Antigen-Specific Immunity and Cross-Priming by Epithelial Ovarian Carcinoma-Induced CD11b+Gr-1+ Cells

Kei Tomihara, Miao Guo, Takako Shin, Xiuhua Sun, Sara M. Ludwig, Michael J. Brumlik, Bin Zhang, Tyler J. Curiel and Tahiro Shin

*J Immunol* 2010; 184:6151-6160; Prepublished online 28 April 2010;
doi: 10.4049/jimmunol.0903519
http://www.jimmunol.org/content/184/11/6151

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/04/28/jimmunol.0903519.DC1

**References**

This article cites 55 articles, 29 of which you can access for free at:
http://www.jimmunol.org/content/184/11/6151.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
Antigen-Specific Immunity and Cross-Priming by Epithelial Ovarian Carcinoma-Induced CD11b+Gr-1+ Cells

Kei Tomihara,1 Miao Guo,1 Takako Shin, Xiuhua Sun, Sara M. Ludwig, Michael J. Brumlik, Bin Zhang, Tyler J. Curiel, and Tahiro Shin

Both innate and adaptive immune systems are considered important for cancer prevention, immunosurveillance, and control of cancer progression. It is known that, although both systems initially eliminate emerging tumor cells efficiently, tumors eventually escape immune attack by a variety of mechanisms, including differentiation and recruitment of immunosuppressive CD11b+Gr-1+ myeloid suppressor cells into the tumor microenvironment. However, we show that CD11b+Gr-1+ cells found in ascites of epithelial ovarian cancer-bearing mice at advanced stages of disease are immunostimulatory rather than being immunosuppressive. These cells consist of a homogenous population of cells that morphologically resemble neutrophils. Moreover, like dendritic cells, immunostimulatory CD11b+Gr-1+ cells can strongly cross-prime, augmenting the proliferation of functional CTLs via signaling through the expression of costimulatory molecule CD80. Adoptive transfer of these immunostimulatory CD11b+Gr-1+ cells from ascites of ovarian cancer-bearing mice results in the significant regression of s.c. tumors even without being pulsed with exogenous tumor Ag prior to adoptive transfer. We now show for the first time that adaptive immune responses against cancer can be augmented by these cancer-induced granulocyte-like immunostimulatory myeloid (CD11b+Gr-1+) cells, thereby mediating highly effective antitumor immunity in an adoptive transfer model of immunity. The Journal of Immunology, 2010, 184: 6151–6160.

The immune system consists of two arms: innate and adaptive immunity. Innate immunity is the Ag-independent arm mediated primarily by macrophages, dendritic cells (DCs), granulocytes, NK cells, and NKT cells. Adaptive immunity is the system that mediates Ag-specific immune responses and creates immunologic memory through both T and B cells. With respect to anticancer immune responses, many studies have shown that host immune competence in both innate immunity and adaptive immunity is important for cancer prevention, cancer immunosurveillance, and the control of cancer progression (1–4). Efficient bridging between innate immunity and adaptive immunity by myeloid-lineage cells, such as DCs, which possess a mechanism for cross-priming CTLs, might be a key mechanism by which an effective anticancer immune response is maintained (5).

Neutrophils are a subset of myeloid leukocytes that are most commonly found in the peripheral blood from mice or humans. In addition to expressing an array of adhesion molecules, neutrophils can also respond to a wide variety of stimuli through surface receptors for diverse molecules, such as cytokines, complement, adenosine, the Fc portion of Abs, and TLRs (6). Neutrophils are recruited to the site of infection, phagocytose pathogens, and destroy them by releasing reactive oxygen species and NO (7–9). In addition to their intracellular granules containing large amounts of peroxidases and proteases, neutrophils can also secrete cytokines and chemokines. Not only are these molecules critical at the front line of the innate immune system for antimicrobial defense, but recent studies have also suggested that they can contribute to the development of adaptive immunity, such as delayed-type hypersensitivity reaction and cross-priming of CD8 T cells (10, 11), by cross-talk with DCs (12). Although the ability of neutrophils to kill cancer cells with reactive oxygen species and NO has been documented (13), the functional contribution of neutrophils in adaptive anticancer immune response remains largely unknown.

Recently, a population of suppressive CD11b+Gr-1+ cells has been designated myeloid-derived suppressor cells (MDSCs) (14). MDSCs are a unique category of the myeloid lineage, and their definition still seems ambiguous because CD11b and Gr-1, the gold standard surface molecules for MDSCs, are expressed in a wide range of myeloid lineages. Therefore, as we will show, it is problematic to automatically consider that CD11b+Gr-1+ cells are MDSCs before their suppressive functions are confirmed. It is therefore necessary to identify surface or intracellular molecules that are absolutely specific for MDSCs.

There is general agreement that MDSCs are a heterogeneous cell population that resembles macrophages or granulocytes at the phenotypic level, depending on the type of tumor and its anatomic location. Various mouse and human studies have found that MDSCs emerge the peripheral blood and tumor microenvironment and often exhibit strong immunosuppression. It has been also shown that the accumulation of MDSCs within either the tumor microenvironment or peripheral blood correlates with a poor prognosis (15, 16). This has been cited as one of the most important issues impeding cancer immunotherapy, as is the case for regulatory T cells (Tregs) (17). In 1995, Dr. Hans Schreiber’s group described the elimination of a Gr-1+ population by a specific anti–Gr-1 mAb that reduced...
the volume of transplanted skin cancer. They further showed that the accumulation of myeloid cells in tumor-bearing mouse decreased antitumor immune responses and promoted tumor growth (18, 19).

Our present study demonstrates the significant accumulation of CD11b+Gr-1+ cells in the spleen and ascites from epithelial ovarian cancer-bearing mice. Although it was anticipated that this population would comprise immunosuppressive MDSCs, our results were not consistent with previous studies (20–22). This population did not represent vascular leukocyte cells based on either phenotypic or functional differences (23–25). In addition, these CD11b+ Gr-1+ cells stimulated Ag-nonspecific and Ag-specific T cells both in vitro and in vivo. Moreover, these immunostimulatory CD11b+ Gr-1+ cells strongly cross-primed CTLs and exhibited therapeutic potential to inhibit cancer progression in an s.c. tumor-bearing mouse model by adoptive transfer.

Materials and Methods

Mice

C57BL/6 and BALB/c mice were obtained from the National Cancer Institute, OT-I and OT-II mice were obtained from Taconic (Hudson, NY). All mice were maintained under specific pathogen-free conditions in accordance with the institutional guidelines of the University of Texas Health Science Center at San Antonio. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Abs and reagents

PE-conjugated anti-mouse B7-H1 (MIH5), B7-DC (TY25), B7-H3 (M3.2D7), B7-H4 (eBioMIH92), B7RP-1 (HK5.3), CD31 (390), CD71 (R1721), CD115 (AFS98), MHC class I (34-1-2S), PerCP-Cy5.5-conjugated anti-mouse CD45R (RA3-6B2), CD90.1 (Thy1.1, H1551), PE-Cy7-conjugated anti-mouse CD4 (GK1.5), CD11c (N418), CD19 (eBioID3), allophycocyanin-conjugated anti-mouse CD11c (N418), CD49b (DX5), CD80 (16-10A1), CD86 (GL1), F4/80 (BM8), MHC class II (M5/114.15.2), allophycocyanin-eFluor780-conjugated anti-mouse CD8a (53-6.7), Gr-1 (RB6-8C5), eFluor505-conjugated anti-mouse CD3 (17A2), CD11b (M1/70), and purified CD16/32 (93) mAbs, functional grade anti-mouse CD3 (145-2C11), CD28 (3H5), CD80 (16-10A1), and purified CD16/32 (93) mAbs or with specific OVA peptide in the presence or absence of CD11b+ Gr-1+ cells, Tregs, or MDSCs. For consistency, we used only CD11b+Gr-1+ cells in spleen or ascites of ID8- or IE9-bearing mice that had developed ascites within 10 wk of tumor challenge and were used within 4 d after EG7 tumor challenge. Tumor size was measured three times per week until tumor size was >100 mm² (length × width).

Statistical analysis

Student t test (one-tailed) was used for data analysis when appropriate.

Results

Accumulation of CD11b+Gr-1+ cells in spleen and ascites of a mouse ovarian cancer model

After the i.p. injection of 10 million ID8 cells, a mouse epithelial ovarian cancer cell line (25, 28), the mice usually began to develop cancerous ascites starting at 7 wk postinjection. Mice that developed ascites were easily distinguished by a brief external examination, because they exhibited abnormal swelling in the abdomen. Approximately 60% of the mice developed ascites within 10 wk after injection (Fig. 1A). The remaining mice eventually developed ascites, although <20% of them took >14 wk to do so. Once the mice developed ascites, tumors grew progressively without treatment and the mice generally died within 1–2 wk thereafter. For consistency, we used only mice that had swollen abdomens and developed ascites within 10 wk postinjection and that were observed within 4 d. Although we tried to use ascites from mice with a similar degree of abdominal swelling, the volume of ascites acquired from each mouse varied, as did the number of hematopoietic cells isolated from ascites (data not shown).

Various minor subsets of lymphocytes and monocyes were observed in the ascites, including CD3+, B220+, DX5+, and CD11c+ cells. The most conspicuous population in ascites was CD11b+Gr-1+ cells (Fig. 1B: maximum 40.5%, minimum 6.3%, average 18.6%). CD11b+Gr-1+ cells also accumulated in the spleens of ID8-bearing mice (maximum 7.5%, minimum 1.9%, average 4.6%) compared with the spleen of naive mice (maximum 2.2%, minimum 1.2%, average 1.7%). Normally, CD11b+Gr-1+ cells could not be observed in the peritoneal cavity of naive mice, because the majority of these were CD11b+Gr-1+ peritoneal macrophages (so called PECs; Fig. 1C). This population tended to decrease over time. In contrast,
CD11b+Gr-1+ cells gradually accumulated in the peritoneal cavity after ID8 challenge and increased dramatically at a late stage when the tumor volume was extremely large. There were few CD11b+Gr-1− cells in the spleen. The majority of the distinct population of CD11b+Gr-1+ cells observed in the peritoneal cavity from 3 wk until obvious ascites production were composed of CD3+CD4+B220lowCD11c− T cells and thus were not studied further.

**CD11b+Gr-1+ cells from malignant ascites are not MDSCs**

We initially assumed that the CD11b+Gr-1+ cells accumulating in the spleen (ID8/spleen) and ascites (ID8/ascites) of the ID8-bearing mice would consist of MDSCs as previously described (20, 21, 29). Surprisingly, CD11b+Gr-1+ cells from both compartments did not suppress but rather enhanced both CD4+ and CD8+ T cell proliferation in vitro in both a nonspecific and an Ag-specific manner.

**FIGURE 1.** CD11b+Gr-1+ cells accumulated in spleen and ascites of ID8-bearing mice are not suppressive. A, The kinetics of ascites accumulation in mice was observed (n = 24 per group) following the i.p. injection of 10 million ID8 or IE9 (OVA-producing ID8) cells. B, The percentage and total number of CD11b+Gr-1+ cells from the indicated sources (10–15 mice per group) are shown. C, Accumulation of CD11b+Gr-1+ cells in ID8-bearing mice is shown. Mice were sacrificed 3, 5, 7, and 9 wk after ID8 challenge. Cells from spleens, PECs, or ascites were collected and stained with anti-CD11b and anti–Gr-1 mAbs. Two different mice were sacrificed 9 wk after ID8 injection. One mouse did not have obvious abdominal swelling but nonetheless developed 0.5 ml of cancerous ascites. The other mouse had a swollen abdomen with 10 ml of ascites. The data are representative of three independent experiments. ***p < 0.001; ****p < 0.0001. D and E, In vitro suppression assays. T cells from naive mice (D) or whole spleen cells from OT-I mice (E) were cultured with or without PECs from naive mice or CD11b+Gr-1+ or CD11b+Gr-1− cells isolated from ID8-bearing mice by sorting using flow cytometry or with CD4+CD25+ Tregs from naive mice in the presence of anti-CD3 and CD28 mAbs. T cell proliferation was measured by CFSE dilution. Alternatively, whole spleen cells from OT-I or OT-II mice were cultured with the appropriate OVA-specific peptides in the presence or absence of purified CD11b+Gr-1+ cells isolated from the indicated sources (n = 5 per group). EG7 cell is an OVA-producing mouse thymoma cell line. Proliferation was measured by [3H] thymidine incorporation and was based on the ratio of the proliferation of T cells incubated in the absence relative to the presence of CD11b+Gr-1+ cells.
specific manner. Regardless, CD11b+Gr-1+ cells isolated from the spleen of EG7-bearing mice, which are considered to be typical MDSCs, consistently suppressed both CD4+ and CD8+ T cell proliferation in vitro (Fig. 1D). This nonsuppressive population coexisted in ascites together with relatively suppressive CD11b+Gr-1− cells that were a mixture of PECs and tumor-associated macrophages (16) (Fig. 1E). PECs that were sorted from the peritoneal cavity of naive mice had slight suppressive function, although the influence of PECs on the generation of CD11b+Gr-1− cells is still not yet clear. In addition, there were too few CD11c+ cells for additional functional studies.

Because suppressive function was not observed in the CD11b+Gr-1− cells from ID8/spleen and ID8/ascites, factors important for suppression by MDSCs were examined. After magnetic purification and sorting by flow cytometry to achieve maximum purity, total mRNA was extracted. The expression of genes encoding inducible NO synthase (iNOS), arginase-1 (Arg-1), vascular endothelial growth factor, matrix metallopeptidase 9 (MMP9), S100A8, and S100A9 were quantitatively measured and compared with the mRNA expression of the housekeeping gene GAPDH. According to previously published data, iNOS and Arg-1 gene expression are elevated in MDSCs arising in several different mouse tumor models including MSC-2-, CT26-, 4T1-, and 3LL-bearing mice (15, 16, 30–32). These transcripts are also reported to be considerably higher in neutrophils relative to other leukocyte populations (6, 11, 33). Interestingly, we observed that CD11b+Gr-1− cells from ID8/ascites demonstrated significantly decreased iNOS, Arg-1, S100A8, and S100A9 mRNA expression compared with CD11b+Gr-1− cells from either naive/spleen or MDSCs from the spleen of EG7-bearing mice (EG7/spleen) (Fig. 2A). This observation might relate to the immunostimulatory property of CD11b+Gr-1− cells.

Microscopic examination of CD11b+Gr-1− cells from the ID8 ascites suggested that an almost pure population of cells possessing segmented nuclei that were morphologically similar to neutrophils (Fig. 2B). In contrast, the vast majority of CD11b+Gr-1− cells from the spleen of ID8-bearing mice resembled immature neutrophils, having band-shaped nuclei.

To further explore the features of CD11b+Gr-1− cells in ID8-bearing mice, we examined surface marker expression. CD11b+Gr-1− cells from naive spleen, ID8/spleen, and ID8/ascites had no detectable expression of CD11c, ICOSL, B7-DC, or B7-H4 (Supplemental Fig. 1). The expression of CD124 and B7-H3 was identical between all CD11b+Gr-1− populations examined. Finally, the expression of CD31, CD115, MHC class II, and F4/80 was significantly decreased, and the expression of CD71, CD80, and B7-H1 was increased in CD11b+Gr-1− cells from ID8/ascites compared with CD11b+Gr-1− cells from naive spleens (Fig. 2C). The expression profile of surface molecules other than CD11b and Gr-1 on MDSCs is still controversial, with many discrepancies and
inconsistencies in the scientific literature (30, 34, 35). Nonetheless, our data demonstrate that the CD11b⁺Gr-1⁺ cells in ID8/ascites are distinct from any known subpopulation of MDSCs or vascular leukocyte cells (CD11c⁺CD11b⁺Gr-1⁺CD31⁺CD115⁺F4/80⁺) (24, 25) described to date. In addition, CD3 expression (Supplemental Fig. 2) and both intracellular and surface expression of CTLA-4 (data not shown) could not be detected in CD11b⁺Gr-1⁺ cells from ID8/spleen or ID8/ascites.

We next investigated whether CD11b⁺Gr-1⁺ cells from ID8-bearng mice could directly stimulate T cells. OT-II CD4⁺ or OT-I CD8⁺ T cells were purified and cultured with or without purified CD11b⁺Gr-1⁺ cells in the presence of specific peptides. Both OT-II CD4⁺ or OT-I CD8⁺ T cells cultured with CD11b⁺Gr-1⁺ cells from ID8/ascites proliferated to a greater extent compared with

CD11b⁺Gr-1⁺ cells from ovarian cancer-bearing mice enhance T cell proliferation

We next investigated whether CD11b⁺Gr-1⁺ cells from ID8-bearing mice could directly stimulate T cells. OT-II CD4⁺ or OT-I CD8⁺ T cells were purified and cultured with or without purified CD11b⁺Gr-1⁺ cells in the presence of specific peptides. Both OT-II CD4⁺ or OT-I CD8⁺ T cells cultured with CD11b⁺Gr-1⁺ cells from ID8/ascites proliferated to a greater extent compared with

FIGURE 3. CD11b⁺Gr-1⁺ cells augmented T cell proliferation both in vitro and in vivo. A, OT-I or OT-II T cells were purified, CFSE-labeled, and cultured with or without purified CD11b⁺Gr-1⁺ cells isolated from the indicated sources in the presence of each respective OVA-specific peptide. T cell proliferation was measured by CFSE dilution. B, Naive CD4⁺ or CD8⁺ T cells were purified from allogeneic mice and cultured with or without CD11b⁺Gr-1⁺ cells purified from the indicated sources (n = 5 per group). CD11c⁺ cells and PECs were isolated from the spleen and the peritoneal cavity of naive mice. T cell proliferation was measured by [³H]thymidine incorporation. C, In vivo proliferation assay was performed by adoptively transferring 1 million purified OT-I CD8⁺ or OT-II CD4⁺ T cells (Thy.1.1⁺) to naive hosts (Thy.1.2⁻). The following day, either purified CD11b⁺Gr-1⁺ cells, isolated from the indicated sources, or purified CD11c⁺ cells from the spleen of naive mice, were injected i.p. after incubation with relevant OVA peptides. Five days later, spleen, mesenteric lymph nodes (mesLN) and peripheral lymph nodes (periLN) of host mice were isolated and transferred OT-I CD8⁺ or OT-II CD4⁺ T cells were examined. D, The summary of in vivo proliferation assays is shown both in terms of the percentage and the total number of transferred cells. A–C, The data are representative of four independent experiments. *p < 0.05; **p < 0.005; ***p < 0.0005; ****p < 0.0001.
these same cells cultured with CD11b\(^+\)Gr-1\(^+\) cells from either naive spleen or ID8/ascites (Fig. 3A).

To evaluate the ability of CD11b\(^+\)Gr-1\(^+\) cells to function as APCs, whole spleen T cells from allogeneic mice were purified and cultured with CD11b\(^+\)Gr-1\(^+\) cells in vitro. After 4 d of culture, CD11b\(^+\)Gr-1\(^+\) cells from ID8/ascites were able to stimulate both CD4\(^+\) and CD8\(^+\) allogeneic T cells, although CD11c\(^+\) DCs remained the most potent inducer of allogeneic T cell proliferation (Fig. 3B).

To evaluate whether CD11b\(^+\)Gr-1\(^+\) cells from ID8-bearing mice were able to stimulate T cells in vivo, purified OT-II CD4\(^+\) or OT-I CD8\(^+\) T cells (both Thy1.1\(^+\)) were adoptively transferred to naive Thy1.2\(^+\) hosts prior to immunization with OV A peptide-pulsed CD11b\(^+\)Gr-1\(^+\) cells isolated from the indicated sources (B) in the absence of OV A-specific peptide. Representative data (based on CFSE dilution) are shown. A summary of proliferation (\(n = 7\) per group) measured by either CFSE dilution or \(^{3}\text{H}\)thymidine incorporation is shown. C, Intracellular production of IL-2 and IFN-\(\gamma\) was measured in the OT-I T cells examined in panel A. FACS panels are representative of four independent experiments. \(p < 0.05; \ast \ast p < 0.01; \ast \ast \ast p < 0.0005; \ast \ast \ast \ast p < 0.0001.

**FIGURE 4.** Immunostimulatory CD11b\(^+\)Gr-1\(^+\) cells were capable of cross-priming in vitro. Purified OT-I T cells were cultured with either PECs from naive mice, CD11b\(^+\)Gr-1\(^+\) or CD11b\(^+\)Gr-1\(^+\) cells from ascites of IE9-bearing mice (A), or purified OT-I T or OT-II T cells were cultured with purified CD11b\(^+\)Gr-1\(^+\) cells isolated from the indicated sources (B) in the absence of OV A-specific peptide. Representative data (based on CFSE dilution) are shown. A summary of proliferation (\(n = 7\) per group) measured by either CFSE dilution or \(^{3}\text{H}\)thymidine incorporation is shown. C, Intracellular production of IL-2 and IFN-\(\gamma\) was measured in the OT-I T cells examined in panel A. FACS panels are representative of four independent experiments. \(p < 0.05; \ast \ast p < 0.01; \ast \ast \ast p < 0.0005; \ast \ast \ast \ast p < 0.0001.

Immunostimulatory CD11b\(^+\)Gr-1\(^+\) cells cross-prime Ag-specific T cells in vitro and in vivo

Cross-priming is an important function of DCs (5) that has also been reported for neutrophils (7). We therefore tested whether ID8 tumor-induced immunostimulatory CD11b\(^+\)Gr-1\(^+\) cells could cross-prime T cells. To evaluate this, we generated a chicken OV A-producing ID8 stable cell line that was designated ID8/OVA (IE9; Supplemental Fig. 3A). OVA expression in IE9 cells was
comparable to other OVA-expressing cell lines (Supplemental Fig. 3 B), and the kinetics of developing ascites by injecting 10 million IE9 cells was likewise comparable to that observed for ID8 cells (Fig. 1 A). In addition, we observed that CD11b+Gr-1+ cells in ascites were highly phagocytic in nature compared with DCs and CD11b+Gr-1− cells (Supplemental Fig. 4): a phenomenon that is often observed for neutrophils (36, 37).

We cultured PECs from naive mice, CD11b+Gr-1−, or CD11b+Gr-1+ cells from IE9/ascites with purified OT-I CD8+ T cells in the absence of any OVA-specific peptide. Interestingly, OT-I CD8+ T cells proliferated significantly when cultured with CD11b+Gr-1+ cells from IE9/ascites (Fig. 4A). We then subsequently cultured CD11b+Gr-1+ cells from naive spleen, IE9/ascites, and IE9/ascites with purified OT-II CD4+ or OT-I CD8+ T cells in the absence of any peptide and confirmed that the proliferation of OT-I CD8+ T cells was induced only by the CD11b+Gr-1+ cells from IE9/ascites (Fig. 4B). Moreover, these OT-I CD8+ T cells produced copious quantities of IL-2 and IFN-γ (Fig. 4C), suggesting that CD11b+Gr-1+ cells were capable of cross-priming functional OT-I CD8+ T cells in vitro.

To evaluate whether CD11b+Gr-1+ cells from IE9-bearing mice could cross-prime CD8+ T cells in vivo, two experiments were performed: an in vivo proliferation assay and a therapeutic assay. Purified OT-I CD8+ T cells (Thy1.1+) were adoptively transferred to naive hosts (Thy1.2). The following day, purified CD11b+Gr-1− cells isolated from the indicated sources were injected by i.p. Five days later, transferred OT-I CD8+ T cells were detected in host mice. B. The summary of in vivo cross-priming assays is shown both in terms of the percentage and the total numbers of transferred Thy1.1+ CD8+ cells (n = 7 per group). C. Cells from the mesLN from each host mouse were cultured with or without OVA-specific peptide to measure IFN-γ production in the culture supernatant. A–C. Data are representative of four independent experiments. *p < 0.05; **p < 0.01; ***p < 0.0005; ****p < 0.0001.

**FIGURE 5.** Immunostimulatory CD11b+Gr-1+ cells were capable of cross-priming in vivo. A, Purified OT-I CD8+ T cells (Thy1.1+) were adoptively transferred to naive hosts (Thy1.2). The following day, purified CD11b+Gr-1− cells isolated from the indicated sources were injected by i.p. Five days later, transferred OT-I CD8+ T cells were detected in host mice. B. The summary of in vivo cross-priming assays is shown both in terms of the percentage and the total numbers of transferred Thy1.1+ CD8+ cells (n = 7 per group). C. Cells from the mesLN from each host mouse were cultured with or without OVA-specific peptide to measure IFN-γ production in the culture supernatant. A–C. Data are representative of four independent experiments. *p < 0.05; **p < 0.01; ***p < 0.0005; ****p < 0.0001.
in the mesenteric lymph nodes of the mice immunized with CD11b+Gr-1+ cells from IE9/ascites, but not from ID8/ascites (Fig. 5A, 5B). In the mesenteric lymph node, CD11b+Gr-1+ cells from IE9/ascites induced nearly 4-fold more transferred OT-I CD8+ T cells to proliferate in both the percentage and in the total cell number than did CD11b+Gr-1+ cells from naïve spleen or from IE9/spleen. Significant IFN-γ production was observed only in mice immunized with CD11b+Gr-1+ cells from IE9/ascites (Fig. 5C) when mesenteric lymph nodes from each host mouse were cultured ex vivo.

In our therapeutic model, adoptive transfers of CD11b+Gr-1+ cells from naïve spleen, IE9/spleen, or IE9/ascites together with purified OT-I CD8+ T cells were performed twice to EG7-bearing mice. Mice that received CD11b+Gr-1+ cells from either naïve or IE9/ spleen quickly developed s.c. tumors, and tumor sizes quickly exceeded 100 mm2 by 20 d after challenge. In stark contrast, mice receiving CD11b+Gr-1+ cells from IE9/ascites exhibited significantly delayed tumor development (Fig. 6A). In fact, one mouse did not develop a tumor until 15 d after challenge, while another mouse initially developed an EG7 tumor that then regressed completely. Four of six mice had significantly delayed tumor progression, although these tumors eventually regained a size >100 mm2. By comparing the time until the EG7 s.c. tumors developed >100 mm2, we observed that the mouse group treated with CD11b+Gr-1+ cells from IE9/ascites showed significant delay in tumor onset when compared with any other treatment groups (Fig. 6B). The therapeutic effect of CD11b+Gr-1+ cells from IE9/ascites was abrogated when mice were injected with EL-4 cells, which have no OVA production (data not shown). Mice treated with CD11b+Gr-1+ cells from EG7/spleen (MDSCs) developed tumors relatively quickly compared with the other groups, although no significant difference was observed.

These in vivo data indicate that immunostimulatory CD11b+Gr-1+ cells from ascites of IE9-bearing mouse effectively cross-prime functional Ag-specific CD8+ T cells. Blocking CD80 or B7-H1 on immunostimulatory CD11b+Gr-1+ cells during the process of cross-priming did not alter the proliferation of CD8+ OT-I T cells. However, blocking CD80 but not B7-H1 resulted in decreased cytokine production in proliferating CD8+ OT-I T cells (Fig. 7). This finding indicates that CD80 expression is required on the surface of immunostimulatory CD11b+Gr-1+ cells for the generation of functional CTLs in cross-priming.

**Discussion**

In this study, we showed the strong immunostimulatory function of CD11b+Gr-1+ cells induced by the epithelial ovarian cancer cell line ID8. To date, the most studied population of cancer-associated CD11b+Gr-1+ cells are MDSCs, which are generally regarded as a heterogeneous population of macrophage-like and neutrophil-like cells that exhibit strong immunosuppressive properties (30, 35). However, the CD11b+Gr-1+ cells that we have identified in ascites from ID8-bearing mice morphologically resemble neutrophils, but with some distinct differences. For example, cross-priming by immunostimulatory CD11b+Gr-1+ cells leads to more functional CTLs and significant regression of s.c. tumors in an Ag-specific manner. Immunostimulatory CD11b+Gr-1+ cells preferentially stimulate CD8+ T cells in vivo, although this population supports the proliferation of both CD4+ and CD8+ T cells in vitro. Reasons for this observation are not completely understood, but could include the greater ease of proliferation of OT-I versus OT-II cell in vivo, and greater MHC class I compared with MHC class II expression on immunostimulatory CD11b+Gr-1+ cells. In addition,
it is possible that the attenuated expression of iNOS, Arg-1, S100A8, and S100A9 transcripts in CD11b+Gr-1+ cells compared with MDSCs might be correlated to the immunostimulatory functions of CD11b+Gr-1+ cells from ID8/ascites (38, 39). The mechanisms governing induction of immunostimulatory CD11b+Gr-1+ cells versus MDSCs, are still unknown. Differentially induced cytokines or other signaling molecules, such as in the immune responses against tumor, could alter the phenotypic and functional differences between CD11b+Gr-1+ cells.

Several observations of nonsuppressive CD11b+Gr-1+ cells have actually been reported. For example, activated NK cells have been shown to induce the conversion of MDSCs to immunogenic APCs, presumably by producing soluble factors from activated NK cells (40). It has also been shown that Gr-1+CD11b+F4/80+ macrophage-like cells suppressed T cell proliferation, but that Gr-1+CD11b+F4/80- neutrophil-like cells were not suppressive in a tumor-bearing mouse model (41). Even suppressive Gr-1+CD11b+F4/80+ cells induced NK cell-mediated killing in an RMA-S tumor, whereas anti–Gr-1 mAb administration resulted in enhanced tumor growth. Interestingly, recent publications have demonstrated a clear distinction between the immunologic function of CD11b+Gr-1int/low cells and CD11b+Gr-1int/low cells isolated from spleens of either naive or tumor bearing mice (42–44). In these cases, CD11b+Gr-1int/low cells exhibited much less immunosuppressive function compared with CD11b+Gr-1int/low cells. Immunostimulatory CD11b+Gr-1+ cells generated in ID8/ascites are morphologically similar to CD11b+Gr-1high cells, but only immunostimulatory CD11b+Gr-1+ cells exhibit strong immunostimulatory properties with cross-priming. Furthermore, the expression pattern of surface molecules and a shift in side scatter in flow cytometry are dissimilar. Therefore, immunostimulatory CD11b+Gr-1+ cells can actually cross-prime in situ (i.e., within the ascites compartment of tumor-bearing mice itself). This is a technically challenging question that we are currently attempting to answer. Presumably, immunostimulatory CD11b+Gr-1+ cells failed to induce an effective antitumor immune response within the ascites compartment itself primarily because of large numbers of cancer cells, as is the case in advanced disease, in addition to the presence of other immunosuppressive factors. It is also likely that other detrimental consequences of cancer (e.g., infiltration of immunosuppressive CD11b+Gr-1+ cells in ascites) outweigh the beneficial role of immunostimulatory CD11b+Gr-1+ cells on antitumor immunity. In addition, we are currently unable to determine whether the small population of CD11b+Gr-1+ cells observed in the peritoneal cavity of ID8-bearing mice at an early stage (e.g., 3–7 wk after ID8 injection) were immunostimulatory or immunosuppressive. If they were immunostimulatory, it is possible that CD11b+Gr-1+ cells contribute to the immunologic editing of immunogenic cancers. CD11b+Gr-1+ cells might control ID8 tumor progression by inducing endogenous CTLs, which could account for the fact that the development of ascites occurs over many weeks in ID8-bearing mice. Alternatively, if these early-stage cells were in fact immunosuppressive MDSCs, it is possible that immunostimulatory CD11b+Gr-1+ cells arose from these MDSCs. In general, stem cells, immature DCs, or other types of immature myeloid cells are more immunosuppressive (50–55) than their fully differentiated or mature counterparts. Using this logic, immunostimulatory CD11b+Gr-1+ cells could differentiate from MDSCs. It is also possible that immunostimulatory CD11b+Gr-1+ cells and MDSCs might simultaneously coexist, but their prevalence might be influenced by factors induced by the host’s immune response and may also depend on the type of cancer and its anatomic location. Establishing a better understanding of the nature of cancer-induced CD11b+Gr-1+ cells is of great importance because such cells could have strong therapeutic potential in treating cancer.

Acknowledgments
We thank Dr. John Feulinger (University of Rochester Medical Center, Rochester, NY) for providing the OVA cDNA construct, Drs. Lieping Chen and Drew Pardoll (Johns Hopkins University) for providing the anti–Gr-1 hybridoma (RB6-8C5), EL-4, EG7, B16, and B16/OVA cell lines, and Dr. Sherry Werner for assistance in pathology.

Disclosures
The authors have no financial conflicts of interest.

References


