Role of Immature Myeloid Gr-1<sup>+</sup> Cells in the Development of Antitumor Immunity

Qingsheng Li, Ping-Ying Pan, Peidi Gu, Dongping Xu, and Shu-Hsia Chen

Carl C. Icahn Institute for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine, New York, New York

ABSTRACT

One of the mechanisms by which tumor cells evade the immune system is the lack of proper antigen-presenting cells. Improvement in host immunity against tumor cells can be achieved by promoting the differentiation of dendritic cells (DCs) from immature myeloid cells (Gr-1<sup>-</sup>Ly-6C<sup>-</sup>CD80<sup>+</sup>) that accumulate in the bone marrow and lymphoid organs of mice with large tumor burdens. The enriched immature myeloid cells inhibit T-cell proliferation and tumor-specific T-cell response, which can be reversed by the differentiation of immature myeloid cells or depletion of F4/80<sup>+</sup> cells. Sorted Gr-1<sup>-</sup>/F4/80<sup>-</sup> immature myeloid cells differentiated into CD11c<sup>+</sup> cells that express CD80 and I-A/E (MHC class II) in the presence of recombinant murine granulocyte macrophage colony-stimulating factor (GM-CSF). Furthermore, intratumoral gene delivery of GM-CSF not only promoted the differentiation of carboxyfluorescein succinimidyl ester-labeled immature myeloid cells into CD11c<sup>+</sup> cells with the characteristics of mature DCs (CD80<sup>+</sup>, I-A/E<sup>-</sup>) but also enhanced innate natural killer and adaptive cytolytic T-cell activities in mice treated with interleukin (IL)-12 and anti-4-1BB combination therapy. More importantly, intratumoral delivery of GM-CSF and IL-12 genes in combination with 4-1BB costimulation greatly improved the long-term survival rate of mice bearing large tumors and eradicated the untreated existing hepatic tumor. The results suggest that inducing the maturation of immature myeloid cells, thus preventing their inhibitory activity and enhancing their antigen-presenting capability, by GM-CSF gene therapy is a critically important step in the development of effective antitumor responses in hosts with advanced tumors.

INTRODUCTION

Tumor cells deploy multiple mechanisms to avoid recognition and elimination by the immune system. One mechanism is inefficient, or lack of, antigen presentation, which is essential for priming an effective antitumor response in a tumor-bearing host (1–3). Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) capable of inducing immunity to foreign antigens (4, 5). Several mechanisms contribute to inadequate antigen presentation in the host with a large tumor burden. First, blockage of the ability of CD34<sup>+</sup> progenitor cells to differentiate into fully functional DCs in the presence of tumor supernatant suggests that tumor cells may actively prevent DC maturation (6). Second, the absence of expression of costimulatory molecules in tumor-associated DCs was observed in cancer patients and tumor-bearing mice (7, 8). Third, tumor cells may induce early apoptosis of DCs (9, 10). Furthermore, DCs from tumor-bearing hosts may be inefficient in lymphoid homing and retention of mature DCs locally (11, 12). Although the mechanism by which tumor cells affect the function of APCs is not understood completely, accumulating evidence suggests that secretion of cytokines, such as vascular endothelial growth factor (12, 13), macrophage-colony stimulating factor (14), interleukin (IL)-6 (14), IL-10 (15), and transforming growth factor β (15), by tumor cells may result in dysfunctional APCs in a tumor-bearing host.

Our and others’ studies have demonstrated recently an accumulation of Gr-1<sup>-</sup> immature myeloid cells in bone marrow (BM), lymphoid organs, and spleens of cancer patients and tumor-bearing mice (16–20). In addition, these cells are associated with immune suppression, such as in mice with viral infection (21, 22) or receiving BM transplantation (23), UV irradiation (24), or cyclophosphamide treatment (18, 25). In our previous studies, we demonstrated that murine colon tumor (MCA-26) growth was accompanied by an increase in the number of immature Gr-1<sup>-</sup>/CD11b<sup>-</sup> myeloid cells, which suppressed CD3/CD28-mediated proliferation of T cells through NO production (26). The immature Gr-1<sup>-</sup> myeloid cell is a myeloid precursor that can differentiate into inhibitory or stimulatory APCs, depending on the in vitro culture conditions (16). However, it has not been determined whether these Gr-1<sup>-</sup>/CD11b<sup>+</sup> cells can differentiate into mature DCs in vivo or what role they play in the antitumor response.

Granulocyte macrophage colony-stimulating factor (GM-CSF) is an important cytokine for generating DCs in vitro and for modulating immune responses in vivo. In our previous study, intratumoral GM-CSF gene delivery enhanced tumor infiltration of DCs and macrophages and the migration of activated DCs to the spleen. Other studies showed that implantation of irradiated GM-CSF-expressing tumor cells resulted in the expansion of DCs (27–29) and antitumor responses (30, 31). GM-CSF gene transfer also resulted in the differentiation and activation of a myeloid DC population in the lung (32). The ability of GM-CSF to augment cellular immunity is believed to result from the stimulation of the maturation and growth of APCs, thus improving antigen presentation (33, 34). These findings suggest that local GM-CSF gene delivery may provide an efficient way to increase the number and function of APCs in a large tumor-bearing host. Furthermore, the ability of GM-CSF to stimulate DC differentiation may provide an avenue for the maturation of immature myeloid cells and overcoming the immune suppression observed in hosts with a large tumor burden.

Recent advances in adenoviral gene therapy enable the more effective application of cytokines, which can result in adverse effects when delivered systemically, to immune-enhancing cancer therapy. IL-12 plays an important role in antitumor responses because of its ability to enhance natural killer (NK) and CTL activities (35–38) and in the antigen presentation capacity of DCs in vivo (39). 4-1BB is an inducible costimulatory molecule that belongs to the tumor necrosis factor receptor superfamily. Cross-linking 4-1BB by agonistic antibodies transmits a distinct and potent costimulatory signal leading to the activation and expansion of activated T cells (40–42) and DCs (43, 44). We showed previously that intratumoral administration of adenovirus, expressing the murine IL-12 (ADV/mlIL-12) gene alone, increased the survival time of tumor-bearing mice significantly (45). Combining IL-12 gene delivery with systemic administration of an agonistic monoclonal antibody (mAb) against 4-1BB resulted in synergistic effects on antitumor responses (46). However, the therapeutic effect is compromised severely in mice with a large tumor burden (47). Because of the accumulation of myeloid Gr-1<sup>-</sup> cells and

Received 6/6/03; revised 10/6/03; accepted 11/10/03.

Grant support: National Cancer Institute ROI Grants CA 70337, CA 75175, and CA 84404, and Sharp Foundation Grant to S.H. Chen.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Shu-Hsia Chen, Carl C. Icahn Institute for Gene Therapy and Molecular Medicine, Box 1496, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Phone: 212-659-8256; Fax: 212-803-6740; E-mail: Shu-Hsia.Chen@mssm.edu.
the ineffectiveness of the IL-12 and anti-4–1BB combination therapy in mice bearing large tumors, we hypothesize that differentiation of immature myeloid cells into mature DCs, induced by intratumoral GM-CSF gene delivery, can reverse immune suppression and improve the efficacy of the ADV/mIL-12 and anti-4–1BB combination therapy in mice with large tumor burdens. The maturation of immature myeloid cells in vivo may result in an enhanced IL-12-activated NK response and 4–1BB engagement on DC and CTL development by NK and DC reciprocal interaction (48–50).

In this study, we examined whether intratumoral delivery of the GM-CSF gene could induce the differentiation of immature myeloid cells into functionally mature DCs and what therapeutic effect the ADV/mGM-CSF + ADV/mIL-12 + anti-4–1BB combination treatment would have in mice bearing large tumors. We found that differentiation of immature myeloid cells is crucial for the combination with IL-12 and 4–1BB activation, which can significantly enhance innate and adaptive immunity against advanced tumors.

**MATeRIALS AND METHODS**

**Experimental Animals.** Eight- to 12-week-old female BALB/c mice and C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD). Influenza hemagglutinin (HA)-specific, H-2Kd-restricted CD8 T-cell receptor (TCR) transgenic mice were obtained from Dr. Linda Sherman (The Scripps Research Institute, La Jolla, CA; Ref. 51). Influenza HA-specific, I-Ea-restricted CD4 TCR transgenic mice were obtained from Dr. Constantin A. Bona (Mount Sinai School of Medicine, New York, NY; Ref. 52). All of the animal experiments were performed in accordance with the animal guidelines at Mount Sinai School of Medicine.

**Media, Reagents, and Staining.** Recombinant murine GM-CSF and recombinant murine tumor necrosis factor α were purchased from ProproTech (Rocky Hill, NJ). The synthetic HA peptide (55) was used for CD8 TCR transgenic T cells and HA peptide (56) for CD4 TCR transgenic T cells. Peptides were purchased from ResGen Invitrogen Corporation (Huntsville, AL).

For antibody production in BALB/c nude mice, 2A (antiimmunotein 4–1BB) hybridoma, a gift from Dr. Lieping Chen (Mayo Clinic, Rochester, MN), was used. The antibodies used for flow cytometric analysis include anti-Gr-1 (RB6-8C5), anti-Ly-6C (AL-21), anti-CD80 (16-10A1), anti-CD11b (M170), anti-CD31 (MEC13.3), anti-I-A/E (2G9), anti-CD11c (HL3), and anti-CD40 (5C11). All of the antibodies and isotype-matched mAbs used in flow cytometry were obtained from BD Biosciences (San Jose, CA) and 3-amino-9-ethyl-carbazole (Sigma Chemical Co.) substrate. Spot numbers were determined by counting spots using a dissection microscope.

**Generation of FrII Immature Myeloid Cell-Derived DCs.** Mice with tumor sizes >10 mm were killed, and their spleens, thymus, and femurs were harvested. Splenocytes or BM cells were fractionated by centrifugation on a Percoll (Amersham Biosciences, Piscataway, NJ) density gradient as described (26). Cells were collected from the gradient interface. Cells banding between 40 and 50% were labeled as fraction (Fr) I, between 50 and 60% as FrII, and between 60 and 70% as FrIII. FrII BM cells were seeded in 100-mm Petri dishes. DCs were generated as described by Lutz et al. (56) with minor modifications. Recombinant murine GM-CSF (200 units/ml) was used throughout the culture period. Cells were maintained for 11 days with the addition of recombinant mouse tumor necrosis factor α (50 units/ml) every 24 h in culture. After 11 days in culture, nonadherent cells, representing the majority of the cells generated in the cultures, were harvested and subjected to surface marker analysis or used as APCs in a T-cell proliferation assay.

**CFSE Labeling and Adoptive Transfer.** BM FrII cells from naive or tumor-bearing mice with liver metastases were labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR). The BM and spleen FrII cells (2 × 10^7/ml) were suspended in RPMI 1640 (without FCS) and incubated with 1 μl of CFSE stock solution (5 mM in DMSO) at 37°C for 10 min, followed by one wash in an equal volume of cold FCS, three times with complete medium, and two times with cold PBS. Each
RESULTS

Significant Accumulation of Gr-1+ Immature Myeloid Cells in Mice with Large Tumors. In our previous studies, a significantly higher number of Gr-1+Mac-1+ (CD11b+) immature myeloid cells was observed in mice with large tumors (tumor > 9 × 9 mm²; Ref. 26). BM cells were fractionated by a Percoll density gradient centrifugation to enrich the low-density (FrII; 1.063–1.075 g/ml) immature myeloid cells. To assess the phenotype, the BM FrII cells derived from naive or tumor-bearing mice were stained with antibodies against various markers. The number and percentage of Gr-1+, Ly-6C+, and F4/80+ were much higher in BM FrII cells derived from MCA-26-tumor-bearing hosts than from naive mice. In contrast, expression of CD40, MHC class II (I-A/I-E), was significantly lower in mice with a large tumor burden than in naive mice (Fig. 1). No difference in CD31 expression was observed between naive and tumor-bearing mice. A similar profile was found in FrII cells derived from the spleen (data not shown). The accumulation of Gr-1+CD11b+ cells is not unique to the MCA-26 tumor model because similar results have been observed in other tumor models (e.g., breast and JC-tumor-bearing mice; data not shown).

Our previous report showed that the Gr-1+ cells in BM and spleen derived from BALB/c tumor-bearing mouse inhibited the CD3+CD28-induced proliferative response of naive BALB/c splenocytes (26). To assess whether Gr-1+ cells were capable of suppressing antitumoral CTL responses, splenocytes from cured long-term surviving mice were stimulated with irradiated parental MCA-26 tumor cells in the presence of FrII Gr-1+ cells, and the CTL activity was analyzed. As shown in Fig. 2A, the enriched BM FrII cells derived from tumor-bearing mice inhibited tumor-specific CTL responses, whereas no inhibition was observed in the coculture with FrII cells. We also tested whether Gr-1+ cells could inhibit the proliferation of T cells derived from HA TCR transgenic mice with HA peptide. We found that these enriched BM FrII cells inhibited HA peptide-stimulated T-cell proliferation. The inhibitory effect of these immature myeloid cells is decreased significantly after they are cultured with GM-CSF for 10 days followed by incubation in the presence of GM-CSF and tumor necrosis factor α for 24 h (Fig. 2B). Similar results were obtained using HA+ CD8 TCR transgenic splenocytes (data not shown).

To identify the cell type involved in immune suppression, FrII cells were depleted of F4/80+ cells in vitro by complement lysis. As shown in Fig. 2C, depletion of F4/80+ cells can restore T-cell proliferation at a high ratio of splenocytes to F4/80-depleted FrII cells, suggesting that the F4/80+ cells in FrII may be one of the effector cell types responsible for the observed suppression. The fluorescence-activated cell-sorted FrII Gr-1+Fr4/80+ cells showed characteristics of myeloid cell morphology with large nuclei and a high nucleus-to-cytoplasm ratio (Fig. 2D).

In Vitro and in Vivo Differentiation of DCs from Immature Myeloid Gr-1+ Cells. To evaluate the capacity of FrII Gr-1+Fr4/80+ cells to differentiate into DCs, the Gr-1+Fr4/80+ sorted cells were cultured with GM-CSF and tumor necrosis factor α as described in “Materials and Methods.” Interestingly, the sorted Gr-1+Fr4/80+ FrII cells differentiated into mature DCs with high levels of CD11c and MHC class II expression (Fig. 3A, bottom panels) when compared with the originally sorted cells without subsequent culture (Fig. 3A, top panels). Moreover, the cultured Gr-1+Fr4/80+ and Gr-1+Fr4/80+ cells induced a strong mixed lymphocyte reaction by allogeneic T cells, whereas the sorted Gr-1+Fr4/80+ cells under the same culture conditions did not (Fig. 3B). These results indicate that Gr-1+Fr4/80+ and Gr-1+Fr4/80+ cells have the potential to differentiate into functional mature DCs under the proper conditions.

Confirming that intratumoral GM-CSF gene delivery can induce the differentiation of Gr-1+ myeloid cells in tumor-bearing mice is essential for devising an effective therapeutic modality for treating hosts with a large tumor burden. To determine whether FrII cells could differentiate into DCs in tumor-bearing mice treated with ADV/mGM-CSF, the FrII cells derived from BM and spleen of large tumor-bearing mice were labeled with CFSE and adoptively transferred into syngeneic mice by tail vein infusion. The recipient tumor-bearing mice were injected with ADV/mGM-CSF or the control vector DL312. Cells from the spleens of all of the treated animals were isolated 5 days after virus injection and stained with anti-CD11c, anti-CD80, and anti-MHC class II (I-A/I-E). A significant number of
CFSE-labeled cells had undergone one or more cycles of proliferation in ADV/mGM-CSF- or DL312-treated recipient tumor-bearing mice as depicted by the twofold decrease in FITC intensity (Fig. 4A). Analysis of divided CFSE-positive cells showed a significant increase in the numbers of \( \text{CD}11c^-\text{T}-\text{cell receptor (TCR)-transgenic SP in the presence of HA peptide for 72 h, and incorporation of } ^{[3]}\text{H}-\text{thymidine was measured. C, suppression of T-cell proliferation mediated by F4/80}^-\text{cells within BM FrII cells. SP (10}^5/\text{well}) from HA}^-\text{CD8 TCR transgenic mice were cocultured with FrII cells or F4/80}^-\text{-depleted FrII cells (0.125–0.5 \times 10^5/\text{well}) in the presence of HA peptide for 72 h. [H]-thymidine uptake was analyzed. Data shown are from a representative of reproducible independent experiments. D, morphology of sorted Gr-1}^-\text{F4/80}^-\text{cells from BM FrII of tumor-bearing mice. Gr-1}^-\text{F4/80}^-\text{cells were sorted by fluorescence-activated cell sorter from BM FrII cells of MCA-26 tumor-bearing mice, stained with Wright-Giemsa, and evaluated by light microscopy (x} \times 1000).}

GM-CSF Gene Delivery Increased Infiltration and Maturation of DCs in Tumor-Bearing Mice. The effect of intratumoral delivery of the GM-CSF gene on the recruitment and maturation of DCs also was evaluated. Mice with hepatic MCA-26 colon tumor (8 \times 9 \text{mm}^2) were injected intratumorally with ADV/mGM-CSF or control vector (DL312). TILs and splenocytes were isolated 5 days after adenoviral injection and stained with a panel of antibodies. As shown in Fig. 5A, more CD11c^-I-A/E^- and CD11c^-CD80^- cells were observed in tumor-bearing mice treated with ADV/mGM-CSF (top panels) than in those treated with control vector DL312 (bottom panels). Similar results were observed in TILs, in which higher frequencies of CD11c^- cells were seen in ADV/mGM-CSF-treated mice (Fig. 5B, top panels) compared with control vector-treated mice (Fig. 5B, bottom panels).

Taken together, the results in Fig. 4 and Fig. 5 indicate that intratumoral gene delivery of GM-CSF promotes differentiation of immature Gr-1^- myeloid cells into DCs and enhances tumor infiltration of DCs with characteristics of mature phenotypes. Therefore, direct GM-CSF gene delivery into the tumor may represent a potential solution for overcoming the immune suppression observed in hosts with a large tumor burden.
Long-Term Survival of Mice Bearing Large Tumors Treated with the Combination Therapy of ADV/mGM-CSF, ADV/mIL-12, and Anti-4-1BB Activation. Although combination treatment with ADV/mIL-12 and anti-4-1BB mAb was successful in treating tumors sized 5 × 5 mm² (45), its efficacy is compromised severely when treating tumors >9 × 9 mm² (47). Because of the ability of ADV/mGM-CSF to increase APC infiltration in tumors and to promote the differentiation of immature myeloid cells in vivo, we hypothesize that intratumoral injection of ADV/mGM-CSF can overcome the immune suppression mediated by immature myeloid cells in mice bearing large tumors. The maturation and activation of APCs mediated by intratumoral gene delivery of GM-CSF followed by the immune activation of ADV/mIL-12 and anti-4-1BB mAb may induce stronger antitumor immunity in large tumor-bearing mice. MCA-26 tumor-bearing mice were given an intratumoral injection of ADV/mGM-CSF or control vector DL312 on day 6. At day 14 after tumor implantation, mice with tumors sized 9 × 9 to 12 × 12 mm² were injected with ADV/mIL-12 or control vector DL312, followed by injection of anti-4-1BB or control antibodies. The results obtained from three separate, reproducible experiments are combined and shown in Fig. 6. The combination therapy with ADV/mGM-CSF + ADV/mIL-12 + anti-4-1BB achieved a 67.7% long-term survival rate, whereas only 20.0% of mice treated with ADV/mIL-12 and anti-4-1BB survived and remained tumor-free at day 120 after tumor implantation. The combination treatment with ADV/mGM-CSF + anti-4-1BB or ADV/mGM-CSF + ADV/mIL-12 did not improve the therapeutic efficacy when compared with the ADV/mIL-12 + anti-4-1BB treatment (28.6% versus 20.0% and 18.7% versus 20.0%). The results indicated that the addition of ADV/mGM-CSF to the ADV/mIL-12 + anti-4-1BB treatment improved the therapeutic efficacy significantly (P < 0.0015, log-rank test) when treating mice with a large tumor burden.

Significant Increase in Innate and Adaptive Immune Responses after Combination Treatment with ADV/mGM-CSF, ADV/mIL-12, and Anti-4-1BB mAb in Large Tumor-Bearing Animals. To further identify the effector mechanisms involved in the various therapeutic paradigms, NK activity and T-cell responses of all of the treatment groups were measured. The nonparenchymal cells derived from liver were isolated at day 3 after combination treatment, and direct NK cytolytic activity was measured using 51Cr-labeled YAC-1 cells as the target cell. As shown in Fig. 7A, a significant increase in NK activity was observed in mice receiving the ADV/mGM-CSF + ADV/mIL-12 + anti-4-1BB combination therapy when compared with other treatment groups (group 1 versus group 2; P < 0.01, repeated measure ANOVA).

The CTL response and IFN-γ-producing cells were evaluated 3 weeks after the treatment. Thirteen of 24 mice treated with the ADV/mGM-CSF + ADV/mIL-12 + anti-4-1BB combination therapy had significant CTL activity (cytolytic activity > 10%) compared with mice treated with ADV/mIL-12 and anti-4-1BB (4 of 24; P = 0.0145, Fisher’s exact test; Fig. 7B), whereas no significant CTL activities were observed in other treatment groups (0 of 19, 1 of 22, and 0 of 16 in ADV/mGM-CSF + ADV/mIL-12 + rat IgG, ADV/mGM-CSF + DL312 + anti-4-1BB, and untreated MCA-26 tumor-bearing mice, respectively). Using ELISPOT assay, only the splenocytes from ADV/mGM-CSF + ADV/mIL-12 + anti-4-1BB combination-treated mice were shown to induce significant IFN-γ secretion against parental tumor MCA-26 compared with splenocytes from the other four groups (P < 0.001, ANOVA; Fig. 7C). These results suggest that the maturation of Gr-1⁺ myeloid cells in conjunction with IL-12 + anti-4-1BB activation may enhance not only the increase of innate NK cell number but also the development of adaptive immunity through DC and T-cell interaction.

Fig. 4. Generation of dendritic cells (DCs) from the immature myeloid cells of tumor-bearing mice. Carboxyfluorescent succinimidyl (CFSE)-labeled bone marrow (BM) fraction (Fr) II cells were adoptively transferred into tumor-bearing mice at 24 h after virus (ADV/mGM-CSF or control vector DL312) injection. Each recipient mouse received 2 × 10⁷ CFSE-labeled FrII cells by tail vein infusion. Splenocytes were isolated 5 days after adoptive transfer and stained with anti-CD11c-PE, anti-MHC class II (I-A/I-E)-biotin, anti-CD80-biotin, or isotype controls, followed by APC-conjugated streptavidin (PharMingen). The CFSE-positive dividing cells from ADV/mGM-CSF-treated or control vector DL312-treated animals were analyzed. A, the Y axis represents the forward scattering intensity of immature myeloid cells; the X axis represents green fluorescence intensity caused by CFSE labeling. B, the expression of CD11c/I-A/I-E or CD11c/CD80 after electronic gating on CFSE⁺-positive cells (R1 regions) from ADV/mGM-CSF-treated (left panels) or control vector DL312-treated (right panels) animals is presented in dot plots. Data shown are representative of four independent experiments. C, summary of DC differentiation from FrII cells after gene delivery. The expression of CD11c⁺/I-A/I-E⁺ and CD11c⁺/CD80⁺ cells were gated on CFSE⁺-positive cells (R1 regions). The numbers of CD11c⁺/I-A/I-E⁺ and CD11c⁺/CD80⁺ cells are significantly higher in ADV/mGM-CSF-treated animals (P < 0.05, Student’s t test).
To determine if a high level of NK and CTL activity led to development of antitumor immunity against pre-existing nontreated hepatic tumor, animals were injected with $1 \times 10^5$ MCA-26 tumor cells s.c. into the flank and $3 \times 10^4$ MCA-26 tumor cells into the liver. At day 6, all of the mice that formed 4–5 mm$^2$ s.c. tumor and 3–5 mm$^2$ hepatic tumor were selected for injection. ADV/mGM-CSF + ADV/mIL-12 + anti-4–1BB or control vector + rat immunoglobulin were injected into s.c. tumor; however, no injection was made in the hepatic tumor, and the long-term survival was followed. As shown in Fig. 8A, animals treated with ADV/mGM-CSF + ADV/mIL-12 + anti-4–1BB displayed a significant prolonged long-term survival than animals treated with ADV/mDL312 + ADV/mDL312 + rat IgG ($P = 0.0305$, log-rank test). All of the control vector- and rat immunoglobulin-treated animals died because of the death of the hepatic and s.c. tumor. There was no evidence of residual tumor cells, inflammatory infiltrates, or hepatitis. Instead, abundant accumulation of ceroid pigment (digested organelles from dead tumor cells) in foamy macrophage was noted at higher magnification (Fig. 8B). The results indicated that the distal untreated hepatic tumor in the ADV/mGM-CSF + ADV/mIL-12 + anti-4–1BB-treated survival animals had undergone complete regression. Similar results also have been obtained in the existing lung and s.c. tumor models (data not shown). The results also indicate that there is a strong systemic antitumor effect on noninjected tumor in vivo.

**DISCUSSION**

The accumulation of Gr-1$^+$ CD11b$^+$ (Mac-1) myeloid cells often is associated with a large tumor burden (16–20) or a state of immune suppression (21–24). Although the underlying mechanism has yet to be determined in detail, several lines of evidence indicate that tumor cells actively suppress immune responses by affecting the development of DCs. It has been shown that tumor cells may secrete cytokines capable of stimulating myelopoiesis, such as GM-CSF (19) and IL-6 (14), and cytokines that inhibit DC differentiation, including vascular endothelial growth factor (12, 13), transforming growth factor β (15), and macrophage-colony stimulating factor (14). Therefore, the accumulation of Gr-1$^+$ CD11b$^+$ myeloid cells may be the combined effect of these mechanisms.
of an increase in myelopoiesis and a blockade in DC development. This represents a significant obstacle to immune-enhancing therapy for advanced cancer. Promoting immature myeloid cells and decreasing their inhibitory activity are paramount to a successful immune-enhancing therapy in a host with a large tumor burden.

Gr-1⁺ myeloid cells have been shown to be involved in the inhibition of T-lymphocyte activation in tumor-bearing animals (16–20, 26, 57). However, the phenotype and the associated in vivo function have not been well defined. In the present study, we found that the majority of Gr-1⁺ cells derived from BM and spleen of tumor-bearing mice also expressed Ly-6C and F4/80. Bulk FrII Gr-1⁺ cells derived from tumor-bearing mice can differentiate into CD11c⁺/MHC class II⁺ and CD11c⁺/CD80⁺ DCs in vitro and in vivo, respectively. The mechanisms by which these immature myeloid cells accumulate in the tumor-bearing host and become involved in antigen presentation are under investigation currently. Thus far, the results suggest that Gr-1 cells accumulated in tumor-bearing mice are immature myeloid cells, which are not able to properly differentiate into mature DCs in tumor-bearing animals. The developmental stage of these FrII Gr-1⁺ or Gr-1⁺F4/80⁺ cells needs to be clarified. Our results indicate that FrII Gr-1⁺F4/80⁺ cells derived from tumor-bearing animals may be related to early immature myeloid cells rather than monocytes because mature monocytes do not exhibit natural suppressor activity (58). The morphology of sorted Gr-1⁺F4/80⁺ cells shows characteristics consistent with myeloid cells. Furthermore, the majority of monocytes are CD43⁻ (59), whereas the Gr-1⁺F4/80⁺ cells found among these immature myeloid cells are CD43⁺ (data not shown).

The depletion of F4/80⁺ cells from the immature myeloid cells partially restored T-cell proliferation, suggesting that these cells are involved in immune suppression. However, other cells also may be involved in the suppression of T-cell activation because the depletion of F4/80⁺ cells only partially restored T-cell proliferation at lower ratios (Fig. 2C). Thus, the presence of other immature myeloid cells capable of inhibiting T-cell responses needs to be explored. We found that, after in vitro culture with GM-CSF, sorted nonadherent BM FrII Gr-1⁺F4/80⁺ cells induced a strong mixed lymphocyte reaction by allogeneic T cells, whereas no significant mixed lymphocyte reaction was observed with GM-CSF-stimulated Gr-1⁺F4/80⁻ cells (Fig. 3B). The evidence indicates that Gr-1⁺F4/80⁺ inhibitory immature myeloid cells can differentiate into DCs in vitro.

The differentiation of immature myeloid cells to mature DCs induced by gene delivery of GM-CSF has been confirmed in vivo. After therapy, a significant increase in the number of CD11c⁺ DCs with the mature phenotype (MHC class II⁺ and CD80⁺) was observed in CFSE-labeled FrII cells in vivo (Fig. 4, B and C). The recruitment and maturation of DC in the TIL and spleen as a result of GM-CSF gene delivery are efficient (Fig. 5). This indicates that local cytokine gene delivery can modulate the tumor environment and initiate the proper immune response. A novel subset of murine CD11c⁺B220⁺Gr-1⁺ plasmacytoid DCs has been identified recently, and this subset of DCs may play a central role in antiviral innate immunity (60–62). Whether the DCs present in the TIL are identical to the murine CD11c⁺B220⁺Gr-1⁺ plasmacytoid DCs remains to be determined.

Using an ADV/mIL-12 and anti-4–1BB agonistic antibody combination therapy, we achieved eradication and long-term remission in mice with tumors measuring 5 × 5 mm², but the therapy was less potent in mice with tumors >9 × 9 mm² (46, 47). The treatment of larger tumor-bearing animals with higher doses (200, 100, and 50 µg) of anti-4–1BB did not statistically improve their survival rate.₁ However, combination therapy with GM-CSF, IL-12, and 4–1BB costimulation improved the therapeutic efficacy dramatically in mice with large tumor burdens compared with the effect observed in those animals receiving only IL-12 and 4–1BB in this study. Furthermore, the combination therapy induced a stronger antitumor response. The enhanced efficacy and stronger antitumor responses can be attributed to several mechanisms. GM-CSF may promote the differentiation of immature myeloid cells into DCs (Fig. 4, B and C), thus contributing to the innate antitumor immunity for NK cells and DCs, as we hypothesized. However, other possible, but not mutually exclusive, mechanisms cannot be ruled out; for example, the recruitment of DCs to the tumor sites may be induced by GM-CSF (Fig. 5B) and/or IL-12 gene delivery (61) and enhanced tumor antigen presentation, which is consistent with the reports by our laboratory and others (63). Second, our previous data demonstrated that IL-12 can activate NK cells, which in conjunction with T-cell activation by 4–1BB ligand or

Fig. 7. Treatment of ADV/mGM-CSF and ADV/mIL-12 plus anti-4–1BB monoclonal antibody (mAb) enhances natural killer (NK) cell activity and tumor-specific CTL response. Tumor-bearing animals received the same treatment regimen as those in Fig. 6 and were divided randomly into five groups: ADV/mGM-CSF + ADV/mIL-12 + anti-4–1BB mAb (group 1, ■■), DL312 + ADV/mIL-12 + anti-4–1BB mAb (group 2, □□), ADV/mGM-CSF + ADV/mIL-12 + rat IgG (group 3, △△), ADV/mGM-CSF + adv-mDL312 + anti-4–1BB mAb (group 4, □□), and DL312 + DL312 + rat IgG (group 5, △△). A, NK cytolytic activity against YAC-1 in liver mononuclear cells derived from the various treated groups. The results presented are representative of reproducible experiments. B, CTL activity against MCA-26 in spleens. Splenocytes were harvested 3 weeks after treatment and cocultured with irradiated MCA-26 cells for 5 days, followed by a standard 4-h ⁵¹Cr release CTL assay against MCA-26 cells. The data represent the number of mice with positive CTL activity (>10% of lysis) versus the total number of treated mice analyzed in each group. The data presented were generated from five independent experiments. C, IFN-γ enzyme-linked immunospot (ELISPOT) data from the same animals 3 weeks after receiving the injection of anti-4–1BB mAb or rat IgG. MCA-26-specific IFN-γ-secreting cells were enumerated by ELISPOT assays. Each bar represents the mean spot number of the triplicates ± SD. The result is a representative of two independent experiments (P < 0.001, ANOVA).
agonistic antibody can induce proliferation of activated T cells (45, 46, 64). In this study, we demonstrated that the combination treatment using gene delivery of GM-CSF, IL-12, and anti-4–1BB costimulation significantly increased the NK cell number, NK cytotoxicity, and tumor-specific T-cell responses. These effects may result from the reciprocal interaction between DCs and NK cells. Mature DCs can activate IL-12-activated NK cells (65), and NK cells may amplify DC responses through direct and indirect interaction (48–50). Third, anti-4–1BB can activate GM-CSF- and IL-12-activated DCs and may promote their final maturation. These matured DCs prime naïve T cells more efficiently, resulting in stronger and more persistent tumor-specific CTL responses and a higher frequency of IFN-γ-producing T cells in tumor-bearing mice treated with the combination of ADV/mGM-CSF, ADV/mIL-12, and anti-4–1BB.

It has been suggested that elimination of Gr-1+ immature myeloid cells in vivo could be a key step to increasing the efficiency of antitumor vaccines because treatment of immunocompetent mice with anti-Gr-1 mAbs reduced the growth of a variety of UV light-induced tumors (66). On the basis of our findings in this study, inducing the differentiation of Gr-1+ immature myeloid cells in vivo is a more efficient way to not only eliminate suppressive activities by immature myeloid cells but also enhance antitumor immune responses.

In conclusion, our results suggest that intratumoral delivery of GM-CSF and IL-12 genes in combination with 4–1BB costimulation may offer a new strategy to reverse the immune suppression associated with advanced tumors and improve therapeutic efficacy in hosts with a large tumor burden.

ACKNOWLEDGMENTS

We thank Mr. Zhan Liu for his excellent technical help; Dr. Lieping Chen, Dr. Gordon Keller, Dr. Gwendalyn Randolph, Dr. Savio Woo, and Mr. Emerson Edwards for their helpful discussion; and Dr. Shinozaki Katsunori for pathologic pictures.

REFERENCES


21. Piccioli, D., Sbrana, S., Melandri, E., and Valiante, N. M. Contact-dependent stimulation through a mechanism dependent on endog-
IMMATURE MYELOID Gr-1+ CELLS AND ANTITUMOR IMMUNITY


Role of Immature Myeloid Gr-1\(^+\) Cells in the Development of Antitumor Immunity

Qingsheng Li, Ping-Ying Pan, Peidi Gu, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/64/3/1130

Cited articles
This article cites 66 articles, 38 of which you can access for free at:
http://cancerres.aacrjournals.org/content/64/3/1130.full.html#ref-list-1

Citing articles
This article has been cited by 20 HighWire-hosted articles. Access the articles at:
/content/64/3/1130.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.