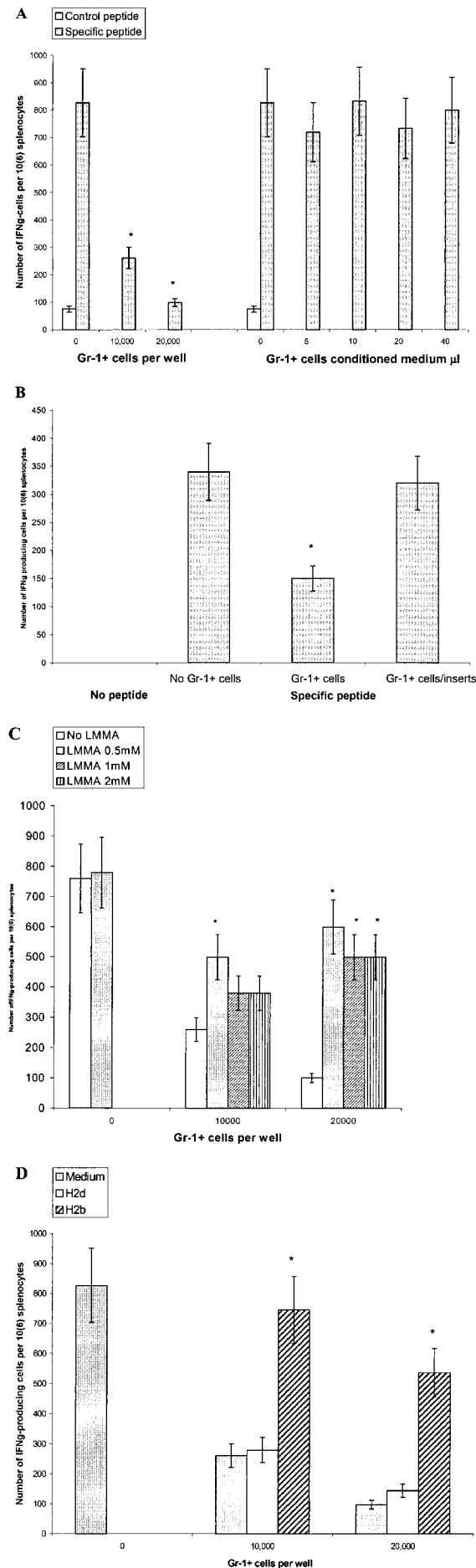
Methods. No inhibition of IFN- γ production was detected under these experimental conditions (Fig. 5B).

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 (H2D^b) were incubated on ice for 30 min with 3 μ g of either specific anti-H2D^b Ab or control anti-H2D^d 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-H2D^b, but not with H2D^d 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 \pm 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 \pm 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 μ M 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 \pm 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.



spleens of tumor-bearing mice and cultured for 5–6 days in complete medium. Less than 10% viable cells were found after that time. This supports the hypothesis that those Gr-1⁺CD11b⁺ cells were in fact immature myeloid cells incapable of surviving without growth factors. Different concentrations of GM-CSF were used to support cell viability and differentiation. In addition to GM-CSF we used IL-4 and ATRA. IL-4 is known to selectively inhibit macrophage differentiation and to promote differentiation of the dendritic cells. ATRA is a natural oxidative metabolite of vitamin A and is known to be a regulator of cell differentiation (21, 22). ATRA induces terminal differentiation of promyelocytes into mature neutrophils in patients with M3 (acute promyelocytic) leukemia (23). Recently, we have shown that ATRA induced differentiation of immature myeloid cells isolated from cancer patients (8). Because Gr-1⁺ cells were represented by immature myeloid cells on early stages of differentiation we hypothesized that these cells can be differentiated into mature cells by ATRA. Five- to 6-day incubation of Gr-1⁺ cells with GM-CSF at optimal concentration (20 ng/ml) provided >90% cell viability. The total number of viable cells remained the same as was used at the start of the culture. Combination of GM-CSF with 10 ng/ml IL-4 or with 1 μ M ATRA showed similar results. Phenotypic analysis of these cells by flow cytometry revealed that all cells retained CD11b and MHC class I expression. 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,

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 10⁶ cells/ml. Supernatants were collected and added immediately in triplicate to 2 × 10⁵ splenocytes at indicated concentrations. The number of IFN- γ -producing cells was analyzed using the ELISPOT assay as described above. Cumulative results (average \pm 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 (10⁵ cells per well) into 96-well Millipore MultiScreen-HA plates precoated with anti-IFN- γ Ab as described in *Materials and Methods*. Gr-1⁺ cells (2 × 10⁴) were placed on top of cell culture inserts (Nunc, Naperville, IL) with a pore size of 0.2 μ m, and inserts were placed in wells with splenocytes. Culture medium contained IL-2 and specific peptide as described in *Materials and Methods*. After 24-h incubation, inserts were removed and an ELISPOT assay was performed as described in *Materials and Methods*. Results are labeled as “Gr-1⁺ cells/inserts”. In parallel wells splenocytes were cultured either alone (no Gr-1⁺ cells) or with Gr-1⁺ cells added directly to splenocytes (Gr-1⁺ cells). Average \pm SD of one experiment is shown. Two experiments with similar results were performed. *, Statistical significant differences from control level (no Gr-1⁺ cells) ($p < 0.05$). *C*, Splenocytes (2 × 10⁵ per well) from immunized mice were incubated with indicated number of Gr-1⁺ cells in the presence of different concentrations of LMMA. Cumulative results of three performed experiments are shown. *, Statistically significant differences from control peptide level ($p < 0.05$). *D*, Gr-1⁺ cells isolated from C3 tumor-bearing mice were treated for 30 min on ice with 3 μ g anti-H2D^d or anti-H2D^b Abs, washed, incubated in complete medium for 1 h at 37°C, washed again, and added to splenocytes isolated from immunized mice. An ELISPOT assay was performed as described in Fig. 4. The number of peptide-specific IFN- γ -producing cells was calculated per 10⁶ splenocytes. Two experiments with similar results were performed. Average \pm SD of one experiment is shown. *, Statistically significant differences from control peptide level ($p < 0.05$).

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 $\alpha\beta$ 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

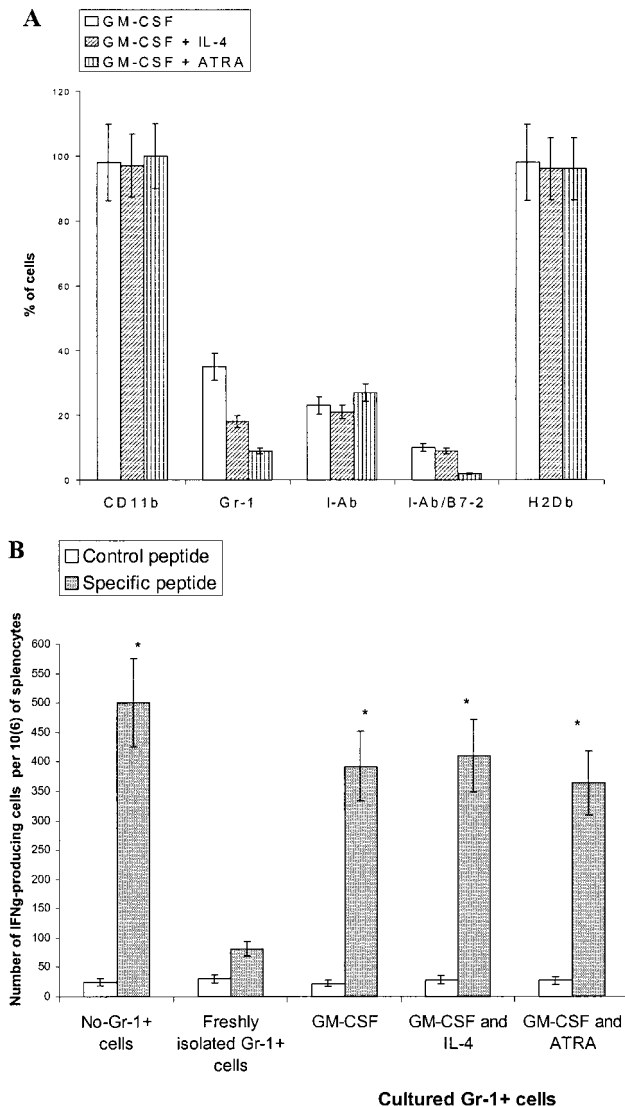


FIGURE 6. Negative effect of Gr-1⁺ cells can be reversed by incubation with growth factors and differentiation agent. Gr-1⁺ cells were isolated from C3 tumor-bearing C57BL/6 mice and incubated for 5–6 days with 20 ng/ml GM-CSF, GM-CSF and 10 ng/ml IL-4, or GM-CSF and 1 μ M ATRA. After that time cells were collected, washed, and used for analysis. **A**, Phenotype of cells was analyzed by flow cytometry using indicated Abs. Proportion of positive cells is shown. Cumulative results of three performed experiments are shown. **B**, ELISPOT assay with splenocytes isolated from immunized C57BL/6 mice was performed as described above. Cultured Gr-1⁺ cells (2×10^4 per well) were added to 2×10^5 splenocytes and incubated in presence of the specific or control peptides. Three experiments with similar results were performed. Average \pm SD of one experiment is shown. *, Statistically significant differences from control peptide level ($p < 0.05$).

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 factor capable of induction of differentiation of human leukemia cell line HL-60 and freshly isolated acute promyelocytic leukemia cells (28, 29). ATRA may also affect the growth of normal hemopoietic progenitors and blast progenitors in acute myelogenous leukemia. However, these effects of ATRA depend on culture conditions (30–32). Because of the nature of Gr-1⁺CD11b⁺ immature myeloid cells we investigated whether addition of ATRA may help in the differentiation of these cells. Addition of ATRA to GM-CSF dramatically reduced expression of Gr-1⁺ on cell surface, but did not significantly affect expression of MHC class II or B7-2. However, these cells also did not inhibit peptide-specific T cell response. Currently we are investigating possible mechanisms of these effects.

Our data indicate that the presence of small numbers of immature myeloid cells may inhibit Ag-specific CD8-mediated T cell

response. Advanced stage cancer is associated with increased accumulation of immature myeloid cells, which are able to block Ag-specific T cell responses (7, 8). This may provide one of the possible explanations of the difficulties in achieving a clear therapeutic effect of tumor vaccines in some preclinical and clinical studies dealing with advanced disease. Our study demonstrates a necessity of a combination of vaccination with a treatment able to eliminate immature myeloid cells. Differentiation of these cells may be one of the approaches. It is possible that positive effects of GM-CSF-producing tumor vaccines (33, 34) may be associated with reduction of immature myeloid cells and elimination of negative signal delivered by these cells. ATRA may be another potential adjuvant for vaccination of patients with advanced stage cancer.

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