Tumor-Induced CD11b<sup>+</sup>Gr-1<sup>+</sup> Myeloid Cells Suppress T Cell Sensitization in Tumor-Draining Lymph Nodes

Satoshi Watanabe, Katsuya Deguchi, Rongxiu Zheng, Hidemasa Tamai, Li-xin Wang, Peter A. Cohen and Suyu Shu

*J Immunol* 2008; 181:3291-3300; doi: 10.4049/jimmunol.181.5.3291
http://www.jimmunol.org/content/181/5/3291

**References**
This article cites **49 articles**, 31 of which you can access for free at: http://www.jimmunol.org/content/181/5/3291.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Tumor-Induced CD11b<sup>+</sup>Gr-1<sup>+</sup> Myeloid Cells Suppress T Cell Sensitization in Tumor-Draining Lymph Nodes<sup>1</sup>

Satoshi Watanabe,* Katsuya Deguchi,* Rongxiu Zheng,† Hidemasa Tamai,‡ Li-xin Wang,* Peter A. Cohen,* and Suyu Shu<sup>2,2</sup>*

Suppression of tumor-specific T cell sensitization is a predominant mechanism of tumor escape. To identify tumor-induced suppressor cells, we transferred spleen cells from mice bearing progressive MCA205 sarcoma into sublethally irradiated mice. These mice were then inoculated subdermally with tumor cells to stimulate T cell response in the tumor-draining lymph-node (TDLN). Tumor progression induced splenomegaly with a dramatic increase (22.1%) in CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid-derived suppressor cells (MDSC) compared with 2.6% of that in normal mice. Analyses of therapeutic effects by the adoptive immunotherapy revealed that the transfer of spleen cells from tumor-bearing mice severely inhibited the generation of tumor-immune T cells in the TDLN. We further identified MDSC to be the dominant suppressor cells. However, cells of identical phenotype from normal spleens lacked the suppressive effects. The suppression was independent of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. Intracellular IFN-γ staining revealed that the transfer of MDSC resulted in a decrease in numbers of tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Transfer of MDSC from MCA207 tumor-bearing mice also suppressed the MCA205 immune response indicating a lack of immunologic specificity. Further analyses demonstrated that MDSC inhibited T cell activation that was triggered either by anti-CD3 mAb or by tumor cells. However, MDSC did not suppress the function of immune T cells in vivo at the effector phase. Our data provide the first evidence that the systemic transfer of MDSC inhibited and interfered with the sensitization of tumor-specific T cell responses in the TDLN. The Journal of Immunology, 2008, 181: 3291–3300.

D

epite the demonstration that antitumor immunity is trig-
gerated in tumor-bearing hosts, tumors often grow pro-
gressively, escaping the attack by the immune system.

Suppression of tumor-specific effector T cell generation by tumor-
induced suppressor cells may be one of the important mechanisms of
tumor escape. Recent evidence has documented that tumor
growth actively induces suppressor cells by several distinct mech-
isms. Studies in animal models have shown that a variety of
suppressor cells in the tumor-bearing host constitute the immuno-
suppressive network (1, 2). Among functional suppressor cells,
CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (Treg<sup>3</sup>) (3), suppressive
macrophages (4, 5), and CD11b<sup>+</sup>Gr-1<sup>+</sup> myeloid-derived suppres-
sor cells (MDSC) (6, 7) have been experimentally implicated as
dominant suppressors. However, mechanisms of immune sup-
pression and their contributions to tumor progression remain
poorly defined. Elucidation of the characteristics of these suppres-
sor cells and their mechanisms of suppression will help develop
new strategies of tumor immunotherapy.

<sup>1</sup> Abbreviations used in this paper: Treg, regulatory T cell; MDSC, myeloid-derived
suppressor cell; TDLN, tumor-draining lymph-node; s.d., subdermally; Foxp3, fork-
head box P3.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

MDSC are precursors of macrophages, dendritic cells, granulo-
cytes, and myeloid cells. MDSC are usually defined as
CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in the mouse. The accumulation of MDSC in
lymphoid organs, tumor masses, and peripheral blood has been
observed in tumor-bearing individuals and is often associated
with large tumor burdens. Tumor cells often produce a variety of
cytokines, such as GM-CSF, M-CSF, IL-3, IL-6, and VEGF, to pre-
vent myeloid differentiation and result in expansion and accumu-
lation of MDSC (8). MDSC are capable of inhibiting antitumor
immune responses of CD4<sup>+</sup> T cells (7, 9), CD8<sup>+</sup> T cells (10, 11),
and NK cells (12, 13). In vitro studies provided evidence that
MDSC can do the following: 1) inhibit T cell proliferation in re-
response to various mitogens and antigenic peptides (9), 2) induce
loss of the expression of TCR ζ-chain (14), 3) suppress CTL in-
duction by allo-Ag (15) and irradiated tumor cells (16), and 4)
down-regulate IFN-γ production by T cells in response to specific
peptides (17). The mechanisms of suppressive function of MDSC
have been well studied in vitro, but there are few in vivo studies
to demonstrate and elucidate the suppressive effects of MDSC di-
rectly. The mechanisms of suppression by MDSC on tumor-spe-
cific T cells are largely unknown. Recently, it was reported
that transfer of MDSC induced T cell anergy in peptide-vaccine drain-
ing lymph-nodes at the early phase of T cell activation (10), and
Marigo et al. (18) suggested that MDSC mediated T cell tolerance
in tumor-draining lymph-nodes (TDLN). It remains unclear where
and which phase of T cell activation is suppressed by MDSC in
vivo.

Previous studies in our laboratory demonstrated that TDLN
played a pivotal role in initiating antitumor immunity (19).
APCs in the growing tumor process Ag and migrate to the
TDLN to initiate a primary immune response. We have repeat-
edly demonstrated that TDLN T cells are immunologically sen-
sitized during early tumor progression, but functionally defi-
cient when tested in adoptive immunotherapy. However, these
“pre-effector” T cells could be matured in vitro by stimulation with irradiated tumor cells in the presence of IL-2 (20, 21). Additional experiments demonstrated that activation of TDLN cells nonspecifically with anti-CD3 Ab followed by culture in low concentrations of IL-2 induced vigorous T cell proliferation and acquired potent antitumor therapeutic function (22). Activated TDLN T cells are capable of mediating immunologically specific regression of established tumors when systemically

**FIGURE 1.** Phenotype of spleen cells from naive and 28-day MCA205 tumor-bearing mice.

**FIGURE 2.** Therapeutic effects of TDLN cells from mice reconstituted with tumor bearers’ spleen cells are diminished. A, Mice were irradiated (500 cGy) and were transferred i.v. with $3 \times 10^6$ spleen cells from either normal or 28-day tumor-bearing mice. These mice were inoculated s.d. with $3 \times 10^6$ MCA205 tumor cells to stimulate TDLN. Twelve days later, TDLN cells were harvested and activated in vitro by the anti-CD3/IL-2 method for 5 days. Activated TDLN cells were then adoptively transferred i.v. into a new group of mice that bore 3-day established pulmonary metastases. The therapeutic effects of TDLN cells between mice reconstituted with normal and tumor-bearers’ spleen cells are significantly different in both experiments ($p < 0.01$). B, Phenotype analyses of freshly harvested TDLN cells from mice reconstituted with normal (□) and tumor bearers’ spleen cells (■). The percentages of Thy1.2, B220, CD4, CD8, CD4⁺CD25⁺, and CD4⁺Foxp3⁺ cells are similar ($p = 0.62, 0.53, 0.63, 0.07, 0.59,$ and 0.82, respectively), however, transfer of tumor bearers’ spleen cells increased the percentage of CD11b⁺Gr-1⁺ cells in TDLN ($p = 0.014$).
transferred. However, normal non-TDLN cells did not possess detectable antitumor reactivity (22, 23). Based on principles established in animal models, we have conducted several clinical adoptive immunotherapy trials with tumor-stimulated LN T cells for the treatment of advanced renal cell carcinomas (24), gliomas (25), and squamous cell carcinomas of the head and neck (26). Although dramatic clinical responses were observed in a few patients, the majority of them did not respond. Thus, understanding regulatory mechanisms may provide new insights into the development of effective immunotherapeutic strategies. The immune response is subjected to regulations mediated by several defined inhibitory circuits. The response in the TDLN is likely also subjected to the immune-suppression mediated by tumor cells and tumor-induced suppressor cells. Although it has been observed that immunological reactivities of TDLN are suppressed as tumors progress (20, 23, 27, 28), the role of tumor-induced suppressor cells has not been well defined.

Tumor cells are capable of inhibiting T cell function through their ability of secreting soluble factors, such as TGF-β, IL-10, VEGF, and PGE₂ (1, 2). The amounts of these tumor-derived factors increase as tumors progress. It is therefore difficult to analyze functions of tumor-induced suppressor cells in vivo in animals with large tumor burdens. To identify tumor-induced suppressor cells that interfere with antitumor immune response in the TDLN, we transferred spleen cells from tumor-bearing mice into sublethally irradiated mice. These reconstituted mice were inoculated subdermally (s.d.) with the MCA205 tumor to stimulate TDLN. The therapeutic effects of activated TDLN cells were then evaluated by the adoptive immunotherapy to seek evidence of immune suppression.

We report in this study that antitumor reactivities of TDLN cells generated from mice that received spleen cells from tumor-bearing mice were severely suppressed when compared with that from mice that received normal spleen cells. In vivo studies demonstrated that the inhibition of T cell sensitization was mediated by MDSC but not Treg. TDLN from MDSC transferred mice showed a reduced number of tumor Ag-specific CD4⁺ and CD8⁺ T cells, compared with that from mice transferred with normal spleen cells. Further analyses revealed

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Suppression of immune response is mediated by Gr-1⁺ MDSC. Various cell preparations from tumor bearers’ spleens were tested for their ability to suppress immune responses. Experimental procedures are outlined in Materials and Methods. A, Phenotype analyses of cell populations used for reconstitution of irradiated mice. B, Identification of suppressor cells by negative selection of Gr-1 (a) and CD25 (b) depleted cell populations (p < 0.01 between groups 4 and 5 in a and p = 0.83 between groups 4 and 5 in b). C, Suppression of immune responses by the transfer of purified Gr-1⁺ cells (p < 0.01 between groups 3 and 4). D, Specificity of MDSC suppression. Spleen cells from either MCA205 or MCA207 tumor-bearing mice were transferred to irradiated mice for tumor specificity study. TDLN were stimulated by MCA205 tumor and tested against MCA205 pulmonary metastases (p = 0.84 between groups 3 and 4).
that MDSC inhibited not only primary sensitization of tumor-specific T cells but also tumor-sensitized T cells responding to a secondary tumor Ag stimulation. MDSC, which are induced during progressive tumor growth, have the ability to regulate the initiation of antitumor immunity in TDLN by suppressing generation of tumor-specific T cells.

Materials and Methods

Animals

Female C57BL/6N (B6) mice were purchased from the Biological Testing Branch, Frederick Cancer Research and Development Center, National Cancer Institute. They were housed in a specific pathogen-free environment and used at an age of 8 to 12 wk. Experimental protocols were approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

Tumors

MCA205 and MCA207 are fibrosarcomas of B6 origin that were induced initially by i.m. injection of 3-methylcholanthrene (29). These tumors have been routinely passaged in vivo and were used between the fifth to eighth passage. Single cell suspensions were prepared from solid tumors by digestion with a mixture of 0.1% collagenase, 0.01% DNase, and 2.5 U/ml hyaluronidase (Sigma-Aldrich) for 3 h at room temperature. The cells were filtered through a 100-μm nylon mesh, washed, and suspended in HBSS for i.v. and s.d. inoculations.

Procedures for analysis of suppressor cells and anti-CD3/IL-2 activation of TDLN

B6 mice were immune depleted by sublethal 500 cGy irradiation. One day later, mice were reconstituted i.v. with 3 × 10⁶ spleen cells from either normal or 21–28 day s.c. tumor-bearing mice. These mice were then inoculated s.d. with 3 × 10⁸ MCA205 tumor cells on both flanks to stimulate TDLN. Twelve days later, tumor-draining inguinal LN were harvested, and single-cell suspensions were prepared mechanically. To generate therapeutic effector T cells, TDLN cells were activated with anti-CD3 mAb immobilized on 24-well plates for 2 days and expanded in complete medium containing 4 U/ml of human rIL-2 (supplied by Chiron Therapeutics) for 3 days as previously described (22). Complete medium consists of RPMI 1640 supplemented with 10% heat-inactivated FBS and antibiotics. These cells were harvested and resuspended in HBSS for evaluation of therapeutic reactivities in adoptive immunotherapy. In this model system, suppressor cell function was demonstrated by the inhibition of therapeutic effects in the activated TDLN cells.

Adoptive immunotherapy of 3-day established pulmonary metastases

To establish pulmonary metastases, mice were inoculated i.v. with 3 × 10⁵ MCA205 tumor cells suspended in 1 ml HBSS because the lung provides the first capillary bed in which i.v. injected tumor cells could lodge and initiate metastases. On day 3 of tumor growth, mice were treated by the i.v. transfer of 3 to 10 × 10⁶ of activated TDLN cells of various sources in 1 ml HBSS. On day 16–18 of tumor growth, all mice were sacrificed. Metastatic tumor nodules on the surface of the lungs were enumerated after counterstaining with India ink as previously described (30).

Cell separation procedures

Spleens of normal or tumor-bearing mice were harvested and single-cell suspensions were prepared mechanically using the plunger of a 10 ml plastic syringe. Red cells were lysed by treatment with ammonium chloride potassium solution (8.29 g of NH₄Cl, 1.0 g of KHCO₃, and 0.372 g of EDTA/L). Cells were suspended in MACS buffer (0.5% BSA in PBS with 2 mM EDTA) and 1.0 ml of cell suspension was incubated with 100 μl of biotinylated anti-Gr-1 mAb or anti-CD25 mAb (BD Biosciences) for 10 min on ice. Cells were washed with cold MACS buffer, resuspended at 3 × 10⁶ cells in 2.4 ml of MACS buffer and then incubated

---

FIGURE 4. MDSC reduce numbers of tumor-specific CD4⁺ and CD8⁺ T cells in TDLN. A, TDLN cells from mice reconstituted with normal spleen cells (30 × 10⁶) only or reconstituted with MDSC (15 × 10⁶) admixed with normal cells (30 × 10⁶) were analyzed for specific immune reactivities by intracellular IFN-γ staining after specific and nonspecific stimulations of T cells as indicated. B, Suppression of specific T cell reactivities mediated by the transfer of either MCA205 or MCA207 tumor bearers’ spleen cells.
at 4°C with anti-biotin microbeads (Miltenyi Biotec) for 15 min. Gr-1<sup>−</sup>/H11002 or CD25<sup>−</sup>/H11002 cell populations were collected as flow through cells from magnetic columns (MACS-LS) and Gr-1<sup>−</sup>/H11001 population was positively isolated after disengaging MACS-LS columns from the magnet. The resulting cells were analyzed by flow cytometry.

**FACS analysis and intracellular IFN-γ staining**

FITC-conjugated mAb to B220 (RA3-6B2), CD11b (M1/70), Gr-1 (RB6-8C5), PE-conjugated mAb to Thy1.2 (53-2.1), CD8 (53-6.7), CD25 (PC61), Gr-1 (RB6-8C5), H-2K<sup>b</sup> (AF6-88.5), I-Ab (AF6-120.1), CD80 (16-10A1), CD86 (3D1), CD40 (16-10A1), CD11c (HL3), IFN-γ (XMG1.2), Cyochrome-conjugated mAb to CD4 (RM4-5), CD8 (53-6.7), and isotype-matched mAb were purchased from BD Biosciences. PE-anti-Forkhead box P3 (Foxp3, FJK-16s) and anti-F4/80 (BM8) mAb were purchased from eBioscience. Cell surface phenotypes were determined by direct immunofluorescence staining with conjugated mAb and analyzed using the FACSCalibur (BD Biosciences). Foxp3 staining was performed using the PE-Foxp3 staining set (eBioscience). Intracellular IFN-γ staining was conducted as previously described (31). In brief, T cells were stimulated with either MCA205 or MCA207 tumor cells prepared from solid tumor tissues at 1:1 ratio. Controls included stimulation with immobilized anti-CD3 mAb (145-2C11). Brefeldin A (10 μg/ml, Sigma-Aldrich) was added at 6 h and cells were harvested at 24 h. The cells were then pretreated with FcR block Ab followed by staining for 30 min with Cy-conjugated anti-CD4 or anti-CD8 mAb. Washed cells were fixed with 2% paraformaldehyde for 20 min, permeabilized with 0.3% saponin, and incubated for 40 min with PE-conjugated IFN-γ. Unbound mAb were removed by two washes with 0.3% saponin in PBS.

**Analyses of suppressor activity of MDSC in vitro**

Splenic mononuclear cells from normal mice used as a source of T cells were labeled with CFSE (Molecular Probes). Cells were stimulated with immobilized anti-CD3 mAb for 3 days in the absence or presence of Gr-1<sup>−</sup> cells or Gr-1<sup>−</sup> cells isolated from spleens of tumor-bearing mice. The responder to suppressor ratio was 1:1. Proliferation of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells was evaluated as CFSE dilution by flow cytometry. The statistical analysis was performed using the Wilcoxon rank-sum test, or Student’s t test. A two-tailed p value of <0.05 was considered significant. All experiments were repeated at least twice.

**Results**

Antitumor efficacy of TD LN cells from mice reconstituted with tumor-bearers’ spleen cells is significantly compromised

Progressive tumor growth may induce suppressor cells that constitute the immunosuppressive network (1, 2). Experimental evidence suggests that accumulation of suppressor cells, such as Treg and MDSC, may down-regulate immune responses and promote tumor progression (3, 6, 7). To investigate systemic immune suppression, spleens from mice bearing s.c. MCA205 tumors for 28 days were harvested. These spleens displayed significant splenomegaly and an increased spleen cellularity (342 ± 20 × 10<sup>6</sup>/spleen) compared with that of normal mice (98 ± 7 × 10<sup>6</sup>). Splenomegaly was a general phenomenon in mice bearing progressive s.c. tumors such as MCA205 and MCA207 fibrosarcomas, B16 melanoma, and 4T1 mammary carcinoma. As shown in Fig. 1, phenotypic analysis revealed...
that splenomegaly was attributed largely by increased Gr-1+ cells from 2.6% in normal animals to 22.1% in tumor-bearing animals, whereas the percentages of CD4+CD25+ Treg were similar between normal (2.6%) and tumor-bearing animals (2.1%). The majority (90.6%) of Gr-1 positive cells also expressed CD11b.

An animal experimental protocol was devised to evaluate whether tumor-induced suppressor cells exhibited in vivo immune suppressive function. In this model, sublethally (500 cGy) irradiated mice were transferred i.v. with spleen cells (3 × 10^6) from tumor-bearing mice followed by s.d. inoculation with MCA205 tumor cells. Twelve days later, TDLN (inguinal) were harvested and cells were activated in vitro with immobilized anti-CD3 mAb for 2 days, followed by culture in the presence of low doses of IL-2 (4 U/ml) for 3 days as previously described (22). The therapeutic effects of these cells were assessed in the adoptive immunotherapy of 3-day established pulmonary MCA205 metastases in separate experiments. As shown in Fig. 2A, TDLN cells generated from mice reconstituted with normal cells were therapeutically efficacious. The transfer of 10 × 10^6 TDLN cells resulted in reducing the average number of metastatic nodules from ≥250 in the no treatment control, to 23 and 7, in experiments 1 and 2, respectively. TDLN cells from mice reconstituted with tumor-bearers’ spleen cells demonstrated minimum or no therapeutic effects. Fig. 2B depicts phenotypes of freshly harvested TDLN cells. The two TDLN cell populations showed similar phenotypes, except that the percentage of CD11b+Gr-1+ cells in that TDLN from mice reconstituted with tumor-bearers’ spleen cells was significantly higher than that from mice reconstituted with normal spleen cells. (3.3 ± 0.5% vs 1.6 ± 0.2%, p = 0.014).

Depletion of MDSC restores the antitumor immune response in TDLN

To identify suppressor cells in the spleens of tumor-bearing hosts, we depleted the Gr-1+ cells or Treg by negative selection using magnetic beads. Flow cytometry analyses showed that CD11b+Gr-1+ cells and CD4+CD25+Foxp3+ cells were reduced from 19.8 to 0.2% and 1.9 to 0.2%, respectively (Fig. 3A). These cells were then transferred systemically into irradiated mice and 12-day MCA205 TDLN were activated by the anti-CD3/IL-2 method. Again, the in vivo antitumor reactivities of activated TDLN cells were evaluated in the adoptive immunotherapy of 3-day established pulmonary metastases. Although there was no discernible phenotype differences in all TDLN cells, depletion of CD11b+Gr-1+ MDSC but not CD4+CD25+Foxp3+ regulatory T cells eliminated the suppressive effects (Fig. 3, Ba and Bb). These
results thus indicated that the suppression of antitumor effects was likely mediated by MDSC. Treg from the tumor-bearing spleens did not seem to play a significant role.

To positively identify suppressor cells, purified CD11b<sup>+</sup>Gr-1<sup>−</sup> MDSC (15 × 10<sup>6</sup>), or Gr-1<sup>−</sup> negatively selected cells from tumor-bearing mice (15 × 10<sup>6</sup>) were studied. In the experiment depicted in Fig. 3Bc, mice received purified cells along with 30 × 10<sup>6</sup> normal spleen cells to reconstitute sufficient number of T cells. Twelve-day TDLN were activated and their antitumor effects were evaluated in adoptive immunotherapy. Based on the reduction of numbers of pulmonary metastases, the transfer of purified CD11b<sup>+</sup>Gr-1<sup>−</sup> MDSC alone was responsible for all the observed suppression.

**Inhibition of T cell sensitization by MDSC is not tumor specific**

We next examined whether inhibition of T cell sensitization by MDSC was tumor specific. Mice were reconstituted with spleen cells from either MCA205 or MCA207 tumor-bearing mice followed by s.d. inoculation with MCA205 tumor cells to stimulate TDLN. After in vitro activation, TDLN cells were again evaluated for their therapeutic effects in adoptive immunotherapy. The results demonstrated that the suppression mediated by MDSC was not tumor specific because both MCA205- and MCA207-induced suppressor cells demonstrated a similar suppressive function for the MCA205 TDLN (Fig. 3Bd).

**Systemic transfer of MDSC inhibits tumor-specific T cell generation**

To quantitatively analyze tumor-specific T cells in the activated TDLN from mice reconstituted with CD11b<sup>+</sup>Gr-1<sup>−</sup> suppressor cells, IFN-γ secretions were evaluated after stimulation with specific MCA205 or nonspecific MCA207 tumor cells. Stimulation of TDLN with anti-CD3 mAb was also included. TDLN from the mice reconstituted with normal spleen cells served as the positive control. As depicted in Fig. 4A, both CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to specific MCA205 stimulation were diminished in mice transferred with MDSC.

Although the MDSC were apparently capable of inhibiting specific T cell generation, we still were confronted with the question whether antigenetically different tumors would stimulate functionally different MDSC. In additional experiments, mice were transferred with spleen cells from either MCA205 or MCA207 tumor-bearing mice. MCA205 tumor-induced TDLN were analyzed for IFN-γ secretion after specific tumor stimulation. As shown in Fig. 4B, inhibition of tumor-specific IFN-γ producing T cells was mediated nonspecifically because both MCA205 and MCA207 tumor bearers’ spleen cells exerted similar suppressive reactivities.

**MDSC inhibit T cell proliferation in vitro, but not IFN-γ secretion by survival cells**

Because of a lack of tumor specificity, we analyzed the ability of MDSC to impact on the proliferation of normal T cells. CFSE-labeled normal spleen cells were stimulated with immobilized anti-CD3 mAb for 3 days in the presence or absence of CD11b<sup>+</sup>Gr-1<sup>−</sup> cells at 1:1 ratio. Proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was evaluated as CFSE dilution. It became apparent that the presence of MDSC inhibited the normal T cell response to anti-CD3 stimulation (Fig. 5A). This inhibition was further confirmed by the fact that there was a severe reduction of numbers of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 5B). By contrast, no suppression was observed with Gr-1<sup>−</sup> cells. To examine the effects of MDSC on IFN-γ secretion by the survived T cells, they were stimulated by anti-CD3 mAb for 3 days. Intracellular IFN-γ staining revealed that despite severe suppression of proliferation, the survival T cells retained the ability to secrete IFN-γ (Fig. 5C).

**MDSC suppress the expansion of tumor-specific T cells in vitro**

To further examine whether tumor-sensitized T cells were suppressed by MDSC, tumor-specific T cells were generated from TDLN of normal mice that were inoculated s.d. with MCA205 tumor cells. These TDLN cells contained tumor-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells (32, 33). Twelve-days after tumor growth, TDLN cells were stimulated by the anti-CD3/IL-2 method in the presence or absence of CD11b<sup>+</sup>Gr-1<sup>−</sup> cells at 4:1 ratio. Again, their antitumor effects were assessed in adoptive immunotherapy as detailed previously. As shown in Fig. 6A, TDLN cells activated in the presence of MDSC showed minimal or no antitumor effects. We therefore, analyzed TDLN T cell proliferation after anti-CD3/IL-2. As shown in Fig. 6, B and C, the
presence of MDSC significantly suppressed proliferation of both CD4+ and CD8+ TDLN T cells. Although some studies have demonstrated that MDSC regulated T cell responses to mitogens (34), Ag (35), allo-Ag (36), and viral Ag (17), there are a few examples suggesting that MDSC have suppressive effects on secondary tumor Ag-specific responses (16, 37). We took a different approach to study this possibility. After being cocultured with MDSC, TDLN T cells were further stimulated with tumor cells or anti-CD3 for an additional day to investigate the suppressive effect of MDSC on the ability of activated TDLN to respond to specific tumor stimulation. Activated TDLN cells contained tumor-specific T cells capable of responding to specific tumor stimulation (Fig. 6D). However, in the presence of MDSC, the numbers of CD4+ and CD8+ T cells that responded to specific tumor stimulation decreased greatly, indicating the suppression by MDSC affected not only primary sensitization of tumor-specific T cells in TDLN but also affected sensitized T cells responding to secondary specific antigenic stimulation. Similar to the Fig. 5C analysis, TDLN T cells were nevertheless capable of secreting IFN-γ in response to CD3 stimulation after cocultured with MDSC.

**MDSC do not inhibit in vivo antitumor function of tumor-specific T cells**

To establish a strategy of tumor immunotherapy, it is important to determine whether suppressor cells affect the therapeutic function of activated tumor-specific TDLN T cells. Twelve-day TDLN from normal mice were activated as described above and they were admixed with MDSC before adoptively transferred into mice bearing 3-day established pulmonary metastases (Fig. 7A) or 3-day established skin tumor (Fig. 7B). The results clearly showed that MDSC coinoculated with effector T cells did not suppress their in vivo antitumor reactivities.

**Discussion**

Tumor-induced immune suppression consists of several functionally distinct mechanisms that are mediated by distinct types of cells (1, 3, 6). Because tumor cells are capable of secreting several soluble factors that directly suppress antitumor immunity, it is particularly difficult to investigate tumor-induced, host derived suppressor cells independently in the absence of other suppressive factors. Therefore, a model system where suppression mechanisms could be individually analyzed in normal mice was designed and used in the current study. In this system, we found that antitumor immune responses that normally occurred in the TDLN were strongly suppressed in mice that were systemically transfected with tumor bearers’ spleen cells. We further identified that MDSC alone from the spleens of tumor-bearing mice possessed all the suppressive activities. These observations are consistent with several previous studies in which MDSC mediate immune suppression (6, 8, 38). In our study, Treg were not involved. Different methods and model systems may allow the preferential identification of particular suppressor cell functions. For example, depletion of Treg by administration of Ab reduced skin tumor growth of RL.δ1 BALB/c derived leukemia and B16 derived melanoma (39), and transfer of Treg abolished antitumor effects of pmel-1 T cell and gp100 encoding vaccinia virus vaccination (40).

It has been reported that MDSC may induce the development of Treg (9) and depletion of Treg hampers accumulation of MDSC (41). Although depletion of Treg from tumor bearers’ spleen cells failed to reverse the suppression, the possibility exists that the transferred MDSC were capable of inducing secondary generation of Treg in vivo. However, our experiments did not support this hypothesis as no evidence of increased numbers of Treg in the recipient mice existed.

Previous studies have shown that MDSC promote tumor progression in vivo, possibly through a mechanism associated with T cell dysfunction (6, 8, 38). However, few studies delineate the mechanism of MDSC-mediated suppression, especially on tumor-specific T cells. Our results clearly showed that systemic transfer of MDSC inhibited initiation of antitumor immune response by decreasing the number of tumor-specific T cells and suppressed antitumor efficacy of activated TDLN cells (Figs. 3Bc and 4A). Consistent with our results, Clark et al. (42) reported that MDSC infiltrated into the pancreatic cancer tissue during early stage of tumor progression. We previously demonstrated that effector T cells were stimulated with specific tumor Ag and proliferated greatly in tumor tissues before tumor regression could occur (43, 44). In the current study, we demonstrated that MDSC had the ability to suppress antitumor reactivities of tumor-sensitized T cells in responding to secondary specific antigenic stimulation (Fig. 6, A and D). Further, we investigated whether MDSC suppressed the function of tumor-specific T cells at the effector phase. We found that the cotransfer of MDSC did not interfere with the therapeutic effects of adoptively transferred T cells (Fig. 7, A and B). It has been suggested that freshly isolated MDSC from tumor-bearing mice required IFN-γ to become fully suppressive (45), and MDSC-infiltrating tumor tissue expressed a distinct phenotype (6). Therefore, it may be possible that the lack of inhibition of effector T cell function may reflect the stage of MDSC maturation and MDSC do not function without further activation. Additional experiments are necessary to determine under what conditions MDSC can suppress in vivo function of T cells at the effector phase.

In the current study, MDSC inhibited T cell proliferation and sensitization in TDLN nonspecifically. Kusmartsev et al. (46) demonstrated that transfer of MDSC from MethA sarcoma-bearing mice promoted the progression of CT-26 colon adenocarcinoma. MDSC from CT-26 bearing mice promoted skin tumor progression of MethA as well. We used MCA205 and MCA207 fibrosarcomas. Both cell lines were chemically induced and characteristically similar, but they had distinct tumor rejection Ag that were recognized by specific T cells (44). Similar to the previous report, tumor-induced generation of MDSC did not show tumor specificity because both MCA205 and MCA207-induced MDSC suppressed the immune response to MCA205 (Figs. 3Bd and 4B).

It has not been determined whether MDSC are able to suppress antitumor effects of CD4+ T cells. Because MDSC represent a heterogeneous population of myeloid precursors, it is no surprise that some MDSC suppressed only CD8+ T cells (46, 47), while others were capable of suppressing both CD4+ and CD8+ T cells (17, 48). In the current study, although pheno- typic analyses of MDSC revealed that MDSC highly expressed MHC class I but not class II (data not shown), transfer of MDSC reduced the number of both tumor-specific CD4+ and CD8+ T cells in TDLN (Fig. 4). In vitro studies also demonstrated that MDSC suppressed both CD4+ and CD8+ T cell proliferation in response to the CD3/IL-2 culture (Figs. 5 and 6) and secretion of IFN-γ from CD4+ and CD8+ T cells after tumor-specific stimulation (Fig. 6D).

Other studies have demonstrated that MDSC suppress T cell activation through 1) production of arginase and inducible NO synthase to reduce amino acid availability (8) and 2) generation of reactive oxygen species to induce the modification of TCR-CD3 complexes (6, 10). In our study, MDSC interfered with IFN-γ
secretion from tumor-specific T cells, but did not inhibit anti-CD3-induced IFN-γ secretion from both naïve T cells and TDLN T cells (Fig. 5C and 6D). Anti-CD3 mAb stimulation is through cross-linking of CD3 complex regardless of defects of TCR signal transduction (23). MDSC seemed to affect binding of specific Ag to TCR or TCR signal transduction after Ag engagement as suggested by other investigators (6, 10). Although cell numbers decreased in the in vitro proliferation assay, due to the presence of MDSC, the survival cells retained their full ability to respond to anti-CD3 stimulation. These results suggest that MDSC neither inhibit the binding of anti-CD3 mAb nor the CD3-mediated signal transduction pathway in T cell activation despite their ability to inhibit T cell proliferation. It was demonstrated that MDSC secreted NO synthase. NO synthase and NO had ability to prevent T cell proliferation by blocking IL-2 signaling pathway (49). Our observations may reflect these reported findings. It is generally perceived that to express full antitumor efficacy, tumor Ag-specific T cells are stimulated with Ag presented by professional APCs to expand and mature. It appears that MDSC strongly regulates antitumor immunity by interfering with both early phase of TCR activation and clonal expansion of tumor-specific T cells following Ag presentation.

To induce an adequate number of tumor-specific T cells is one of the goals of studying tumor immunotherapy, especially for cancer vaccine development. Approaches to control MDSC may increase the number of tumor-specific T cells and enhance therapeutic efficacy of tumor immunotherapy.

Acknowledgments
We thank Christopher C. Paustian and Edward I. Cho for comments on the paper.

Disclosures
The authors have no financial conflict of interest.

References

Downloaded from http://www.jimmunol.org/ by guest on May 29, 2017


