Cytosine Deaminase-Uracil Phosphoribosyltransferase and Interleukin (IL)-12 and IL-18: A Multimodal Anticancer Interface Marked by Specific Modulation in Serum Cytokines

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Abstract

Purpose: To test the effects of a new combination, cytosine deaminase (CD) + uracil phosphoribosyltransferase (UPRT) – mediated gene-directed enzyme prodrug therapy (GDEPT) with interleukin (IL)-12 and IL-18, on (a) growth of murine prostate and remote tumor deposits, (b) mouse survival, and (c) T helper (Th) 1/Th2 serum cytokine balance with a special focus to assess correlation with tumor burden/survival.

Experimental Design: Efficacy of intraprostatic administration of adenovirally delivered murine IL-12 and IL-18 against orthotopic RM1 tumors and lung pseudometastases was assessed in C57BL/6 mice. At necropsy, tumor growth, lung colony counts, effects on immune cell infiltration, vasculature, apoptosis, and proliferation were estimated. Next, CDUPRT-GDEPT + cytokines were tested at suboptimal doses in mice with RM1CDUPRT prostate tumors/RM1 lung deposits and analyzed as above. Effects on mouse survival were also assessed. Host immune responses to different treatments were assessed by monitoring 11 serum cytokines using Luminex technology.

Results: Our data show that IL-12 and IL-18, when combined with CDUPRT-GDEPT, caused significant reduction in local RM1 tumors and lung colonies with enhanced long-term survival versus individual treatments. A dramatic enhancement of tumor infiltration by a wider repertoire of immune cells and disruption of vasculature implied the combination to be more immunostimulatory and antiangiogenic. Remarkably, lowering of serum IL-4 and monocyte chemoattractant protein-1 (MCP-1) was consistently associated with lower tumor burden (local and systemic), and this, rather than an increase in Th1 cytokines, better predicted treatment efficacy. In addition, mouse survival correlated with substantially higher cytokine (Th1/Th2) levels after treatment.

Conclusion: Locoregional application of CDUPRT-GDEPT and IL-12/IL-18 was effective against local and systemic prostate cancer and improved survival. Monitoring serum levels of IL-4 and MCP-1 may accurately reflect tumor burden and, hence, host response to therapy.

Heterogeneity of clinical prostate cancer has culminated in inadequacy of single-treatment regimens and inefficient patient management. An urgent need has emerged for (a) new combination regimens that can be given into the primary tumor and without toxicities and (b) design of preclinical screening to generate clinically relevant data.

Immunostimulatory strategies have the potential to combat cancer and generate long-term anticancer effects. However, poorly immunogenic tumors and immunocompromised patients pose a challenge. The in situ amplification of cytotoxicity via local and distant bystander effects by locally delivered gene-directed enzyme prodrug therapies (GDEPT) is shown to be inflammatory [T cells, macrophages, and natural killer (NK) cells], and several studies support the concept that further enhancement is possible using cytokines (1). This was first shown when IL-2 combined with thymidine kinase–based GDEPT (HSV-tk) was found to be more potent against a metastatic carcinoma model than either alone and induced antitumor immunity (2). Since then, several clinical and preclinical studies have shown the safety and enhanced potency of similar combination regimens and, importantly, their association with systemic immune responses (3–6). Three clinical trials conducted using HSV-tk-GCV or valacyclovir in patients with prostate cancer have shown that systemic T-cell responses are generated after HSV-tk-GDEPT and these were enhanced (up to 12 months) when combined with radiation and androgen ablation (7). Despite the promise, the clinical data suggest that potency of the treatments needs to be elevated.

A novel GDEPT based on a fusion gene from Escherichia coli, cytosine deaminase-uracil phosphoribosyltransferase...
Th1 phenotype was also confirmed by studies of mice deficient (20). That both are required for significant expression of the suppress antigen (ovalbumin)–specific oral tolerance in mice tumor properties of IL-12 and IL-18 are well documented in IL-18 genes into the primary organ–confined tumor. Anti-(18, 19), and importantly, the IFN-\(\gamma\) expression from differentiating Th1 cells (13, 14) is another example of synergy between cytokine production from Th1 cells and the prodrug 5-fluorocytosine have established clinical safety. Individually, recombinant IL-12–based and IL-18–based therapies have been proven clinically safe at biologically effective doses. Effective local and long-term systemic responses were generated at suboptimal doses with no cumulative toxicities, ideal for clinical application. We are the first to identify that monocyte chemoattractant protein-1 and IL-4 together could be potential serum biomarkers to assess local and systemic tumor burden; after validation in retrospective clinical trials in cancer patients undergoing treatment, this can be followed up to moniter clinical response. Remarkably, only mice that displayed substantially elevated serum cytokine levels soon after treatment survived; once validated in the clinic, this could assess potential efficacy of immunostimulatory therapies and, hence, individualize treatments.

**Translational Relevance**

This study is specifically designed to target heterogeneity of clinical prostate cancer and inefficient patient management. This is the first demonstration of synergy between cytokine deaminase–uracil phosphoribosyltransferase (CDUPRT) gene-directed enzyme prodrug therapy (GDEPT) and immunostimulatory cytokines interleukin (IL)-12 and IL-18. CDUPRT-GDEPT has clear potential in the clinic; CD and the prodrug 5-fluorocytosine have established clinical safety. Individually, recombinant IL-12–based and IL-18–based therapies have been proven clinically safe at biologically effective doses. Effective local and long-term systemic responses were generated at suboptimal doses with no cumulative toxicities, ideal for clinical application. We are the first to identify that monocyte chemoattractant protein-1 and IL-4 together could be potential serum biomarkers to assess local and systemic tumor burden; after validation in retrospective clinical trials in cancer patients undergoing treatment, this can be followed up to monitor clinical response. Remarkably, only mice that displayed substantially elevated serum cytokine levels soon after treatment survived; once validated in the clinic, this could assess potential efficacy of immunostimulatory therapies and, hence, individualize treatments.

(CDUPRT) converts the nontoxic prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) and cytoxic pyrimidine antimetabolites that inhibit DNA and RNA synthesis. The potential and novelty of this system relate to (a) use of the two enzymes that circumvents resistance to 5-FU and sensitizes cancer cells to low doses of 5-FU and 5-FC (8, 9), recently confirmed through its efficacy against primary cancer cells from pleural fluids/ascites of patients with 5-FU–resistant metastases (10); (b) the ability to kill dividing and nondividing cells; and (c) the use of Food and Drug Administration–approved 5-FU/5-FU as prodrug. Although this system is yet to be explored in the clinic, preclinical efficacy against local and metastatic prostate cancer was recently proven in our laboratory (11).

We also found that local and distant bystander effects of CDUPRT-GDEPT were associated with dose-dependent tumor infiltration by macrophages and CD4+ and NK cells. Relevant to this, an increase in the therapeutic efficacy of CD-GDEPT was achieved by combining it with T helper (Th) 1–promoting cytokines [e.g., granulocyte macrophase colony-stimulating factor (GM-CSF; ref. 12), IFN-\(\gamma\) (13), IL-2 (14), IL-12 (4), and IL-18 (15)]. To augment the systemic anticancer immune responses possibly triggered through CDUPRT-GDEPT and to overcome potential immunsuppression due to 5-FU release in tumor microenvironment, we combined CDUPRT/5-FC with ectopic concomitant delivery of immunostimulatory IL-12 and IL-18 genes into the primary organ–confined tumor. Anti-tumor properties of IL-12 and IL-18 are well documented in preclinical and clinical studies (16, 17). Further, the two synergize in IFN-\(\gamma\) production from differentiating Th1 cells (18, 19), and importantly, the IFN-\(\gamma\) released was shown to suppress antigen (ovalbumin)–specific oral tolerance in mice (20). That both are required for significant expression of the Th1 phenotype was also confirmed by studies of mice deficient in IL-12 and IL-18, which display remarkably reduced IFN-\(\gamma\) production, impaired NK cell activity, and defective Th1 cell responses (21). We report here the effects of this combination treatment on local and remote tumor growth and mouse survival in an orthotopic model of murine prostate cancer (22). The growth kinetics (cell proliferation and apoptosis), tumor vasculature, and immune cell infiltration in tumors were also assessed.

Exhaustive assessment of responses to therapy in humans is compromised due to limited noninvasive tests and heterogeneity of the population. The latest trend is to analyze multiple biomarker panels in patient sera to assess response and disease progression. Cytokine panels are especially attractive because development and progression of most cancers involves dysregulated/aberrant immune responses, signified by changes in cytokine serum levels. Indeed, an altered Th1/Th2 cytokine balance with increased Th2 cytokine levels was found in patients harboring different tumor types, and a correlation between cytokine levels and cancer patient responses to therapy has also been shown (23, 24). Although now a standard in many recent clinical trials, preclinical evaluations rarely include such analyses. Hence, we included a cytokine profile end point to our study; it may help to evaluate the deviation of immune response from normal as in the case of humans and hence yield greater clinical relevance to these preclinical data. The effects of different treatments on the Th1/Th2 balance of serum cytokines are reported in correlation with tumor growth and survival.

**Materials and Methods**

**Cell lines and mice.** Androgen-refractory murine prostate cancer cell line (RM1; ref. 25) and the transformed derivatives (RM1CDUPRT) were cultured in DMEM containing 10% fetal bovine serum with hygromycin (800 \(\mu\)g/ml) for the latter (all three from Invitrogen). Male C57BL/6 (6-8 wk) mice were bought from Laboratory Animal Services (Perth, Western Australia, Australia) and maintained under specific pathogen-free conditions. All animal care and experiments were conducted under the guidelines of University of New South Wales Animal Care and Ethics committee and Australian Office of the Gene Technology Regulator.

**Adenoviruses.** Replication-defective adenovirus expressing murine IL-12 (AdmIL-12) was constructed using Virapower Adenoviral kit (Invitrogen) as per instructions (details in Supplementary Data). Briefly, the gene was amplified from a commercial vector pORF-mIL-12 (p35p40) v12 (Invitrogen) using primers modified to enable cloning into the entry vector 1A (Invitrogen), which was then recombined with the destination adenoviral vector using Gateway technology (Invitrogen) to generate AAd.cmv.mIL-12. This was subsequently transfected into 293 cells to rescue AdmIL-12 (BD Pharmingen). Replication-defective adenovirus expressing secreted murine IL-18 (mIL-18) was purchased from RIKEN BRC DNA Bank (RIKEN Biosource Center). Secretion of mIL-18 and mIL-12 was confirmed in Ad5mIL-12/Ad5mIL-18–infected 293A cells by optE1A Mouse IL-12/18 ELISA (BD Pharmingen).

**In vivo studies.** RM1 cells implanted into the prostate or tail vein of C57BL/6 mice induce tumor formation and experimental lung metastasis, respectively, in an aggressive and reproducible manner (22). Intraprostastic injections were done with \(5 \times 10^5\) RM1CDUPRT or RM1 cells in the subcapsular region of the prostate surgically, whereas \(2.5 \times 10^5\) RM1 cells were injected i.v. to generate lung pseudometa-

**Evaluation of effects of treatment with AdmIL-12 and/or AdmIL-18 on RM1 prostate cancer in C57BL/6 mice.** To assess the effects of the
cytokine therapy (n = 10) alone, intraprostatic RM1 tumors (4 × 4 mm) were injected with 2 × 10^7 plaque-forming units (pfu) of Adm-IL-12 and/or AdmIL-18 on day 5; on day 6, RM1 cells were given i.v. to establish lung pseudometastases. Mice were euthanized on day 17, and tumors and other organs (kidney, lungs, liver, heart, and spleen) were frozen or paraffin embedded for subsequent histologic and immunohistochemical studies. Mouse sera were stored at -80°C until further analysis.

The experimental groups were (a) RM1/AdGFP (4 × 10^7 pfu), (b) RM1/AdGFP/Adm-IL-12 (2 × 10^7 pfu per virus), (c) RM1/AdGFP/AdmIL-18 (2 × 10^7 pfu per virus), and (d) RM1/Adm-IL-12/AdmIL-18 (2 × 10^7 pfu per virus).

The following analyses were done at necropsy (day 17):

Evaluation of tumor growth/lung colony formation. Harvested prostate cancers were weighed and volumes were determined \[ V = \frac{\pi}{6}d_1d_2^2 \], where \( d_1 \) and \( d_2 \) are diameters at right angles. Harvested lungs were weighed and fixed in Bouin's fixative to visualize lung lesions using a dissecting microscope and counted as described (see Supplementary Data for details; ref. 11).

Immunohistochemical analysis of orthotopic prostate tumors. Tumors were frozen or formalin fixed and 5 μm sections were stained for infiltrating T lymphocytes (CD4+ and CD8a+ (BD Pharmingen) and macrophages (F4/80+, BD Pharmingen)) and NK cells (AsialoGM1+ (Wako) and endothelial cells (CD31+)) and positive stained cells were scored by light microscopy as described (11).

Cytokine profiles in treated mice. Sera harvested from mice were analyzed (n = 5) using a multiplex mouse cytokine/chemokine LINCOplex kit (Luminex Corp.). Murine cytokines/chemokines IL-2, IL-4, IL-5, IL-10, IL-12, GM-CSF, IFN-γ, tumor necrosis factor (TNF)-α, regulated on activation, normal T expressed and secreted (RANTES), and MCP-1 were assessed. Briefly, 25 μL of mouse serum

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**Fig. 1.** Effects of combining Adm-IL-12 and AdmIL-18 therapy against RM1 prostate cancer. A, schematic of general outline of the *in vivo* experimentation in the orthotopic mouse model of RM1 prostate cancer carried out in this study. B, graphical representation of effects of intraprostatic administration of Adm-IL-12 (2 × 10^7 pfu) and/or AdmIL-18 (2 × 10^7 pfu) on growth of prostate tumors (mass, g) and on number of lung colonies. Control mice were given AdGFP (4 × 10^7 pfu). C, micrographs of CD31+ endothelial cells in the prostate tumors of mice given different treatments. Each treatment panel shows ×10 magnified image on the left and ×40 magnified image on the right. B and C, mice with tumors treated with AdGFP (1), Adm-IL-12 (2), AdmIL-18 (3), and AdmIL-12 and AdmIL-18 (4). D, cytokine levels in mice with low tumor burden in the prostate (tumors <700 mm^3) or lungs (<20 colonies), and control group is represented by mice given AdGFP only. *, the trend observed was significant compared with the control mice (P < 0.005 was considered statistically significant). The cytokine levels are represented as percentage of values for the healthy untreated reference C57BL/6 mice (a value of 100).
Evaluation of combination of CDUPRT-GDEPT with cytokine therapy (n = 10) on RM1 prostate cancer in C57BL/6 mice. Intracranial RM1CDUPRT tumors (5 x 104 cells; 4 x 5 mm) were injected with AdmIL-12 + AdmIL-18 at 2 x 107 pfu each (day 5). A day later, 5-FC or saline was administered i.p. at 250 mg/kg/d for 11 consecutive days. The same day, RM1 cells were given i.v. to establish lung colonies. The protocol at necropsy was as above. Different treatment groups (n = 10) were as follows: (a) RM1CDUPRT/AdmGFP/saline, (b) RM1CDUPRT/AdmGFP/5-FC, (c) RM1CDUPRT/AdmIL-12/AdmIL-18/saline, and (d) RM1CDUPRT/AdmIL-12/AdmIL-18/5-FC. In another experiment (n = 10), survival of similarly treated mice was assessed. Mouse weights were recorded twice weekly. Sera harvested from these mice 24 h after treatment were analyzed as above.

Data analysis. A one-way ANOVA was done (GraphPad Prism v4.0) if the data in multiple groups were normally distributed followed by Tukey’s post test if significance was indicated (P < 0.05). The survival data were analyzed using Kaplan-Meier analyses and a log-rank test to assess significance.

Results

That CDUPRT-GDEPT led to tumor infiltration with immune cells in a dose-dependent manner (11) suggested that the immune system is involved and further augmentation may be possible on combination with immunostimulatory murine cytokines mIL-12 and mIL-18. Both agents (cytokines and CDUPRT) can potentially activate immune cells or modulate tumor-host interactions. Hence, an orthotopic model of syngeneic RM1 prostate cancer in immunocompetent C57BL/6 mice was considered apt to reproduce the tumor microenvironment in the host organ in the context of the host (26). To show the benefit of combination therapy, treatments were given at suboptimal doses: GDEPT-treated mice were given 5-FC at the suboptimal dose of 250 mg/kg/d, and AdmIL-12 or AdmIL-18 was given at 5 to 20 times lower than commonly used [doses explored for most studies fall within the range 107-108 pfu of adenoviral vector, generally given more than once (4, 27, 28)].

The combination of mIL-12 and mIL-18 therapy alone led to a significant reduction in growth of intraprostatic RM1 tumors and lung pseudometastases in C57BL/6 mice.

Local RM1 prostate cancer growth. The data clearly show the efficacy of combining AdmIL-12 and AdmIL-18 treatment on local prostate cancer growth compared with AdmGFP-treated or AdmIL-12–treated or AdmIL-18–treated mice (P = 0.003; Fig. 1B). There was none to negligible tumor growth in 3 of 10 mice compared with 1 of 10 mice in IL-12–treated group and none in mIL-18–treated or GFP-treated mice. Treatment with either cytokine alone did not show any significant advantage over the control AdmGFP-treated group. Interestingly, in mIL-18–treated group, tumor growth seemed to be enhanced but this was not statistically significant [average volume: 3,453 mm3 (mIL-18) versus ~2,168 mm3 (mIL-12), 2,754 mm3 (GFP), and 1,271 mm3 (mIL-12 + mIL-18)].

Lung pseudometastases. Mice treated with AdmIL-12 + AdmIL-18 showed a clear reduction in lung colonies compared with those receiving either cytokine alone or control treatments (P < 0.0001; Fig. 1B). Less than 10 colonies were counted in 5 of 10 mice in this group. Notably, despite insignificant effects on intraprostatic RM1 tumor growth, individual cytokine treatments led to a significant reduction in the number of lung colonies [mIL-12 (~0.1, P = 0.0003) and mIL-18 (~33, P = 0.0003)] in comparison with AdmGFP-treated mice (~98). This trend was further reinforced by lung mass data (P < 0.0001; Supplementary Fig. S1A and B).

Proliferation and apoptosis in prostate tumors. Changes in growth kinetics (cell death and proliferation) of a tumor during therapy are indicative of treatment efficacy. Tipping the balance between cell death (apoptosis and necrosis) and proliferation in favor of cell survival may result in tumor formation. Immunohistochemical assessment of apoptosis in tumors from different treatment groups showed the presence of more (but not significantly) apoptotic cells in all treatment groups compared with the control (AdmGFP) group (Supplementary Table S1). However, analysis of cell proliferation using anti–Ki-67 antibody staining in tumors did not show any significant differences between groups.

Vascular density of prostate tumors. The antitumor effects of both IL-12 and IL-18 are partly mediated through their antiangiogenic activity (29, 30). Immunohistochemical scoring of tumors for vasculature changes [numbers of endothelial cells (CD31+); Fig. 1C; Supplementary Table S1] showed a significant reduction in the number of CD31+ cells in all treatment groups compared with control tumors (P = 0.003). This also indicated that cytokines, individually, had an effect despite no obvious effect on local tumor growth. Further, this reduction was more in tumors from IL-18 (P = 0.0008) and combination treatment groups (P = 0.006), suggesting that IL-18–mediated antangiogenic effects may be more potent in comparison with those of IL-12.

Immune infiltration of tumors. Assessment of the extent of infiltration of tumors with macrophages (F4/20+), CD4+, CD8α-, and NK cells (AsialoGM1+; Supplementary Table S1) showed no significant differences between treatment groups. However, an enhancement in tumor infiltrates of CD4+, CD8+, and NK cells was observed when AdmIL-12 + AdmIL-18 were injected, which correlated with the prostate tumor reduction in combination-treated mice.

Serum cytokine profiles in treated mice. Detection of specific cytokines reveals the state of the immune response at that given time; a significant application of this has been in monitoring cancer progression and its response to therapy. Recently, simultaneous testing using a multiplexed panel of cytokines has proven to be accurate and reproducible using very small volumes of biological samples (31).

For these analyses, the cytokine/chemokine panel was divided into Th1 [IL-2, IL-12, IL-18, TNF-α, IFN-γ, GM-CSF, and Th1-polarizing RANTES (32)] and Th2 [IL-4, IL-5, IL-10, and Th2-polarizing MCP-1 (33)] groups. Sera analyses at necropsy showed that AdmIL-12 + AdmIL-18–treated mice showed a significant increase in Th1 [IFN-γ (P = 0.0005) and IL-18 (P = 0.02)] and a decrease in Th2 [IL-10 (P < 0.0001) and IL-4 (P < 0.001)] cytokines (Supplementary Fig. S2) compared
with AdGFP-treated mice. A general, vector/treatment-related increase in the levels of IL-4, IL-10, IL-12, TNF-α, GM-CSF, RANTES, and MCP-1 was observed in experimental mice versus reference healthy C57Bl/6 mice (a value of 100; Table 1A; Supplementary Fig. S2; Supplementary Table S1). An important observation was that mice with low lung colony counts (<20) showed a clear reduction in Th2 cytokine levels (IL-4 and IL-10) with a significant increase in IFN-γ levels (Fig. 1D).

**Combining CDUPRT-GDEPT with mIL-12 + mIL-18 therapy led to a further reduction in growth of both local prostate cancers and lung colonies and a significant survival advantage.** Having shown the effectiveness of IL-18 + IL-12 gene therapy in targeting local and distant RM1 tumors, we then evaluated the potential of coupling cytokine therapy with CDUPRT-GDEPT (11) for possible synergistic effects.

**Local tumor growth.** In contrast to the control-treated mice, a significant reduction in intraprostatic tumor growth was noted in all other treatment groups (P < 0.0001; Fig. 2A), with almost complete abrogation of tumor growth in CDUPRT-GDEPT + AdmIL-12 + AdmIL-18–treated mice (four of eight showing no tumor; Fig. 2A). Neither the combination of cytokines alone nor GDEPT alone achieved similar efficacy.

**RM1 lung colony growth.** A clear reduction of lung colony numbers was observed in all treatment groups (P < 0.0001; Fig. 2B; Supplementary Fig. S3) with the cytokines + CDUPRT-GDEPT combination being the most effective. Although care was taken to use the cells at the same passage numbers and monitor cell viability (minimum 95%) for all experiments, a variation in overall numbers of lung colonies was seen between experiments. This could be due to technical variability in the injections, lung colony counts, cell count/viability, or mouse batch variation. Although not statistically significant, an enhanced reduction in lung colonies (495) was noted in cytokine-treated mice than those treated with GDEPT alone (636) or AdGFP/saline (761.5). Lung mass analysis further corroborated these data (P = 0.003; Supplementary Fig. S4). Histologic analyses of lung sections showed a significant reduction in the tumor growth area with increasing healthy lung area in the order combination > cytokine > GDEPT > control (Supplementary Fig. S4).

**CDUPRT-GDEPT + cytokine therapy led to a general lowering of Th2 type cytokines.** Assessment of cytokine profiles in mouse sera at necropsy (Table 2B; Supplementary Fig. S5) showed that with the exception of TNF-α, no Th1 cytokines displayed a statistically significant trend; however, some trends were of interest. The IL-2, GM-CSF, and TNF-α (P = 0.02) levels were greater and IL-18 levels were lower in GDEPT and combination-treated groups than in other groups, suggesting a correlation with GDEPT treatment. Among the Th2 cytokines (Table 1B; Supplementary Fig. S5), IL-4 was significantly reduced (P = 0.0001) in cytokine-treated and combination-treated mice (P = 0.0001) compared with controls. A significant decrease in Th2-polarizing MCP-1 levels was observed in mice that received cytokines alone or in combination with GDEPT (P = 0.002). In contrast, Th2, IL-5 levels were higher in GDEPT and combination-treated mice (P = 0.003), which was not surprising given a consistent increase in IL-5 levels has been observed after IL-2 applications (34), leading to the postulation that IL-5 may be a downstream mediator of IL-2–triggered antitumor responses.

Overall, IFN-γ levels were generally lower and GM-CSF, IL-18, MCP-1, and RANTES levels were greater than the reference healthy mouse levels.

To identify any generic trends correlating cytokine profiles with local or distant prostate cancer growth, the profiles of mice with tumor volumes <500 mm³ or <500 lung colonies were compiled (Fig. 2C). Again, only Th2 IL-4 and MCP-1 showed a

### Table 1. Summary of relative serum cytokine levels in tumor-bearing mice treated with AdmIL-12 and/or AdmIL-18 (A), GDEPT, and/or AdmIL-12 + AdmIL-18 [B (at necropsy) and C (24 h after treatment)]

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>IL-12</th>
<th>IL-18</th>
<th>IL-12 + IL-18</th>
<th>IL-12 + IL-18 + GDEPT</th>
<th>IL-12 + IL-18 + GDEPT + P*</th>
</tr>
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<tbody>
<tr>
<td>IL-2</td>
<td>DD</td>
<td>U</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>D</td>
<td>D</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>U</td>
<td>D</td>
<td>0.33</td>
<td></td>
<td></td>
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<tr>
<td>IL-10</td>
<td>U</td>
<td>D</td>
<td>0.65</td>
<td></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>U</td>
<td>D</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>U</td>
<td>D</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANTES</td>
<td>D</td>
<td>D</td>
<td>0.0033</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>D</td>
<td>D</td>
<td>0.002</td>
<td></td>
<td></td>
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<tr>
<td>IL-18</td>
<td>D</td>
<td>D</td>
<td>0.19</td>
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*P < 0.05 (significance).

1 D = reduction in serum cytokine levels compared with control mice (RM1/AdGFP for A and RM1CDUPRT/AdGFP/saline for B and C). Number of "D" represents the extent of reduction relative to control mice.

1 U = increase in serum levels compared with control mice. Number of "U" represents the extent of increase relative to control mice.

1 Value above reference value (a value of 100 for a healthy mouse).

1 No significant change in comparison with the control mice.
substantial reduction in these mice, implying that inhibition of tumor growth more closely correlated with reduction of Th2 cytokines/chemokines.

**Immune infiltration in prostate tumors.** Evaluation of the effects of different treatments on immune cell infiltration in local tumors revealed that the combination (CDUPRT-GDEPT + cytokines) therapy led to extensive tumor infiltration by CD4+ T (P = 0.007, 5-fold) and CD8a+ T (P = 0.02, 3-fold) cells, macrophages (P < 0.0001, 4.7-fold), and NK cells (P < 0.0001, 11.2-fold; Fig. 3; Table 2; Supplementary Fig. S6) versus controls. This was substantially greater than that observed in tumors given either cytokines or CDUPRT-GDEPT alone.

To examine potential mechanisms of treatment-related antitumor effects, we assessed effects on apoptosis, cell proliferation, and the tumor vasculature.

**Proliferation and apoptosis.** M30 CytoDEATH protein staining of the tumor sections showed a significant increase in the apoptotic cells in CDUPRT-treated group (P < 0.0001; Fig. 3; Table 2; Supplementary Fig. S6) compared with all other groups. Surprisingly, despite maximal antitumor efficacy, very
few apoptotic cells were noted in tumors treated with combination therapy. Possibly, because the prostate was almost intact, there was minimal presence of tumor cells. Consistent with this, when analyzed for proliferating cells, the combination treatment group showed the least number of Ki-67+ cells ($P < 0.0001$; Fig. 3; Table 2; Supplementary Fig. S6). The trend (combination < GDEPT < cytokines < control) implied that the antiproliferative effects of GDEPT are more potent than those induced by cytokines alone in local RM1 tumors.

**Vasculature.** Previously, we have shown that CDUPRT-GDEPT is associated with disruption of vasculature, which may have contributed to its cytotoxic effects. Vascular analysis of tumors from treatment groups showed a clear reduction in CD31+ endothelial cells in all treatment groups; compared with control mice, ~5-fold more disruption occurred in the combination treatment group (Fig. 3; Table 2; Supplementary Fig. S6). The trend (combination < cytokines < GDEPT < control; $P = 0.0008$) strongly indicated that the extent of vasculature disruption may play a direct role in cytotoxic effects of different treatments.

**Effects on mouse survival.** This experiment was done as above but this time the mice ($n = 10$) were not implanted with cells to produce lung pseudometastases and were monitored for survival. Mice treated with the combination regimen had a significant survival advantage with the median survival increase to 34 versus 29.5 days for cytokines alone–treated mice and 25 days for control and GDEPT alone–treated mice (Fig. 4A). Statistical comparison (log-rank test) showed a significant difference ($P = 0.011$) and a significant trend between treatment groups ($P = 0.0019$).

**Serum levels of cytokines in mice 24 hours after treatment.** The immediate effects of different treatments on the immune status of treated mice were analyzed. Assessment of cytokine levels 1 day after the treatment began (Fig. 4C; Tables 1C and 2; Supplementary Data) showed a general increase in most cytokine levels compared with reference healthy mice, except for TNF-α and GM-CSF, which were significantly reduced ($P = 0.01$ and $P < 0.0001$, respectively) in combination-treated mice. Lower serum levels of Th2 cytokines [IL-4 ($P = 0.0008$) and IL-10] were observed in mice treated with GDEPT or GDEPT + cytokines. The most important observation was the remarkable increase in serum levels of most cytokines (except IL-18) in surviving mice compared with the group averages (Fig. 4C); this suggested that mice with greater immediate stimulation of the immune system (Th1 and Th2) had a significantly greater chance of survival.

**Discussion**

Cancer is a complex aggressive disease and, unfortunately, still poses a formidable challenge; a multifaceted approach with a potential to target the diverse array of cancer cells locally and systemically was evaluated. Our data reinforce the significance of using combination regimens rather than single modalities against cancer. The combined efficacy of local delivery of CDUPRT-GDEPT and AdmIL-12 and AdmIL-18 against local and remote deposits of hormone-refractory RM1 prostate cancer was unequivocally proven. Further, the potential of individual dose reduction in the combination regimen (GDEPT and mIL-12 + mIL-18) was clearly shown; despite the suboptimal doses, the cell killing effects of combination far exceeded those observed with either alone. Importantly, the combined cytotoxicity of the two treatments did not lead to any adverse effects; mice given combination treatment displayed minimal loss of condition and weight (only 10% of these mice displayed ≤5% weight loss in comparison with 18% weight loss in 95% of the control group). In addition, the combination treatment led to a survival benefit: mice given combination treatment had the longest median survival (34 days) versus mice given AdmIL-12 + AdmIL-18 (29 days).

The enhancement of immune stimulation by the combination treatment was clearly shown by significantly enhanced

**Table 2.** Immunohistochemical analyses of prostate tumor sections showing effects of different treatments on tumor infiltration by immune cells and apoptosis, vasculature, and proliferation

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Immunohistochemical scores* ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Th cells (CD4+)</td>
</tr>
<tr>
<td>RM1CDUPRT</td>
<td>16.3 ± 2.6</td>
</tr>
<tr>
<td>Saline/AdGFP</td>
<td>17.6 ± 3.5</td>
</tr>
<tr>
<td>RM1CDUPRT/5-FC</td>
<td>28.4 ± 5.7</td>
</tr>
<tr>
<td>AdGFP</td>
<td></td>
</tr>
<tr>
<td>Saline/AdmIL-12/AdmIL-18</td>
<td>80.7 ± 18.4</td>
</tr>
</tbody>
</table>

*After initial scanning under ×100 magnification, positive stained cells in 10 fields under ×400 (0.15 mm²) magnification were counted and the mean number/high-power field (SE) was determined.

1 Any stained endothelial cells or clusters separated from adjacent microvessels were included and counted as one microvessel, whereas infrequent CD31+ macrophages and plasma cells were excluded from the analysis. Neither vessel lumens nor RBCs were used to define a microvessel.

2 M30 CytoDEATH antibody-positive cells detect early apoptotic events in cells by detecting specific epitope of cytokeratin-18 that is presented after cleavage by caspases.
tumor infiltration and by a wider repertoire of immune cells (CD4\(^+\) T, CD8\(^+\) T, and NK cells and macrophages). This was also shown in the superior vaccination effects generated by the treatment (Supplementary Fig. S7). We were interested in examining the long-term vaccination effects of the treatment within the constraints of this experiment to partially simulate the clinical situation. Measurement of s.c. RM1 tumors on days 6, 8, and 12 after implantation in surviving mice and in eight

![Image of tumor infiltration and immune cell infiltration](Image)

**Fig. 3.** Effects of different treatments on tumor infiltration by immune cells, apoptosis, proliferation, and vasculature in intraprostatic RMICDUPRT tumors. The images display the extent of infiltration by T cells (CD4\(^+\), CD8\(^+\)), macrophages (F4/80\(^+\)), NK cells (AsialoGM\(^+\)), proliferation (Ki-67), apoptosis (M30 CytoDEATH), and vasculature disruption (endothelial cells, CD31\(^+\)) in RMICDUPRT tumors in mice treated with AdGFP + saline (A), AdGFP + 5-FC (B), AdmIL-12 + AdmIL-18 + saline (C), and AdmIL-12 + AdmIL-18 + 5-FC (D). Again, each treatment panel shows ×40 magnified image (corresponding ×10 images are shown in Supplementary Fig. S3). The positive cells were scored through light microscopy; after initial scanning under ×100 magnification, positive stained cells in 10 fields under ×400 (0.15 mm\(^2\)) magnification were counted and the mean number of stained cells/high-power field (±SE) was averaged over 10 fields.
age-matched untreated C57BL/6 mice showed that tumors were smaller in mice given cytokine or combination therapy. This antitumor activity was evident despite being challenged with a high dose (2.5 × 10^5 cells) of highly aggressive parental RM1 cells (22). Although this was more evident in the GDEPT + cytokine–treated mice, the long-term effects of treatment would need to be exhaustively analyzed in another series of experiments using larger cohorts to yield statistical significance. These data were encouraging and will underpin future optimization/vaccination studies.

The mechanism of action most likely involved apoptosis, decreased proliferation, and disruption in the vasculature; the loss of vasculature was a remarkable feature of cytokine (IL-12 + IL-18) and cytokine + CDUPRT-GDEPT therapies. Both cytokines act through release of IFN-γ, which impedes angiogenesis. Given that IFN-γ levels were high in cytokine-treated mice suggested that this was a contributing mechanism (Table 1A; Supplementary Fig. S2). Although no increase in IFN-γ was seen in RM1CDUPRT/IL-12/IL-18–treated mice (Table 1B), the loss of vasculature and efficacy of the regimen was clear. The IFN-γ released within the tumor microenvironment or the cytokine activity through IFN-γ–independent pathway (35) may have contributed to these results. CDUPRT-GDEPT may be involved based on our previous data showing CDUPRT-GDEPT dose-dependent disruption of the vasculature in local tumors (11). It was postulated that this was mediated by the cytokines released due either to the stimulation of the immune system (1) or to the toxicity of 5-FU released in the tumor microenvironment against endothelial cells (36). However, exhaustive elucidation of the mechanism of this disruption was beyond the scope of this study.

Efficacy of the combination (GDEPT + IL-12 + IL-18) suggested a positive interaction between the treatments; the efficacy was obtained despite the mild immunogenicity of RM1 cells (37). Although we did not explore possible mechanisms, CDUPRT/5-FC–based molecular chemotherapy could have induced the sensitization of tumor cells to immunotherapy. Chemotherapy-induced sensitization of tumor cells to immunotherapy has been shown; previously, cisplatin and etoposide were shown to sensitize prostate cancer cells to the lytic effects induced by CTLs, tumor-infiltrating lymphocytes, and leukocyte-activated killer cells (38, 39). Particularly relevant was the sensitization of colon and breast carcinoma cells to antigenspecific CTLs after 5-FU treatment through increased expression of carcinoembryonic antigen and thymidylate synthetase antigens (CTL target antigens; refs. 40, 41). Of note is the fact that these mice were challenged with s.c. RM1 cells (2.5 × 10^5) 14 d after the treatment was stopped (also see Discussion and Supplementary Data) and were euthanized on day 12 after s.c. implantations. In addition, there were no long-term surviving mice in control and GDEPT alone–treated groups.

**Fig. 4.** Effects of combination of CDUPRT-GDEPT and AdmIL-12 + AdmIL-18 on survival of mice. Mice implanted with RM1CDUPRT tumors were given different treatments and were monitored twice weekly until the end of the experiment. A, survival plot representing the Kaplan-Meier analysis of mice given different treatments. B, effects of different treatments on serum levels of Th1 cytokines (top), Th2 cytokines (middle), and chemokines (bottom) in mice on day 7 (24 h after treatment began). C, comparison of percentage reference values of serum cytokines/chemokines in surviving mice (mice with no tumor growth from cytokine alone and GDEPT + cytokine groups) with the respective group averages and control mice (AdGFP + saline treated). Note that these mice were challenged with s.c. RM1 cells (2.5 × 10^5) 14 d after the treatment was stopped (also see Discussion and Supplementary Data) and were euthanized on day 12 after s.c. implantations. In addition, there were no long-term surviving mice in control and GDEPT alone–treated groups.
that thymidylate synthetase is the enzymatic target of 5-FU and 5-FU monophosphate, which are the key metabolites of 5-FC through the actions of CDUPRT. In another study, MHC1 expression was enhanced in tumor cells after AdCD treatment and both MHC1 and the costimulatory molecule B7.1 were enhanced on combination with GM-CSF (12). It was postulated that AdCD treatment creates cell debris, which is then processed through antigen-presenting cells. Hence, we postulate that combining CDUPRT-GDEPT and cytokines may have acted through CDUPRT/5-FC-mediated cytodestruction apoptosis and antiangiogenesis (see data), overexpression of relevant antigens (e.g., thymidylate synthetase), and sensitization of tumor cells to the cytolytic activity of effector lymphocytes. Concomitant or subsequent treatment with IL-12 and IL-18 could stimulate antigen presentation (enhanced infiltration by macrophages; Fig. 3) and expand tumor-specific CTL clones (increase in CD8a+ infiltration; Fig. 3), thus initiating and supporting an immune response with potential antitumor activity. Although not explored in this study, effects of tandem treatments of the two modalities in different orders may modulate the effects.

That the two modalities are functional was obvious from our data. In addition, the ability of RM1CDUPRT cells used in this study to metabolize 5-FC in vivo has also been shown (11).

Although we did not specifically test the IL-12 and IL-18 cytokine secretion by the tumor cells in situ, we have previously confirmed that the viruses used in this study produce bioactive cytokines (42). This was further confirmed by the significant reduction in vasculature of local tumors in IL-12–treated or IL-18–treated mice (P = 0.003) and systemic effects against RM1 lung pseudometsastases.

An interesting aspect of our study was the effects of different treatments on serum cytokine profiles, which were examined at two time points, soon after treatment to assess immediate responses and at necropsy, when mice in most groups would have tumors growing in the prostate or in the lungs, albeit at different rates. In the latter scenario, cytokine levels would reflect the host immune status based on tumor-host interactions and host response to the therapy. Especially with regard to mIL-12 and/or mIL-18, exogenous production is unlikely to have contributed to the serum levels of these cytokines; according to our in vitro data (data not shown), 100% infection of the implanted cells would correlate to production of ~3.24 pg/mL mIL-12 and 383 pg/mL mIL-18, which is unlikely to make significant contribution to endogenous levels produced in healthy mice (134.86 pg/mL mIL-12 and 2,228 pg/mL mIL-18; Tables 2 and 3; Supplementary Data). It is possible though that their in situ production indirectly stimulated production of these and other cytokines. This coupled with the fact that elevated serum levels of cytokines IL-12 and/or IL-18 have been correlated with tumor activity and disease progression suggested that an elevation in IL-18 levels in IL-18–treated mice, which also had the largest primary tumor mass, may indicate tumor activity (Fig. 1B and D). Further, a lowering of these in combination group suggested lowered tumor activity, which was reaffirmed by our tumor growth data. Importantly, when GDEPT was combined with cytokines (Figs. 2 and 4), efficacy was significantly enhanced despite lowering of or no effects on systemic IL-12 or IL-18 levels, suggesting synergistic interactions between modalities leading to downstream processes such as immunosensitization by GDEPT together with immunostimulation by combination of cytokines themselves, which is effective even under immunodeficient conditions (43). The general increase observed in the serum levels of IL-4, IL-10, IL-12, TNF-α, GM-CSF, RANTES, and MCP-1 in experimental animals compared with the healthy untreated control could be due to innate and adaptive immune responses to adenoviral vectors and cellular responses to transgenes (44). Several interesting observations were made.

The paradigm that cytokine imbalance skews the Th2 response, which favors tumor growth, roughly holds in this preclinical setting: analyses of sera at the end of each experiment indicated that a decrease in Th2 cytokines is consistent with a good response (tumor reduction and survival) and is a better predictor of treatment efficacy. Abnormally high levels of Th2 cytokines (e.g., IL-10 and IL-4) with concomitant decrease in Th1 (e.g., IFN-γ and IL-2) have been described in humans and rodents with growing tumors (45–47).

Specifically, reduction in Th2 cytokines/chemokines, IL-4 and/or IL-10, and MCP-1, correlated with the efficacy of the treatment/s. For example, in these experiments, although the Th1 profile in RM1/IL-12 + IL-18–treated mice was different from that generated by RM1CDUPRT/IL-12/IL-18 treatment (lower IFN-γ levels and unchanged IL-18 levels in the latter), the Th2 cytokines (IL-4 and IL-10) showed a substantial reduction with both treatments. A similar pattern in the survival studies in GDEPT + cytokine–treated mice suggested that a reduction in Th2 cytokine levels rather than an increase in Th1 serum cytokines was a better predictor of treatment response and of survival benefit. This was confirmed when a general analysis of mice with low tumor burden (irrespective of treatment; intraprostatic and lung colonies) clearly showed decreased levels of Th2 cytokine (Figs. 1D and 2C). In particular, lowering of MCP-1 (a potent angiogenic factor that contributes to tumor migration and metastases; significantly lower in mice with small intraprostatic tumors) and IL-4 (significant lowering in mice with low lung colony counts) consistently indicated treatment efficacy. This is consistent with studies showing that anti–MCP-1 neutralizing antibodies inhibited tumor angiogenesis and metastases in several cancer types (48, 49) and with the association of high IL-4 levels with advanced stages of cancer (50, 51). Lowering of IL-4 was particularly noted in mice with low lung colony numbers, suggesting a positive correlation between low serum IL-4 levels and inhibition of metastases.

Although a consistent reduction of Th2 cytokines was associated with treatment efficacy, the Th1 cytokine increase was variable for different treatments (Table 1A–C). For example, cytokine treatment led to an increase in IFN-γ levels (Table 1A and B; Supplementary Fig. S2), and combination with GDEPT led to involvement of other Th1 cytokines, such as IL-2, along with downstream mediators such as IL-5, suggesting different mechanisms of immune stimulation.

Interestingly, despite a general decline in most cytokine levels in mice given the CDUPRT-GDEPT + IL-12 + IL-18 therapy, the efficacy was maximal. It is unlikely that this decline is due to toxicity of metabolites of CDUPRT/5-FC treatment to circulating cytokine-producing cells; we have shown previously that 5-FC given at twice the dose was nontoxic and led to undetectable serum levels of 5-FU in CDUPRT/5-FC–treated mice (11). In light of this, it is plausible then that the profiles
may represent the actual immune status in response to the treatments and tumor growth.

Finally, that the extent of overall immune stimulation soon after treatment is crucial for better survival was clearly shown. A dramatic increase in levels of all cytokines (Th1 + Th2) in surviving mice compared with the rest of the group (Fig. 4C) implied that, initially, adequate stimulation of the immune system (Th1 and Th2) may be a prerequisite to achieve any long-term benefits. In concert with this, clinical studies have clearly shown that patients with a deteriorated immune system are less likely to benefit from immunotherapies. Use of agents that synergize with respect to overall stimulation of the immune system and targeting the disease in early stages, when the immune system is relatively robust, would help achieve this aim.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Rosetta Martinello-Wilks for her intellectual contribution toward the initiation of this study.

References

Cytosine Deaminase-Uracil Phosphoribosyltransferase and Interleukin (IL)-12 and IL-18: A Multimodal Anticancer Interface Marked by Specific Modulation in Serum Cytokines

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