Intratumoral Delivery of Dendritic Cells Engineered to Secrete Both Interleukin (IL)-12 and IL-18 Effectively Treats Local and Distant Disease in Association with Broadly Reactive Tc1-type Immunity

Tomohide Tatsumi, Jian Huang, William E. Gooding, Andrea Gambotto, Paul D. Robbins, Nikola L. Vujanovic, Sean M. Alber, Simon C. Watkins, Hideho Okada, and Walter J. Storkus


ABSTRACT

Dendritic cells (DCs) were adenovirally engineered to constitutively and durably secrete the potent Th1-biasing cytokines interleukin (IL)-12 (AdIL12DC) and/or IL-18 (AdIL18DC) and evaluated for their ability to promote therapeutic antitumor immunity in murine sarcoma models. Injection of either AdIL12DC or AdIL18DC into day 7 CMS4 or MethA tumors resulted in tumor rejection or slowed tumor growth when compared with control cohorts. Importantly, intratumoral injection with DCs engineered to secrete both IL-12 and IL-18 (AdIL12/IL18DC) resulted in complete and the most acute rejection of any treatment group analyzed. This strategy was also effective in promoting the regression of contralateral, untreated tumors. Both CD4+ and CD8+ T cells were required for tumor rejection. CD8+ splenic T cells from mice treated with AdIL12/IL18DC produced the highest levels of IFN-γ in response to tumor rechallenge in vitro and displayed the broadest repertoire of Tc1-type reactivity to acid-eluted, tumor-derived peptides among all treatment cohorts. This apparent enhancement in cross-presentation of tumor-associated epitopes in vivo may result from the increased capacity of engineered DCs to kill tumor cells, survive tumor-induced apoptosis, and present immunogenic MHC/tumor peptide complexes to T cells after intratumoral injection. In support of this hypothesis, cytokine gene-engineered DCs expressed higher levels of MHC and costimulatory molecules, as well as Fas ligand and membrane-bound tumor necrosis factor α, with the latter markers associated with elevated tumoricidal activity in vitro. Cytokine gene-engineered DCs appeared to have a survival advantage in situ when injected into tumor lesions, to be found in approximation with regions of tumor apoptosis, and to have the capacity to ingest apoptotic tumor bodies. These results support the ability of combined cytokine gene transfer to enhance multiple effector functions mediated by intralesionally injected DCs that may concertedly promote cross-priming and the accelerated immune-mediated rejection of tumors.

INTRODUCTION

DCs3 effectively elicit primary and boost secondary immune responses to self and foreign antigens (1, 2). Because these specialized antigen-presenting cells can induce the generation of both antigen-specific CTLs and T helper cells, DC-based vaccines are attractive strategies for the treatment of cancer. In this regard, DCs pulsed with tumor-associated antigens in various forms, including whole cell lysates (3, 4), peptides (5, 6), proteins (7), RNA (8), or DNA (9, 10), have proven effective in eliciting protective and therapeutic antitumor immunity in murine models. The results of several DC-based tumor vaccine trials have also recently been reported in the setting of B-cell lymphoma, melanoma, prostate cancer, and renal cell carcinoma, among others (11–14). Although tumor-specific T cells were promoted by vaccination in most patients, objective clinical responses have thus far only been observed in a minority of treated individuals. These modest current clinical successes for DC-based cancer vaccines would be expected to improve if study designs were modified for optimal DC promotion of Th1-type immunity in cancer-bearing hosts.

IL-12 exhibits a number of immunologically important activities, including the ability to enhance natural killer and CTL activities (15–17) and polarize CD4+ T-cell responses by supporting Th1/Tc1-type and suppressing or repolarizing Th2-type immunity (18, 19). We and others have reported potent antitumor effects associated with IL-12 gene therapy using IL-12 gene-modified tumor cells (20–22) and DCs (23) or systemic administration of IL-12 protein (24, 25) in murine tumor models. Based on these results, Phase I/II clinical trials of IL-12 gene therapy have been performed, with significant but transient objective clinical responses reported to date (26).

IL-18 is a member of the IL-1 family of proinflammatory cytokines, produced by activated macrophages and DCs, that also appears to play an important role in driving Th1/Tc1-dominated immune responses (27–29). Recently, IL-18 has also demonstrated potential as a biological “adjuvant” in murine tumor models, with systemic administration of recombinant IL-18 or direct intratumoral injection of IL-18 adenoviral vector inducing significant antitumor effects in multiple murine tumor models (30–32). Indeed, we have recently reported that intratumoral delivery of IL-18 gene-transduced DCs can elicit antitumor Th1-type immunity in association with enhanced therapeutic efficacy in the CMS4 tumor model (33).

IL-12 acts synergistically with IL-18 by enhancing IFN-γ production from Th1/Tc1-type T cells (34, 35), thereby providing a strong rationale for the use of these factors in combined CGT approaches. Whereas the coordinate administration of these two cytokines (as recombinant proteins) in murine tumor models has resulted in more potent antitumor responses than that observed for the single agents, coadministration has also been associated with lethal organ damage and septic shock-like toxicities that appear attributable to the extremely high systemic levels of IFN-γ evoked by this strategy (31). To overcome such systemic toxicities, we examined the effectiveness of therapies based on the injection of genetically transduced DCs to provide paracrine secretion of IL-12 and/or IL-18 in the tumor-associated microenvironment. We demonstrate that intratumoral delivery of DCs genetically modified to secrete both IL-12 and IL-18 safely induces accelerated tumor rejection, in association with stron-
ger type 1 immunity and a more diverse “therapeutic” repertoire of tumor-reactive, Tc1-type T cells in situ.

MATERIALS AND METHODS

Mice. Female 6–8-week-old BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in microisolator cages. We generated the BALB/c:EGFP Tg mice by eight cycles of backcrossing C57BL/6-TgN(ActbEGFP)1Osb mice (Jackson) onto the BALB/c background within the Central Animal Facility at the University of Pittsburgh. Animals were handled under aseptic conditions per an Institutional Animal Care and Use Committee-approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

Cell Lines and Culture. C57BL/6 and MethA are chemically induced BALB/c sarcomas and have been described previously (36). Cell lines were maintained in CM (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μM l-glutamine (all reagents from Life Technologies, Inc., Grand Island, NY)) in a humidified incubator at 5% CO2 and 37°C.

Generation of DCs in Vitro from BM. The procedure used in this study was described as described by Son et al. (37). Briefly, BALB/c or BALB/c:EGFP Tg BM was cultured in CM supplemented with 1000 units/ml recombinant murine granulocyte/macrophage colony-stimulating factor and recombinant IL-4 (Schering-Plough, Kenilworth, NJ) at 37°C in a humidified, 5% CO2 incubator for 7 days. DCs were then isolated at the interface of 14.5% (w/v) metrizamide (ICN Biomedicals, Cleveland, OH), or appropriate isotype-matched controls. Second, the DCs were labeled with PE-conjugated anti-CD11c, CD40, CD54, CD80, CD86, H-2Kd, I-A d (all from BD PharMingen), or appropriate isotype-matched controls. Third, the DCs were labeled with PE-conjugated streptavidin (Jackson Immunotech). In all cases, 99% of the targeted cell subset was specifically analyzed by flow cytometry of splenocytes using PE-conjugated anti-CD4 and anti-CD8 mAbs (PharMingen). In all cases, 99% of the targeted cell subset was specifically depletes (data not shown).

Mouse IL-18 and IL-12 Production from Adenoviral Transduced DCs. Five million (day 7 cultured) DCs were infected with recombinant Ads encoding mouse IL-12 (AdIL12; MOI = 50), mouse IL-18 (AdIL18; MOI = 200), both AdIL12 and AdIL18, or mock vector (AdΔ5; MOI = 200), as reported previously (33). After 48 h, adenoviral infected DCs were harvested and analyzed for phenotype and function. Culture supernatants were also collected for measurement of mouse IL-12 and mouse IL-12 production using species-specific IL-12 ELISA kits (BD Pharmingen, San Diego, CA), with lower levels of detection of 31.5 and 62.5 pg/ml, respectively.

Flow Cytometry. For phenotypic analysis of adenovirally infected DCs, PE- or FITC-conjugated mAbs against mouse cell surface molecules [CD11b, CD11c, CD40, CD54, CD80, CD86, H-2Kd, I-A d (all from BD Pharmingen)] and appropriate isotype controls were used, and flow cytometric analysis was performed using a FACScan (Becton Dickinson, San Jose, CA) flow cytometer. Cell surface expression of TNF family ligands was assessed using a previously described, highly sensitive, three-step flow cytometry technique (39). First, adenovirally infected DCs were stained with antimouse TRAIL Ab (eBioscience, San Diego, CA), anti-mouse Fasl Ab (MBL, Medical & Biological Laboratories, Nagoya, Japan), antimouse TNF-α Ab (Endogen, Woburn, MA), or appropriate isotype-matched controls. Second, the DCs were labeled with biotin-conjugated secondary Abs (Vector Laboratories, Burlingame, CA). Third, the DCs were labeled with PE-conjugated streptavidin (Jackson Immunotech, West Grove, PA). The results of flow cytometric analysis of TNF family ligand expression are reported in arbitrary MFI units.

MTT Assays. To evaluate the cytotoxicity of control or genetically engineered DCs against tumor cells, 24-h MTT assays were performed as described previously (40). For blocking the interaction between the TNF family and its ligands, DCs were preincubated for 60 min with antagonist Abs against TRAIL, FasL, or TNF-α (final concentration, 20 μg/ml). Effector DCs and targets were then mixed in a 5:1 (DC:tumor cell) ratio, and cytotoxicity assays were performed as described above (40).

Animal Experiments. BALB/c mice received s.c. injection with 2 × 10⁵ CMS4 or 5 × 10⁵ MethA cells in the right flank on day 0. On day 7, tumor size reached approximately 20–30 mm². On days 7 and 14, BALB/c mice were treated with intratumoral immunization of 1 × 10⁶ adenoviral transduced DCs in a total volume of 100 μl of PBS. Tumor size was assessed every 3 or 4 days and recorded in mm² by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area ± SD. To assess the impact of systemic immunity from vaccination, we examined the growth of contralateral untreated tumors. For the latter models, BALB/c mice received s.c. injection with 2 × 10⁵ CMS4 cells in both flanks on day 0. On days 7 and 14, 1 × 10⁶ AdIL-12 and AdIL-18 coinjected DCs (AdIL12/IL18DC) were injected in the tumor on the right flank, and both tumors were measured every 3 or 4 days.

To assess the fate and function of injected DCs, we generated day 7 BM-derived DCs from BALB/c:EGFP Tg mice and infected them with the AdΔ5, AdIL-12, AdIL-18, or AdIL-12 + AdIL-18 viruses as indicated above. Forty-eight h later, 1 × 10⁵ control or virally infected DCs were harvested, washed in PBS, and injected into day 7 CMS4 tumors established in syngeneic BALB/c mice. After 1 additional day, tumors were resected, fixed for 1 h in 2% paraformaldehyde (in PBS), and then cryoprotected in 30% sucrose in PBS before being shock frozen in liquid nitrogen-cooled isopentane. Five-μm frozen sections were then generated and incubated in a reaction mixture containing 1 mg Cy3-conjugated UTP, 250 units/ml terminal transferase in 200 mM potassium cacodylate, 25 mM Tris, and 20 mM cobalt chloride (Boehringer Mannheim, Indianapolis, IN). After a 45-min incubation at 37°C, the reaction was terminated by washing with PBS and counterstained with 2 mg/ml Hoechst 33258 (Sigma) for 3 min. The washed sections were then mounted in Gelvatol (Monsanto) and observed using an Olympus BX51 microscope equipped with a cooled charge-coupled device color camera. Images of TUNEL-, EGFP-, and Hoechst-stained nuclei were collected.

T-cell Depletion Experiments. On days 1 and 6, 11, and 16 after tumor inoculation, mice received i.p. injection with 100 μl of PBS (control) or ascorbic acid (0.05% ascorbic acid) (GK1.5 hybridoma; ATCC, Manassas, VA), antimouse CD8 (53-6-72 hybridoma; ATCC), or isotype control Ab (H22-15-5 hybridoma; ATCC). The efficacy of specific subset depletions was validated by flow cytometry analysis of splenocytes using PE-conjugated anti-CD4 and anti-CD8 mAbs (PharMingen). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

Spleenic CD8+ T-cell Responses against CMS4 Tumors and Eluted Naturally Processed Peptides Derived from CMS4 Cells. Peptides were acid-eluted from viable CMS4 cells and separated on reverse-phase HPLC, as described previously (41). Individual HPLC fractions were lyophilized to remove organic solvent and then reconstituted in 200 μl PBS and stored at −20°C until use. Pooled CD8+ T cells were isolated to a purity of >95% from the spleens of 2 treated mice/group 7 days after the second DC injection (i.e., day 21 after tumor inoculation) using magnetic bead cell sorting (MACS; Miltenyi Biotech, Auburn, CA) and then cocultured (1 × 10⁶/well) with 1 × 10⁴ irradiated (10,000 rads) CMS4 cells or syngeneic DCs (2 × 10⁴) cells/well) and HPLC-fractionated peptides in 96-well tissue culture plates. After a 48-h incubation, culture supernatants were collected and analyzed for IFN-γ release using a commercial ELISA (BD Pharmingen) with a lower limit of detection of 31.5 pg/ml. Data are reported as the mean ± SD of triplicate determinations.

Statistical Analyses. All experiments with three or more groups in which treatment was applied as a completely randomly design were first analyzed by a one-way or two-factorial ANOVA. If the resulting P was <0.05, specific pairwise contrasts were tested with a t test with Welch’s correction for unequal variance as needed. Data were checked for distributional properties, and appropriate transformations were applied. Cytotoxicity was determined in repeat experiments in which results were expressed as a percentage of target cells killed. These data were arcsin transformed and analyzed by a two-way mixed model ANOVA using the model: treatment + mouse. Data were randomly stratified as a random factor for between-group differences were subsequently stratified by experiments. Analysis of IFN-γ production from splenocyte-derived T cell and expression of TNF family ligands were conducted with the exact Kruskal-Wallis test. If the P for the Kruskal-Wallis test was <0.05, a priori contrasts were evaluated with the Wilcoxon test. The analysis of therapeutic single tumor inoculation murine treatment models was conducted with mixed linear models. Data were log transformed, within-mouse covariance was estimated, and fixed effects of treatment were adjusted for random mouse effects. Raw Ps for comparing pairs of groups at a single time were adjusted by bootstrap resampling. The growth of bilateral inoculated tumors was analyzed by a two-way fractional fixed
RESULTS

Cytokine Production by and Phenotype of Adenovirally Infected DCs. We initially validated IL-12 and IL-18 production from adenovirally infected DCs (Table 1). As expected, DCs infected to produce both IL-12 and IL-18 (AdIL12/IL18DC) secreted significant quantities of both mIL-12 and mIL-18, respectively (Table 1). The culture medium of mIL-18 cDNA-transfected DCs (AdIL18DC) contained mIL-18 but also contained a significantly elevated quantity of IL-12p70. In contrast, the culture medium of IL-12-transfected DCs (AdIL12DC) contained significant quantities of IL-12, but no detectable IL-18. Finally, the supernatants derived from control AdΔ5DC did not contain detectable levels of either IL-12 or IL-18. Analyses of the supernatants harvested from engineered DCs over time indicated that DCs secreted peak levels of IL-12 and/or IL-18 two days after infection with AdIL12 or AdIL18, but DCs continued to produce statistically elevated levels of these cytokines for up to 10 days after transfection (data not shown).

A flow cytometric examination of the impact of IL-12 and/or IL-18 cDNA insertion on DC phenotype was then performed 48 h after adenoviral infection. AdIL12/IL18DCs displayed significantly elevated levels of the MHC class I/II and CD86 costimulatory molecules, and AdIL18DCs exhibited elevated levels of the MHC class I/II, CD80, and CD86 molecules, when compared with either AdΔ5DC control virus-infected DCs or noninfected DCs, with these two control groups yielding indistinguishable results (Table 2). In contrast, among the markers analyzed, AdIL12DCs expressed increased levels of MHC class I and class II but not costimulatory molecules when compared with control DCs (Table 2).

Increased Expression of TNF Family Ligands on Adenoviral Infected DCs. Human DCs express several TNF family ligands, including FasL, TRAIL, TNF-α, and LT-α/β2, and can implement these in mediating the apoptotic death of tumor cells in vitro and in vivo (40, 42). Our pilot studies indicated that mouse DCs have similar properties and that Th1-biasing cytokine (such as IL-12 or IL-18) gene transfer into DCs might enhance the antitumor effector function of DCs (23, 33). Based on these findings and considerations, we evaluated the impact of cytokine gene insertion on DC expression of FasL, TRAIL, and TNF-α. As shown in Fig. 1, noninfected DCs express a basal level of TRAIL, FasL, and TNF-α on their cell surface; the expression level of TRAIL is very low, whereas FasL and TNF-α are expressed at comparatively moderate levels. Whereas AdΔ5 infection of DCs did not result in any modulation of TRAIL, FasL, or TNF-α expression, both FasL and TNF-α were increased in intensity on the cell membrane of AdIL12/IL18DCs, AdIL18DCs, and AdIL12DCs when compared with AdΔ5 or noninfected DCs (P = 0.0286). TRAIL expression by AdIL12/IL18DCs was also significantly elevated versus noninfected DCs (P = 0.0286), whereas AdIL12 or AdIL18 infection of DCs did not result in statistically significant alteration in expression of TRAIL.

Cytokine Gene-engineered DCs Exhibit Improved Tumoricidal Activity in Vitro and Enhanced Survival/Effector Function in Vivo. We next examined the tumoricidal activity of adenovirally infected DCs against CMS4 cancer cells using 24-h MTT assays. Noninfected DCs or AdΔ5DCs displayed approximately the same levels of killing activity (Fig. 2A), suggesting that adenoviral infection alone did not modulate this DC function. In contrast, AdIL12DCs and AdIL12/IL18DCs (but not AdIL18DCs) were significantly better killers of CMS4 target cells on a cell-per-cell basis than AdΔ5DCs or noninfected DCs (all P < 0.01). Combined infection of DCs with both AdIL-12 and AdIL-18 yielded the highest level of cytotoxicity observed for any DC effector cell group tested in these analyses, and this increased cytotoxicity was significantly better than that of the AdIL-18DC, AdΔ5DC, or control DC cohorts (all P ≤ 0.05), but not of the AdIL12DC group (P = 0.71), at the 5:1 (DC:tumor cell) ratio evaluated. We observed a similar order: AdIL12/IL18DC > AdIL12DC > AdIL18DC > AdΔ5DC or DC at various times in a 42-day experiment evaluating the in vitro tumoricidal activity of DC effector cells against the MethA sarcoma (data not shown).

To determine the role of TNF family ligands in DC-mediated cytotoxicity, we performed 24-h MTT assays in the presence of antagonist Abs (Fig. 2B). AdIL12/IL18DC-mediated cytotoxicity against CMS4 cells was significantly decreased when anti-TRAIL or anti-TNF-α Abs were applied (P < 0.0001), whereas blocking anti-Fasl Ab had little effect. When all three blocking Abs were added, DC-mediated cytotoxicity was decreased by approximately 50% (P < 0.0001). These results demonstrate that AdIL-12 and/or AdIL-18 infection enhances the cytotoxic activity of DCs against CMS4 cells, with both TRAIL and TNF-α playing significant roles in this effector function.

To assess the impact of cytokine gene engineering on DC stability and function within the tumor microenvironment in situ, we first generated BM-derived DCs from BALB/c:EGFP Tg mice; infected these DCs with AdΔ5, AdIL-12, and/or AdIL-18; and injected 1 × 10⁶ of these engineered (or control uninfected) DCs into the lesions of syngeneic BALB/c mice bearing day 7 CMS4 tumors. Twenty-four h later, tumors were resected, fixed, sectioned, and counterstained for TUNEL + apoptotic cells. As shown in Fig. 3, A–C, tumors injected with PBS, uninfected DCs, or AdΔ5DCs failed to contain EGFP+ (green) DCs and exhibited only a limited number of TUNEL+ (red) apoptotic tumor cells. In marked contrast, we were able to detect EGFP+ DCs in tumors if these DCs had been infected with AdIL12 and/or AdIL18 before injection (Fig. 3, D–F). These

Table 1

<table>
<thead>
<tr>
<th>DCs evaluated</th>
<th>mIL-12</th>
<th>mIL-18</th>
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</thead>
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<tr>
<td>Control DC (no Ad)</td>
<td>&lt;0.06</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>AdΔ5DC</td>
<td>&lt;0.06</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>AdIL12DC</td>
<td>85.6 ± 1.1</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>AdIL18DC</td>
<td>0.86 ± 0.03</td>
<td>63.5 ± 0.2</td>
</tr>
<tr>
<td>AdIL12/IL18DC</td>
<td>85.3 ± 3.2</td>
<td>9.6 ± 0.8</td>
</tr>
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</table>

* In ng/5 × 10⁶ DCs/48 h.

Table 2

<table>
<thead>
<tr>
<th>DCs evaluated</th>
<th>MHC I</th>
<th>MHC II</th>
<th>CD40</th>
<th>CD80</th>
<th>CD86</th>
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<td>242</td>
<td>59</td>
<td>108</td>
<td>652</td>
</tr>
<tr>
<td>AdΔ5DC</td>
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<td>244</td>
<td>64</td>
<td>103</td>
<td>644</td>
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<tr>
<td>AdIL12DC</td>
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<td>339</td>
<td>51</td>
<td>106</td>
<td>685</td>
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<tr>
<td>AdIL18DC</td>
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<td>394</td>
<td>65</td>
<td>168</td>
<td>1026</td>
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<tr>
<td>AdIL12/IL18DC</td>
<td>359</td>
<td>414</td>
<td>66</td>
<td>93</td>
<td>918</td>
</tr>
</tbody>
</table>

* Values represent the mean of MFI values obtained in three independent experiments.

* DCs were harvested at day 7 of culture and either not infected or infected with the indicated adenoviral vectors at MOIs of 50 (for AdIL18) and/or 200 (for AdΔ5 or AdIL-12). Forty-eight h later, DCs were recovered from culture and phenotyped by expression of the indicated markers.

* P < 0.05 compared with mock-infected DCs.
viable EGFP+ DCs were typically localized in or proximal to regions of increased tumor apoptosis, with some injected DCs containing apoptotic tumor bodies (Fig. 3D).

**Intratumoral Delivery of DCs Engineered to Secrete IL-12 and/or IL-18 Is Therapeutic in the CMS4 Tumor Model.** Based on our observation that cytokine gene-engineered DCs exhibit increased survival and tumoricidal activity, and hypothesizing that this might yield a mechanism that would enhance the cross-priming of antitumor T cells in vivo, we next examined whether IL-12 gene and/or IL-18 gene transfer enhanced the therapeutic potential of DCs delivered intratumorally in the CMS4 tumor model. BALB/c mice received s.c. injection with $10^5$ CMS4 cells. On day 7, these tumors exhibited a mean tumor area of 20–30 mm$^2$. Groups of six tumor-bearing mice were then treated by intratumoral injection of $10^6$ AdIL12/IL18DC, AdIL12DC, AdIL18DC, AdIL5DC, or PBS, and cross-sectional tumor areas were measured through day 42. As shown in Fig. 4A, the growth of CMS4 tumors in mice treated with AdIL12/IL18DC, AdIL12DC, or AdIL18DC was significantly inhibited when compared with tumors in the mice treated with the control protocols ($P \leq 0.05$ on days 14–42 versus AdIL5DC or PBS). AdIL12/IL18DC therapy was superior to AdIL18DC at all time points and to AdIL12DC therapy until day 21. From day 21 onward, tumor regression in AdIL12DC-treated mice became similar to that of the AdIL12/IL18DC-treated group and produced significantly greater antitumor effects than AdIL18DC treatment ($P \leq 0.05$). As shown in Fig. 4B, all six mice treated with AdIL12/IL18DC rejected their tumors by day 42 (95% confidence interval for the percentage of rejected tumors = 54–100%). When data from a second 28-day experiment were combined with the data from the 42-day experiment, 83% (i.e., 10 of 12) of mice treated with AdIL12 DC and 33% (i.e., 4 of 12) of mice treated with AdIL18DC also ultimately rejected their tumors ($P = 0.0075$). These results demonstrated that either IL-12 or IL-18 gene transfer can enhance the therapeutic effects of DC-based therapy against established CMS4 tumors and that injection of DCs engineered to secrete both IL-12 and IL-18 accelerates the therapeutic effect of IL-12/IL18DC treatment.
effectiveness of this treatment strategy. We obtained similar results in a homologous treatment model for the MethA sarcoma, with AdILI12/ILI18DC-based therapies proving statistically superior ($P < 0.05$) to AdILI12DC-, AdILI18DC-, or Adp5DC-based therapies until day 28 (Fig. 4C).

To prove that the therapeutic benefit of our AdILI12/ILI18DC-based regimen in the CMS4 tumor model was T cell dependent, we performed T-cell subset depletion studies (Fig. 4D). Both CD4+ and CD8+ T-cell depletions significantly inhibited the therapeutic efficacy of intratumoral injections with AdILI12/ILI18DCs ($P < 0.05$ at all time points).

**DCs Are Required for the Observed Efficacy of AdILI12/ILI18DC-based Intratumoral Therapy.** To prove that DCs play a requisite role in the observed therapeutic benefit associated with combined IL-12 + IL-18 CGT, we performed additional control experiments in the CMS4 tumor model. Mice bearing established day 7 CMS4 tumors received injection with PBS, AdILI12/ILI18DCs, or AdILI12 (5 $\times$ 10$^7$ pfu) + AdILI18 (2 $\times$ 10$^8$ pfu) Ads. The amount of each Ad injected was equivalent to the total amount of each virus used to generate the AdILI12/ILI18DCs applied in the comparator cohort. As shown in Fig. 5, AdILI12/ILI18DCs, but not the combined Ads, promoted the rapid rejection of CMS4 tumors after intratumoral administration.

**Intratumoral Injection of AdILI12/ILI18DC Enhances Tc1-type Antitumor T-cell Responses.** Based on our in vivo depletion data, we next evaluated whether the therapeutic benefits observed in our DC-based treatment regimens were associated with the degree of anti-CMS4 effector CD8+ T-cell generation in treated animals. CD8+ T cells were isolated from splenocytes using magnetic bead cell sorting (MACS) 7 days after the final intratumoral injections of DCs and then cocultured with irradiated CMS4 tumor cells for 2 additional days (Fig. 6). CD8+ T-cell production of IFN-γ in response to CMS4 tumors differed significantly among treatment groups ($P = 0.0011$, exact two-tailed Kruskal-Wallis test). CD8+ T cells obtained from mice treated with the AdILI12/ILI18DC regimen produced higher levels of the Tc1-associated cytokine IFN-γ in response to tumor rechallenge in vivo versus CD8+ T cells obtained from mice treated with any other DC-based regimen or with PBS only, suggesting an association between IFN-γ production and the degree of therapeutic effects observed in this system.
IMMUNOTHERAPY USING IL-18 AND IL-12 GENE-TRANSDUCED DCs

Splenic CD8+ T Cells Isolated from Mice Treated with Intratumoral Injections of AdIL12/IL18DC React against an Expanded Array of CMS4-derived Peptide Epitopes. To evaluate the repertoire of CMS4-derived peptide epitopes recognized by Tc1-type T cells in treated tumor-bearing mice, IFN-γ production was measured by ELISA after coculture of splenic CD8+ T cells with syngeneic DCs pulsed with peptides that had been acid-eluted from CMS4 cells and subsequently separated using reverse-phase HPLC. As shown in Fig. 7, CD8+ T cells harvested from mice treated with the AdIL12/IL18DC regimen reacted against a wide range of HPLC fractions containing CMS4 peptides. CD8+ T cells isolated from animals treated with AdIL12DC or AdIL18DC injections reacted against a more limited set of HPLC fractionated CMS4 peptides, and in cases where common fractions were recognized by T cells from mice in all of the cytokine gene-engineered DC treatment cohorts, the magnitude of IFN-γ production in the AdIL12/IL18DC group was typically highest. CD8+ T cells from mice treated with AdIL12DC or PBS responded poorly to CMS4 fractionated peptides. These results suggest that the therapies based on intratumoral delivery of DCs modified by IL-12 and IL-18 gene transfer can amplify and expand the repertoire of Tc1-type, antitumor CD8+ T-cell responses in association with increased therapeutic benefit.

Both Locoregional and Systemic Therapeutic Antitumor Immunity Are Induced by Intratumoral Injection with AdIL12/IL18DC. Because the strongest and most diverse Tc1-type, antitumor T-cell responses were induced in AdIL12/IL18DC-treated animals, we next chose to analyze whether the treatment of a CMS4 lesion in one flank would impact the progression of contralateral, untreated CMS4 tumors. Bilateral tumors were established in BALB/c mice for 7 days. On days 7 and 14, tumors established in the right flank of these animals were injected with AdIL12/IL18DC, AdIL18DC, or PBS. Tumors on the left flank remained untreated. As shown in Fig. 8, both the treated and nontreated tumors in mice receiving the AdIL12/IL18DC regimen were significantly smaller on days 14 through the chosen end point of these experiments on day 28 (all P < 0.01) when compared with tumors in mice treated with either PBS or the AdIL12DC regimen, and in two of five cases, animals in the AdIL12/IL18DC treatment group were rendered tumor free by day 21.

DISCUSSION

In the current study, mice bearing established CMS4 or MethA sarcomas were treated with intratumoral injections of syngeneic DCs engineered to constitutively and durably secrete the Th1-biasing cytokines IL-12 ± IL-18. We theorized that cytokine gene-engineered DCs would survive longer and be capable of mediating tumoricidal activity, tumor apoptotic body uptake, and subsequent cross-priming of tumor-reactive T cells in the tumor-draining lymph nodes and...
Our results demonstrate that established CMS4 or MethA tumors may be therapeutically treated by intratumoral injection with IL-12 and/or IL-18 cDNA-transfected DCs (but not control DCs), suggesting that Th1-cytokine gene transfer into DCs enhances their antitumor efficacy in this tumor model. Importantly, tumors injected with DCs engineered to secrete both IL-12 and IL-18 regress most acutely of all treatment groups and were statistically superior to either the AdIL12DC or AdIL18DC group until day 24, when the AdIL12DC (but not the AdIL18DC) cohort was provided a comparable level of therapeutic benefit. Whereas we would hypothesize that the superior (but not the AdIL18DC) cohort was provided a comparable level of AdIL12DC or AdIL18DC group until day 24, when the AdIL12DC promotes the regression of both treated and nontreated contralateral tumors, supporting the ability of this treatment protocol to induce systemic antitumor immunity as noted above. It should be noted, however, that contralateral tumors regressed at a slower rate than treated lesions, suggesting that additional DC- and/or T-cell-dependent effects beyond those linked to the induction of systemic antitumor effector T cells were in play within tumors directly injected with AdIL12/IL18DC. Clearly the impact of locally coproduced IL-12 and IL-18 would be expected to be multifunctional, and one must consider the ability of these cytokines to inhibit angiogenesis, promote the production of IFN-γ-dependent chemokines and lymphocytic infiltration, and maintain immune effector function(s) within the typically immunosuppressive or proapoptotic tumor microenvironment (43–46). Indeed, we have observed that injected cytokine gene-engineered DCs exhibit improved viability within the tumor site in vivo, arguably allowing these cells to generate and acquire apoptotic tumor bodies and to extend the window of time during which they may productively cross-prime antitumor T cells in situ. We are currently performing extensive kinetic experiments to determine the fate and migration patterns of control versus engineered DCs in/to draining lymph nodes after their injection into tumor lesions to partially address this issue. One would also hypothesize, given the extended durability in cytokine production by injected AdIL12/IL18DC within the treated lesion, that increases in inflammatory cell infiltrates and the resistance of these cells to tumor-induced apoptosis would be likely, based on the underlying immunobiologies of IL-12 and IL-18 (47–49). Interestingly, our finding that AdIL18DCs produce elevated levels not only of IL-18 but also of IL-12 may prove to be important in discerning the mechanisms by which AdIL18DC-based therapy is at least partially effective in our sarcoma models. Currently, there is no literature to support or refute the ability of IL-18 to elicit IL-12 production from DCs. However, DCs isolated from mice deficient in functional p38 mitogen-activated protein kinase are impaired in their ability to produce IL-12 (50), and IL-18 is known to activate p38 mitogen-activated protein kinase in murine DCs (51). Hence, it is conceivable that IL-18 produced by AdIL18DCs may act in an autoimmune fashion to facilitate IL-18 receptor-positive DC production of IL-12p70. Alternatively or additionally, AdIL18DC may produce more IL-12 due to indirect effects, such as IL-18-induced production of IFN-γ-dependent chemokines and lymphocytic infiltration, and maintain immune effector function(s) within the typically immunosuppressive or proapoptotic tumor microenvironment (43–46). Indeed, we have observed that injected cytokine gene-engineered DCs exhibit improved viability within the tumor site in vivo, arguably allowing these cells to generate and acquire apoptotic tumor bodies and to extend the window of time during which they may productively cross-prime antitumor T cells in situ. We are currently performing extensive kinetic experiments to determine the fate and migration patterns of control versus engineered DCs in/to draining lymph nodes after their injection into tumor lesions to partially address this issue. 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Hence, it is conceivable that IL-18 produced by AdIL18DCs may act in an autoimmune fashion to facilitate IL-18 receptor-positive DC production of IL-12p70. Alternatively or additionally, AdIL18DC may produce more IL-12 due to indirect effects, such as IL-18-induced production of IFN-γ from contaminant (<5%) activated T or natural killer cells, with subsequent IFN-γ promotion of a more DC1-type phenotype (52).

In addition to their well-publicized roles in antigen cross-presentation, DCs injected directly into tumor lesions may mediate direct tumoricidal activity as a result of their expression of TNF family ligands, including TNF-α, LT-α/β, TRAIL, and FasL. Indeed, in the current study, we have demonstrated that DCs engineered to secrete IL-12 and/or IL-18 (but not control DCs) not only express significantly higher levels of MHC and costimulatory molecules but also express elevated levels of membrane-bound TNF-α and FasL. MTT assays revealed that DCs infected with AdIL-12 and/or AdIL-18, especially DCs coinfected with AdIL-12 and AdIL-18, mediated

spleen of treated animals. Therapy-associated, cross-primed T cells would exhibit an expanded repertoire of antitumor specificities that could theoretically be more effective in mediating tumor regression. Our results demonstrate that established CMS4 or MethA tumors may be therapeutically treated by intratumoral injection with IL-12 and/or IL-18 cDNA-transfected DCs (but not control DCs), suggesting that Th1-cytokine gene transfer into DCs enhances their antitumor efficacy in this tumor model. Importantly, tumors injected with DCs engineered to secrete both IL-12 and IL-18 regressed most acutely of all treatment groups and were statistically superior to either the AdIL12DC or AdIL18DC group until day 24, when the AdIL12DC (but not the AdIL18DC) cohort was provided a comparable level of therapeutic benefit. Whereas we would hypothesize that the superior impact of AdIL12/IL18DC therapy requires cotransfection of DCs to produce IL-12 and IL-18, we have not yet formally evaluated whether the intratumoral injection of AdIL12DC and AdIL18DC (i.e., single cytokine cDNA transfectants) yields a similar favorable outcome.

Subsequent analyses revealed that the antitumor efficacy associated with intraleSION AdIL12/IL18DC therapy requires both CD4+ and CD8+ T cells based on the results of T-cell subset depletion experiments, requires DCs and cannot be reproduced by simple intratumoral injection of AdIL-12 + AdIL-18 viruses, and appears to be associated with the induction of stronger, more diverse Tc1-type immunity. These latter Tc1-type responses were polyclonal in nature based on the ability of isolated immune CD8+ T cells to recognize IFN-γ on a broad array of HPLC-resolved CMS4 peptides when presented by syngeneic DCs in vitro. Whereas a surprising number of HPLC fractions containing CMS4-derived peptides were recognized by CD8+ T cells from AdIL12/IL18DC-treated mice when presented by DCs, these T cells did not react against all fractions, nor did they react against control, nonpulsed DCs in IFN-γ secretion assays. We are currently in the process of determining which peptide-containing fractions recognized by these “therapeutic” T cells are idiotypic to the CMS4 sarcoma and which fractions contain shared sarcoma epitopes by analyzing a corresponding peptide fractionation derived from alternate H-2b tumors, including the MethA sarcoma, the Renca renal cell carcinoma, and the TS/A mammary carcinoma.

Importantly, experiments in a bilateral tumor model suggest that treatment of a single lesion by intratumoral delivery of AdIL12/IL18DC promotes the regression of both treated and nontreated contralateral tumors, supporting the ability of this treatment protocol to induce systemic antitumor immunity as noted above. It should be noted, however, that contralateral tumors regressed at a slower rate than treated lesions, suggesting that additional DC- and/or T-cell-dependent effects beyond those linked to the induction of systemic antitumor effector T cells were in play within tumors directly injected with AdIL12/IL18DC. Clearly the impact of locally coproduced IL-12 and IL-18 would be expected to be multifunctional, and one must consider the ability of these cytokines to inhibit angiogenesis, promote the production of IFN-γ-dependent chemokines and lymphocytic infiltration, and maintain immune effector function(s) within the typically immunosuppressive or proapoptotic tumor microenvironment (43–46). Indeed, we have observed that injected cytokine gene-engineered DCs exhibit improved viability within the tumor site in vivo, arguably allowing these cells to generate and acquire apoptotic tumor bodies and to extend the window of time during which they may productively cross-prime antitumor T cells in situ. We are currently performing extensive kinetic experiments to determine the fate and migration patterns of control versus engineered DCs in/to draining lymph nodes after their injection into tumor lesions to partially address this issue. One would also hypothesize, given the extended durability in cytokine production by injected AdIL12/IL18DC within the treated lesion, that increases in inflammatory cell infiltrates and the resistance of these cells to tumor-induced apoptosis would be likely, based on the underlying immunobiologies of IL-12 and IL-18 (47–49). Interestingly, our finding that AdIL18DCs produce elevated levels not only of IL-18 but also of IL-12 may prove to be important in discerning the mechanisms by which AdIL18DC-based therapy is at least partially effective in our sarcoma models. Currently, there is no literature to support or refute the ability of IL-18 to elicit IL-12 production from DCs. However, DCs isolated from mice deficient in functional p38 mitogen-activated protein kinase are impaired in their ability to produce IL-12 (50), and IL-18 is known to activate p38 mitogen-activated protein kinase in murine DCs (51). Hence, it is conceivable that IL-18 produced by AdIL18DCs may act in an autoimmune fashion to facilitate IL-18 receptor-positive DC production of IL-12p70. Alternatively or additionally, AdIL18DC may produce more IL-12 due to indirect effects, such as IL-18-induced production of IFN-γ from contaminant (<5%) activated T or natural killer cells, with subsequent IFN-γ promotion of a more DC1-type phenotype (52).
enhanced cytotoxicity against CMS4 cells in vitro and that this killing was partially blocked with anti-TRAIL and anti-TNF-α antagonist mAbs. The residual DC-mediated killing of tumor cells that cannot be blocked by the mixture of antagonist anti-TNF-α, anti-FasL, and anti-TRAIL Abs may be due to the influence of DC-expressed LT-α/β that was not evaluated in this study. These findings suggest that IL-12 and IL-18 gene transfection into DCs can enhance the ability of these cells to direct kill cancer cells via certain TNF family ligands and that this mechanism may be relevant to the effective generation of apoptotic tumor bodies (as evidenced in our CMS4 imaging studies), providing tumor antigen for subsequent DC cross-presentation to specific T cells in vitro. We are currently evaluating this issue in the CMS4 and MethA models using intratumorally injected DCs generated from gld (Fas-L deficient), LT-α/β−/−, LT-B−/−, TNF-α−/−, and LT-α/β-TNF triple knockout mice. Preliminary evidence continues to support a dominant role for TNF-α in DC-mediated killing of these tumors in vitro and in situ. In this context, it is important to delineate the potential importance of DC membrane-associated versus secreted TNF-α as a tumoricidal effector molecule. Whereas maximal levels of approximately 30% CMS4 apoptosis and 10% MethA apoptosis were observed in 24-h MTT assays using recombinant TNF-α concentrations of ≥100 units/ml (data not shown), this level of killing was far inferior to that mediated by cytokine gene-modified DCs in the current report, suggesting that additional potency may be associated with DC membrane-bound TNF-α.

Despite recent progress and some early success reported for DC-based cancer immunotherapies, there is a great need to improve this therapeutic strategy. We have shown here that combinational adenoviral-based IL-12 and IL-18 gene transfer into DCs results in an improved therapeutic reagent capable of promoting enhanced antitumor efficacy in vivo when injected directly into tumor lesions. This paracrine delivery strategy was chosen because significant toxicities have been reported previously for combined rIL-12 + rIL-18 systemic therapy in murine tumor models (31), and similar complications might be anticipated in prospective human clinical trials. Whereas serum IFN-γ levels became transiently elevated in mice 2 days (i.e., at the peak of transduced DC production of IL-12/IL-18 in vitro) after each intratumoral injection of AdIL-12/IL18DC (when compared with Ad5/DC- or control PBS-injected mice), this only approached a maximal level of 400 pg/ml in serum (data not shown). This degree of IFN-γ production and our inability to discern any treatment-associated modulation in animal behavior or physical appearance, we believe that intraskeletal AdIL12/IL18DC therapy is not only very effective but also safe. Our findings support intraskeletal delivery of DC-based, IL-12/IL-18 gene therapies as therapeutic regimens for cancer.

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REFERENCES


Intratumoral Delivery of Dendritic Cells Engineered to Secrete Both Interleukin (IL)-12 and IL-18 Effectively Treats Local and Distant Disease in Association with Broadly Reactive Tc1-type Immunity

Tomohide Tatsumi, Jian Huang, William E. Gooding, et al.


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