Combinalional FLt3 Ligand and Granulocyte Macrophage Colony-Stimulating Factor Treatment Promotes Enhanced Tumor Infiltration by Dendritic Cells and Antitumor CD8+ T-Cell Cross-priming but Is Ineffective as a Therapy

Aklile Berhanu,1 Jian Huang,1 Sean M. Alber,2 Simon C. Watkins,2 and Walter J. Storkus1,3,4

Departments of1Immunology, 2Cell Biology and Molecular Physiology, and 3Dermatology, University of Pittsburgh School of Medicine and 4The University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania

Abstract

Dendritic cells play significant roles in the development and maintenance of antitumor immune responses. Therapeutic recruitment of dendritic cells into the tumor microenvironment has the potential to result in enhanced antitumor T-cell cross-priming against a broad array of naturally processed and presented tumor-associated antigens. We have observed that the treatment of BALB/c mice bearing syngeneic CMS4 sarcomas with the combination of recombinant Flt3 ligand and recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) for five sequential days is sufficient to optimize the number of tumor-infiltrating dendritic cells (TIDC). However, despite the significant increase in the number of TIDCs, the therapeutic benefit of Flt3 ligand and GM-CSF treatment is minimal. Therapy-associated TIDCs do not exhibit a “suppressed” or “suppressor” phenotype in vitro, and their enhanced numbers in cytokine-treated mice were associated with increased levels of peripheral antitumor CD8+ T effector cells and with an augmented population of CD8+ tumour-infiltrating lymphocytes (TIL). These data suggest that Flt3 ligand + GM-CSF therapy of murine tumors fails at a mechanistic point that is downstream of specific T-cell priming by therapy-induced TIDCs and the recruitment of these T cells into the tumor microenvironment. Based on the enhanced infiltration of tumors by CD4+CD25+ TIL in Flt3 ligand + GM-CSF–treated mice, this could reflect the dominant influence of regulatory T cells in situ. (Cancer Res 2006; 66(9): 4895-903)

Introduction

The induction and maintenance of an effective antitumor immune response is critically dependent on dendritic cells. The ability of dendritic cells to capture and process tumor-derived antigens and to migrate to secondary lymphoid organs to cross-prime T cells is essential in developing protective immunity against pathogens or malignancy (1). Immunogenic dendritic cells are equipped to provide (at least) three signals needed for the activation of naive T cells and their development and polarization into specific subsets of effector T cells: peptides complexed with MHC molecules on the surface of dendritic cells (i.e., signal 1; ref. 2); T-cell costimulatory molecules, such as CD80 and CD86 (i.e., signal 2; ref. 3); and secreted cytokines, such as interleukin-12 (IL-12) and IL-10, that play important roles in the functional polarization of activated T cells into type 1, type 2, or regulatory type effector cells (i.e., signal 3; refs. 4, 5). The provision of signal 1 by dendritic cells without accompanying costimulatory signals has been shown to result in specific T-cell deletion and anergy (6). Dendritic cells that express high MHC complexes and costimulatory molecules but do not produce IL-12p70 have also been shown to be “tolerogenic” or to lack the capability of inducing Th1-type immune responses that seem critical for the eradication of tumors and intracellular pathogens (7).

Given the central role of dendritic cells in initiating and maintaining antigen-specific immune responses, it is not surprising that in some diseases, such as cancer, that alterations in dendritic cells development and function are associated with tumor escape from immune-mediated surveillance (8–10). Typically, tumor-infiltrating dendritic cells (TIDC) express low levels of MHC class II molecules and lack expression of costimulatory molecules (11, 12). Dendritic cells isolated from tumor-bearing mice and humans show significantly reduced abilities to activate peptide-specific CD8+ T cells and to stimulate allogeneic T cells when compared with normal control dendritic cells (13, 14). In contrast, T cells isolated from cancer patients or tumor-bearing mice that are stimulated with dendritic cells from normal controls generate normal T-cell responses (13, 14), suggesting that defects in dendritic cell–mediated cross-priming of tumor-reactive T cells in situ may be a major limitation for immunotherapy-based approaches in the cancer setting.

In our studies, the treatment of mice bearing syngenic s.c. CMS4 sarcomas with Flt3 ligand + granulocyte-macrophage colony-stimulating factor (GM-CSF) results in the recruitment of large numbers of TIDCs, in the absence of discernable therapeutic benefit. TIDCs in the treated mice do not exhibit a phenotype consistent with the induction of T-cell anergy or deletion, and indeed, we noted elevated levels of tumor-specific CD8+ T cells in the spleens of mice and enhanced CD8+ T-cell infiltration into treated tumors in vivo. These data suggest additional rate-limiting processes (such as that mediated by regulatory T cells) must be circumvented to permit the effective immunotherapy of tumor-bearing animals.

Materials and Methods

Mice. Six- to 8-week-old female BALB/cJ and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were...
maintained in the pathogen-free animal facility of the Hillman Cancer Center at the University of Pittsburgh Cancer Institute. All animal work was done in accordance with an Institutional Animal Care and Use Committee–approved protocol.

**Tumor establishment.** The CMS4 sarcoma, CT26 colon carcinoma, and RENCA renal adenocarcinoma cell lines (all H-2d; ref. 15) were cultured in complete medium (10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 mmol/L L-HEPES, and 2 mmol/L L-glutamine in RPMI 1640; all from Invitrogen Corp., Carlsbad, CA). CMS4 expresses H-2Kd class I molecules but exhibits a negative phenotype for the CD11c, CD80, CD11b, B220, CD86, and I-A6 markers. All cell lines were negative for known mouse pathogens. Cultured tumor cells were washed twice with PBS, and 5 × 10⁶ tumor cells were then resuspended in 100 μL of PBS and injected s.c. in the right flank of BALB/c mice. Tumor growth was monitored every 2 to 3 days by measuring the width and length of the tumors using a DigiMax slide caliper (Bel-Art Products, Pequannock, NJ), and area was calculated as the product of these values. Treatment groups included three to five mice, as indicated.

**Combinational cytokine therapy.** For in vivo dendritic cells expansion, mice were injected with 20 μg each of rFlt3 ligand and rGM-CSF (16) for three to seven consecutive days, as indicated in the text and figure legends. Injections were given s.c. in the scruff of the neck, with cytokines diluted in a total volume of 100 μL PBS. Both cytokines were the kind gifts of Pharmacia Corp. (St. Louis, MO).

**Analysis of single-cell preparations from tumor and spleen.** Single-cell suspensions were generated from resected spleens and tumors as previously described (15). For isolating CD11c+ dendritic cells, spleen or tumor-infiltrating lymphocyte (TIL) cells were E4 blocked with anti-CD64/32 antibody (BD PharMingen, San Diego, CA), incubated with CD11c MicroBeads (Miltenyi Biotec, Inc., Auburn, CA), and then positively selected on MiniMACS magnetic columns per the manufacturer’s instructions. CD4+ T cells were isolated from the CD11c– fraction of spleen or tumor cell suspensions by using CD4 MicroBeads (Miltenyi Biotec). Because tumors from cytokine-untreated mice contain only very rare infiltrates of CD11c+ dendritic cells or CD4+ T cells, it was logistically impossible to isolate enough cells in this cohort size to perform comparative analysis with CD11c+ dendritic cells or CD4+ T cells isolated from the tumors of Flt3 ligand + GM-CSF–treated mice.

**Flow cytometry.** The following antibodies and their corresponding isotype controls (all purchased from BD Pharmingen) were used for staining: biotinylated CD11c, CD80-PE, CD11b-PE, B220-FTC, CD86-PE, CD80-PE, I-A6-FITC, biotinylated-CD4, and CD25-FTC. After adding the appropriate antibody, the cells are incubated in the dark at 4 °C for 30 minutes and washed twice by centrifugation using fluorescence-activated cell sorting (FACS) buffer. For the detection of apoptosis, FACS-conjugated pan caspase inhibitor, Z-VAD-FMK (Promega Corp., Madison, WI), was used to stain cells using our previously reported protocol (15). Cells stained with primary antibodies were then incubated with Streptavidin-PerCP, washed twice, fixed in 1% paraformaldehyde, and analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, Inc., Fullerton, CA). Data were analyzed by using WinMDI (The Scripps Research Institute, La Jolla, CA) or EXPO32 (Beckman Coulter) software.

**Confocal immunofluorescence staining and morphomet quantita- tion.** Tumor tissue samples were embedded in ornithine carbamyl transferase medium (Tissue-Tek Sakura Finetek U.S.A., Inc., Torrance, CA), frozen, and stored at −80°C. Five-micrometer tissue sections were prepared using a cryostat microtome, mounted on Superfrost Plus (Fisher Scientific, Pittsburgh, PA) slides, and stored at −80°C. For staining, the sections were fixed in 2% paraformaldehyde (Sigma-Alrich, St. Louis, MO) at room temperature for 40 minutes and blocked with normal goat serum for 40 minutes. The sections were then incubated with unconjugated (CD11c), biotinylated (CD11c, CD3, and I-Aa), or PE-conjugated (CD11b, CD80, and B220) primary antibodies or matching isotype controls (all from PharMingen) for 1 hour. This was followed by incubation with goat antihamster Cy3 (Jackson Immunoresearch, West Grove, PA) or streptavidin-Alexa 488 (Invitrogen) for 1 hour. Finally, Hoechst or Sytox Green (Invitrogen) was applied to stain nuclei. Images were acquired using Olympus Provis fluorescence or Olympus Fluoview 500 confocal microscopes (Olympus, Melville, NY).

Numbers of CD11c+ dendritic cells in tumor sections were quantitated using Metamorph v.6.1 software (Universal Imaging Corp., Downingtown, PA). For each tissue section, both the CD11c+Cy3 and Sytox Green nuclear staining images were captured sequentially to avoid bleed through between the channels. For each tissue section, ≥10 images were captured from nonoverlapping image field areas. The percentage of CD11c+ cells was calculated as follows: (CD11c+ staining count per field / total nuclei count per field) × 100.

For analysis of particle uptake by dendritic cells in situ, tumor-bearing mice that were either untreated or treated with Flt3 ligand + GM-CSF were injected i.t. on the day following cessation of cytokine therapy with 0.2-μm yellow-green (505/515 nm) Fluorospheres (Invitrogen) in a total volume of 50 μL PBS. Twenty-four hours later, animals were sacrificed, tumors were resected, and 10-μm cryosections prepared, as noted above, then blocked in 2% bovine serum albumin (BSA: 40 minutes), incubated in hamster anti-mouse CD11c (PharMingen) for 1 hour, and incubated in goat anti-hamster Cy3 (Jackson Immunoresearch) and phallolidin-Alexa 647 (Invitrogen) for 1 hour. Images were taken using an Olympus Fluoview 500 Scanning Confocal Microscope.

**In vitro activation of dendritic cells.** CD11c MicroBead-selected dendritic cells were resuspended in 200 μL complete medium containing 5 × 10⁵ cells. The following concentrations of dendritic cell activation stimuli were used: 10 ng/mL lipopolysaccharide (LPS; Sigma), 75 μg/mL Staphylococcus aureus Cowan strain I (SAC; EMD Biosciences, San Diego, CA), or 100 μg/mL polyriboinosinic polyribocytidylic acid (poly [Ei]; Sigma). Each stimulation condition was done in duplicate wells in 96-well plates. The plates were placed in a humidified incubator at 37°C and 5% CO₂ for 24 hours. At the end of the incubation period, the plates were centrifuged, and the cell-free supernatants were collected in Eppendorf tubes and stored frozen at −80°C until used in ELISA.

**Analysis of dendritic cell stimulation of alloreactive T cells in vitro.** H-2d CD11c+ dendritic cells isolated from the spleens of untreated normal mice or Flt3 ligand + GM-CSF–treated tumor-bearing mice, or from single-cell digests of resected CMS4 tumors were cocultured with H-2d CD4+ splenic T cells at T cell ratios of 1:1, 1:10, or 1:100 in 96-well round-bottomed plates. The plates were then incubated at 37°C and 5% CO₂ for 3 days. On the 4th day, CD4+ T-cell proliferation was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay according to the manufacturer’s procedure. In brief, MTT solution was added to each well, and the plates were incubated at 37°C for 2 hours. After the incubation period, the MTT formazan crystals were dissolved in 0.1 N HCl in anhydrous isopropanol. The absorbance was measured at 570 versus 690 nm background.

**DQ-Ovalbumin uptake assay.** CD11c+ dendritic cells were resuspended at 50 × 10⁵ per 100 μL of complete medium, with 100 μL of the cell suspension transferred to each of four polypropylene tubes. DQ-Ovalbumin (Invitrogen, Carlsbad, CA) was added to two of the tubes at a concentration of 10 μg/mL. One set of tubes (one containing and one lacking DQ-Ovalbumin) was placed on ice, whereas a second set of tubes was incubated at 37°C for 1 hour. At the end of the incubation period, the cells were washed with FACs buffer, stained with anti-CD11c antibody, washed, and evaluated immediately by flow cytometry.

**In vitro stimulation of CD8+ T cells.** CD8+ T cells were isolated by MACS selection from the CD11c+CD4+ fraction of splenocytes or TILs. Then, 2 × 10⁷ CD8+ T cells were cocultured with 5 × 10⁸ irradiated (100 Gy) CMS4 sarcoma cells or Flt3 (H-2d) mammary carcinoma cells in 96-well round-bottomed plates in a humidified incubator at 37°C and 5% CO₂ for 72 hours. At the end of the culture period, the plates were centrifuged to pellet the cells, and the cell-free supernatants were collected and stored at −80°C.

ELISA. ELISAs were done on cell supernatants using IL-12p70, IL-10, or IFN-γ specific OptEIA ELISA sets (BD Biosciences) according to the manufacturer’s instructions. The lower limit of detection for these assays were 62.5, 31.3, and 31.3 pg/mL, respectively.
Statistical analysis. Statistical analyses were done using an unpaired two-tailed Student’s t test. SPSS for windows (SPSS, Inc., Chicago, IL) software was used to conduct the analysis. Comparative differences yielding \( P < 0.05 \) were considered significant.

Results

Combined treatment of mice with Flt3 ligand + GM-CSF leads to an increase in systemic dendritic cell numbers in vivo.

One of our goals was to define and apply a cytokine treatment regimen capable of systemically expanding optimal numbers of dendritic cells and to subsequently mobilize these cells into tissues, including tumors. In initial experiments, we treated mice with GM-CSF alone, Flt3 ligand alone, or a combination of the two cytokines and assessed their effect on the splenic dendritic cells. As shown in Fig. 1A, whereas the percentage of CD11c+ splenic dendritic cells in control, untreated mice was \( \sim 2.4\% \), this percentage increased to \( \sim 5.1\% \) and \( \sim 15.3\% \) when mice were treated with 20 \( \mu g/d \) of GM-CSF or Flt3 ligand alone for seven consecutive days, respectively, consistent with previous reports (17–19). However, the highest increase in the number of splenic dendritic cells resulted from the treatment with Flt3 ligand + GM-CSF (16). The increase in dendritic cells numbers paralleled the spleen size, with the organs harvested from mice treated with Flt3 ligand + GM-CSF being the largest and most cellular among all cohorts evaluated (data not shown). Overall, the absolute numbers of CD11c+ splenic dendritic cells were increased \( \sim 3\, , \, 10\, , \, \text{and} \, \sim 30\)-fold (versus untreated mice) after treatment with GM-CSF, Flt3 ligand, or both cytokines, respectively, supporting the preferred use of Flt3 ligand + GM-CSF to maximally increase systemic dendritic cells numbers in vivo.

Significantly enhanced infiltration of tumor tissues by dendritic cells is observed after treatment with both Flt3 ligand + GM-CSF. Because tumors may negatively affect dendritic cells development and function in situ (8, 20), we next determined whether Flt3 ligand + GM-CSF treatment altered dendritic cell numbers in the tumor microenvironment. We treated BALB/c mice bearing day 7 CMS4 sarcomas with daily injections of Flt3 ligand + GM-CSF using a (initially empirical) 7-day course. On the day following the last cytokine administration, mice were sacrificed, and their tumors were examined by immunofluorescence microscopy for CD11c+ dendritic cell infiltration. Although there were very few
CD11c+ dendritic cells in tumor sections isolated from untreated mice, those mice receiving cytokine treatment displayed high levels of CD11c+ TIDCs, which were distributed throughout the lesion (Fig. 1B). Optimal levels of cytokine-enhanced TIDCs and splenic dendritic cell numbers occur with slightly different kinetics. The presence of optimal numbers of dendritic cells (at least transiently) in the tumor microenvironment would be presumed important for the initiation and maintenance of tumor-reactive T cells in secondary lymphoid organs, as well as the tumor microenvironment itself (21, 22). Within tumor lesions, dendritic cells would be envisioned to acquire tumor debris via direct (23, 24) or indirect (25–27) killing/death of tumor cells and to consequently cross-present processed tumor antigens to T cells in the spleen and lymph nodes. Hence, we studied the kinetics of dendritic cells infiltration into the tumor to define an optimal schedule for cytokine administration. Mice bearing day 7 CMS4 tumors were treated with Flt3 ligand + GM-CSF for 3, 5, or 7 consecutive days, and the number of CD11c+ dendritic cells infiltrating the tumors was quantitated by confocal microscopy. In parallel, the frequencies of splenic CD11c+ dendritic cells were quantitated by single-cell flow cytometric analysis. As shown in Fig. 1C, the average percentage of TIDCs in control, untreated mice was <1% of total cells, and the highest level of TIDCs observed in any confocal image field was 1.7%. Treatment with Flt3 ligand + GM-CSF resulted in significant increases in TIDC frequencies at all time points evaluated (versus control untreated lesions), with the maximal increase observed for a 5-day course of cytokine administration (mean = 17.6% TIDCs; \( P = 0.021 \) versus untreated or day 3 treated; \( P = 0.733 \) versus day 7 treated). The kinetics of dendritic cell mobilization into the spleen varied from that of the tumor (Fig. 1D). Average splenic CD11c+ dendritic cells frequencies continued to increase throughout the treatment period [i.e., 7.9% (3-day treatment), 16.1% (5-day treatment), and 27% (7-day treatment)]. However, no additional increase was observed if cytokine treatment were extended through 9 days (data not shown).

Enhancement of CD8α+ dendritic cells in the spleen of Flt3 ligand + GM-CSF–treated mice is not mirrored in the tumor. CD11c+ dendritic cells contain several phenotypic and functionally distinct subpopulations that have been linked with contrasting states of immunity (4, 28), including CD8α+CD11c+ (previously referred to as “lymphoid”), CD8α–CD11b+CD11c+ (previously referred to as “myeloid”), and B220+CD11c+ (“plasmacytoid”) dendritic cells (16, 18, 29). As shown in Fig. 2, the spleens of untreated, non–tumor-bearing mice contained ~22% CD8α+CD11c+, ~11% B220+CD11c+, and ~62% CD11b+CD11c+ dendritic cells. Treatment of control or tumor-bearing mice with Flt3 ligand + GM-CSF principally affected the mobilization/expansion of CD8α+ dendritic cells into/within the spleen, resulting in an approximate doubling in lymphoid dendritic cell frequencies (i.e., ~46% versus ~22% in controls). On the contrary, the relative composition of the B220+CD11c+ dendritic cell subset was lower in the treated mice (~5% versus 11% in controls; \( P = 0.043 \)), but this cell population seemed to express a relatively higher level of the CD11c marker than control B220+ dendritic cells. Within the CD11c+ dendritic cell subpopulation, there were two largely distinct cohorts defined in the treated mice CD11bhigh and CD11bmid in an approximate 1:2 ratio.

When compared with the spleen, tumors in treated mice contained ~8- and 4-fold lower frequencies of CD8α+CD11c+ dendritic cells (i.e., 6% versus 46%; \( P = 0.004 \)) and B220+CD11c+ dendritic cells (i.e., 3% versus 11%; \( P = 0.035 \)), respectively. These data suggest that combined cytokine therapy results in a preferentially skewing towards CD11b+CD11c+ versus CD8α+CD11c+ or B220+CD11c+ TIDCs.

Flt3 ligand + GM-CSF–induced TIDCs exhibit normal phenotype and function. Although “myeloid” CD11b+CD11c+ dendritic cells can be strong stimulators of primary CTL responses (30, 31), it is clear that “immature” or “mature” CD11b+CD11c+ dendritic cells exhibit very different immunostimulatory indices (32). Immature dendritic cells express low levels of costimulatory molecules on their surface, whereas mature dendritic cells have up-regulated levels of these markers, and tumors have been reported to induce or restrict TIDCs towards an immature phenotype (8, 11, 33). As shown in Fig. 3A, the majority of freshly isolated TIDCs from Flt3 ligand + GM-CSF–treated mice consistently expressed the I-A^d, B7.1 (CD80), and B7.2 (CD86) molecules on their cell surface at levels consistent with a mature dendritic cell phenotype. Notably, TIDCs expressed higher levels of these markers when compared with CD11c+ dendritic cells isolated from the
spleens of these same animals (Fig. 3B) or untreated, control mice (Fig. 3C). These data suggest that the CMS4 microenvironment does not preclude the presence of TIDCs bearing a mature phenotype in situ.

Because the tumor microenvironment can promote the apoptotic death of dendritic cells (34), we assessed TIDCs for evidence of a proapoptotic phenotype using a fluorescent, cell-permeable derivative of z-VAD-FMK (which irreversibly binds to activated caspases in cells undergoing apoptosis) as a probe. As shown in Fig. 3A, no significant FITC-VAD-FMK staining intensity difference was detected in TIDCs versus splenic dendritic cells isolated from the cytokine-treated or untreated mice.

We also did in situ staining analyses of TIDCs in tumor tissue sections resected from mice treated with Flt3 ligand + GM-CSF to confirm our in vitro phenotype data. As shown in Fig. 3D, TIDCs were easily distinguished by CD11c+ staining and by their hair-like projections (dendrites; see inset). Similar to the data obtained by flow cytometry (Fig. 2), virtually all of the CD11c+ TIDCs coexpressed CD11b and I-Ad class II molecules on their surfaces (Fig. 3D). Staining with anti-CD8a or anti-B220 antibodies did not reveal costaining with CD11c+ TIDCs in tumor sections but was readily detectable in spleen sections (data not shown). We also noted minimal cell staining with the FITC-VAD-FMK probe in tumor sections, and those cells reactive with this reagent were CD11c- (data not shown).

TIDCs produce higher levels of IL-10 rather than IL-12 p70 upon activation in vitro. Despite an immunogenic phenotype expressed by cytokine-induced TIDCs in vitro, the presence of such cells in situ did not lead to “clinical benefit.” Given the reported importance of IL-12p70 and IL-10 as immunostimulatory versus immunosuppressive dendritic cell–produced cytokines that may differentially affect the functional outcome of T-cell cross-priming (35, 36), we analyzed the ability of TIDCs to produce these cytokines after in vitro activation. TIDCs and splenic dendritic cells isolated from untreated or Flt3 ligand + GM-CSF–treated mice did not spontaneously secrete IL-12p70 when cultured in the absence of exogenous stimuli (Fig. 4A). However, the addition of the TLR ligands LPS, SAC, or poly (I:C) resulted in IL-12p70 production from all dendritic cell populations, with the sole exception of poly (I:C)–stimulated splenic dendritic cells isolated from mice treated with Flt3 ligand + GM-CSF. There was no significant difference in IL-12p70 production levels between LPS- and SAC-stimulated TIDCs versus untreated splenic control dendritic cells; the TIDCs produce comparatively higher than normal levels of IL-10 in response to TLR-ligands [i.e., 17.5-, 10.8-, and 8.8-fold higher in response to LPS, SAC, and poly (I:C), respectively].

TIDCs mediate soluble antigen/particle uptake and processing and effectively stimulate allogeneic T-cell responses in vitro and in vivo. Lack of TIDC efficacy in our model could relate to the inability of these cytokine-induced TIDCs to uptake and cross-present antigens (37) to antitumor T cells in vitro. To test whether their antigen uptake and processing functions were intact, we cultured freshly isolated CD11c+ TIDCs with dye-quenched

Figure 3. CD11c+ TIDCs exhibit the surface phenotype of (semi)mature dendritic cells and do not exhibit a “suppressed” or highly apoptotic phenotype. The TIDC (A) and splenic dendritic cell (SPDC; B and C) populations described in Fig. 2 were stained with antibodies specific for MHC class II, and the indicated costimulatory molecules and CD11c+ gated cells were analyzed by flow cytometry. In each case, the empty histogram represents isotype control staining, whereas the filled profiles represent specific staining. Representative of three independent experiments. Inset numbers, mean fluorescence intensity staining for the indicated marker. D, immunofluorescence staining of tumor sections, allowing for confirmation of the most prevalent TIDC phenotype as CD11b+CD11c+ and I-Aα+CD11c+. Representative of tumor sections analyzed from two treated mice.
T-cell proliferation induced by TIDCs was consistently higher than that observed for untreated H-2$$^d$$ splenic dendritic cells. However, splenic dendritic cells isolated from tumor-bearing mice treated with Flt3 ligand + GM-CSF were comparatively better than TIDCs in their ability to activate alloantigen-reactive T cells ($P = 0.039$).

To show that the TIDCs in the Flt3 ligand + GM-CSF–treated animals were capable of taking up particulates in situ, 1 day after the cessation of cytokine treatment, fluorescent 0.2-μm beads were injected i.t., and biopsy sections were evaluated 24 hours later by confocal microscopy. As depicted in Fig. 4D, CD11c$$^+$$ TIDCs were more prevalent in cytokine-treated versus nontreated tumors, and these cells were clearly able to bind/endocytose the injected beads, supporting the likelihood that these cells can effectively acquire exogenous antigens within the tumor microenvironment.

**Therapeutic effect of Flt3 ligand and GM-CSF treatment is minimal in controlling tumor growth in vivo.** Although our data clearly suggest a 5-day cytokine regimen to be effective for the promotion of maximal TIDC numbers, treated CMS4-bearing mice did not display statistically significant therapeutic benefits from cytokine administration (Fig. 5A). The lack of therapeutic benefits by this regimen was not restricted to CMS4 tumors but was also observed in BALB/c mice bearing syngeneic CT26 (Fig. 5B) or RENCA (Fig. 5C) tumors.

Flt3 ligand + GM-CSF treatment results in the systemic cross-priming of antitumor T cells and enhanced T-cell infiltration of tumor lesions in situ. Given the lack of treatment efficacy, we directly assessed whether or not combined cytokine therapy–associated TIDC enhancement translated into the improved cross-priming and i.t. recruitment of specific CD8$$^+$$ T cells in vivo. We first evaluated splenic CD8$$^+$$ T cells for evidence of tumor-specific (CMS4 versus irrelevant 4T1) production of IFN-γ. Although no IFN-γ production was detected from splenic CD8$$^+$$ T cells isolated from untreated, CMS4-bearing mice upon stimulation with CMS4 or 4T1 tumor cells, splenic CD8$$^+$$ T cells isolated from Flt3 ligand + GM-CSF–treated mice produced high levels of IFN-γ when cultured with CMS4 but not 4T1 cells (Fig. 6A). This suggests that TIDCs in cytokine-treated mice are competent to cross-prime peripheral tumor-specific CD8$$^+$$ T cells in vivo.

**Figure 4.** TIDCs isolated from Flt3 ligand (F) + GM-CSF (G)–treated mice produce elevated levels of IL-10 but normal levels of IL-12p70 and are competent to uptake and process soluble antigens and to stimulate allogenic T-cell proliferation in vitro and to uptake particulates in vivo. A, TIDC (black histograms) and splenic dendritic cells (Flt3 ligand + GM-CSF treated, open histograms; untreated, hatched histograms) described in Fig. 2 were cultured for 24 hours with or without the indicated TLR ligands, and the supernatants were assayed by cytokine-specific ELISA. Columns, mean of three independent experiments; bars, SD. B, TIDCs and splenic dendritic cells (SPDC) were assayed using DQ-Ovalbumin as a soluble antigen. Tubes to which DQ-Ovalbumin was added or not added were incubated at 4°C and 37°C for 1 hour, washed, and analyzed by flow cytometry. Bold black histograms, dendritic cells incubated without DQ-Ovalbumin; gray histograms, dendritic cells incubated with DQ-Ovalbumin (inset numbers, mean fluorescence intensity values). Derive from one of two experiments done with similar results obtained. C, the H-2$$^d$$ TIDC and splenic dendritic cells were cocultured with CD4$$^+$$ splenic T cells isolated from C57BL/6 (H-2$$^b$$) mice for 3 days and assayed for MLR responses using a 4-hour MTT assay as described in Materials and Methods. Columns, mean of three independent experiments; bars, SD. **, $P < 0.05$. D, animals were prepared and treated as outlined in Fig. 2. One day after the cessation of Flt3 ligand + GM-CSF treatment, fluorescent beads were injected i.t. into untreated or Flt3 ligand + GM-CSF–treated mice. Tumors were resected 24 hours later, and 3-color confocal microscopy was done on cryosections (green, beads; red, CD11c; blue, phalloidin). Larger field images reflect 60 magnifications and represent summations of 20 orthogonal section images obtained for each specimen. Insets, mid-cell slices to document bead internalization by TIDCs (magnification, ×125). Representative of lesions obtained from two animals evaluated in each treatment cohort.
As shown in Fig. 6B, very few CD8\(^+\) (CD3\(^+\)) T cells could be imaged in tumor sections generated from untreated mice. In marked contrast, tumor sections isolated from Flt3 ligand + GM-CSF–treated mice were infiltrated by large numbers of CD3\(^+\)CD8\(^+\) T cells. These T cells were located throughout the tumor lesion and were not confined to peripheral areas of the tumor stroma (as reported for some cancers; ref. 38). Similar to what we observed for splenic CD8\(^+\) T cells isolated from Flt3 ligand + GM-CSF–treated animals, the corresponding CD8\(^+\) TIL also produced high levels of IFN-\(\gamma\) against CMS4 but not 4T1 tumor cells \emph{in vitro}. These data suggest that the therapeutic failure of Flt3 ligand + GM-CSF treatment was not associated with an inability to promote CD8\(^+\) TIL exhibiting CMS4 specificity \emph{in situ}.

**Figure 5.** The effect of combined Flt3 ligand + GM-CSF cytokine therapy on the growth of tumors \emph{in vivo} is minimal. BALB/c (3-5 per cohort) mice bearing established CMS4 (A; sizes 14-18 mm\(^2\)), CT26 (B; sizes 29-30 mm\(^2\)), or RENCA (C; sizes 20-33 mm\(^2\)) tumors were treated with 20 \(\mu\)g/d of each cytokine for five consecutive days, and tumor measurements were taken every 2 to 3 days. Arrows, days in which the Flt3 ligand + GM-CSF treatment was administered. Points, mean; bars, SD. Similar results were obtained in repeat experiments, with 6 to 10 total mice analyzed per cohort. — untreated; — FL + GM.

**Figure 6.** Flt3 ligand (FL) + GM-CSF (GM) treatment results in enhanced infiltration of specific CD8\(^+\) T cells and CD4\(^+\)CD25\(^+\) T cells into CMS4 tumors. A, CD8\(^+\) T cells were isolated by MACS selection from the CD11c\(^-\)CD4\(^-\) fraction of splenocytes or TIL of Flt3 ligand + GM-CSF–treated animals on day 14. IFN-\(\gamma\) production was measured by specific ELISA after coculture of CD8\(^+\) T cells with irradiated CMS4 (relevant target) or control 4T1 (irrelevant target) tumor cells for 72 hours. When splenic CD8\(^+\) T cells isolated from untreated mice were stimulated with irradiated CMS4 or control 4T1 cells, <50 pg/mL of IFN-\(\gamma\) was detected. Columns, mean of three independent experiments; bars, SD. B, 5-\(\mu\)m sections of established day 14 CMS4 tumors described in Fig. 2 were stained and analyzed by confocal microscopy. CD3\(^+\) T cells (green) and CD8\(^+\) T cells (red). Representative of tumor sections analyzed from two treated mice in each case. C, CD4\(^+\) T cells were positively selected from the CD11c\(^-\) fraction of TILs or splenocytes isolated from the Flt3 ligand + GM-CSF–treated BALB/c mice described in Fig. 2. The cells were then costained with anti-CD4 and anti-CD25 antibodies and analyzed by flow cytometry. Inset, percentage of cells in each quadrant. Representative of two independent experiments.
Treatment with Flt3 ligand + GM-CSF results in high levels of CD4+CD25+ TIL. Because CD4+CD25+ regulatory T cells have been reported to be enriched in the tissues of patients with cancer (39, 40) and may contribute to the suppression of the antitumor immune responses in vivo, this could explain why effective cross-primming and recruitment of anti-CMS4 CD8+ TIL in Flt3 ligand + GM-CSF–treated mice fails to be associated with antitumor efficacy in vivo. As shown in Fig. 6C, we found that whereas only ~10% of splenic CD4+ T cells coexpressed CD25 in tumor-bearing mice treated with Flt3 ligand + GM-CSF, that nearly half (42 ± 4%) of the CD4+ TIL were CD25+. These data support a therapy-associated enrichment of CD4+ T cells bearing a regulatory T-cell phenotype that could be directly related to anti-CMS4 CD8+ TIL dysfunction in situ.

Discussion

Because most solid tumors express variable levels MHC class I molecules and fail to express T-cell costimulatory molecules (i.e., B7.1 and B7.2), it is imperative that functionally appropriate dendritic cells access the tumor microenvironment and consequently cross-prime (40) specific CD8+ T cells in the periphery that have the potential to infiltrate tumors and regulate their growth (or mediate their regression) in vivo. The ability of in vitro generated dendritic cells to mediate antitumor efficacy when used in vaccines and therapies (41, 42) suggests that these adoptively transferred cells support effective antitumor T-cell cross-priming. Furthermore, the ability of constitutive cross-priming mediated by TIDCs to regulate tumor progression is circumstantially supported by direct correlations reported between elevated numbers of TIDCs and improved patient prognosis (43) or reduction in the establishment of metastatic disease (44).

With the intent of promoting enhanced TIDC numbers and improved cross-primming of tumor-reactive T cells, we did this preliminary assessment of combined Flt3 ligand + GM-CSF therapy in the s.c. CMS4 sarcoma model. Previous studies have shown that the treatment of mice (17) or humans (45) with the hematopoietic cytokine Flt3 ligand results in significant expansion of dendritic cells in both lymphoid and nonlymphoid tissues in vivo. In some cases, the treatment of mice with Flt3 ligand resulted in complete rejection or reduction in tumor growth rate, with this effect shown to be dependent on CD8+ T and/or natural killer cells (46, 47). In our studies, we treated CMS4-bearing mice with Flt3 ligand + GM-CSF because this combined treatment synergistically expanded dendritic cells in the spleen and in tumors. However, such treatment did not affect tumor progression. To determine why this therapy failed, and given numerous reports that tumors can interfere with dendropoisesis (8, 20, 34) or with dendritic cells function (11, 14), we focused our attention on the tumor microenvironment to determine whether TIDCs in cytokine-treated animals were functionally competent to cross-prime specific T effector cells. We found that CMS4 tumors in animals treated with Flt3 ligand + GM-CSF were highly infiltrated by dendritic cells (versus nontreated tumors), with optimal TIDCs observed after 5 days of cytokine administration. Because the number of dendritic cells in tumors versus spleens of mice stabilizes or diminishes between days 5 and 7 of cytokine treatment, and given no evidence for increased apoptosis among any of the dendritic cell populations assessed, it is possible that TIDCs are leaving the tumor and trafficking to the draining lymph nodes during this interval to activate specific T cells.

Consistent with their ability to cross-prime CD8+ T cells in situ, Flt3 ligand + GM-CSF–mobilized TIDCs uniformly expressed high levels of I-A<sup>+</sup>, CD86, and CD80, a comparatively mature phenotype. In extended analyses, these TIDCs were also found to be effective in taking up and processing DQ-BSA protein and in stimulating the proliferation of alloreactive T cells in vitro. Furthermore, TIDCs isolated from mice treated with Flt3 ligand + GM-CSF seemed to maintain their ability to produce normal levels of IL-12p70 when stimulated with TLR ligands. In situ analyses showed that (a) TIDCs in animals treated with Flt3 ligand + GM-CSF were capable of taking up subcellular sized fluorescent beads, consistent with their capacity to acquire exogenous antigens in the tumor microenvironment; and (b) anti-CMS4 CD8+ T cells were effectively primed to a greater extent in tumor-bearing mice treated with Flt3 ligand + GM-CSF; and that these cells were present within TIL populations.

Disappointingly, Flt3 ligand + GM-CSF administration failed to effectively treat established CMS4 (or CT26 or RENCA) tumors, despite the ability of this strategy to promote enhanced (largely CD11b+CD11c+) TIDC populations that seem effective in cross-priming peripheral antitumor CD8+ T cells that exhibit the capacity to infiltrate tumor lesions. In this regard, the ability of cytokine therapy-induced TIDCs to produce significantly up-regulated levels of IL-10 may be a relevant to our lack of clinical effect. Given their high expression levels of I-A<sup>+</sup> and costimulatory molecules, as well as their IL-10 production capabilities, our described TIDCs are similar to semimature dendritic cell populations described by Akbari et al. (5) and McGuirk et al. (48), that seem capable of inducing CD4+CD25+IL-10+ T regulatory (Tr1) cells in vivo. The presence of such regulatory T-cell populations in our tumor models could clearly impede the efficacy of our applied therapy.

Consistent with this hypothesis, we found that CD4+CD25+ T cells are enriched ~4-fold in TIL versus spleens of mice treated with Flt3 ligand + GM-CSF. Therefore, the combined cytokine regimen seems to result in increased numbers of TIDCs that are functionally competent to induce not only antigen-specific CD8+ Tc1 cells, but also T cells bearing a CD4+ regulatory T-cell phenotype, that may neutralize the antitumor activity of the CD8+ T-cell population in situ. Tumor-infiltrating regulatory T cells may also reinforce the semimature phenotype of therapy-induced TIDCs via elaboration of IL-10 and/or transforming growth factor-β and/or instigate dendritic cell production of the tryptophan-catabolizing enzyme IDO that is associated with enhanced effector T-cell apoptosis, as well as, the potentiation of regulatory T-cell responses (49, 50). This would establish a positive-feedback circuit favoring immune suppression in the tumor microenvironment. Prospective evaluation of these possibilities in our model system is clearly warranted to characterize the potential suppressive nature of the observed therapy-induced CD4+CD25+ T cells on dendritic cell and antitumor CD8+ T effector cells, and to determine whether novel Flt3 ligand + GM-CSF–based combinational therapies that incorporate modalities to silence or delete regulatory T cells in vivo define an effective cancer therapy.

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References

Combinational FLt3 Ligand and Granulocyte Macrophage Colony-Stimulating Factor Treatment Promotes Enhanced Tumor Infiltration by Dendritic Cells and Antitumor CD8+ T-Cell Cross-priming but Is Ineffective as a Therapy

Aklile Berhanu, Jian Huang, Sean M. Alber, et al.