Abstract

Dendritic cells are professional antigen-presenting cells capable of inducing and regulating innate and antigen-specific immune responses. Therapeutic cancer vaccines using ex vivo engineered or in vivo targeted dendritic cells are being evaluated in clinical trials. T-helper type-1 (Th1)–skewed immune responses are characterized by the preferential induction of antigen-specific IFN-γ–secreting CD4+ T cells and correlate with effector mechanisms important for tumor and viral immunity. Methods to “polarize” human monocyte-derived dendritic cells for the preferential induction of Th1-skewed immune responses have been developed, and polarized dendritic cells (DC1s) are being evaluated in preclinical and clinical studies. Here, we show that stimulation of bone marrow–derived murine dendritic cell populations with poly(I:C) and CpG8 results in phenotypic maturation of dendritic cells and synergistic induction of durable, high-level IL-12p70 secretion characteristic of human type-1 polarized dendritic cells. Functionally, these dendritic cells induce antigen-specific Th1-type CD4+ T-cell activation in vitro and in vivo. Dendritic cell maturation and polarization are not inhibited by the presence of live B16 melanoma tumor cells, and tumor-loaded DC1s induce delayed-type hypersensitivity responses in vivo. DC1s loaded with B16 melanoma cells and injected into tumor-bearing mice induce Th1-skewed tumor-specific CD4+ T cells and a significant reduction in tumor growth. Tumor infiltrates in DC1-immunized animals are characterized by the presence of CD4+ T cells and activated macrophages. These results show a murine model of DC1 function and suggest an important role for CD4+ T cells and macrophages in DC1-induced antitumor immune responses. They have implications for the future development of DC1-based immunotherapies and strategies for clinical immune monitoring of their effectiveness. (Cancer Res 2005; 65(21): 10059-67)

Introduction

Dendritic cells are professional antigen-presenting cells (APCs) with the unique ability to initiate and control antigen-specific immune responses. In peripheral tissues, dendritic cells reside in an "immature" state with high antigen uptake and processing ability. Upon activation, dendritic cells migrate from peripheral tissues to draining lymph nodes, displaying antigenic peptides in the context of MHC class I or MHC class II for presentation to CD8+ or CD4+ T cells, respectively. Fully mature dendritic cells influence the type of immune response they stimulate through expression of a combination of cell surface and secreted "polarizing" signals referred to collectively as "signal 3" (1, 2). Although many factors contribute to T-cell polarization, secretion of T-helper type-1 (Th1)–driving cytokines, such as interleukin-12 (IL-12), IL-18, IL-23, and IL-27, by dendritic cells, favors the induction of a Th1-biased response. Dendritic cells secreting Th1-driving cytokines have been referred to as type-1 polarized dendritic cells or DC1s (1).

Considerable effort is being made to develop strategies to use dendritic cells to induce tumor-specific immunity, including nearly 100 clinical trials designed to evaluate safety or efficacy in humans (3–5). Recent evidence suggests Th1-type immune responses have the potential to mediate tumor therapy through multiple effector mechanisms. CD8+ CTLs are well established as important effector cells for tumor immunity, and Th1-skewed CD4+ T-helper cells are known to support effective and durable CD8+ T-cell immunity (6, 7). It has long been established that activated CD4+ T cells can have a direct role in tumor rejection, potentially through a mechanism involving recruitment and activation of macrophages (8–10). In addition, IFN-γ secreted by Th1 CD4+ T cells can have direct antitumor and antiangiogenic activities (10, 11). Very recent studies support the role of CD4+ T cells in tumor immunity by providing evidence that effector CD4+ T cells mediate immunity against cervical cancer and myeloma in the apparent absence of tumor-specific CD8+ T cells and suggest that this effect is mediated at least in part by macrophages activated by T cell–derived IFN-γ (12–14). Taken together, these observations provide rationale for the use of type-1 polarized dendritic cells to induce Th1-skewed immune responses for the treatment or prevention of cancer.

Although T-cell polarization is a result of the convergence of several factors, secretion of IL-12p70 has been a useful surrogate marker for DC1-type function. Dendritic cells in peripheral tissues can be stimulated to secrete Th1-driving cytokines, including IL-12p70, by "danger signals" including ligands that stimulate toll-like receptors (TLR; ref. 15). Depending on the specific TLRs ligated, dendritic cells are polarized toward DC1 or DC2 function and stimulate Th1- or Th2-biased T-cell immunity, respectively. Importantly, for the purpose of tumor immunotherapy, TLR ligation also stimulates dendritic cell maturation, making TLR agonists attractive adjuvants for the generation of fully functional DC1s. In preclinical studies, various cocktails of cytokines, commonly including IL-1β, tumor necrosis factor-α (TNF-α), IL-6, prostaglandin E2, and/or IFN-γ have been developed to generate dendritic cells with type 1 function from peripheral blood–derived precursors (16). Most recently, it has been shown that the addition of poly(I:C), a TLR3 agonist, to IL-1β, TNF-α, and IFN-γ generates a potent type-1 polarized dendritic cell characterized by higher levels
of production of IL-12p70 and improved functional capacity for T-cell activation (16, 17). As yet, a murine equivalent of the human monocyte–derived DC1s being evaluated in clinical trials has not been developed, limiting efforts to define the mechanisms and limitations of DC1-induced tumor immunity in vivo.

We sought to investigate the ability of in vitro TLR ligation to induce type-1 polarized function in murine bone marrow–derived dendritic cells and their capacity to induce therapeutic responses against the nonimmunogenic melanoma B16 following adoptive transfer. The B16 model was chosen because it is a formidable model tumor for the evaluation of immunotherapeutic strategies. B16 has multiple described mechanisms of immune evasion. These include (a) down-regulation of MHC class I molecules and antigen-processing machinery (18); (b) production of vascular endothelial growth factor that inhibits dendritic cell function and T-cell immunity (19); and (c) production of galectin-1, a negative regulator of T-cell activation and survival (20). In addition, recent studies show that CD4+CD25+ regulatory T cells can prevent induction of effective anti-B16 CD8+ T-cell responses (21–24). Taken together, these features suggest that the B16 melanoma models the most challenging tumor escape mechanisms thus far described for a variety of human tumors.

To load dendritic cells with tumor antigen, we used a modified version of an autologous whole cell coculture strategy we described previously (25). We have shown that cellular vaccines consisting of tumor cells and dendritic cells that have been cocultured overnight can elicit effective tumor immunity (25). Using two relatively nonimmunogenic murine tumor models, we have shown that this immunization strategy can induce tumor-specific CTLs and protective tumor immunity in vivo and is capable of causing regression of established tumors, resulting in persistent antitumor immunity (25). Importantly, dendritic cell tumor cell immunization has the potential to simultaneously stimulate CD4+ and CD8+ T-cell–mediated immunity against multiple tumor antigens. Because the autologous tumor cell is the source of antigen, immunization does not depend on the prior identification of unique or “shared” tumor antigens and is not limited to individuals expressing a particular corresponding MHC allele (as in the case of synthetic tumor peptide epitopes). Furthermore, because the immunization is patient specific, it has the potential to stimulate immunity against uniquely expressed (polymorphic or mutated) tumor antigens that may be an important component of an effective “regressor” antitumor response.

Studies we describe here combine two novel approaches to dendritic cell–based tumor immunotherapy: in vitro TLR ligation to induce effective DC1 function and whole tumor cell loading of dendritic cells to enable presentation of a broad range of autologous tumor antigens through multiple processing pathways. We show that stimulation of bone marrow–derived murine dendritic cell populations with poly(I:C) and CpGs results in phenotypic maturation of dendritic cells and synergistic induction of durable, high-level IL-12p70 secretion characteristic of human type-1 polarized dendritic cells. Functionally, these dendritic cells induce antigen-specific Th1-type CD4+ T-cell activation in vitro and in vivo characterized by secretion of IFN-γ. Dendritic cell maturation and polarization are not inhibited by the presence of live tumor cells, and dendritic cells exposed to tumor cells induce DTH responses in vivo. Polarized dendritic cells loaded with tumor cells and injected into tumor-bearing mice induce Th1-skewed tumor-specific CD4+ T cells and a significant reduction in tumor growth. Tumor infiltrates in DC1-immunized animals are charac-

terized by the presence of CD4+ T cells and activated macrophages, and the apparent absence of CD8+ T cells. These results show a murine model of DC1 function and suggest an important role for CD4+ T cells and macrophages in DC1-induced antitumor immune responses. They have implications for the future development of DC1-based immunotherapies and strategies for relevant clinical immune monitoring of their effectiveness.

Materials and Methods

Mice and cell lines. Female C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the Central Animal Facility at the University of Pittsburgh and used according to institutional guidelines. B16 is a C57BL/6-derived murine melanoma obtained from American Type Culture Collection (Rockville, MD). The CD40L-transfected cell line J558 was a generous gift from Dr. Pawel Kalinski (University of Pittsburgh). OT-II T cells were obtained from OT-II Rag2−/− transgenic mice (Taconic, Germantown, NY) and recognize an I-A^d-presented COOH-terminal peptide derived from chicken egg ovalbumin, OVA 323-339 (ISQA-VHAAHAEINEAGR).

Reagents and antibodies. Chicken egg ovalbumin (OVA; Sigma Chemical Co., St. Louis, MO) was dissolved in AIM-V medium (Life Technologies, Carlsbad, CA) at 30 mg/mL and sterile filtered. CpG is phosphorothioate CpG-ODN 1668 with sequence 5′-TCTATGGCTTCTT-GATGCT-3′ (Sigma Genosys, The Woodlands, TX), which was previously described to activate murine immune cells (26). poly(I:C) (Sigma, St. Louis, MO) is a double-stranded RNA mimic known to stimulate maturation in both murine and human dendritic cells. IFN-γ (PeproTech, Rock Hill, NJ) was used at a concentration of 20 μg/mL. Lipopolysaccharide (LPS, Sigma) is used at a concentration of 1 μg/mL to induce maturation of dendritic cells.

Phenotypic analysis and cytokine production by dendritic cells. Dendritic cells were generated from bone marrow as previously described (25). Briefly, bone marrow cells were depleted of RBC and lymphocytes and cultured for 5 days in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS, 1-glutamine, 2-ME, HEPES, and antibiotics and containing 10^5 units/mL of granulocyte macrophage colony-stimulating factor and IL-4 (dendritic cell medium). Seventy-five percent of cell cytokine supplemented culture medium was replaced every other day, and loosely adherent cells were collected on day 5 and dendritic cells purified by immunomagnetic bead cell sorting using anti-CD11c-conjugated magnetic beads (purity >80% as shown by flow cytometry analysis Miltenyi Biotec, Bergisch Gladbach, Germany). CD11c^+ cells expressed MHC class I, MHC class II, CD40, CD80, and CD86 but did not express B220 as determined by flow cytometry.

Purified dendritic cells were suspended in dendritic cell medium at a concentration of 5 × 10^6 cells/mL and cultured 18 hours with or without LPS or a combination of CpGs (1 μmol/L), poly(I:C) (20 μg/mL), and/or IFN-γ (20 ng/mL). Staining was done using PE-conjugated anti-CD11c antibody and either FITC-conjugated anti-I-Α^d, anti-CD40, or anti-CD86 antibodies (all from PharMingen, San Diego, CA) and cells were analyzed by flow cytometry. Marker expression was assessed on gated CD11c^+ cells. Cytokine measurement was determined by ELISA (PharMingen) according to manufacturer protocols. Plates were developed with TMB substrate (Sigma) and absorbance read using a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA). In some experiments, B16 tumor cells were cocultured with dendritic cells at a dendritic cell/B16 cell ratio of 1:1. In some experiments, J558 cells were added at 5 or 24 hours at a dendritic cell/B16 cell ratio of 3:1. In some experiments, J558 cells were added at 5 or 24 hours at a dendritic cell/B16 cell ratio of 3:1. In some experiments, J558 cells were added at 5 or 24 hours at a dendritic cell/B16 cell ratio of 3:1. In some experiments, J558 cells were added at 5 or 24 hours at a dendritic cell/B16 cell ratio of 3:1.

To determine the effect of the length of stimulation with DC1 cocktail (1 μmol/L, CpGs, 20 μg/mL, poly(I:C), and 20 ng/mL IFN-γ), dendritic cells were suspended in DC1 cocktail at a concentration of 10^6 cells/mL. Cells were maintained in this cocktail or washed after 2 or 3 hours of stimulation and resuspended in dendritic cell medium. At 4 hours, all samples were washed and resuspended in dendritic cell medium (2-, 3-, and 4-hour stimulation groups) or DC1 medium (8-hour stimulation group) and...
incubated for four additional hours. Supernatants, containing cytokines secreted between 4 and 8 hours, were then collected and frozen for ELISA. For controls, cells were maintained in dendritic cell medium or DC1 medium for 18 hours before collecting supernatants for ELISA.

Ovalbumin-based assays. Purified dendritic cells were loaded with OVA (1 mg/mL) for 6 hours in dendritic cell medium with DC1 cocktail added after 2 hours to some groups. The proliferative response of OT-II T cells to dendritic cells was determined by titrating the dendritic cells in duplicate in a 96-well round-bottomed plate and OT-II T cells in RPMI were added at a concentration of 5 × 10^4 cells per well, with the final volume being 200 μL per well. Thymidine (1 μCi per well) was added on day 2 of incubation and plates harvested on day 3. Proliferation is reported as mean cpm of duplicate microcultures. For cytokine production OT-II T cells and dendritic cells were cultured at a ratio of 10:1 in a 96-well round-bottomed plate in 200 μL RPMI. Following 3 days of incubation, supernatants were collected and frozen for ELISA.

For in vivo immunizations, dendritic cells were suspended in PBS at a concentration of 10^5 cells/mL. Naive C57BL/6 mice were immunized by footpad/haunch injections on days 1, 7, and 14 with a total of 3 × 10^5 dendritic cells delivered per immunization. Spleens were harvested on day 21 and CD4^+ T cells purified with magnetic beads. Splenic APCs from naive C57BL/6 were loaded with OVA (1 mg/mL) for 2 hours, washed thrice in PBS, and used as target cells for 24-hour IFN-γ or 48-hour IL-5 ELISPOT assays (both plates and antibody sets from PharMingen) and for 48-hour ELISA assays (PharMingen). For both assays, 4 × 10^5 APCs per well and 2 × 10^5 CD4^+ T cells per well were suspended in either 150 μL (ELISPOT) or 200 μL (ELISA) of AIM-V medium. ELISPOT plates were incubated at 37°C for the indicated time and developed as described in the manufacturer’s protocols. For ELISA, supernatants were collected after 48 hours of incubation, pooled for each group, and frozen for later analysis.

Measurement of antitumor responses in vivo. For generation of tumor-loaded dendritic cells, B16 tumor cells were suspended in PBS at a concentration of 10^6 cells/mL and placed in a T-75 culture flask. Cells were treated with 17 μL/mL UVADEX (Therakos, Exton, PA) and irradiated with 4.5-J UVA using an Ultralite phototherapy process controller (Ultralite Enterprises, Inc., Lawrenceville, GA) and then washed thrice with PBS and suspended in dendritic cell medium. Purified dendritic cells were suspended in dendritic cell medium and loaded with tumor antigen by coculture at a dendritic cell/tumor ratio of 3:1.

For ELISPOT assays, 3 × 10^5 B16 cells in 50 μL PBS were injected i.d. into the shaved abdomen of naive female C57BL/6 mice on day 0. Tumor-bearing mice were immunized with 3 × 10^6 tumor-loaded dendritic cells by footpad/haunch injections on days 7 and 14. Spleens were harvested on day 21 and CD4^+ T cells purified using magnetic beads and used for ELISPOT assays. B16 were centrifuged and pellets subjected to three rounds of freezing and thawing using liquid nitrogen to generate tumor lysate. Naive splenic APCs were pulsed with B16 lysate at an APC/tumor equivalent ratio of 3:1 for 2 hours and washed thrice in PBS for use as target cells.

For DTH responses, naive female C57BL/6 mice were primed by injecting 5 × 10^5 dendritic cells, in 150 μL PBS into the left footpad and haunch. Five days later, DTH responses were elicited by injecting 5 × 10^5 dendritic cell, tumor-loaded dendritic cells, or tumor-loaded DC1s in 50 μL PBS into the contra lateral footpad, using the same cells to elicit the response that were used to prime the mice. After 48 hours, footpad thickness was measured using a dial thickness gauge and compared with footpad thickness just before elicitation.

Immunotherapy and microscopy. Naive female C57BL/6 mice were challenged on day 0 with 5 × 10^5 B16 cells in 50 μL PBS by i.d. injection into the shaved abdomen. Mice were then immunized on day 1 and every 7 days thereafter. Tumor size was measured thrice weekly using digital display calipers and the tumor was calculated by multiplying the widest diameter in of the tumor by the diameter 90 degrees to that measurement. Mice were sacrificed when tumor size reached 20 mm in diameter or mice became moribund.

For microscopy, some mice were sacrificed at day 8 and tumors were excised and fixed in 4% paraformaldehyde in PBS. Samples were processed and frozen sections stained with H&E or stained with rat antibodies to detect cells expressing F4/80-like receptor (macrophages, PharMingen), iNOS/NOS2 (PharMingen), CD4 (BIODESIGN International, Saco, ME), or CD8 (BIODESIGN International) and visualized using Alexa Fluor–conjugated secondary antibodies (Molecular Probes, Eugene, OR). Nuclei were visualized by staining with Hoechst stain (Sigma). Staining and imaging was done by the Center for Biologic Imaging facility at the University of Pittsburgh.

Statistical analysis. Data are expressed as means ± SE. Statistical significance of differences was assessed by a one-way ANOVA followed by Tukey’s multiple comparison test to evaluate differences between specific groups. An unpaired t test was used to evaluate differences when only two groups were being compared. For tumor growth analysis, a one-way ANOVA was used to compare tumor size on day 21. P < 0.05 was considered significant. Statistical analyses were done using Prism 4.02 software (GraphPad Software, San Diego, CA).

Results

Poly(I:C) and CpGs activate dendritic cells synergistically to induce high-level IL-12p70 secretion. First, we evaluated the effects of DC1-driving stimuli on the activation and polarization of murine bone marrow–derived dendritic cells. CD11c-purified dendritic cells were cultured in dendritic cell medium in the presence or absence of IFN-γ, poly(I:C), and/or CpGs. We determined the levels of IL-10 and IL-12p70 in 18-hour culture supernatants (Fig. IA). Individually, IFN-γ and poly(I:C) were unable to induce significant levels of spontaneously secreted IL-12p70, and CpGs alone stimulated low/intermediate levels of IL-12p70 secretion. However, simultaneous stimulation of dendritic cells with a combination of poly(I:C), CpGs, and IFN-γ, or poly(I:C) and CpGs without IFN-γ, induced very high levels of IL-12p70 secretion.

We next addressed the effects of ligand dose, either individually or in combination, on IL-12p70 secretion. Higher doses of either CpGs or poly(I:C) alone did not result in an increase of IL-12p70 secretion compared with that observed with low-dose CpG stimulation (Fig. 1B). However, the combination of CpGs and poly(I:C) resulted in over a 2-fold increase in IL-12p70 secretion compared with any single ligand dosing, showing a potent synergistic effect of simultaneous TLR3 and TLR9 stimulation (Fig. 1B). The inclusion of IFN-γ with poly(I:C) and CpGs significantly reduced IL-10 secretion, which has been implicated in down-regulation of IL-12p70 secretion and preferential Th2 skewing (27). This observation is consistent with previously published human and murine studies suggesting that exposure of dendritic cells to IFN-γ increases Th1 and down-regulates Th2-promoting capacities of dendritic cells (27–31). Importantly, stimulation of dendritic cells with this combination also resulted in phenotypic dendritic cell maturation/activation as shown by increased levels of cell surface expression of important antigen presentation molecules MHC class II, CD86, and CD40 similar to that seen with LPS stimulation, a gold standard for murine bone marrow–derived dendritic cell activation (Fig. 1C). Based on these results, we used a combination of poly(I:C) (20 μg/mL), CpGs (1 μmol/L), and IFN-γ (20 ng/mL) in subsequent experiments and refer to this as a “DC1 cocktail.”

Characterization of IL-12p70 secretion by dendritic cells stimulated with DC1 cocktail. Previous studies with human dendritic cells show that potential for dendritic cells to produce IL-12p70 is limited to a narrow window of time, a phenomenon referred to as dendritic cell “exhaustion” (32). Similarly, under our experimental conditions, secretion of IL-12p70 by DC1s occurred in a narrow window of time. High IL-12p70 secretion was observed...
Figure 1. CpGs and poly(I:C) act synergistically to induce dendritic cell (DC) maturation and high levels of IL-12p70 secretion. A, purified dendritic cells were stimulated by culture with IFN-γ (20 ng/mL), poly(I:C) (20 μg/mL), and/or CpGs (1 μmol/L) for 18 hours at 37°C as indicated after which IL-10 (open columns) and IL-12p70 (solid columns) secretion was determined by ELISA. B, purified dendritic cells were suspended in dendritic cell medium supplemented with IFN-γ (20 ng/mL) and stimulated with varying concentrations of CpGs alone, poly(I:C) alone, or both as indicated; IL-12p70 concentration was determined in 18 hours supernatants. C, purified dendritic cells were suspended in dendritic cell medium and stimulated with LPS (1 μg/mL; heavy line), DC1 cocktail (heavy line), or left unstimulated (thin line) for 18 hours; expression of the indicated maturation markers on the CD11c+ gated cell population was determined by flow cytometry. Representative of at least three similar experiments. Columns, means; bars, ± SE.

... during the first 4 hours of stimulation, was increased between 4 and 8 hours of stimulation, and then significantly diminished after 8 hours (Fig. 2A). To evaluate IL-12p70 secretion in relationship to the length of stimulation with DC1 cocktail, dendritic cells were cultured in DC1 cocktail for 2, 3, or 4 hours and then washed and recultured in normal dendritic cell medium. To directly compare IL-12 secretion over the 4- to 8-hour time period, all groups of dendritic cells were again washed at the 4-hour time point and then recultured in dendritic cell medium or DC1 medium for an additional 4 hours before collecting supernatants for cytokine ELISA. In general, longer incubation with DC1 cocktail correlated with higher secretion of IL-12p70, reaching a maximum with 8 hours of stimulation (Fig. 2B). Dendritic cells stimulated for 8 hours secreted nearly the same level of IL-12p70 as that secreted by dendritic cells for 18 hours with ligand continuously present. Previous studies suggest that dendritic cells are able to stimulate Th1 immune responses, whereas they secrete IL-12p70 but promote Th2 responses following exhaustion (32). Based on previous human studies showing significant IL-12p70 secretion by DC1s following CD40 ligation, we hypothesized that IL-12p70 secretion by murine DC1s could be sustained through dendritic cell-T cell contact (33). To address this, we determined the duration of IL-12p70 secretion by DC1s following CD40 ligation. DC1 cultures were prepared as described, except that CD40L-expressing J558 cells were added 5 or 24 hours after the addition of DC1 driving cocktail. Signaling DC1s through CD40 5 hours after initial TLR ligation resulted in sustained high-level secretion of IL-12p70 through the initial 24-hour time period and significant but lower level expression from 24 to 48 hours (Fig. 2C). In contrast, CD40 ligation 24 hours after initial TLR ligation neither rescued nor sustained high level IL-12p70 secretion (Fig. 2D). Both DC1s and dendritic cells that had not been exposed to TLR ligands secreted similar, smaller amounts of IL-12p70 following CD40 ligation at 24 hours. Together, the data suggest a benefit for Th1 skewing if polarized dendritic cells interact with CD40L-expressing T cells within several hours of initial TLR ligation. In subsequent experiments, TLR-ligated DC1s were used for in vitro and in vivo analysis 4 hours after initial TLR ligation.

Polarized DC1s induce CD4+ T-cell proliferation and Th1 differentiation. Although IL-12p70 secretion is frequently used as a surrogate marker of the ability of dendritic cells to induce Th1-skewed T-cell immunity, the ultimate definition of DC1s is functional. We sought to determine the ability of these murine DC1s to stimulate Th1-skewed antigen-specific T-cell immunity in vitro and in vivo. OVA-pulsed DC1s were used to stimulate naive OT-II T cells in in vitro cultures. OT-II T cells recognize OVA peptide 323-339 (ISQAVHAAHAEINEAGR) when presented by I-Ab in vitro OT-II T cells in antigen-dependent manner. Specifically, we compared OT-II T-cell responses stimulated by DC1s or nonpolarized dendritic cells that were antigen loaded or not by pulsing with soluble OVA. As expected, stimulation of OT-II T cells with OVA-pulsed dendritic cells (● and ○) resulted in significant and comparable proliferation of OT-II T cells (● and ○) compared with unpulsed dendritic cell/DC1 stimulators (■ and ●; Fig. 3A). However, OVA-pulsed DC1s induced significantly greater IFN-γ secretion by responder OT-II T cells compared with OVA-pulsed nonpolarized dendritic cells, resulting in a predominance of IFN-γ production relative to IL-5 by responding T cells indicative of strong Th1 bias in responders stimulated by the polarized DC1s (Fig. 3B). In these experiments, IFN-γ secreted by dendritic cells alone, DC1s alone, or OT-II T cells alone was negligible (data not shown). Under the same experimental conditions, we were unable to detect secretion of IL-5 by ELISA (data not shown).

We next evaluated the ability of DC1s to stimulate a CD4+ Th1-polarized OVA-specific immune response in vivo. Mice were immunized i.d. with dendritic cells or DC1s pulsed with soluble...
OVA. CD4+ T cells isolated from the splenocytes of immunized mice were incubated with OVA-loaded splenic target cells, and antigen-specific IFN-γ and IL-5 secretion was determined using ELISPOT (Fig. 3C) and ELISA (Fig. 3D). Polarized DC1s induced a clear Th1-biased immune response, as indicated by pronounced IFN-γ production. Nonpolarized dendritic cells induced a mixed Th1/Th2 response characterized by significantly less IFN-γ and more IL-5 production by antigen-specific CD4+ T cells. The number of IFN-γ-secreting T cells observed in ELISPOT was consistent with the amount of secretion of cytokines determined by ELISA. Together, these data indicate that dendritic cells stimulated with DC1 cocktail drive Th1-biased CD4+ T-cell immune responses in vitro and in vivo.

Tumor cell–loaded DC1s induce Th1-skewed antitumor immunity. We sought to determine the capacity of DC1s loaded with whole autologous tumor cells to induce Th1-skewed tumor-specific immunity. B16 melanoma cells have multiple immune escape mechanisms, including their ability to directly inhibit dendritic cell maturation and function. Based on the potent dendritic cell activation and polarization we observed with synergistic TLR ligation, we hypothesized that the potential inhibitory effects of cocultured B16 melanoma cells on dendritic cells in the vaccine would be overcome by TLR ligation. To address this issue, we compared dendritic cell maturation and IL-12p70 production by DC1s cultured in the presence or absence of B16 melanoma cells. Dendritic cells were cocultured with or without B16 tumor cells and stimulated with DC1/CD11c-driving cocktail or LPS (1 μg/mL) as a positive control. Cells and supernatants were collected after 18 hours of culture and analyzed for expression of phenotypic markers (Fig. 4A) and secretion of IL-12p70 (Fig. 4B), respectively. As determined by comparison of MHC class II, CD86, and CD40 expression, stimulation of dendritic cells with TLR3 and TLR9 ligation induces dendritic cell activation even in the presence of B16 tumor cells. Furthermore, the presence of B16 tumor cells did not inhibit the ability of DC1s to secrete high levels of IL-12p70 in response to synergistic TLR ligation (Fig. 4B).

Th1-type immune responses support the induction of delayed-type hypersensitivity (DTH) reactions (35). To initially evaluate the in vitro immunogenicity of B16 loaded DC1s, we evaluated the capacity of this DC1/melanoma vaccine to induce DTH reactions. We immunized mice by i.d./s.c. injection of dendritic cells alone, dendritic cells loaded with B16 melanoma by coculture (as described above; dendritic cell/melanoma), or dendritic cells loaded with B16 melanoma by coculture in the presence of DC1-driving cocktail (DC1/melanoma). Five days later, immunized animals were challenged (elicitation) with the vaccines by i.d. injection into the contralateral footpads. Injection of the vaccines alone did not induce increases in footpad swelling at the immunization site (data not shown). DC1/melanoma-immunized groups induced a strong DTH response as shown by a 2-fold increase in contralateral footpad thickness at the elicitation site 48 hours after elicitation compared with the response induced by nonpolarized dendritic cells (Fig. 4C).

To directly evaluate the immunotherapeutic effect of DC1-based immunization, groups of tumor-bearing mice were immunized by i.d./s.c. injection of dendritic cells loaded with B16 tumor cells for 6 hours, in the presence (DC1/melanoma) or absence (dendritic cell/melanoma) of poly(I:C), CpGs, and IFN-γ over the final 4 hours of incubation. We observed a significant delay in tumor growth in mice receiving the DC1/melanoma immunization, whereas immunization with nonpolarized dendritic cell/melanoma resulted in tumor growth similar to that seen in untreated controls (Fig. 5A). CD4+ T cells from splenocytes were isolated and used to evaluate tumor-specific T-cell responses by ELISPOT. Because B16 tumor cells do not express MHC class II molecules,
we used B16 lysate–loaded splenic APCs from nonimmunized mice as targets. Unloaded splenic APC were used as a control. Mice immunized with the DC1/melanoma coculture vaccine showed a high level of IFN-γ-secreting tumor-specific CD4+ T cells (Fig. 5B). DC1/melanoma-immunized mice showed much lower but significant levels of IL-5-secreting tumor-specific CD4+ T cells. In comparison, mice immunized with dendritic cell/melanoma showed only low levels of tumor-specific IFN-γ-secreting CD4+ T cells, which were comparable with the levels of IL-5-secreting tumor-specific CD4+ T cells generated. These results are consistent with the induction of a tumor-specific Th1-skewed response by DC1s as shown by a significant and predominant induction of tumor-specific IFN-γ-secreting Th cells.

Interestingly, despite the significant inhibition of tumor growth and the potent Th1-skewed CD4+ T-cell responses observed in DC1/melanoma-immunized animals, we were unable to detect tumor-specific CTL activity in these animals using standard 51Cr release assays (data not shown). This was not completely unexpected, given the previously reported results of others and the multiple well-described mechanisms of immune evasion observed with the B16 tumor model (18–24). To further characterize the antitumor response in responding animals, we used microscopy with H&E stains (Fig. 5C) and immunofluorescence (Fig. 5D) to characterize tumor infiltrates. Tumors from responding DC1-immunized mice showed a moderate CD4+ T-cell infiltrate and extensive macrophage infiltration compared with naive mice or mice immunized with nonpolarized dendritic cell. Macrophages infiltrating tumors in DC1-immunized mice expressed iNOS/NOS2, indicative of macrophage activation (Fig. 5D, C). This pattern is consistent with an IFN-γ-mediated response similar to that seen in classic DTH responses. As predicted by our inability to detect a tumor-specific CTL response, the infiltrates in tumors of responding animals did not seem to contain significant numbers of CD8+ T cells.

Discussion

Recent advances in our understanding of dendritic cell biology and increasing evidence that dendritic cell vaccines can induce tumor-specific immune responses in cancer patients are leading to renewed optimism for the development of therapeutic dendritic cell–based cancer vaccines, and are providing rationale for a new generation of adoptive transfer and in vivo targeted dendritic cell vaccines (3–5). Whereas immunization strategies that target and manipulate dendritic cells in vivo offer considerable theoretical advantages over adoptive transfer therapies, currently, ex vivo manipulation of dendritic cells enables better monitoring and control of dendritic cell function. Ex vivo engineered dendritic cell vaccines potentially offer both increasing therapeutic benefit and a unique means to develop a better understanding of human immunoregulatory mechanisms, including those in cancer patients. Information obtained from the clinical application of adoptive transfer dendritic cell therapies is uniquely contributing to the development of in vivo targeted dendritic cell vaccines and other evolving immunotherapies.

As an example of this, ex vivo engineered dendritic cell vaccines now in clinical trials are testing the hypothesis that more efficacious antitumor immunity may be obtained by inducing Th1-skewed antitumor immunity. Several lines of evidence support this approach. In cancer patients, Th1 skewing has been correlated with improved clinical outcomes (36, 37). Substantial evidence from murine models and human preclinical studies suggest that Th1-skewed CD4+ T cells support potent CD8+ T-cell responses important for tumor and viral immunity (6, 7, 38). In addition, both historical evidence and several very recent studies suggest that effector mechanisms other than CD8+ T cells can play an important role in tumor regression (8, 12, 13). To induce Th1-skewed immunity, human dendritic cells have been engineered ex vivo using cytokine cocktails and/or TLR ligands to express high levels of cell surface molecules associated with T-cell activation and to
secrete high levels of IL-12p70, an indicator of Th1-skewing function (1). These human DC1s have been shown to stimulate both CD4+ T-cell responses dominated by IFN-γ secretion and potent CD8+ T-cell inducing activity in vitro (17, 33). Surrogate markers of DC1 function can readily be monitored ex vivo before dendritic cells are injected into cancer patients, enabling future correlations with results from immune monitoring and clinical outcomes.

The development of DC1 therapies is currently limited by the lack of a representative murine model. Much of the effort to develop and characterize DC1s to date has focused on ex vivo manipulated human dendritic cells. Because of differences between human and murine dendritic cells, including differences in subset characteristics and TLR expression between subsets, extrapolating DC1-driving protocols designed to polarize human dendritic cells to murine bone marrow–derived dendritic cells has not been straightforward. Here, we present a murine model of ex vivo engineered DC1s developed by monitoring the same indicators used as surrogates for human DC1 function. Using bone marrow–derived murine dendritic cells, we found that a DC1-polarizing cocktail that included poly(I:C), CpGs, and IFN-γ induced both phenotypic maturation and IL-12p70 secretion consistent with that observed with polarized human monocyte–derived DC1s. Although the mechanism of synergy we observe remains unclear, very recent studies show synergy between TRIF-derived DC1s. Although the mechanism of synergy we observe remains unclear, very recent studies show synergy between TRIF-derived DC1s.

In ex vivo studies, human monocyte–derived DC1s have been shown to secrete high levels of IL-12p70 over a narrow window of time, peaking 8 to 12 hours after stimulation and then returning to baseline levels, a phenomenon referred to as exhaustion (17, 32, 42). Previous reports suggest that the capacity of DC1s to prime Th1 immunity is lost following exhaustion, resulting in the generation of Th2-skewed immunity (32). This imposes a theoretical limit on the use of DC1s for immunization; that is, there is concern that IL-12p70 secretion may exhaust before injected DC1s engage T cells in the relevant lymphatic tissues. Like human dendritic cells, polarized murine DC1s used in this study secret high levels of IL-12p70 early in response to TLR ligation, with exhaustion occurring after 12 hours. Interestingly, we found that engagement of CD40 before exhaustion leads to extended high level IL-12p70 secretion, suggesting that DC1s will continue to secrete a high level of IL-12p70 if they encounter antigen-specific T cells, further promoting Th1 responses. Given the transit times reported for the trafficking of skin injected dendritic cells to the draining lymph nodes in murine and human systems, it is plausible that cutaneously given dendritic cells will engage CD40L-expressing T cells before exhaustion (43, 44). In addition, direct intranodal injection of dendritic cells would likely enable even more timely interaction with resident T cells, obviating exhaustion concerns. Importantly, the levels of IL-12p70 secretion we observe are very high and likely superphysiologic. This potentially enables targeted cytokine therapy, whereby polarized DC1s serve first as targeted delivery vehicles and then as a lymph node resident source of IL-12p70. High-level secreted IL-12p70 may affect surrounding responding T cells in addition to those directly engaging the polarized DC1, enabling amplification of Th1 skewing by resident dendritic cells that may be presenting or cross-presenting tumor antigens without having been directly exposed to polarizing stimuli.

To evaluate tumor immunotherapy, we chose the B16 melanoma, a tumor with multiple well-established mechanisms of tolerance induction and immune escape (18–20). We have previously shown that dendritic cells similarly loaded with tumor antigens by coculture with live tumor cells, even without

\[ \text{Figure 4. DC1-driving cocktail stimulates dendritic cell (DC) maturation and type-1 function in the presence of tumor cells. A, dendritic cells were cultured in the presence or absence of B16 tumor cells (dendritic cells/tumor cell, 3:1), stimulated with LPS (1 \mu g/mL), and/or DC1 cocktail (heavy lines) for 18 hours and CD11c+ cells were phenotypically compared with CD11c+ dendritic cells cultured in dendritic cell medium alone (thin line). Shaded regions, isotype control. B, dendritic cells were cultured in the presence or absence of B16 tumor cells as in (A); IL-12p70 concentration in the supernatants was determined after 18 hours. Representative of at least three similar experiments. C, dendritic cells were loaded with tumor antigens as in (A) with or without DC1 cocktail. Naive mice received 5 \times 10^6 dendritic cells per immunization via left footpad/left haunch injections on day 0 (sensitization) and via right footpad injections on day 5 using the same dendritic cells used for sensitization (elicitation). Footpad thickness was measured just before and 48 hours following elicitation injections. Representative of at least two similar experiments. Columns, means; bars, ±SE. *** P < 0.0001.} \]
on day 21, CD4+ T cells were purified from spleens and used for ELISPOT
A, i.d. into naive mice. Tumor-bearing mice were then immunized via footpad/
DC1 cocktail added after 2 hours. On day 0, B16 melanoma cells were implanted
cocultured with B16 melanoma cells for 6 hours in the presence or absence of
experiments (total of 14 mice per group).
growth curves were generated by combining the results from three separate
1 and every 7 days thereafter. Tumor area was measured using calipers; tumor
in vivo
Tumor cell–loaded polarized DC1s induce Th1 tumor-specific
immunity in vivo that inhibits growing tumors. Dendritic cells (DC) were
cocultured with B16 melanoma cells for 6 hours in the presence or absence of
DC1 cocktail added after 2 hours. On day 0, B16 melanoma cells were implanted
i.d. into naive mice. Tumor-bearing mice were then immunized via footpad-
haunch injections on days 7 and 14 with tumor-loaded dendritic cells or DC1s.
A, on day 21, CD4+ T cells were purified from spleens and used for ELISPOT
assays to detect tumor-specific production of IL-5 (open columns) and IFN-γ
(solid columns). B, tumor-bearing mice were immunized or not (control) with
51Cr release assays nor by localization of CD8+ T cells in tumor
infiltrates. This is consistent with recent results showing partial
tumor inhibition in a B16 protection model, without evidence of
CD8+ T cells in 51Cr release assays (24). These and other studies
suggest that induction of more efficacious B16 melanoma rejection
and potent CD8+ T-cell responses may require elimination of
naturally occurring CD4+CD25+ regulatory T cells (24, 45). The
need to deplete regulatory T cells or to systemically administer
adjuvants and/or CD40 ligands to overcome regulatory T-cell
mediated suppression has been shown in several tumor systems
(21–24, 38, 45, 46).

Recent studies show that activated CD4+ T cells and macro-
phages can inhibit tumor growth in the absence of CD8+ T-cell
responses, and that this immunity depends in large part on
IFN-γ production (12–14). In our studies, inhibition of tumor
growth is associated with IFN-γ production by antigen-specific
CD4+ T cells and tumor infiltrates dominated by CD4+ T cells
and activated macrophages. Although the DC1/melanoma
vaccine we describe did not seem capable of overcoming
suppressiv/evasive effects of B16 tumors, as evidenced by only
partial tumor growth inhibition and the lack of tumor-specific
CD8+ effector T-cells, the vaccine did stimulate Th1-skewed
tumor-specific CD4+ T-cell immunity and corresponding IFN-γ
secretion that likely contributed to macrophage activation. The
importance of these DC1-inducible tumor control mechanisms
may be underappreciated. These studies suggest that nonCD8+
T-cell-dependent Th1-skewed immunity can significantly con-
tribute to tumor therapy, and that measurement of these
responses should be included in clinical immune monitoring.
A rational approach to the further development of DC1-based
tumor vaccines should include strategies to maintain the
benefits of Th1-skewed tumor immunity observed here and
add enhancements designed to improve stimulation of tumor-
specific CD8+ T cells in the setting of immunosuppression or be
combined with strategies to eliminate regulatory T-cell activity.
The murine polarized DC1 model we describe may provide a
useful tool to achieve these goals.

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Figure 5. Tumor cell–loaded polarized DC1s induce Th1 tumor-specific
immunity in vivo that inhibits growing tumors. Dendritic cells (DC) were
cocultured with B16 melanoma cells for 6 hours in the presence or absence of
DC1 cocktail added after 2 hours. On day 0, B16 melanoma cells were implanted
i.d. into naive mice. Tumor-bearing mice were then immunized via footpad-
haunch injections on days 7 and 14 with tumor-loaded dendritic cells or DC1s.
A, on day 21, CD4+ T cells were purified from spleens and used for ELISPOT
assays to detect tumor-specific production of IL-5 (open columns) and IFN-γ
(solid columns). B, tumor-bearing mice were immunized or not (control) with
tumor-loaded dendritic cells or DC1s via footpad and haunch injections on day
1 and every 7 days thereafter. Tumor area was measured using calipers; tumor
growth curves were generated by combining the results from three separate
experiments (total of 14 mice per group). Columns, means; bars, ± SE. C-D, for
image analysis, tumors were excised on day 8, fixed with 4% paraformaldehyde,
and frozen for sectioning. Nuclei were stained using Hoechst stain (blue).
Inset, some sections were double-stained for the macrophage marker F4/80
(green) and iNOS/NOS2 (red). Magnification, × 200 with × 1,000 inset (H&E)
and × 200 with × 600 inset (immunofluorescence). Representative of two similar
experiments.

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References


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