CD80 in Immune Suppression by Mouse Ovarian Carcinoma–Associated Gr-1$^+$CD11b$^+$ Myeloid Cells

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Abstract

An elevated number of Gr-1$^+$CD11b$^+$ myeloid cells has been described in mice bearing transplantable tumors, and has been associated with immune suppression. We examined the role of such myeloid suppressor cells in mice bearing the spontaneously transformed syngeneic mouse ovarian surface epithelial cell line, 1D8. We observed high levels of CD80 expression by Gr-1$^+$CD11b$^+$ cells from spleen, ascites, and tumor tissue of mice bearing 1D8 ovarian carcinoma, whereas CD40 and CD86 were absent. CD80 expression was not detected on Gr-1$^+$CD11b$^+$ cells from naïve mice. However, the expression of CD80 by Gr-1$^+$CD11b$^+$ cells from naïve mice was promoted by coculture with 1D8 cells. Because irradiated 1D8 cells, but not 1D8-conditioned medium, up-regulate CD80 expression by Gr-1$^+$CD11b$^+$ cells, this phenomenon likely requires direct interaction. Gr-1$^+$CD11b$^+$ cells derived from 1D8 tumor–bearing mice provided significant suppression of antigen-specific immune responses, but Gr-1$^+$CD11b$^+$ cells from naïve mice did not. Both short interfering RNA–mediated knockdown and genetic knockout of CD80 expression by Gr-1$^+$CD11b$^+$ cells of 1D8 tumor–bearing mice alleviated the suppression of antigen-specific immune responses. Suppression via CD80 on Gr-1$^+$ CD11b$^+$ myeloid cells was mediated by CD4$^+$CD25$^+$ T regulatory cells and required CD152. CD80 knockout or antibody blockade of either CD80 or CD152 retarded the growth of 1D8 tumor in mice, suggesting that expression of CD80 on Gr-1$^+$CD11b$^+$ myeloid cells triggered by 1D8 ovarian carcinoma suppresses antigen-specific immunity via CD152 signaling and CD4$^+$ CD25$^+$ T regulatory cells. Thus, CD80-dependent responses to myeloid suppressor cells may contribute to tumor tolerance and the progression of ovarian carcinoma. (Cancer Res 2006; 66(13): 6807-15)

Introduction

The interaction of T cells with immunosuppressive versus activated regulatory antigen-presenting cells triggers antigen-specific T cell tolerance as opposed to priming (1–3). Recently, specific subsets of dendritic cells (DC) such as regulatory DC (4), tolerogenic DC (1), and Gr-1$^+$CD11b$^+$ myeloid cells (5, 6) have been found to play a critical role in inducing immunosuppression. Tumor progression is associated with the accumulation of myeloid suppressor cells (MSC) that include immature macrophages, granulocytes, DCs, and myeloid cells (7–10). These MSCs share common features such as myeloid origin, macrophage-like morphology, the phenotype of surface receptors, and the ability to suppress T cells after culture *in vitro* (7–10). The Gr-1$^+$CD11b$^+$ surface markers are most often associated with a population of immature myeloid cells in the spleen of tumor-bearing mice (5, 11, 12). Such immature myeloid cells are present in the bone marrow and spleen of healthy mice, and differentiate into mature myeloid cells under normal conditions (12). The accumulation of Gr-1$^+$CD11b$^+$ cells in large numbers of tumor-bearing mice probably results from various tumor-derived factors. Release of interleukin-10 (13), transforming growth factor-β (14), interleukin-6 (15), vascular endothelial growth factor (16), prostanoids (17), prostaglandin E_2 (18), and stromal-derived factor α (19) by tumors have been implicated in preventing the differentiation and maturation of immunoregulatory cells and hampering the induction of antitumor immunity. These cells are also associated with immune suppression during viral infection, transplantation, UV irradiation, and cyclophosphamide treatment (20).

Receptors and their ligands on T cells and antigen-presenting cells are critical in delivering inhibitory or stimulatory signals that enable immune cells to remain dormant or to respond effectively to various stimuli (21). The CD28-B7 and TNFR/TNF superfamilies contain many of these molecules. B7 family members, including B7-1, B7-2, ICOS ligand, PD-L1, PD-L2, B7-H3, and B7-H4, are expressed on professional antigen-presenting cells as well as on cells within nonlymphoid organs, providing regulation of T cell activation and tolerance in peripheral tissues. The interaction of CD28 with CD80 (B7-1) or CD86 (B7-2) not only promotes initial T cell activation but also regulates self-tolerance by supporting CD4$^+$CD25$^+$ T regulatory (T_{reg}) cell homeostasis. B7 both enhances the development of Ag-specific CD4$^+$CD25$^+$ T_{reg} in the thymus in response to self-Ag recognition and also maintains these cells in peripheral lymphoid organs (22). A low-level expression of CD80/CD86 is required for CD4$^+$CD25$^+$ T_{reg} survival. CD152 (CTLA-4), which binds with higher affinity to CD80/CD86 than CD28, is predominantly expressed on CD4$^+$CD25$^+$ T_{reg} cells (23, 24).

Several mechanisms have been described for the induction of tolerance. These include release of soluble mediators like NO, lack of costimulation, Fas/FasL interaction and the induction of several types of regulatory T cells including Tr1, Th3, NKT cells, and CD4$^+$CD25$^+$ T_{reg} cells (25, 26). Tumor-associated myeloid cells may inhibit T cell responses by inducing the apoptosis of activated T cells via up-regulation of NO production and arginase activity (12, 27). Prostaglandin E_2 and tumor necrosis factor-α function in

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doi:10.1158/0008-5472.CAN-05-3755

macrophage-mediated T cell suppression (28, 29). Although a number of studies show that strict cell-cell contact between MSCs and T cells is required for T cell suppression in mice and humans (30–34), the mechanism by which direct cell-cell contact with such tumor-associated Gr-1$^+$CD11b$^+$ cells suppress CD8$^+$ T cells in an antigen-specific manner is not clear. Herein, we show that the murine model of ovarian carcinoma, mouse ovarian surface epithelial cell (MOSEC) line 1D8 triggers the accumulation of Gr-1$^+$CD11b$^+$ myeloid cells and CD4$^+$CD25$^+$ T_{reg} cells can be found in spleen, ascites, and tumor tissue in mice. Gr-1$^+$CD11b$^+$ cells from tumor-free mice do not detectably express CD80, CD40, or CD86. However, Gr-1$^+$CD11b$^+$ cells in mice bearing MOSEC tumor express high levels of CD80, although neither CD40 nor CD86 are detected. We show that direct contact of Gr-1$^+$CD11b$^+$ cells with MOSEC ovarian carcinoma cells causes the expression of CD80. CD80 expressed by these Gr-1$^+$CD11b$^+$ MSCs plays a critical role in immunosuppression induced by MOSEC tumors. The action of CD4$^+$CD25$^+$ T_{reg} cells and signaling via CD152 are also critical to immunosuppression by MOSEC tumors.

Materials and Methods

Mice. Six- to 8-week-old male C57BL/6 (NCI) and CD80−/− (B6, 129S4-CD80^{TM1SHR}) mice were maintained in a pathogen-free animal facility at least 1 week before use. Experiments were done in accordance with institutional guidelines. For *in vivo* blockade studies, mice were injected i.p. with 250 μg of anti-CTLA-4 monoclonal antibody (UC10-4F10-11; BD PharMingen, San Diego, CA) or anti-CD80 (mB7-1 affinity-purified goat IgG, AF740; R&D, Minneapolis, MN) on days −6, −4, −2, and +1.

MOSEC ovarian carcinoma model. A syngeneic mouse model for epithelial ovarian cancer based on a spontaneously transformed MOSEC cell line (1D8) has been previously described (35). MOSEC did not express the costimulatory molecules CD40, CD80, CD86, CD22.2, CD72, CTLA-4, or other markers CD5, B220, CD11b, and CD11c (data not shown). However, the MOSEC 1D8 cells expressed CD44 and very low levels of costimulatory molecules B7-H1, PD-1, and MHC class II (data not shown). Multiple tumors formed within 60 days after the i.p. injection of 1×10^7 MOSEC (data not shown) in syngeneic C57BL/6 mice. Tissue samples were collected 2 month after the i.p. MOSEC injection. To monitor tumor growth rate, 1×10^7 MOSEC cells were injected s.c. in the right leg.

Flow cytometric analysis and isolation of Gr-1+CD11b+ myeloid cells from ascites of mice with ovarian carcinoma. FITC-conjugated anti-CD11c (N418), anti-mouse CD86 (GL1), anti-mouse CD80 (16-10A1), anti-mouse CD40 (3/23), and phycoerythrin (PE)-labeled anti-CD4 (L3T4), anti-mouse CD8α (53-6.6), anti-mouse CD45 R/B220 (RA36B2), anti-mouse CD11b (M1/70), as well as purified anti-mouse I-A^b (25-9-17), and FITC-labeled anti-mouse Ly-6G (RB6-8C5), allophycocyanin anti-mouse Ly-6G (RB6-8C5), PE or FITC-labeled anti-CD25 (7D4), and PE-labeled CTLA-4 (UC10-4F10-11) were purchased from PharMingen. Single or multiple staining was done using different monoclonal antibodies. For each analysis, isotype-matched control monoclonal antibody was used a negative control. Cells were then washed twice, resuspended in PBS containing 1% paraformaldehyde and 1% FCS and kept at 4°C prior to flow cytometric analysis (FACScan, Becton Dickinson, San Diego, CA).

Ascites or splenic cells from mice with ovarian carcinoma were directly stained with FITC- or allophycocyanin-labeled anti-Gr-1 antibodies and PE-labeled anti-CD11b. After washing, cells were gated for myeloid cell characteristics, i.e., high forward and side scatter and bright staining for CD11b. These selected cells were then sorted using FACScan based on the staining for CD11b and for Gr-1 or the absence of staining for both. The purity of Gr-1$^+$CD11b$^+$ cells was >95%. CD4$^+$CD25$^+$ T_{reg} cells were also sorted using FACScan by staining with FITC-labeled anti-CD4 and PE-labeled CD25 antibodies.

Preparation of bone marrow cells. Bone marrow cells (BMC) were prepared as we have previously described (36). Briefly, BMCs were collected by removing the femur bones of mice, cutting off each end, and flushing out the bone marrow with RPMI 1640 using a syringe. The pooled cells were harvested by centrifugation at 1,600 rpm for 10 minutes and resuspended in 2 mL of ACK buffer for 5 minutes at room temperature to lyse RBC. These cells were washed in medium and cultured in RPMI 1640 with 10% FCS and 1% penicillin and streptomycin. To determine the effect of ovarian carcinoma cells on the BMCs, 5×10^4 ovarian carcinoma cells with or without irradiation (10^4 Rad) or 50% (final concentration) supernatant from tumor cultures were added into BMCs, respectively. The Gr-1$^+$CD11b$^+$ cells were isolated and surface markers were analyzed by flow cytometry.

Short interfering RNA design, synthesis, and transfection. Short interfering RNA (siRNA) sequences used for targeted silencing of human CD80 were designed according to protocols in the Ambion Inc., (Austin, TX) web site, http://www.ambion.com, and had the following sequences: sense 5′-UGGAAGAGAAUUACCUGGCTT and antisense 5′-GCCAGGUAAUUCUCUUCCATT. Nonsilencing control siRNA is an irrelevant siRNA with random nucleotides (5′-ACUATCUAAGUUACTACCCCTT). Sequences were synthesized and annealed by the Johns Hopkins University Genetics Core Facility (Baltimore, MD). Searches of the mouse genome database (BLAST) were carried out to ensure that the sequence would not target other gene transcripts. Cells were transduced with the siRNAs using GeneSilencer (Gene Therapy Systems, San Diego, CA), which was used according to the manufacturer's protocol.

Inhibition of antigen-specific responses by ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells. Gr-1$^+$CD11b$^+$ cells obtained from mice with or without MOSEC tumor, as indicated, splenocytes from mice immunized with human papillomavirus type 16 virus-like particles (VLP), and VLPs were, respectively, used as suppressor, responders, and stimulator. To investigate suppressive role of ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells in the antigen-specific response, 2×10^6 splenocytes were cocultured with VLP (final concentration, 25 μg/mL) in triplicate in 24-well flat-bottomed plates (Falcon, BD Biosciences, Franklin Lakes, NJ) in 200 μL of RPMI 1640 according to our prior methods (36). CD4$^+$ cell and CD8$^+$ beads were prepared according to the described protocol (MACS). Gr-1$^+$CD11b$^+$ MSCs (5×10^4 per well unless otherwise stated) were added into culture of splenocytes during VLP stimulation. To determine the suppressive effect of MSCs on VLP-induced splenocyte proliferation, splenocytes from mice immunized with VLPs were labeled with 2.5 μmol/L of CFSF for 10 minutes at room temperature (with gentle agitation every 2-3 minutes), and then washed thrice with PBS. The CSFE-labeled splenocytes were stimulated with VLP and cocultured with Gr-1$^+$CD11b$^+$ MSCs. The cells and supernatants were harvested separately at 4 days. The cells were analyzed by flow cytometry and IFN-γ in the supernatant was assayed by capture ELISA. To determine the effect of CD4$^+$CD25$^+$ T_{reg} cells on the VLP-specific responses, CD25$^+$ T_{reg} cells were depleted using magnetic beads or the sorted CD25$^+$ T_{reg} cells by FACScan were added into culture. CD4$^+$CD25$^+$ T_{reg}s (1×10^4) were used in all experiments unless otherwise stated. After 3 days, the supernatant was collected and IFN-γ was analyzed.

Blocking experiments were used to determine the role of costimulatory molecules on the ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells in which various neutralizing antibodies were added into the culture to 10 μg/mL, including anti-CD80 (goat IgG) and anti-mouse CTLA-4 (UC10-4F10-11, hamster IgG_1). The supernatants were collected after 3 days and release of IFNγ was analyzed. All monoclonal antibodies had an endotoxin level of <1 endotoxin units/mL.

RT-PCR analysis. Total cellular RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) followed by RNA clean-up with RNeasy Mini kit (Qiagen, Valencia, CA). RT-PCR was done by SuperScript one-step RT-PCR with Platinum Taq according to the protocol provided (Invitrogen). For cDNA synthesis and predenaturation, we used 1 cycle (50°C for 15 minutes, 94°C for 2 minutes); and for PCR amplification, we used 40 cycles (denature, 94°C for 15 seconds, anneal, 55°C for 30 seconds, extend 72°C for 1 min/kb); and for final extension, we used 1 cycle (72°C for 10 minutes). The primers included: mouse Foxp3, sense 5′-ATGCCCAACCCTAGGCCAGC

and antisense 5′-TCAAGGGCAGGGATTGGAGCC; mouse CD80, sense 5′-ATGGCTTGCAATTGTCAGTTG and antisense 5′-CTAAAGGAAGACGG-TCTG; GAPDH, sense 5′-ATGGTGAAGGTCGGTGTGAACGGATTTGGC and antisense 5′-CATCGAAGGTGGAAGAGTGGGAGTTGCTGT.

ELISA. Commercial sandwich ELISA kits were used for the quantitation of IFNγ (Pierce Endogen, Rockford, IL), The absorbance of each of the samples were measured at 450 nm using a SpectraMax 190 ELISA plate reader. Cytokine levels were quantified from two to three titrations using standard curves, and expressed in pg/mL.

Results

Murine model of ovarian carcinoma induces Gr-1$^+$CD11b$^+$ MSCs. Previous studies of mice bearing transplantable tumors (37) have shown the accumulation of a Gr-1$^+$CD11b$^+$ MSC population. We find that mice bearing the transplantable model of ovarian carcinoma MOSEC 1D8 exhibit a distinct Gr-1$^+$CD11b$^+$ population of myeloid cells present in the spleen, ascites, and also tumor tissue that was absent from tumor-free mice (Fig. 1*A*). Consistent with their surface marker phenotype, the Gr-1$^+$CD11b$^+$ population of cells exhibited myeloid features by Giemsa staining (data not shown). This phenomenon also occurs in mice bearing Lewis lung carcinoma (LLC) but not the cervical carcinoma model TC-1 (data not shown).

To determine whether the Gr-1$^+$CD11b$^+$ population of cells suppressed antigen-specific T cell responses as previously described, we used a potent heterologous antigen from our previous studies; human papillomavirus type 16 L1 VLPs. This xenogenic antigen induces potent L1-specific antibody and T cell responses in mice. When VLPs are added to the splenocytes of vaccinated mice, antigen-presenting cells such as DCs and B cells in splenocytes could capture, process, and present VLP and then induce strong VLP-specific T cell responses. We observed that the Gr-1$^+$CD11b$^+$ myeloid cells derived from mice bearing ovarian carcinoma suppressed VLP-specific T cell responses *in vitro*, including both proliferation and release of IFNγ (Fig. 1*B-D*). Importantly, antigen-specific T responses were suppressed by Gr-1$^+$CD11b$^+$ cells from mice bearing MOSEC 1D8 tumor but not those of tumor-free mice (Fig. 1*B-D*). This suggests that Gr-1$^+$CD11b$^+$ cells from mice bearing

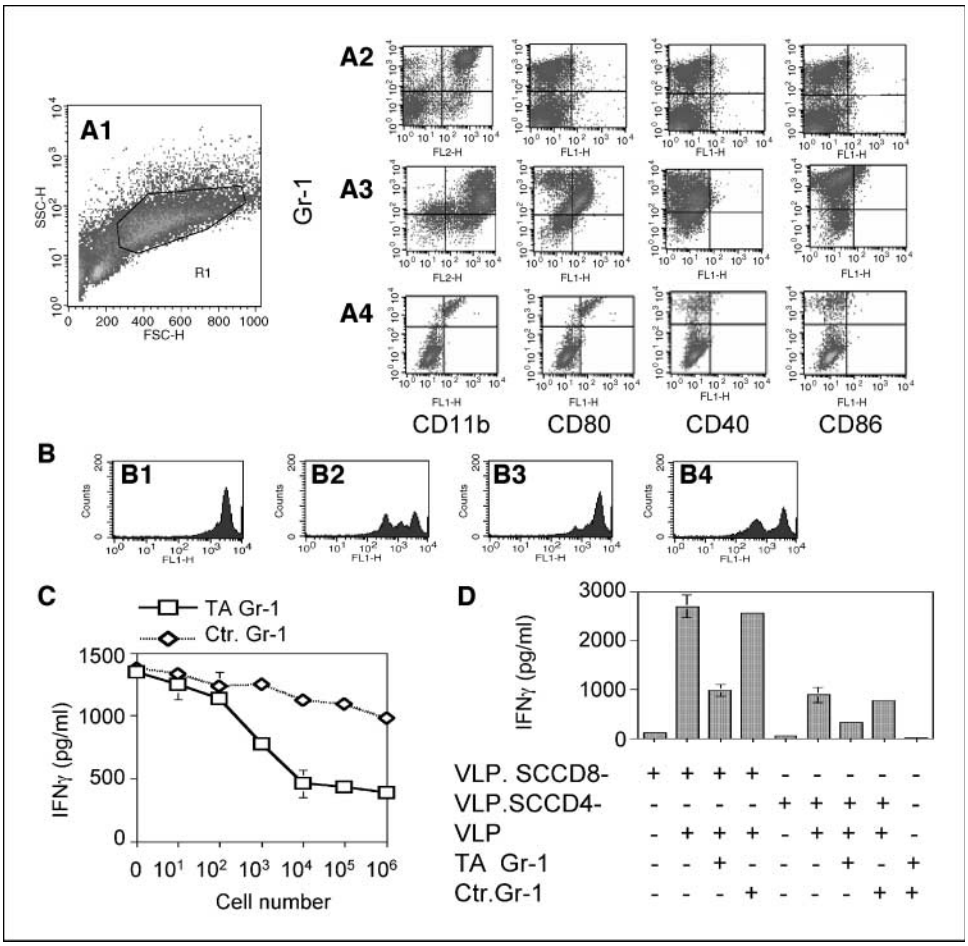

Figure 1. Characteristics of MOSEC 1D8 ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ MSCs. *A,* flow cytometric analysis of splenocytes from a tumor-free mouse. Gating on forward and side scatter (*1*), and staining for Gr-1 versus CD11b, CD80, CD86, and CD40 (*2*); *3,* similar to (*2*), but using splenocytes of mice bearing MOSEC 1D8 tumors; *4,* flow cytometric analysis of MOSEC 1D8 ascites cells. The cells were gated on side and forward scatter, and larger cells were analyzed. The cells were stained by FITC-labeled antimouse CD11b, CD80, CD40, and CD86, PE-labeled antimouse Gr-1 antibodies. *B,* mouse ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells inhibited VLP-specific proliferation of CFSE-labeled splenocytes: *1,* splenocytes from mice immunized by VLPs without stimulation; *2,* splenocytes from mice immunized by VLPs stimulated with VLPs; *3,* similar to (*2*), but MOSEC 1D8 tumor-associated Gr-1$^+$CD11b$^+$ cells (5×10^4) were added into the culture; *4,* similar to (*2*) but Gr-1$^+$CD11b$^+$ cells isolated from tumor-free mice were added into the culture. Splenocytes from mice immunized with human papillomavirus type 16 L1 VLPs (36) were labeled with 2.5 μmol/L of CFSE for 10 minutes and then stimulated by VLP. *C,* mouse ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells inhibited VLP-specific production of IFNγ. The indicated numbers of Gr-1$^+$CD11b$^+$ cells were sorted by flow cytometry from mice bearing MOSEC 1D8 tumor (*TA-Gr1*) or tumor-free mice (*Ctr.Gr-1 cells*) and were cocultured with 2×10^6 VLP-specific splenocytes (*VLP.SC*). *D,* splenocytes from VLP-vaccinated mice, depleted for either CD8$^+$ (*VLP.SCCD8*$^-$) or CD4$^+$ cells (*VLP.SCCD4*$^-$), and either stimulated with VLP or not, were cultured with Gr-1$^+$CD11b$^+$ cells from either control or 1D8 tumor–bearing mice.

MOSEC 1D8 tumor are functionally different from those of naïve mice. Furthermore, Gr-1$^+$CD11b$^+$ cells from mice bearing LLC suppress ovalbumin-specific T cell release of IFNγ, suggesting that this phenomenon is neither unique to VLP-specific T cell responses nor Gr-1$^+$CD11b$^+$ cells from mice bearing MOSEC 1D8 tumor (data not shown).

Some prior studies indicate that MSCs inhibit MHC class I-restricted CD8$^+$ T cells and have no effect on CD4$^+$ T cells (16, 38), whereas others find that Gr-1$^+$CD11b$^+$ MSCs also inhibit CD4$^+$ T cell function (39). We addressed this issue in the MOSEC 1D8 model. When ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells were added into CD8-depleted or CD4-depleted VLP-specific splenocytes, VLP-dependent production of IFNγ was significantly reduced in both splenocyte preparations (Fig. 1*D*). Again, suppression was not observed using Gr-1$^+$CD11b$^+$ cells from naïve mice. This suggests that mouse ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells inhibit antigen-specific release of IFNγ by both CD4$^+$ and CD8$^+$ cells.

Bronte et al. (5) described that Gr-1$^+$CD11b$^+$ myeloid cells expressed MHC class II, B220, F4/80, CD86, CD16/32, and DEC205. In contrast, Gabrilovich et al. (16, 40) reported the expression of MHC class I and the absence of MHC class II and costimulatory molecules, suggesting that there may be significant differences in the phenotypes and functions of Gr-1$^+$CD11b$^+$ myeloid cells induced by different tumors. Therefore, we examined the surface markers for Gr-1$^+$CD11b$^+$ MSCs from mice with 1D8 ovarian carcinoma and found no detectable expression of MHC class II, CD14, B220 (data not shown), or the CD40 and CD86 costimulatory molecules (Fig. 1*A*).

Direct contact of mouse ovarian carcinoma with Gr-1$^+$CD11b$^+$ myeloid cells promotes CD80 expression. A number of tumor-derived signals (17, 40) have been implicated in preventing the differentiation and maturation of immunoregulatory cells and hampering the induction of antitumor immunity. Our findings suggest that Gr-1$^+$CD11b$^+$ cells from mice bearing MOSEC 1D8 tumor are functionally different from those of disease-free mice. Thus, it is possible that 1D8 ovarian carcinoma–derived factors could render Gr-1$^+$CD11b$^+$ cells of naïve mice immunosuppressive. CD80 expression has been described as a surface marker of MSCs expanded in mice with disseminated candidiasis, and its depletion increased IFNγ-mediated antifungal resistance (20, 41). This finding suggested a possible suppressive role for CD80 in the activity of MSCs. Therefore, we examined the levels of CD80 on Gr-1$^+$CD11b$^+$ cells from splenocytes of naïve and MOSEC 1D8 tumor–bearing mice (Fig. 1*A*). CD80 expression was present in the Gr-1$^+$CD11b$^+$ cells of tumor-bearing (Fig. 1*A3*), but not naïve mice (Fig. 1*A2*). Likewise, Gr-1$^+$CD11b$^+$ cells in 1D8 ascites also expressed CD80 (Fig. 1*A4*).

To address whether CD80 expression on Gr-1$^+$CD11b$^+$ cells was a direct effect of the 1D8 tumor cells, we isolated Gr-1$^+$CD11b$^+$ cells from the bone marrow of tumor-free mice and cocultured them for 3 days with 1D8 cells at a ratio of 1:10 (1D8 cells/Gr-1$^+$CD11b$^+$ cells), irradiated 1D8 cells at a ratio of 1:10 (1D8 cells/Gr-1$^+$CD11b$^+$ cells), or in 25% supernatant conditioned using 1D8 *in vitro* cultures. Total RNA from Gr-1$^+$CD11b$^+$ cells with each different exposure was analyzed by RT-PCR for CD80 transcript expression. The semiquantitative RT-PCR analysis suggested that CD80 transcripts were significantly up-regulated when Gr-1$^+$CD11b$^+$ cells from the bone marrow of tumor-free mice were cocultured with 1D8 tumor cells (Fig. 2*A*). A similar effect was observed upon coculture with irradiated 1D8 cells. However, 25% (v/v) conditioned supernatant from 1D8 cultures had a lesser effect on CD80 transcript expression.

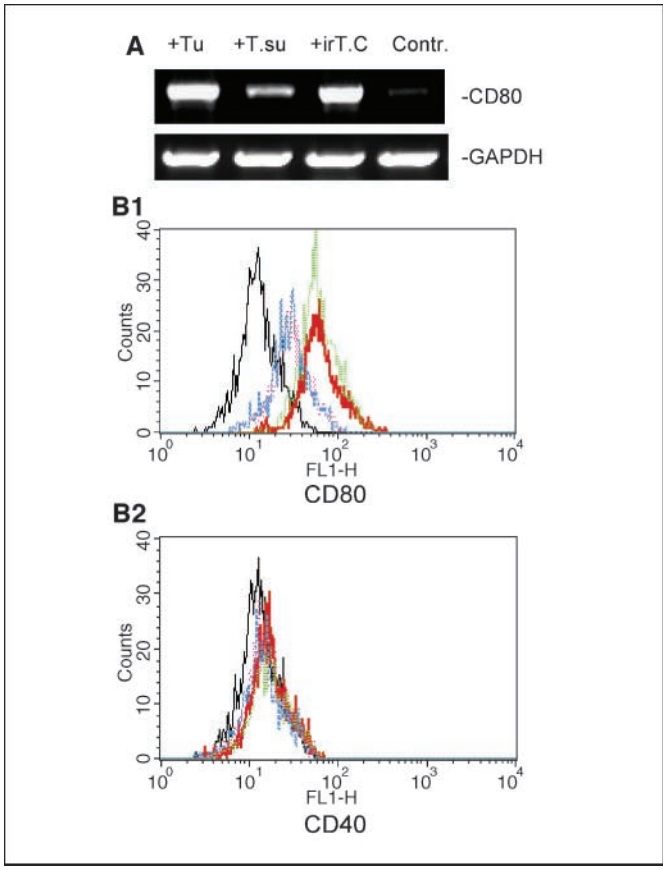

Figure 2. Direct contact between MOSEC 1D8 cells induces expression of CD80 on Gr-1$^+$CD11b$^+$ cells. *A,* semiquantitative RT-PCR showing the effect of cocultured live (*T.C*) or irradiated MOSEC 1D8 cells (*irT.C*) or MOSEC 1D8 cell-conditioned medium (*T.Su*) versus control medium (*Contr.*) on CD80 transcript levels in Gr-1$^+$CD11b$^+$ cells. *B,* flow cytometric analysis of surface CD80 (*1*) and CD40 (*2*) expression on Gr-1$^+$CD11b$^+$ cells cultured in the presence of tumor cells (*red solid line*), irradiated cells (*green stippled line*), 1D8 cell-conditioned medium (*red stippled line*), medium alone (*blue line*), or isotypic control (*solid black line*).

We examined the up-regulation by 1D8 cells of CD80 on Gr-1$^+$CD11b$^+$ cells from the bone marrow of tumor-free mice at the protein level using flow cytometry (Fig. 2*B*). In parallel with the RT-PCR findings for CD80 transcripts, surface expression of CD80 (Fig. 2*B1*), but not CD40 (Fig. 2*B2*), on Gr-1$^+$CD11b$^+$ cells from the bone marrow of tumor-free mice was up-regulated in those cultured with both live and irradiated 1D8 cells, but not cells cultured in 25% 1D8-conditioned medium. Thus, MOSEC 1D8 tumor cells not only cause the accumulation of Gr-1$^+$CD11b$^+$ MSCs but also induce the expression of CD80. This phenomenon depends on direct contact between MOSEC 1D8 ovarian carcinoma cells and Gr-1$^+$CD11b$^+$ MSCs, but not tumor cell proliferation (Fig. 2).

Antibody blockade of CD80 decreases the suppressive potential of mouse ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ MSCs. CD80 is a prototypic member of the B7-CD28 family of costimulatory molecules. However, reports differ in the outcome of CD80-dependent signaling in regulating T cell activity (42). Thus, we hypothesized that CD80 expression by the ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ MSCs might contribute to the suppression of antigen-specific immunity and the induction of

tumor immunotolerance (20, 41). To address the role of CD80 in the suppressive effects of Gr-1$^+$CD11b$^+$ MSCs on antigen-specific T cell responses, we again used splenocytes from mice vaccinated with the potent heterologous antigen, HPV16 L1 VLPs. The presence of CD80-specific neutralizing antibody, but control antibody, inhibited the suppressive effect of ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ MSCs upon VLP-specific and T cell–dependent IFNγ release (Fig. 3*A*). Furthermore, CD80-specific neutralizing antibody did not alter VLP-specific and T cell–dependent IFNγ release in the absence of Gr-1$^+$CD11b$^+$ MSCs suggesting that the effect is mediated by binding of CD80 antibody to CD80$^+$Gr-1$^+$CD11b$^+$ MSCs. Because antibody blockade suggests that CD80 signaling contributes to the suppression of antigen-specific immune responses by ovarian carcinoma–associated CD80$^+$Gr-1$^+$CD11b$^+$ MSCs, we reasoned that blockade of the CD80 ligand CD152 might produce a similar effect. As shown in Fig. 3*A*, antibody blockade of CD152 also significantly decreased the suppression of VLP-specific and T cell–dependent IFNγ release by ovarian carcinoma–associated CD80$^+$Gr-1$^+$CD11b$^+$ MSCs. CD152 antibody produces a significantly stronger inhibition than CD80 antibody ($P < 0.05$) and their effects seem to be additive. CD152 functions as a negative signaling molecule not only expressed by T_{reg} cells, but also in many different kinds of lymphocytes. In contrast, CD80 is mainly expressed in monocyte-derived cells such as antigen-presenting cells. This may account for stronger inhibition of T cell suppression by antibody blockade of CD152 as compared with CD80 blockade.

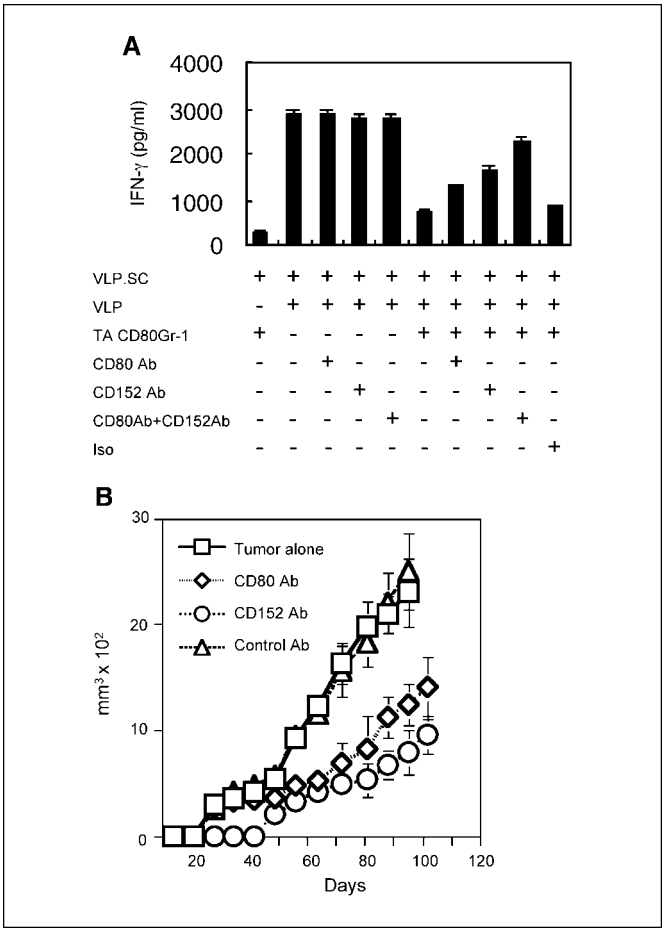

Figure 3. Blockade of CD80 or CD152 reduced antigen-specific T cell suppression by mouse ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells and slowed tumor growth. *A,* effect of neutralizing antibody (10 μg/mL) to CD80 or CD152 on the generation of IFNγ by VLP-specific splenocytes (*VLP.SC*) in the presence of mouse ovarian carcinoma–associated CD11b$^+$Gr-1$^+$ cells (*TA CD80Gr-1*) and CD4$^+$CD25$^+$ cells. Supernatants of cultures were collected after 24 hours and IFNγ was assayed by capture ELISA. *Iso,* control antibody. *B, in vivo* injection of neutralizing antibody to CD80 or CD152 significantly slowed and retarded tumor growth. Mice (six/group) were injected using MOSEC 1D8 cells (s.c. with 5×10^6) and i.p. with 250 μg of control antibody or antibody specific for CD152 or CD80 on days −6, −4, −2, and +1.

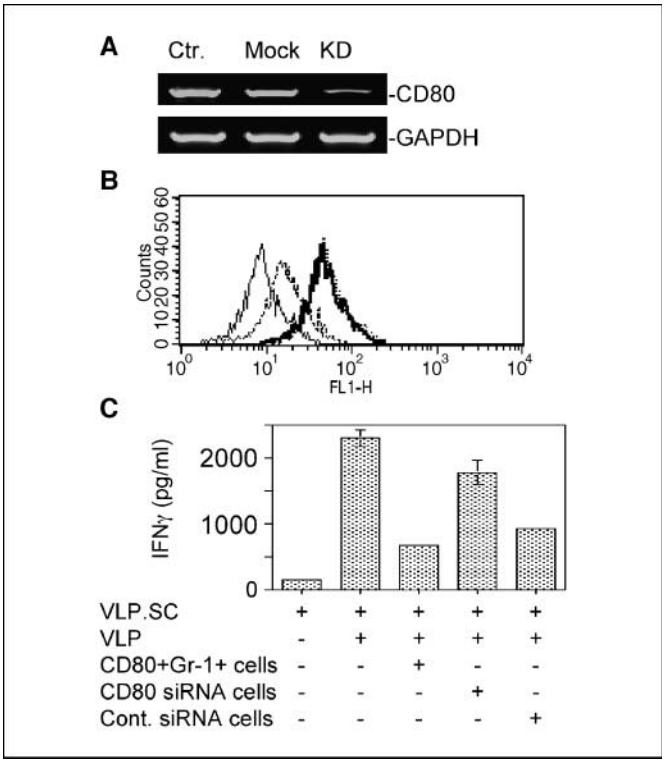

Figure 4. Knockdown of CD80 by siRNA decreased the suppressive potential of ovarian carcinoma–associated CD80$^+$Gr-1$^+$CD11b$^+$ MSCs. *A,* CD80 siRNA reduced the level of CD80 but not GAPDH transcript expression in transfected ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells, as assessed by RT-PCR. *B,* CD80 siRNA, but not nonspecific siRNA, reduced the level of surface CD80 expression in transfected ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells, as assessed by flow cytometric analysis: isotype control (*thin black line*), expression of CD80 on ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ MSCs (*thick black line*), expression of CD80 on ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells by control siRNA (*dotted line*), and expression of CD80 on ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells transfected with CD80-specific siRNA (*dashed line*). *C,* CD80 siRNA inhibited the suppression by ovarian carcinoma–associated Gr-1CD11b$^+$ cells of VLP-induced generation of IFN-γ in VLP-specific splenocyte cultures (*VLP.SC*). *CD80$^+$Gr-1$^+$ cells,* cells sorted from the ascites of mice with MOSEC 1D8 ovarian carcinoma; *CD80siRNA cells,* CD80$^+$Gr-1$^+$ cells transfected with CD80 siRNA; *Cont. siRNA cells,* CD80$^+$Gr-1$^+$ cells transfected with control siRNA.

To further address the role of CD80-dependent signaling on tumor tolerance, we examined the effect of both CD80- and CD152-specific neutralizing antibodies on the growth of the MOSEC 1D8 ovarian carcinoma model in C57BL/6 mice. The administration of either CD80- or CD152-specific neutralizing antibody (250 μg i.p.) on days −6, −4, −2, and +1 around the s.c. inoculation with 10^7 1D8 cells delayed the growth of tumor with respect to mice that did not receive antibody, whereas control antibody failed to retard tumor growth (Fig. 3*B*). When CD80 or CD152 neutralizing antibodies are injected into mice 1 week after inoculation with LLC cells (data not shown), the delay in tumor growth was similar to that seen with the protocol for CD80 antibody injection on days

−6, −4, −2, and +1 observed in mice challenged with 1D8 tumor (Fig. 3*B*). Furthermore, the infusion of $CD80^+Gr\text{-}1^+CD11b^+$ MSCs from mice with large carcinoma burden into naïve mice produces more rapid tumor outgrowth upon subsequent LLC tumor challenge (data not shown). This suggests that expansion of the $CD80^+Gr\text{-}1^+CD11b^+$ MSC population suppresses immunity against both tumor types. Furthermore, stronger inhibition of T cell suppression by antibody blockade of CD152 as compared with CD80 blockade (Fig. 3*A*) is consistent with the greater reduction of 1D8 and LLC tumor growth by treatment of mice with CD152 antibody versus CD80 antibody (Fig. 3*B*; data not shown).

CD80 knockdown by siRNA decreased the suppressive potential of ovarian carcinoma–associated $Gr\text{-}1^+CD11b^+$ MSCs. Antibody blocking studies suggest that signaling via CD80 contributes to the induction of immunosuppression by MOSEC 1D8 ovarian carcinoma. Because antibody treatment can induce nonspecific effects, we sought to further confirm the role of CD80 in the suppression of antigen-specific T cell responses by $Gr\text{-}1^+CD11b^+$ MSCs using CD80-targeted and nonspecific control siRNA. Upon transfection, the CD80-targeted siRNA reduced the CD80 transcript (Fig. 4*A*) and surface protein (Fig. 4*B*) levels with respect to untreated ovarian carcinoma–associated $Gr\text{-}1^+CD11b^+$ MSCs, whereas the control siRNA did not. Flow cytometric analysis showed that neither CD80-targeted nor nonspecific control siRNA altered the level of CD40 or CD86 in these cells (data not shown). Ovarian carcinoma–associated $Gr\text{-}1^+CD11b^+$ MSCs transfected with CD80-targeted siRNA or control siRNA were added into the culture of VLP-specific splenocytes with VLPs. Ovarian carcinoma–associated $Gr\text{-}1^+CD11b^+$ MSCs transfected with CD80-targeted siRNA exhibited significantly reduced suppressive potential for VLP-specific and T cell–dependent induction of IFNγ release as compared with control siRNA–treated cells.

Growth of MOSEC 1D8 tumor is retarded in CD80-deficient mice. Both antibody blockade and siRNA knockdown studies support the involvement of CD80 on the $Gr\text{-}1^+CD11b^+$ cells in inducing immune suppression against MOSEC 1D8 tumor. Therefore, we explored this phenomenon further in CD80-deficient mice. When MOSEC 1D8 cells were injected s.c. into CD80−/− and CD80+/+ mice, tumor appeared later and growth was clearly slower in CD80−/− as compared with CD80+/+ mice, further supporting a role for CD80-dependent signaling in the induction of immune suppression (Fig. 5*A*).

CD80 deficiency decreases the suppressive potential of ovarian carcinoma–associated $Gr\text{-}1^+CD11b^+$ cells. Because MOSEC 1D8 tumor appeared later and growth was clearly slower in CD80-deficient as compared with wild-type mice, we isolated the $Gr\text{-}1^+CD11b^+$ cells derived from these CD80−/− mice and wild-type mice bearing tumors, and examined their ability to suppress antigen-specific T cell responses. $Gr\text{-}1^+CD11b^+$ cells derived from wild-type mice bearing MOSEC 1D8 tumor exhibited significant suppression on the VLP-specific immune responses, whereas those isolated from CD80−/− mice with the equivalent tumor load showed a reduced effect (Fig. 5*B*).

Recent findings suggest that TLR signaling, notably TLR9 activation by CpG, in the appropriate context, could overcome immune tolerance. Using RT-PCR, we determined that $Gr\text{-}1^+CD11b^+$ MSCs express transcripts for TLR2, TLR4, and TLR9 (data not shown). To explore this possibility, $Gr\text{-}1^+CD11b^+$ MSCs isolated from CD80−/− or CD80+/+ mice bearing ovarian carcinomas were stimulated by the CpG ODN2006 (or lipopolysaccharide) to determine the effect of TLR9 activation on antigen-specific T cell suppression. Although control and lipopolysaccharide-treated (data not shown) $Gr\text{-}1^+CD11b^+$ MSCs suppressed VLP-specific and T cell–dependent release of IFNγ, CpG-treated $Gr\text{-}1^+CD11b^+$ cells

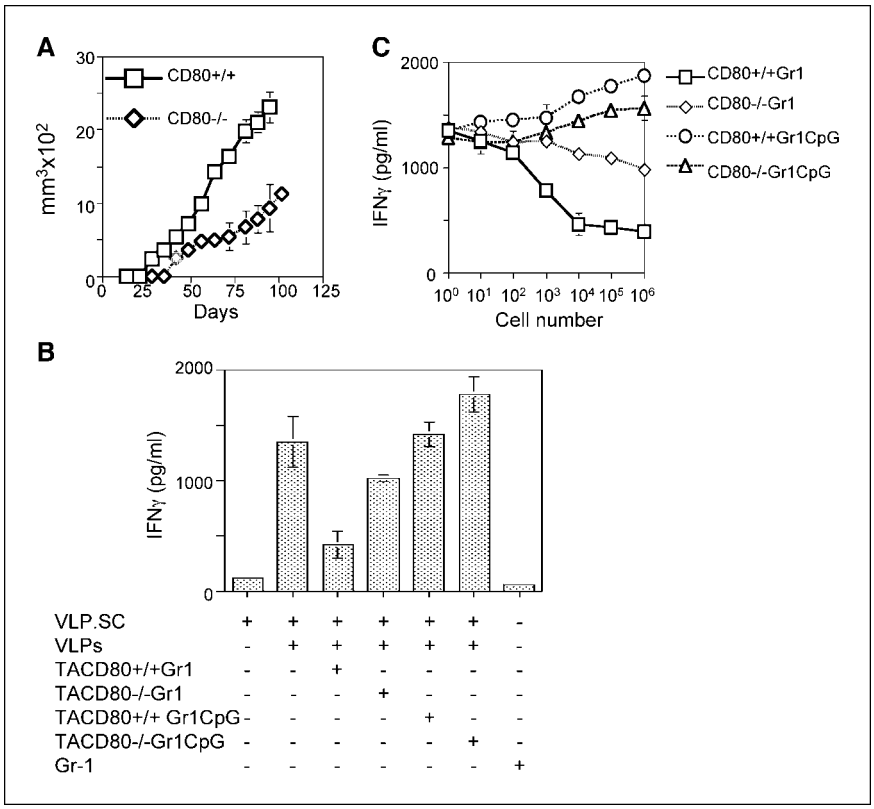

Figure 5. Effect of CD80 deficiency on ovarian carcinoma–associated $Gr\text{-}1^+CD11b^+$ cell suppression of T cell responses and tumor tolerance. *A,* the growth of MOSEC 1D8 tumor is delayed and slower in CD80 knockout mice as compared with wild-type mice (six/group). *B* and *C,* $Gr\text{-}1^+CD11b^+$ cells from CD80−/− mice with ovarian carcinoma exhibited a decreased suppression of VLP-induced production of IFNγ in VLP-specific splenocyte cultures. CD80−/− $Gr\text{-}1^+CD11b^+$ cells and CD80+/+ $Gr\text{-}1^+CD11b^+$ cells were sorted from splenocytes of CD80−/− mice and wild-type mice with ovarian carcinoma, respectively. Isolated $Gr1^+CD11b^+$ cells were cocultured with or without CpG (5 nmol/L, ODN2006; InvivoGen, San Diego, CA). These cells were washed and then added into the culture to assess the influence of CpG treatment of $Gr\text{-}1^+CD11b^+$ cells on their suppression of VLP-specific T cell production of IFNγ. Supernatants of T cell cultures were collected after 24 hours and IFNγ assayed by capture ELISA.

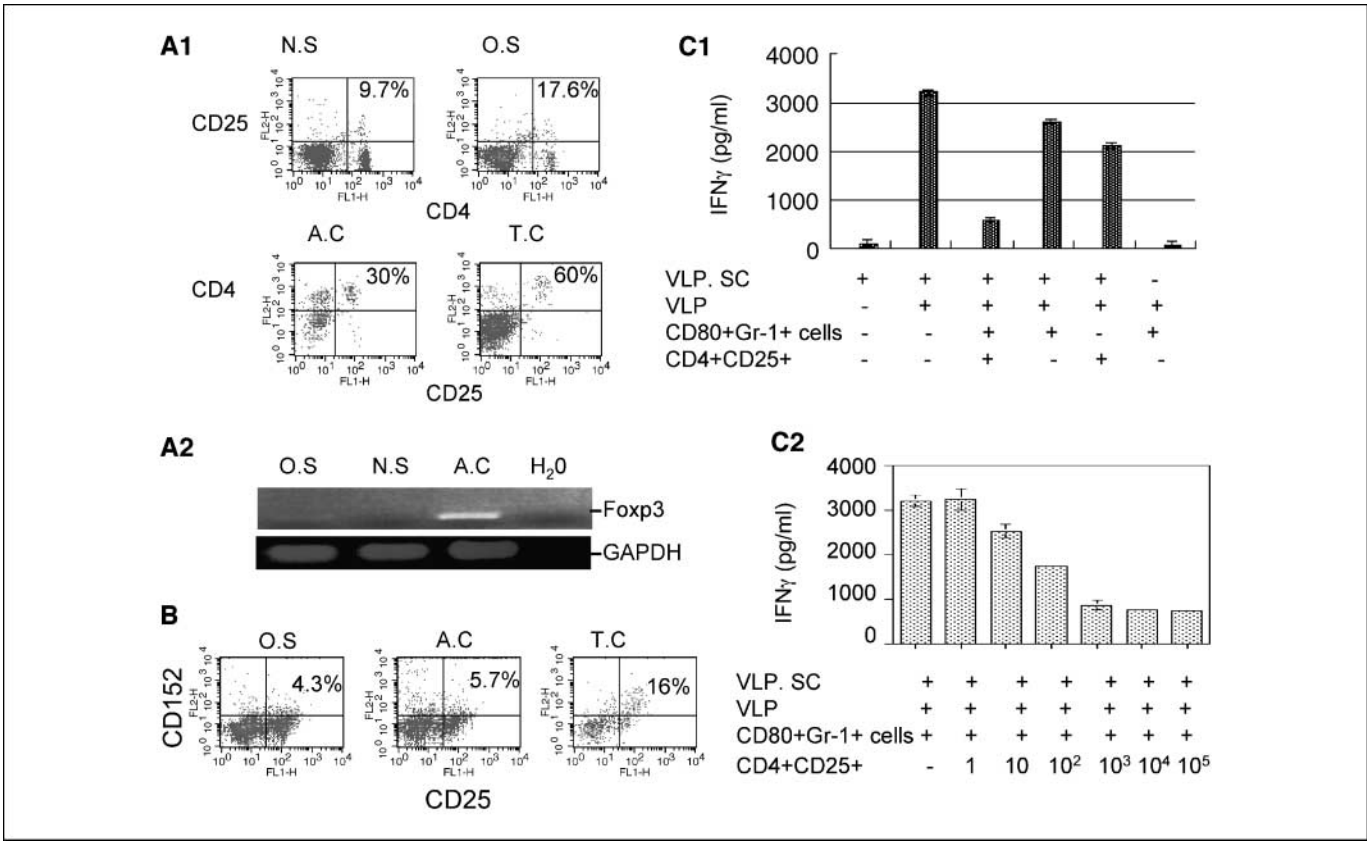

Figure 6. Inhibition of antigen-specific responses by $CD80^+Gr\text{-}1^+CD11b^+$ cells via $CD4^+CD25^+$ T_{reg} cells. *A,* flow cytometric analysis (*1*) of $CD4^+CD25^+$ T_{reg} cells isolated from splenocytes of tumor-free mice (*N.S*), splenocytes from mice with ovarian carcinoma (*O.S*), ascites cells from mice with ovarian carcinoma (*A.C*), and ovarian tumor–infiltrating lymphocytes (*T.C*). The figures reflect the percentage of $CD4^+$ T cells that express CD25. *2,* the above cells were analyzed for Foxp3 and GAPDH transcript expression by RT-PCR. *B,* expression of CD152 on $CD25^+$ T cells isolated from mice bearing MOSEC 1D8 ovarian carcinoma. *C,* $CD4^+CD25^+$ T_{reg} cells are required for the suppression of VLP-induced production of IFNγ in VLP-specific splenocyte cultures by MSCs. VLP-specific splenocytes were depleted of $CD25^+$ cells using microbeads (*VLP.SC*) and stimulated with VLP. $CD80^+Gr\text{-}1^+$ cells and $CD4^+CD25^+$ cells were isolated from the ascites of mice with MOSEC 1D8 ovarian carcinoma by flow cytometry and then added into culture at the numbers indicated.

from tumor-bearing CD80+/+ mice failed to effectively suppress this response. Notably, CpG-treated $Gr\text{-}1^+CD11b^+$ cells from CD80−/− mice bearing ovarian carcinoma significantly enhanced VLP-specific and T cell–dependent release of IFNγ (Fig. 5*B*). These phenomena were dependent on the number of MSCs added to the VLP-specific splenocyte cultures (Fig. 5*C*).

Role of $CD4^+CD25^+$ T_{reg} cells in the suppression of antigen-specific responses by $Gr\text{-}1^+CD11b^+$ MSCs. $CD4^+CD25^+$ T_{reg} cells suppress antitumor immunity and are expanded in patients with ovarian cancer and correlate with poor clinical outcome (43). We also observed an increase in the splenic population of $CD4^+$ $CD25^+$ T cells in mice bearing MOSEC 1D8 tumor (Fig. 6*A1*). Furthermore, a greatly increased proportion of $CD4^+CD25^+$ T_{reg} cells were observed in MOSEC 1D8 ascites (30% of total $CD4^+$ T cells) and tumor tissue (60% of total $CD4^+$ T cells; Fig. 6*A1*). The expression of transcripts of the T_{reg} marker Foxp3 were detected in $CD4^+CD25^+$ cells isolated from MOSEC 1D8 ascites (Fig. 6*A2*). Weak Foxp3 transcript expression was also detected in $CD4^+CD25^+$ splenocytes from mice bearing MOSEC 1D8 tumors, but not those from tumor-free mice (Fig. 6*A2*). We also observed a higher proportion (18.2%) of $CD4^+$ splenocytes of mice bearing ovarian tumor stains for FoxP3 using antibody FJK-16s (eBioscience, San Diego, CA) as compared with $CD4^+$ splenocytes of control tumor-free mice (only 5.7%). These numbers are consistent with the 17.6% and 9.7% of $CD4^+$ splenocytes staining with CD25 in splenocytes from mice bearing ovarian tumor versus those of control tumor-free mice, respectively (Fig. 6*A1*), suggesting that the majority of $CD4^+CD25^+$ cells detected are indeed $FoxP3^+$ T_{reg} rather than activated conventional T cells. A significant fraction of $CD25^+$ T cells from tumor tissue were CD152-positive (Fig. 6*B*), which may account for the ability of CD152 antibody blockade to blunt the suppression of antigen-specific T cell responses (Fig. 3*A*) and retard tumor growth (Fig. 3*B*).

We sought to address the role of $CD4^+CD25^+$ T_{reg} cells in ovarian carcinoma–associated $Gr\text{-}1^+CD11b^+$ cell–mediated suppression of antigen-specific T responses. Thus, $CD25^+$ cells were depleted from the splenocytes of VLP-vaccinated mice. Treatment of these CD25-depleted splenocyte cultures with VLP induced a robust IFNγ response (Fig. 6*C1*). Notably, the addition of both $CD4^+CD25^+$ T cells and $Gr\text{-}1^+CD11b^+$ MSCs from tumor-bearing mice were required for the suppression of VLP-induced release of IFNγ by CD25-depleted splenocyte cultures of VLP-vaccinated mice. To address whether T_{reg} cells require $Gr\text{-}1^+CD11b^+$ MSCs to inhibit the function of T cells in our model, we compared the immunosuppressive effect of $CD4^+CD25^+$ T cells either with or without $Gr1^+$ $CD11b^+$ cells. Although 10^5 $CD4^+CD25^+$ T cells without $Gr1^+CD11b^+$ cells could slightly reduce the T cell response (Fig. 6*C1*), the immunosuppression was much more potent in the presence of $Gr1^+CD11b^+$ cells. Because this suppression was not seen in the absence of $CD4^+CD25^+$ T cells (Fig. 6*C1*), this experiment suggests

a critical role for T_{reg} in the suppression of antigen-specific immunity by Gr-1$^+$CD11b$^+$ MSCs of tumor-bearing mice. Finally, suppression of VLP-induced release of IFNγ by CD25-depleted splenocyte cultures of VLP-vaccinated mice could be titrated with the addition of increasing numbers of CD4$^+$CD25$^+$ T cells (Fig. 6*C2*).

Discussion

In this study, we describe the accumulation of Gr-1$^+$CD11b$^+$ MSCs and CD4$^+$CD25$^+$FoxP3$^+$ T_{reg} cells in the spleen, ascites, and tumor tissue of mice bearing the MOSEC 1D8 model of ovarian carcinoma. Mice bearing LLC, but not the cervical carcinoma model, TC-1 (data not shown), also accumulate Gr-1$^+$CD11b$^+$ MSCs, implying that this phenomenon is neither restricted to the 1D8 model, nor common to all tumor models. The absence of Gr-1$^+$CD11b$^+$ MSC accumulation from the spleens of mice bearing TC-1 suggests that other mechanisms are responsible for tolerance of this tumor. It is clear that tumors use multiple mechanisms to induce immune tolerance involving T_{reg} cells, tolerant DCs, and the tumor cell itself.

We show that the mouse ovarian carcinoma–associated Gr-1$^+$ CD11b$^+$ MSCs expressed a high level of CD80, whereas other costimulatory molecules, i.e., CD40 and CD86, are not detected. We find that direct interaction of MOSEC 1D8 ovarian carcinoma cells causes Gr-1$^+$CD11b$^+$ cells to up-regulate surface expression of CD80. We show that CD80 expressed by ovarian carcinoma–associated Gr-1$^+$CD11b$^+$ cells plays a critical role in immunosuppression induced and maintained by Gr-1$^+$CD11b$^+$ cells.

How does direct contact between tumor and splenic Gr-1$^+$CD11b$^+$ cells occur for CD80 up-regulation? One possibility is via circulating tumor cells that seed metastases. This is consistent with the requirement of extensive disease burden for this effect and the previous description of metastasis of both MOSEC 1D8 and LLC, but interestingly, not TC-1 tumor. It is also possible that blood cells, especially bone marrow stem cells, pass through tumor tissue before entering the spleen (44). Another possibility is that membrane fragments shed from 1D8 tumor cells trigger CD80 up-regulation on splenic MSCs because viable tumor cells are not required for this phenomenon *in vitro* (Fig. 2). Indeed, ovarian cancer sheds high concentrations of membrane fragments into the peripheral blood of patients (45). These shed tumor membrane fragments have been shown to suppress CD3-ζ expression and induce T cell apoptosis *in vitro* (45). Clearly there are multiple opportunities for contact between blood and tumor cells, but defining the mechanism of Gr-1$^+$CD11b$^+$ spleen cell expansion and CD80 up-regulation requires further study.

Recently, several studies have addressed the roles of CD80 and CD86 in regulating immune responses. CD80 and CD86 are sometimes considered interchangeable costimulators. However, it has been proposed that CD80 is the initial ligand responsible for maintaining aspects of immune tolerance through interaction with CD152 (46). These inhibitory functions might then be overridden by the up-regulation of CD86 on DCs as a result of inflammatory stimuli, leading to immune activation. In addition, CD80 and CD86 may have distinct preferences for CD28 and CD152. Our studies strongly support a role for CD80 as the initial ligand responsible for maintaining aspects of immune tolerance through interaction with CD152. CD80 expressed on the Gr-1$^+$CD11b$^+$ cells induces antigen-specific immunosuppression by modulating the activity of T_{reg} cells. Indeed we find that a significant fraction of CD4$^+$CD25$^+$ T_{reg} cells isolated from mice bearing the MOSEC 1D8 ovarian carcinoma express CD152. Others have also found a high level of CD152 on CD4$^+$CD25$^+$ T cells in different systems (47). Furthermore, interference with this role of CD152 suffices to elicit autoimmune disease in otherwise normal animals, presumably through affecting CD4$^+$CD25$^+$ T cell–mediated control of self-reactive T cells (24). CD4$^+$CD25$^+$ T_{reg} cells produce interleukin-10, transforming growth factor-β, interleukin-6, and IFN-γ to induce the T cell immunotolerance (48). This unique function of CD152 could be used to modulate T cell–mediated immunoregulation, and induce immunologic tolerance, or control autoimmunity.

Binding of CD80 and CD152 may affect the function of Gr-1$^+$ CD11b$^+$ cells. Indeed, binding of CD152-Ig to DCs induces the expression of indoleamine-2,3-dioxygenase (IDO), an enzyme that degrades the essential amino acid tryptophan (49). Tryptophan metabolites suppress T cell response *in vitro* and *in vivo* (50) as well as T cell clonal expansion (49). Recent studies suggest that triggering of IDO requires the ligation of CD80/CD86 molecules displayed on DCs by CD152/CD28 expressed on T cells (49). Subpopulations of CD4$^+$CD25$^+$ T_{reg} may constitutively express CD152. Indeed, both mouse CD4$^+$CD25$^+$ T_{reg} cells and a CD152-transfected cell line induced IDO activity in mouse DCs *in vitro* in a CD152-dependent manner. The adaptive immune system could promote tolerance through signals from T_{reg} cells that express CD152 and induce IDO-competent DCs to express IDO, thereby suppressing T cell responses and promoting tolerance. The IDO-catalyzed tryptophan metabolites are toxic for T cells, especially activated T cells. IDO-expressing DC might also promote the development of T_{reg} cells in a feed-forward mechanism (49). Further study is necessary to determine the role of IDO and tryptophan metabolites in immunosuppression produced by ovarian carcinoma–associated CD80$^+$Gr-1$^+$CD11b$^+$ MSCs.

Acknowledgments

Received 10/18/2005; revised 3/13/2006; accepted 4/14/2006.

Grant support: Department of Defense funding, OC010017. Congressionally Directed Medical Research Program, PHS grant RO1 CA122581 and the Richard TeLinde Endowment (R.B.S. Roden), and by the University of Nankai and NSFC grant 30540022 (R. Yang).

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We thank the HERA foundation and Sean Patrick for their encouragement and support of our research program.

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