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Endogenous Granulocyte-Macrophage Colony-Stimulating Factor Overexpression In Vivo Results in the Long-Term Recruitment of a Distinct Dendritic Cell Population with Enhanced Immunostimulatory Function

George Miller,* Venu G. Pillarisetty,* Alaap B. Shah,* Svenja Lahrs,* Zhou Xing,† and Ronald P. DeMatteo2*

GM-CSF is critical for dendritic cell (DC) survival and differentiation in vitro. To study its effect on DC development and function in vivo, we used a gene transfer vector to transiently overexpress GM-CSF in mice. We found that up to 24% of splenocytes became CD11c+ and the number of DC increased up to 260-fold to $3 \times 10^8$ cells. DC numbers remained substantially elevated even 75 days after treatment. The DC population was either CD8α+CD4− or CD8α−CD4+ but not CD8α+CD4+ or CD8α−CD4+. This differs substantially from subsets recruited in normal or Flt3 ligand-treated mice or using GM-CSF protein injections. GM-CSF-recruited DC secreted extremely high levels of TNF-α compared with minimal amounts in DC from normal or Flt3 ligand-treated mice. Recruited DC also produced elevated levels of IL-6 but almost no IFN-γ. GM-CSF DC had robust immune function compared with controls. They had an increased rate of Ag capture and caused greater allogeneic and Ag-specific T cell stimulation. Furthermore, GM-CSF-recruited DC increased NK cell lytic activity after coculture. The enhanced T cell and NK cell immunostimulation by GM-CSF DC was in part dependent on their secretion of TNF-α. Our findings show that GM-CSF can have an important role in DC development and recruitment in vivo and has potential application to immunotherapy in recruiting massive numbers of DC with enhanced ability to activate effector cells. The Journal of Immunology, 2002, 169: 2875–2885.

Dendritic cells (DC) are potent APC that initiate T cell-mediated immune responses. DC are ubiquitous and may be isolated from a variety of organs including the spleen, liver, kidney, heart, and lymph nodes. The diversity of DC subsets in each organ is complex and their phenotypic and functional characterization is the subject of considerable current investigation. In the mouse spleen, at least three distinct DC subsets have been identified. CD11c+CD8α+CD11b–/–DEC205+ DC (putative lymphoid related) are located in the T cell-rich areas of the periarteriolar lymphatic sheaths (PALS), whereas CD11c+CD8α+CD11b+DEC205+ DC (putative myeloid related) reside in the marginal zone (1–6). The latter group may be subdivided further into a 33D1+CD4+ subset and a 33D1−CD4− subset. Regardless of subset, freshly isolated splenic DC express low to moderate levels of MHC class II, CD54, and the costimulatory molecules CD40, CD80, and CD86. After overnight culture, all DC subsets mature considerably. CD8α+ DC populations are known to express high levels of IL-12 upon exposure to a variety of bacterial pathogens or LPS (4, 7–9). Furthermore, IL-12 secretion from CD8α+ DC acts in an autocrine fashion to induce DC release of IFN-γ (7). However, CD8α− DC are also capable of IL-12 secretion depending on the antigenic stimulus (10, 11). Both DC subsets take up and process soluble Ags but CD8α+ DC are more efficient at capturing particulate Ag (4, 6, 12). The relative capacity of CD8α+ and CD8α− DC to stimulate T cells is controversial. Although both potently stimulate allogeneic T cells (12), the CD8α− subset induces apoptosis in CD4+ T cells in vitro (13) and is unable to induce cytokine production from CD8+ T cells (14). In vivo, both DC subsets can prime Ag-specific CD4+ T cells; however, CD8α+ DC generally elicit a Th1 response while CD8α− DC mediate a Th2 or Th0 response (8).

Although the classical stratification of DC into myeloid (CD8α+CD11b+high) and lymphoid (CD8α−CD11b+dull−) subsets has a phenotypic, anatomic, and functional basis (15), recent findings have led investigators to question whether the subsets are developmentally distinct. Traver et al. (16) showed that both CD8α+ and CD8α− DC can arise from clonogenic common myeloid progenitors in the spleen and thymus. Martinez et al. (17) also showed that CD8α− DC can transform into CD8α+ DC in vivo. The precise factors that regulate the commitment of DC precursors to a CD8α+ or CD8α− pathway are unknown (18).

GM-CSF is a critical growth factor for DC in vitro. GM-CSF has also been shown to enhance the ability of DC to present Ag and to stimulate T cells in culture (19, 20). However, the role of GM-CSF in DC generation and function in vivo is less certain. Initial studies using mice deficient in GM-CSF or GM-CSF-transgenic mice suggested that GM-CSF was not vital for DC development in vivo (5, 21). Similarly, Maraskovsky et al. (22) found only modest elevations in splenic DC after daily injection of GM-CSF protein. However, daily injections of GM-CSF conjugated to...
polyethylene glycol, which has a half-life of several hours, produced a short-lived 8-fold increase in splenic DC, suggesting that a threshold serum level of GM-CSF may be required to exert biologic effects on DC recruitment (12). Similarly, administration of tumors transduced to express GM-CSF have been shown to recruit DC locally (23–25).

Because GM-CSF plays a critical role in DC generation from bone marrow in vitro and has been shown to recruit DC in mice, we examined the effects of continuous endogenous secretion of GM-CSF on DC development in vivo. We found that transient (<2 wk) overexpression of GM-CSF in mice produced massive recruitment (up to 3 × 10^8 DC) of a CD11c^+ CD4^- DC population to the spleen. Strikingly, recruited DC secreted high amounts of TNF-α. Other murine DC populations are not known to constitutively produce elevated levels of TNF-α. Recruited DC also had an enhanced ability to capture Ag and to stimulate T cells and NK cells. Their enhanced immunostimulation depended in part on their secretion of TNF-α. Our findings show that endogenous GM-CSF recruits a distinct DC population that has altered secretory properties but is highly functional. Additionally, because of its large and sustained (>75 days) effect on DC recruitment, endogenous overexpression of GM-CSF may have an important role in immunotherapy.

**Materials and Methods**

**DC isolation and flow cytometry**

Single-cell suspensions of splenocytes were prepared by splenic injection of collagenase (Sigma-Aldrich, St. Louis, MO) and mechanical disruption. DC were then purified using anti-CD11c immunomagnetic beads and high-gradient LS separation columns (Miltenyi Biotec, Auburn, CA). The purity of isolated DC was >85% by CD11c staining. DC were either used immediately or after culture in complete medium (RPMI 1640 with 10% heat-inactivated FBS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.05 mM 2-ME). Flow cytometry was performed on an EPICS-XL flow cytometer (Beckman Coulter, Fullerton, CA) after incubating 5 × 10^6 DC/tube with 1 μg of Fc block (anti-CD16/CD32 (2.4G2), Monoclonal Ab Core; Sloan-Kettering Institute, New York, NY) and then labeling with 1 μg of FITC- or PE-conjugated Ab. Splenocytes were stained for DC (CD11c (HL3)), B cells (B220 (RA3-6B2) and CD19 (RA3-6B2)), and MHC class I (H-2Kb) and class II (I-A^b), intercellular adhesion molecule (CD54 (ICAM-1 (3E2)), CD40 (3/23), CD80 (B7-1 (1G10)), CD86 (53–67.7); all from BD PharMingen, Franklin Lakes, NJ). Splenocytes or purified DC were also stained for MHC class I (H-2K^b) and class II (I-A^K) and CD32 (2.4G2; all from BD PharMingen).

**Recombinant adenoviruses**

Recombinant adenoviruses were propagated, purified, and stored as previously described (26). Adenovirus encoding the murine GM-CSF transgene (AdGM) (27) and another Ad vector encoding green fluorescent protein (AdGFP; Quantum Biotechnologies, Montreal, Quebec, Canada) each contain the CMV promoter. Ad encoding murine Flt3 ligand (AdFlt3L) was constructed as follows: a plasmid containing the Flt3L cDNA was obtained from the National Gene Vector Laboratories (Ann Arbor, MI). The SalI fragment was ligated into the SalI site of the shuttle plasmid pDC316 (Microbi, Toronto, Ontario, Canada) that contains a portion of the adenoviral genome and the murine CMV promoter (26). Cre-mediated homologous recombination at specific loxP sites was then performed on 293 human embryonic kidney cells (American Type Culture Collection (ATCC), Manassas, VA) with pBHGlox (Microbi) that contains the adenovirus type 5 genome with E1 and E3 region deletions. DNA was isolated from candidate viral plaques and screened with the restriction enzymes to confirm the presence of the Flt3L transgene. Dose for all viruses was calculated as multiplicity of infection, which is the number of virus particles per target cell. Endotoxin was undetectable in viral stocks using the Limulus amebocyte lysate clot test (sensitivity 6 pg/ml; Associates of Cape Cod, Woods Hole, ME).

**Immunohistochemistry**

Tissue was placed in OCT medium (Miles, Elkhart, IN) and frozen in an isopentane/dry ice bath. Cryostat sections (8 μm) were fixed in Formalin, rinsed with PBS, blocked with 0.3% H_2O_2, and then stained for DC using a biotin-conjugated anti-CD11c Ab (BD PharMingen) for 1 h at room temperature. Afterward, sections were incubated with streptavidin-HRP for 30 min at room temperature and visualized using diaminobenzidine substrate solution (BD PharMingen) for 5 min. Slides were counterstained in hematoxylin and dehydrated in ethanol and mounted. Photographs were taken with an Olympus SZH10 stereo microscope (Olympus, Melville, NY) and a Zeiss Axioplan 1 microscope (Zeiss, Thornwood, NY). For cytoxins, DC were spun at 500 rpm onto ProbeOn slides (Fischer Scientific, Pittsburgh, PA), fixed in Formalin and then in 100% methanol, and visualized with Giemsa stain.

**Cytokine measurement and Ag uptake assays**

Serum or cell culture supernatant was tested by ELISA for IL-4 (BD PharMingen), IL-6, IL-10, IL-12 (p70), TNF-α, GM-CSF, Flt3L, and IFN-γ (all R&D Systems, Minneapolis, MN) according to the respective manufacturer’s protocol. For in vitro cytokine assays, freshly isolated DC were cultured in 24-well dishes at a concentration of 1 × 10^6 cells/ml. Supernatant was harvested after 24 h of culture. LPS (Sigma-Aldrich) was used at 10 ng/ml and TNF-α (R&D Systems) was used at 100 ng/ml. For in vitro Ag uptake assays, DC (2 × 10^6) were incubated with FITC-dextran, FITC-albumin, or FITC-mannose albumin (both Sigma-Aldrich) for various durations at 37°C at a concentration of 1 mg/ml (28). To stop reactions, cells were harvested and placed on ice. In vivo Ag uptake assays were performed by i.v. injecting 2.5 mg of FITC-dextran into mice. After 30 min, splenic DC were harvested and analyzed by flow cytometry.

**T cell proliferation and CTL assays**

For MLR, DC were irradiated (3000 rad) and added at various amounts to 3 × 10^5 syngeneic or allogeneic T lymphocytes (purified using Thy1.2 (CD90.2) immunomagnetic microbeads (Miltenyi Biotec)) in 96-well plates and then pulsed with thyridmine (1 μCi/well) on day 3 for 20 h. For some assays, TNF-α blockade was performed using the clone 2E2 (29) at 50 μg/ml. 2E2 was produced in the Monoclonal Ab Core Facility at Memorial Sloan-Kettering Cancer Center. For Ag-specific T cell stimulation assays, an H-2K^b-restricted CD8^+ T cell hybridoma specific for OVA, 257–264 peptide was used (30). DC were incubated with either OVA (10 μg/ml; Peptide Synthesis Core, Sloan-Kettering Institute) or OVA protein (2 mg/ml; Sigma-Aldrich) for 90 min before being irradiated (3000 rad) and plated at various concentrations with 5 × 10^5 OVA-restricted T cells in a 96-well plate for 2 days. T cell stimulation was determined by assessing IL-2 levels in its supernatant by ELISA (BD Pharmingen). CTL assays were performed as described with modifications (31). Briefly, splenocytes from treated and control animals were harvested and plated at 5 × 10^5 wells in 24-well plates with 10 μg/ml OVA for 5 days. Afterward, effectors were harvested and tested against 1 × 10^5 51Cr-labeled EL-4.OVA (2876 GM-CSF EXPANDS DC 2876 GM-CSF EXPANDS DC)/EL-4 (both from ATCC) target cells for 6 h in 96-well plates. After the incubation, 30 μl of supernatant was transferred to Luma plates (Packard Bioscience, Meriden, CT) and radioactivity was read in a TopCount NXT gamma counter (Packard Bioscience). Percent lysis was calculated according to the following formula: percent specific lysis = ((cpm experimental – cpm spontaneous release) × 100)/(cpm maximum release – cpm spontaneous release). Spontaneous release was always <10% of maximum release. All assays were done in triplicate and repeated at least three times.

**NK culture and cytotoxicity assays**

DC-NK culture assays were performed as described previously (32) with slight modifications. Briefly, 1 × 10^6 splenic NK cells were isolated using DX5 immunomagnetic microbeads and high-gradient LS separation columns (Miltenyi Biotec) and plated with 2 × 10^6 DC in 24-well plates in a total of 700 μl of complete RPMI 1640 medium for 18 h. The purity of the isolated NK cell populations was >85% by FACS analysis using PE-conjugated NK1.1 and DX5 Abs (both BD PharMingen). After culture, NK cells were harvested and tested against 3 × 10^5 51Cr-labeled Yac-1 cells (ATCC). Spontaneous release and maximum release were assayed in a manner similar to the CTL assays.

**Animals procedures and tumor models**

Male C57BL/6 (H-2^k) and BALB/c (H-2^k) mice (6–10 wk old) were purchased from Taconic Farms (Germantown, NY). All procedures were approved by the Institutional Animal Care and Use Committee. Animals were given a single tail vein injection of recombinant adenovirus or saline.

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All adenovirus injections were at a dose of $8 \times 10^{10}$ particles unless otherwise indicated. For some tumor experiments, mice were administered an intrasplenic injection of $2.5 \times 10^5$ B16F10 murine melanoma cells (B16) or $5 \times 10^5$ CT26 colorectal tumor cells (both from ATCC) via a left flank incision followed by splenectomy. For other tumor experiments, mice were challenged with a s.c. flank inoculation of $1 \times 10^5$ CT26 or $3.5 \times 10^5$ EL4.OVA cells. For DC immunization experiments, mice were given two i.p. immunizations of $5 \times 10^5$ DC pulsed with OVA at 1-wk intervals and then 1 wk later sacrificed for CTL assays or challenged with a flank injection of $3.5 \times 10^5$ EL4.OVA cells.

**Results**

**AdGM administration produces transiently high serum GM-CSF levels**

To determine the amount of GM-CSF produced in vivo after i.v. administration of AdGM, we tested several viral doses (Fig. 1A). Serum levels of GM-CSF became detectable (>8 pg/ml) after injection of $5 \times 10^5$ virus particles and markedly higher GM-CSF levels were noted as the dosage was increased. Three days after injection of $2 \times 10^{11}$ viral particles, GM-CSF levels reached 55 ng/ml. However, this dose produced fatal toxicity in nearly 50% of mice within 2 wk. A dose of $8 \times 10^{10}$ particles was therefore chosen for further studies. This dose is known to accomplish gene transfer to ~90% of murine hepatocytes (31). Next, we determined the time course and duration of GM-CSF expression. After inoculation of $8 \times 10^{10}$ particles of AdGM, serum GM-CSF levels peaked at 8 ng/ml by day 3, but dropped precipitously to 0.5 ng/ml by day 7 and were no longer detectable by 2 wk (Fig. 1B). Comparatively, recombinant GM-CSF protein has a half-life of only ~5 h (12).

**GM-CSF recruits massive numbers of DC to the spleen**

To determine whether endogenous secretion of GM-CSF results in DC recruitment to the spleen, we inoculated mice with various doses of AdGM and determined the number of splenocytes that stained for CD11c by flow cytometry (Fig. 2A). At doses below $4 \times 10^{10}$ particles, the total number of splenocytes increased 2- to 4-fold and modest increases were also noted in the percentage of CD11c+ cells (from 1–2% to 7–9%). Conversely, at doses of $8 \times 10^{10}$ particles or higher, the number of splenocytes increased >10-fold and the percentage of CD11c+ cells increased to 14–24%. This resulted in a 200- to 260-fold increase (depending on the experiment) in the total number of splenic DC. In contrast, the percentage of T cells, NK cells, and macrophages were unchanged while there was a sharp decrease (66–38%) in the percentage of splenic B cells (Table I). However, the absolute numbers of all cell types was increased. This resulted in an 8-fold increase in splenic weight compared with saline-treated mice. Only a small fraction of the effects was due to adenovirus alone (Table I).

To determine the time course and duration of DC recruitment after AdGM administration, we treated a cohort of mice with $8 \times 10^{10}$ viral particles and analyzed their splenocytes at weekly intervals for DC recruitment. The number of splenic DC was greatest at 1 wk after AdGM administration (2–3 × 10^5 DC). However, DC levels remained elevated by >15-fold over baseline for >75 days after a single dose of virus (Fig. 2B). This was striking especially considering that serum GM-CSF levels returned to baseline by day 14 (Fig. 1B). In contrast, in mice treated with AdFlt3L, which produced a peak day 3 serum level of 1300 ng/ml murine Flt3L, DC numbers returned to normal by 5 wk after injection. Notably, AdGFP injection alone resulted in only a 2-fold increase in the number of splenic DC and this lasted ~4 wk. The minimal DC recruitment effect of systemic adenovirus alone did not appear to be from adenoviral-induced secretion of GM-CSF or Flt3L because serum levels of these cytokines were undetectable after AdGFP injection. In addition to expanding the splenic DC population for an extended duration, treatment with AdGM also increased the absolute numbers of all splenic leukocytes for a comparable period (Fig. 2C). In contrast, mice treated with AdGM had fewer bone marrow cells 7 days later (data not shown). Subsequently, these bone marrow progenitors generated only 18% as many bone marrow-derived DC as saline controls by 8 days of culture.

**AdGM recruits distinct DC subsets**

We next compared the phenotype of saline, AdGFP, AdFlt3L, and AdGM recruited splenic DC. There were major differences in the DC subsets that were recruited among the groups (Fig. 3A). GM-CSF recruited DC were mostly CD8α−CD11bhigh consistent with a myeloid origin while Flt3L DC were predominantly CD8α−CD11bdim with a smaller population that was CD8α−CD11bhigh. Cross-staining of CD4 and CD8α revealed additional differences between the groups (Table II). GM-CSF DC were 72% CD8α−CD4− and the remainder were essentially CD8α−CD4+. In contrast, 20–

![FIGURE 1](http://www.jimmunol.org/)
GM-CSF EXPANDS DC

DC had minimal staining (Fig. 3B). In contrast, GM-CSF recruited DC had high staining for CD16/CD32, the FcγRIIb receptor (33), compared with controls necessitating blockade of this receptor to reduce nonspecific Ab binding during flow cytometric analysis.

We next examined the maturation level of recruited DC (Fig. 4). Freshly isolated GM-CSF-recruited DC were similar to DC from saline-treated animals in terms of expression of MHC class I, class II, CD40, and CD54 but they had slightly higher CD80 (93 vs 76%) and CD86 (86 vs 50%) expression. However, treatment with AdGFP alone resulted in small elevations in DC MHC and costimulatory molecule expression. Compared with AdFlt3L-recruited DC, GM-CSF DC had weaker MHC class I, CD40, CD54, and CD86 expression but expressed slightly stronger CD80. The flow cytometry results were similar when the DC were isolated without using collagenase. Furthermore, the DC phenotype was similar on days 7, 14, 21, 42, and 75 after treatment with AdGM.

AdGM DC are located in the marginal zone

The predominantly CD8α+ DC population recruited by endogenously produced GM-CSF was located in the marginal zone of the spleen consistent with previous reports of myeloid-related DC (Fig. 5, A and B) (4). On cytospin analysis, AdGM-recruited DC were slightly larger and had more pronounced dendritic processes than DC from untreated or AdGFP-treated mice (Fig. 5, C–E). The larger size of the GM-CSF-recruited DC was further evidenced on flow cytometry. In a representative experiment (of >10) in which 20,000 cells were counted per group, AdGM DC had a mean forward scatter of 360 compared with 250 for DC from untreated mice and 270 for DC from AdGFP-treated animals.

Endogenous GM-CSF recruited DC secrete high TNF-α

To determine whether there were other differences between GM-CSF-recruited DC and controls, we tested their cytokine secretory profiles at baseline and after stimulation. We cultured freshly isolated DC (10^6 cells/ml for 24 h) either alone or with LPS or TNF-α. We used ELISA to quantify supernatant cytokine levels. Strikingly, GM-CSF-recruited DC secreted 2000 pg/ml TNF-α by 24 h compared with only 80 pg/ml or lower for saline or AdGFP DC (Fig. 6B). In contrast, Flt3L-recruited DC did not secrete any detectable TNF-α even after stimulation by LPS or CD40 ligand (data not shown). The production of TNF-α by GM-CSF-recruited DC occurred regardless of whether they were cultured in GM-CSF. In addition, GM-CSF-recruited DC secreted up to 500% higher levels of IL-6 compared with controls when stimulated with either LPS (10 ng/ml) or TNF-α (100 ng/ml; Fig. 6B). In contrast to their elevated production of TNF-α and IL-6, DC from mice treated with AdGM produced dramatically lower IFN-γ compared with DC from saline-treated mice. IL-10 expression was similar for all DC groups. However, DC secretion of IL-10 decreased after stimulation with LPS or TNF-α (Fig. 6C). IL-4, IL-12, or GM-CSF production was not detected from any of the DC groups even after stimulation.

Recruited DC have enhanced Ag capture and T cell stimulatory capacity

We postulated that GM-CSF-recruited DC would have an enhanced ability to capture Ag since GM-CSF has been shown to increase Ag uptake in splenic DC and in peritoneal cells (12, 19, 34). DC macropinocytosis was assessed by uptake of both albumin and dextran while specialized endocytosis via the mannose receptor was tested by uptake of mannosylated albumin. In all cases, DC recruited by GM-CSF had both a faster rate of Ag capture (based on the percentage of fluorescent cells) and a higher total amount of Ag taken up per cell (represented by median fluorescence) (Fig. 7).
mice. To determine whether GM-CSF-recruited DC had increased Ag capture in vivo, mice were inoculated i.v. with 2.5 mg of FITC-dextran and then their splenic DC were harvested and analyzed for Ag uptake by flow cytometry. Again, GM-CSF-recruited DC had a far greater rate of Ag capture than DC from saline- or AdGFP-treated mice (Fig. 7D). However, in consort with our in vitro data, DC from mice treated with AdFlt3L demonstrated the highest uptake (data not shown).

Since GM-CSF-recruited DC produce increased levels of activating cytokines, we postulated that they would have an enhanced ability to stimulate effector cells. We first tested their T cell allostimulatory capacity in an MLR. GM-CSF-recruited DC induced considerably higher alloimmune T cell proliferation than DC from saline- or adenovirus-treated mice (Fig. 8A). Since TNF-α is a potent T cell activator, we postulated that the enhanced allostimulation induced by GM-CSF-recruited DC was a result of their TNF-α secretion. In consort with this hypothesis, TNF-α blockade partially abrogated their increased T cell stimulation (Fig. 8B). We next assessed the ability of the DC to induce Ag-specific T cell stimulation. Consistent with our allogeneic data, freshly isolated GM-CSF-recruited DC loaded with either OVA257–264 peptide (Fig. 8C) or OVA (Fig. 8D) induced markedly higher stimulation of OVA-restricted T cells than DC from saline- or AdGFP-treated controls. GM-CSF-recruited DC also induced higher alloimmune and Ag-restricted T cell stimulation than Flt3L-recruited DC (data not shown).

**Immunization with Ag-pulsed GM-CSF-recruited DC induces CTL**

To determine whether the distinct DC population recruited by endogenously produced GM-CSF could stimulate cytotoxic T cells in vivo, we immunized naive mice with GM-CSF-recruited DC or controls that had been loaded in vitro with OVA257–264 peptide (DC.OVA). After two immunizations, we isolated splenocytes from immunized mice, restimulated them in vitro with OVA, and then tested them against EL4.OVA cells in a CTL assay. Splenocytes from mice immunized with DC.OVA, regardless of DC origin, induced similarly potent CTL-mediated lysis of EL4.OVA target cells (Fig. 9A). Lysis against EL4 targets was 75% lower, confirming the specificity of the assay (data not shown). However, in contrast to our MLR and Ag-restricted T cell stimulation data, immunization with Ag-pulsed GM-CSF-recruited DC did not induce higher CTL than Ag-pulsed DC from control mice. Nonetheless, splenic T cells from mice immunized with GM-CSF-recruited DC.OVA secreted nearly 50% higher levels of IFN-γ than controls (Fig. 9B).

**GM-CSF-recruited DC activate NK cells via secretion of TNF-α**

In addition to their primary role in stimulating T cells, DC have recently been shown to directly enhance NK cell lytic activity by cellular contact (32). Since TNF-α can have pleiotropic effects on a variety of effector cells, we postulated that GM-CSF-recruited

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Table I. Composition of splenocytes 1 wk after treatment with saline or virus (8 × 10^10 particles)*

<table>
<thead>
<tr>
<th>Splenic Weight (mg)</th>
<th>DC</th>
<th>B Cells</th>
<th>T Cells</th>
<th>NK Cells</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline 80</td>
<td>2</td>
<td>1 × 10^6</td>
<td>66</td>
<td>3 × 10^7</td>
<td>35</td>
</tr>
<tr>
<td>AdGFP 190</td>
<td>4</td>
<td>4 × 10^6</td>
<td>58</td>
<td>6 × 10^7</td>
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<td>AdGM 710</td>
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<td>38</td>
<td>4 × 10^7</td>
<td>36</td>
</tr>
<tr>
<td>AdFlt3L 280</td>
<td>40</td>
<td>1 × 10^7</td>
<td>21</td>
<td>6 × 10^7</td>
<td>22</td>
</tr>
</tbody>
</table>

* Representative data from eight experiments.
DC may have an enhanced ability to activate NK cells. To test this, we cocultured GM-CSF DC and controls with freshly isolated NK cells and then harvested the NK cells and plated them against 51Cr-labeled Yac-1 target cells. NK cells that had been cultured with GM-CSF DC induced considerably higher lysis of targets compared with controls at all dilutions tested (Fig. 10). Moreover, consistent with our hypothesis, TNF-α blockade completely abrogated the effects.

Endogenous secretion of GM-CSF does not confer tumor protection

Since endogenous secretion of GM-CSF recruits massive numbers of DC with enhanced immunostimulatory function, we postulated that treatment with AdGM would confer tumor protection in vivo. However, it failed to protect against tumor in a variety of models including s.c. CT26 colorectal cancer, CT26 liver metastases, B16 melanoma liver metastases, and s.c. EL4-OVA lymphoma (Table II). Neither administering AdGM simultaneously to tumor challenge nor extending the interval between viral inoculation and tumor challenge to 14 days changed the outcome. Similarly, and in consort with the CTL data, immunization of naïve mice with OVA-pulsed GM-CSF-recruited DC failed to provide additional tumor protection against EL4.OVA tumor growth compared with immunization with peptide-pulsed DC from saline- or AdGFP-treated mice (Fig. 11).

Discussion

We have shown the most dramatic DC recruitment to date. A single treatment with a gene transfer vector encoding GM-CSF increased the number of splenic DC by 200- to 260-fold. In contrast, daily injections of 10⁸ µg of rFlt3L have been reported to increase DC populations by ~15- to 20-fold (35). Daily administration of GM-CSF conjugated to polyethylene glycol resulted in a nearly 8-fold increase in splenic DC (12). Minimal effects have been achieved with unmodified GM-CSF. Even ProGP-1, which is an agonist of both the Flt3 and GM-CSF receptors, only increased the total number of splenic DC to 5 × 10⁷ cells (36). In contrast, we recruited up to 3 × 10⁹ DC to the spleen. Furthermore, DC expansion after daily administration of any of the above proteins was only sustained for a few days after the cessation of treatments (36). In contrast, we have shown a greater than 15-fold increase in the number of splenic DC over normal even 75 days later. Considering that serum GM-CSF levels returned to baseline by 2 wk after AdGM injection (Fig. 1B), it is surprising that the number of splenic DC remained elevated for so long. It is uncertain whether a large number of cells are recruited shortly after treatment and persist in the spleen or whether there is continued recruitment of DC for an extended period. Using bromodeoxyuridine-labeling experiments, Kamath et al. (35) showed that all DC subtypes have a rapid turnover in the spleen with a half-life of just 2–3 days. This would suggest that there is continuous ongoing DC recruitment despite normalized serum GM-CSF levels. The precise derivation of the recruited DC is uncertain. The difficulty in tracking DC in vivo makes such an analysis difficult. However, our finding that bone marrow cellularity and the number of bone marrow-derived DC were decreased in AdGM-treated animals suggest that the recruited DC originated from the bone marrow.

Distinct DC subsets were recruited by endogenous overexpression of GM-CSF when compared with splenic DC subsets found in normal or Flt3L-treated mice. The vast majority of DC had classical myeloid staining (CD8α−CD11bbrht). In contrast, Flt3L recruits a higher percentage of lymphoid DC (CD8α+CD11bblw).
FIGURE 6. DC recruited by endogenously produced GM-CSF secrete high levels of TNF-α and IL-6 but low IFN-γ. Mice were treated with saline or \(8 \times 10^{10}\) particles of AdGFP or AdGM and then 7 days later their splenic DC were purified and cultured at a concentration of \(1 \times 10^6\) cells/ml in complete medium only or with LPS (10 ng/ml) or TNF-α (100 ng/ml) for 24 h. Supernatants were then tested for TNF-α (A), IL-6 (B), IFN-γ (C), and IL-10 (D). Averages of triplicates are shown. Data are representative of experiments repeated three separate times.

FIGURE 7. DC recruited by endogenously GM-CSF have enhanced Ag uptake in vitro and in vivo. Mice were treated with saline or \(8 \times 10^{10}\) particles of AdGFP, AdGM, or AdFlt3L and then 7 days later their splenic DC were purified. DC were incubated with FITC-dextran for 15 min (A), FITC-albumin for up to 60 min (B), or FITC-mannose albumin for up to 60 min (C), and the percentage of cells that were fluorescent (A and C) or the median fluorescence (B) was determined by flow cytometry at various time points. D, To assess Ag uptake in vivo, FITC-dextran was injected i.v. into mice 7 days after treatment. Splenic DC were harvested 30 min later and tested for fluorescence by flow cytometry. AdGM DC had higher Ag uptake than saline or AdGFP controls in both in vitro and in vivo models. Averages of triplicates are shown.
However, AdGM also expanded the lymphoid DC population, albeit to a much lesser extent than the myeloid population. This contrasts with a previous report in which pegylated GM-CSF was shown to significantly increase the number of DC. However, the increased number of DC did not translate into enhanced immune responses.

**FIGURE 8.** GM-CSF-recruited DC induce higher stimulation of allo- geneic and Ag-specific T cells. Mice were treated with saline or $8 \times 10^8$ particles of AdGFP or AdGM and then 7 days later their splenic DC were purified. An MLR was performed by mixing irradiated DC with allogeneic (BALB/c) splenic T cells that had been purified with immunomagnetic beads. Triplicate wells were plated using $3 \times 10^5$ T cells/well. Syn- geneic T cells had minimal proliferation (data not shown). Blockade of TNF-$\alpha$ partially abrogated the higher allogeneic proliferation caused by GM-CSF-recruited DC. Ag-specific T cell proliferation was measured by incubating DC with either OVA peptide (C) or OVA (D) and then mixing them with $5 \times 10^4$ OVA-specific T cells and then measuring supernatant IL-2 48 h later. DC plated alone and T cells plated alone did not produce detectable IL-2. All T cell assays were done in triplicate and repeated more than three times with similar results.

**FIGURE 9.** Immunization of mice with Ag-pulsed DC recruited by endogenous GM-CSF-induced CTL activity and elevated T cell IFN-$\gamma$ secretion. DC were harvested from mice 7 days after treatment with saline or $8 \times 10^8$ particles of AdGFP or AdGM. Immediately after harvest, DC were loaded in vitro with OVA for 60 min and used to immunize naive mice. After two immunizations, splenocytes were harvested from immunized mice, stimulated in vitro with OVA, and tested in a CTL assay (A) and for cytokine production (B). Splenocytes from mice immunized with GM-CSF-recruited DC. OVA induced similar CTL lytic activity in controls but secreted higher levels of IFN-$\gamma$. CTL and cytokine assays were repeated twice and done in triplicate wells.

However, AdGM also expanded the lymphoid DC population, albeit to a much lesser extent than the myeloid population. This contrasts with a previous report in which pegylated GM-CSF...
reportedly expanded only the CD11b<sup>bright</sup> population but not CD11b<sup>dim</sup> DC (12). Even more striking, virtually all of the GM-CSF-recruited DC were CD4<sup>+</sup>. This is distinctly different from Fli3L-recruited or normal DC and further contrasts with DC recruited by GM-CSF protein injections which reportedly do not have reduced CD4<sup>+</sup> staining (36). Other distinctive phenotypic characteristics of AdGM-recruited DC included high expression of the FcγRII/II receptor CD16/CD32 but minimal B220 staining. The functional importance of each of these phenotypic differences remains unresolved.

Beside the phenotypic differences, there are numerous functional differences between DC recruited by endogenous overexpression of GM-CSF and previous reports using daily GM-CSF protein injections. DC recruited by endogenous GM-CSF overexpression had increased Ag capture in vitro and in vivo consistent with its effects reported previously on peritoneal macrophages (34). These findings are also compatible with those of Daro et al. (12) who showed that DC from mice treated with pegylated GM-CSF captured and processed Ag more efficiently than controls. However, unlike Daro et al. (12), we found that DC recruited by endogenous GM-CSF actually had lower Ag uptake than Fli3L-recruited DC. Furthermore, DC recruited by endogenous GM-CSF stimulated allogeneic and Ag-specific T cells to a greater extent than controls. Conversely, DC recruited by either recombinant or pegylated GM-CSF protein did not have enhanced immunostimulation (12, 36). The functional differences that we observed using DC recruited by endogenous GM-CSF secretion compared with protein injections may simply reflect the disparity in the phenotype and secretory profile of the DC subsets recruited.

Although the production of IL-12 and several other cytokines by DC has been investigated, there are few data regarding DC secretion of TNF-α. In mice, TNF-α matures bone marrow-derived DC. By virtue of its maturational effects, TNF-α renders bone marrow-derived DC more effective in generating antitumor immunity after adoptive transfer (37). However, the effects appeared to be due primarily to maturation and not TNF-α per se because CpG and CD40, potent stimulators of DC maturation, achieve even greater antitumor effects (37, 38). Although the maturational effects of TNF-α on DC are known, the importance of TNF-α production by DC has not been defined. In fact, there have been scarce reports of any constitutive TNF-α secretion by DC subsets. Lu et al. (39) reported that various murine liver DC populations secrete high levels of TNF-α only when stimulated by high-dose LPS (10 μg/ml) for 48 h. Human Langerhans cells can also be induced to produce TNF-α by virus or LPS (40–45). Human monocyte-derived DC produce TNF-α upon exposure to LPS or Escherichia coli (46, 47). In our system, normal splenic DC only produced low levels of TNF-α while Fli3L DC did not make detectable quantities. This is consistent with the report by Puleldran et al. (48), who demonstrated that CD8α<sup>+</sup> splenic DC do not secrete TNF-α while CD8α<sup>−</sup> DC subsets produce very low levels (<100 pg/ml by 24 h).

This appears to be the first report of unstimulated DC producing markedly elevated quantities of TNF-α. The regulation of TNF-α secretion by GM-CSF-recruited DC is uncertain. The consequences of TNF-α production by DC on their capacity to stimulate effector cells have also not been delineated. We showed that the enhanced allostimulatory capacity of GM-CSF-recruited DC is partially mediated by their high TNF-α secretion. TNF-α also accounted for the increased ability of GM-CSF-recruited DC to stimulate NK cells. Thus, TNF-α may enable GM-CSF-recruited DC to link innate and adaptive immunity. The minimal production of IFN-γ by GM-CSF-recruited DC compared with controls may also be a relevant factor in their enhanced immunostimulation. DC production of IFN-γ has been shown to mediate tolerogenic effects on T cells in certain DC subsets (49).

In addition to TNF-α, GM-CSF-recruited DC expressed high levels of IL-6 compared with controls. Both the regulation of DC secretion of IL-6 as well as its effect on DC immune function are not completely understood. Chomarat et al. (50) reported that IL-6 is a critical factor in the molecular control of APC development and that IL-6 release from fibroblasts switches the differentiation of monocytes from DC to macrophages. Nevertheless, the relevance of IL-6 produced by the DC or monocytes themselves in this process is not defined. Grohmann et al. (49) reported that IL-6 plays a critical role in mediating the effects of CD40 ligation in CD8α<sup>+</sup> DC and enhancing their immunogenicity. The role of IL-6 included down-regulation of the IFN-γ receptor expression on CD8α<sup>+</sup> DC and correlated with the reduced ability of these DC to initiate T cell apoptosis in vitro. However, considering that the DC recruited by GM-CSF were largely CD8α<sup>−</sup>, the potential role of

![FIGURE 11.](http://www.jimmunol.org/) Immunization with peptide-pulsed GM-CSF-recruited DC protects against tumor growth. C57BL/6 mice were treated with either saline or 8 × 10<sup>10</sup> particles of AdGFP or AdGM. Their splenic DC were harvested 7 days later, loaded in vitro with OVA, and used to immunize naive mice. After two weekly immunizations, mic were challenged 7 days later with a s.c. flank injection of 3.5 × 10<sup>6</sup> EL4.OVA cells. Immunization with DC.OVA, regardless of DC origin, slowed tumor growth. Consistent with the CTL lysis data, but unlike the MLR and Ag-specific T cell stimulation data, GM-CSF-recruited DC did not confer additional protection over peptide-pulsed control DC. Averages of five mice per group are shown.
IL-6 in mediating their enhanced immunostimulatory function requires further study.

Considering that DC recruited by endogenously GM-CSF have enhanced T cell and NK cell stimulatory properties, it was somewhat surprising that recruitment of large numbers of these DC did not confer tumor protection in any of the models tested. However, it is possible that GM-CSF-recruited DC only acquire enhanced immunostimulatory function after in vitro culture where they became more mature and express higher levels of costimulatory and accessory molecules (51, 52). In contrast, these DC may be relatively quiescent in situ. Similarly, it is uncertain whether recruited DC actually produce activating cytokines such as TNF-α in vivo or only after isolation. The failure of GM-CSF-recruited DC to stimulate tumor protection raises considerable interest in the potential for substances that can directly activate DC in vivo to harness the immune potential of this massive DC recruitment. Merad et al. (53) recently reported that systemic administration of immunostimulatory DNA activates DC in vivo and augments the antitumor effects of Flt3L. Application of other in vivo DC activators to our model holds considerable promise for the immunotherapy of cancer.

References


