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“Enhancement of Anti-Telomerase Immunity against Prostate Cancer”

a. Introduction

The overall objective of this proposal is to enhance the efficacy of cancer vaccines by selectively eliminating or reducing CD4+ regulatory T cells (T_{reg}) expressing the high affinity CD25 IL-2-specific receptor (IL-2R) in patients with metastatic prostate cancer. Preclinical and clinical data from our laboratory (see Reference 1 and Appendix A) and others (2, 3) have shown that CD4+/CD25+ T_{reg} play an important role in the suppression of T cell responses and that elimination of T_{reg} is capable of enhancing T-cell proliferation and cytolytic activity in vitro. We have also demonstrated that human T_{reg} can selectively be depleted in cancer patients using the IL-2/diphtheria toxin conjugate denileukin diftitox, without inducing toxicity on other cellular subsets with intermediate or low expression of CD25 (1). Most importantly, denileukin diftitox-mediated elimination of T_{reg} followed by vaccination with tumor RNA-transfected DC significantly improved the stimulation of tumor-specific T-cell responses in RCC patients, when compared to vaccination alone.

These findings formed the basis of this proposal aimed to augment a vaccine-induced T cell responses by pretreatment of prostate cancer patients with agents that can lead to the preferential depletion of the CD4+/CD25+ regulatory T cells, such as agents which target and kill cells expressing the IL-2 receptor CD25 subunit.

b. Body

In this application, we are seeking to translate this novel concept into a clinical trial setting in which patients with metastatic, hormone-refractory prostate cancer will be enrolled. For T_{reg} depletion, patients will receive a single dose of the diphtheria/IL-2 fusion protein denileukin diftitox (4) followed by intradermal injections of LAMP hTERT mRNA-transfected DC (5). A second cohort is receiving vaccination with LAMP hTERT mRNA-transfected DC alone. In a previous study, DC expressing the chimeric gene product LAMP hTERT have previously shown to effectively elicit potent and hTERT-specific CD4+ and CD8+ T-cell responses in metastatic prostate cancer patients (5). Specifics regarding our progress on the clinical trial and our preliminary data arising from this study are outlined below:

b-1. Clinical Trial Progress

A total of 15 subjects with metastatic prostate adenocarcinoma, stage (T_{1-4}, N+, M_0) or (T_{1-4}, N_0-1, M+) have been enrolled and treated in this Institutional Review Board (IRB) and Food and Drug Administration (FDA)-approved study. Six subjects, enrolled on dose Schedule A, treatment arm A, received a single intravenous dose of denileukin diftitox (18µg/kg) four days prior to vaccination with LAMP hTERT mRNA-transfected DC, while a second cohort (Treatment arm B) of 6 subjects was treated with the vaccine alone. Treatment on both study arms was generally well tolerated with only one adverse event (grade III anemia, likely tumor mediated) reported to regulatory agencies. Two patients were treated on dose Schedule B treatment arm A (n=1) and B (n=1). In one patient there was progression of disease following the third vaccination and was therefore withdrawn from treatment. The PI has relocated to the University of Florida (UF) effective July 1st 2006 and is seeking to re-initiate this trial at UF to reach our targeted goal of enrolling 24 study subjects.


In order to determine whether a single dose of denileukin diftitox is capable of reducing the levels of T_{reg} in prostate cancer patients, PBMC were collected from all subjects prior to (Pre ONT), four days after denileukin diftitox administration (Post ONT), and one month after vaccine treatment (Post vaccine). For in vivo T_{reg} identification and tracking, CD4 positive T-cells were initially isolated from the PBMC of study patients by
magnetic bead separation, then stained with CD25 or FoxP3 antibodies, and finally analyzed for the presence of triple positive T\textsubscript{reg} using flow cytometry.

As shown in Figure 1, denileukin diftitox administration resulted in a significant reduction of CD4\textsuperscript{+}/CD25\textsuperscript{high}/FoxP3\textsuperscript{+} triple positive T\textsubscript{reg} in 3 of 3 patients analyzed thus far. The levels of reduction were 21% (FSH-02), or 33% in subject (HWH-06), and 36% (SBS-04). As observed in a prior study, the T\textsubscript{reg} nadir was reached as early as 4 days after denileukin diftitox administration. In ongoing studies, we are seeking to further corroborate these results by performing additional analyses to quantify FoxP3 transcripts from the peripheral blood of all study subjects and to perform functional analyses to determine potential improvement of anti-TERT immunity after T\textsubscript{reg} depletion. Importantly, we are collecting follow up PBMC samples from all patients to accurately determine the T\textsubscript{reg} nadir after denileukin diftitox administration.

![Figure 1. Depletion of CD4\textsuperscript{+}/CD25\textsuperscript{+}/FoxP3\textsuperscript{+} T\textsubscript{reg} in patients with metastatic prostate cancers.](image)

Peripheral blood samples (PBMC) were collected from 3 study subjects who received a single dose of denileukin diftitox (18µg/kg). PBMC were collected prior to (Pre ONT), four days after denileukin diftitox administration (Post ONT), and one month after denileukin diftitox administration (Post vaccine). To quantitate T\textsubscript{reg} in peripheral blood samples, CD4\textsuperscript{+} T-cells were first isolated from PBMC by magnetic bead separation, then stained with CD25 or FoxP3 antibodies, and finally analyzed for the presence of triple positive T\textsubscript{reg} using flow cytometry. The results obtained from 3 subjects are shown.

**b-3. Immunological Response to Vaccine Therapy**

In order to determine vaccine-mediated stimulation of hTERT-specific T-cell responses in subjects treated on both study arms, we first analyzed and compared directly the numbers of hTERT-specific T cells from pre- and post-therapy PBMC samples using ELISPOT analyses. At this point, we were able to analyze vaccine-induced T-cell reactivities from 2 subjects who received the vaccine alone (without denileukin diftitox).

In preparation for this trial, we have developed a modified Interferon-\gamma spot forming assay, in which mRNA-transfected DC were used for *in vitro* antigenic stimulation: Aliquots of PBMC obtained at baseline and 2
weeks after the sixth vaccination were thawed, T cells were isolated by negative depletion and cultured overnight with DC that had been transfected with hTERT or LAMP-hTERT mRNA. As controls, unloaded DC (data not shown) or green fluorescent protein (GFP) mRNA-transfected DC were used as stimulators. As shown in Figure 2, the numbers of hTERT-specific IFN-γ secreting cells was expectedly low or undetectable when T cells isolated from pre-therapy PBMC samples were analyzed, while after vaccination all 2 patients exhibited significant expansion of hTERT-specific T cells. The magnitude of the observed increases in the numbers of hTERT-specific T cells following vaccination is notable in view of the fact that analysis was performed directly from peripheral blood cells without the need for repeated in vitro restimulations. In stark contrast, no reactivity was observed against unloaded DC (not shown) or DC transfected with GFP RNA (data not shown) in any PBMC sample obtained from study subjects prior or post vaccination.

Figure 2. T-cell induction after vaccination with LAMP hTERT mRNA-transfected DC. PBMC from pre- and post vaccination peripheral blood samples were incubated overnight with RNA loaded DC (targets) as demonstrated in the text. Following short-term antigenic stimulation (using RNA transfected DC), cells were plated onto IFN-γ capture antibody-coated nitrocellulose plates. Visible spots were enumerated using an automated ELISPOT reader. As control targets either unloaded DC (DC) or GFP RNA loaded DC were used (data not shown).

c. Key Research Accomplishments

a) Successful translation of the T reg depletion concept into the clinic by executing a complex phase I/II study and treating study subjects with a combined T reg depletory/vaccine regimen.

b) Our preliminary data suggest that administration of a single dose of denileukin diftitox (18µg/kg) is capable of selectively eliminating CD25-expressing regulatory T cell subsets from the PBMC of prostate cancer patients.

c) Immune analysis demonstrated significant expansion of hTERT-specific T cells in the peripheral blood of all vaccine candidates analyzed to date. Further experiments are underway to provide more an in depth analysis on the vaccine-mediated T-cell response by characterizing their functional ability to lyse tumor cells and determine the exact phenotype and cytokine profiles of vaccine-induced CD4+ and CD8+ T-cell subsets.

d. Conclusions

We have madder considerable progress in the execution and analysis of a complex phase I/II study that will serve as a basis for subsequent phase II clinical trials that would receive input specifications arising from the biologic and clinical results of this ongoing phase I/II trial.
f. References


e. Publications.

Ongoing.
Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells

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In this study, we investigated whether elimination of CD4+CD25+ Tregs using the recombinant IL-2 diphtheria toxin conjugate DAB389IL-2 (also known as denileukin diftitox and ONTAK) is capable of enhancing the immunostimulatory efficacy of tumor RNA-transfected DC vaccines. We show that DAB389IL-2 is capable of selectively eliminating CD25-expressing Tregs from the PBMCs of cancer patients without inducing toxicity on other cellular subsets with intermediate or low expression of CD25. DAB389IL-2–mediated Treg depletion resulted in enhanced stimulation of proliferative and cytotoxic T cell responses in vitro but only when DAB389IL-2 was omitted during T cell priming. DAB389IL-2 significantly reduced the number of Tregs present in the peripheral blood of metastatic renal cell carcinoma (RCC) patients and abrogated Treg-mediated immunosuppressive activity in vivo. Moreover, DAB389IL-2–mediated elimination of Tregs followed by vaccination with RNA-transfected DCs significantly improved the stimulation of tumor-specific T cell responses in RCC patients when compared with vaccination alone. Our findings may have implications in the design of immune-based strategies that may incorporate the Treg depletion strategy to achieve potent antitumor immunity with therapeutic impact.

Introduction

We have previously shown that vaccination with RNA-transfected DCs is an effective strategy to stimulate potent T cell responses in patients with metastatic cancers (1, 2). In this study, we investigated whether the immunostimulatory properties of RNA-transfected DC vaccines can be further enhanced by disrupting regulatory pathways that suppress the activation and function of tumor-specific T effector cells in the cancer patient. Recent research has shown that CD4+ T cells constitutively expressing the IL-2 receptor α-chain (CD25) act in a regulatory capacity by suppressing the activation and function of other T cells (3). Their physiological role is to protect the host against the development of autoimmune responses to antigens expressed by normal tissues (4, 5). Since tumor antigens are largely self antigens, Tregs may also prevent the tumor-bearing host from mounting an effective antitumor immune response. Previous studies have shown that elevated numbers of CD4+CD25+ Tregs can be found in advanced cancer patients (6) and that high Treg frequencies are associated with reduced survival (7). The important role of CD4+CD25+ Tregs in controlling tumor growth was further highlighted by the demonstration that depletion of Tregs using anti-CD25 antibodies can evoke effective antitumor immunity in mice (8, 9). Moreover, anti-CD25 therapy enhanced the therapeutic efficacy of GM-CSF–secreting B16 tumor cells and prolonged survival of tumor-bearing animals (10). Cumulatively, these experimental data suggest that the efficacy of cancer vaccination could be enhanced by treatment with agents that lead to the preferential depletion of CD4+CD25+ Tregs, such as compounds that target the IL-2 receptor CD25 subunit.

In this study, we used the recombinant IL-2 diphtheria toxin conjugate DAB389IL-2 (also known as denileukin diftitox and ONTAK) to eliminate CD25-expressing Tregs in metastatic renal cell carcinoma (RCC) patients. DAB389IL-2 contains the catalytic and membrane translocation domain of diphtheria toxin (11). The binding domain for the diphtheria toxin receptor, however, is deleted and replaced by the human IL-2 gene, which allows for targeting of CD25-expressing cells. The cytotoxic action of DAB389IL-2 occurs as a result of binding to the high-affinity IL-2 receptor, subsequent internalization, and enzymatic inhibition of protein synthesis, ultimately leading to cell death.

Here we show that DAB389IL-2 is capable of selectively eliminating Tregs from human PBMCs in a dose-dependent manner without apparent bystander toxicity to other PBMCs or CD4+ T cells with intermediate- or low-level expression of CD25. Treg depletion resulted in enhanced stimulation of proliferative and cytotoxic T cell responses in vitro but only when DAB389IL-2 was used prior to and omitted during the T cell priming phase. Depletion of Tregs in RCC patients followed by vaccination with tumor RNA-transfected DCs led to improved stimulation of tumor-specific T cells when compared with vaccination alone. To our knowledge, we provide the first clinical evidence that in vivo elimination of Tregs is capable of enhancing the magnitude of vaccine-mediated, tumor-specific T cell responses in humans.

Nonstandard abbreviations used: 7-AAD, 7-amino-acridine dye; CTLA-4, cytotoxic T-lymphocyte antigen 4; DAB389IL-2, recombinant IL-2-diphtheria toxin conjugate; FluM1, influenza virus matrix protein 1; FoxP3, forkhead box P3 transcriptional regulator; GITR, glucocorticoid-induced TNF receptor family gene; hTERT, human telomerase reverse transcriptase; MART-1, melanocyte antigen recognized by T cells 1; MLR, mixed-lymphocyte reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt; OVA, ovarian carcinoma; RCC, renal cell carcinoma; RE, renal epithelium.

Conflict of interest: The authors have declared that no conflict of interest exists.

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The definition and enumeration of human Tregs is complicated by the fact that CD25 is a marker of T cell activation (12, 13). Human CD4+ T cells expressing CD25 represent a heterogeneous cell population containing not only regulatory but also effector/memory...
T cells (14). Analysis of PBMCs from healthy donors and RCC patients revealed the presence of CD4+ T cell populations that express increasing levels of CD25 (14). As shown in Figure 1A, 1 major subset of CD4+ T cells, isolated from the PBMCs of an RCC patient, lacked CD25 expression while a second population was characterized by intermediate levels of CD25, and a third, albeit small portion, exhibited high CD25 cell surface expression levels. To further characterize these 3 subsets, CD4+/CD25low, CD4+/CD25int, and CD4+/CD25high T cells were isolated from the PBMCs of RCC patients by FACS and functionally analyzed in vitro (Figure 1B). CD4+/CD25low cells expressed cell surface markers characteristic of naive/resting T cells and demonstrated reduced proliferative activity following exposure to tetanus toxoid, renal tumor RNA, benign renal epithelium (RE) RNA, and PBMC RNA-loaded DCs. In contrast, CD4+/CD25int cells produced a strong proliferative response against tetanus toxoid, and a significant, albeit weaker, response, against RCC RNA-encoded antigens. No proliferative response against RE RNA- or PBMC RNA-transfected DCs (latter not shown) was observed. CD4+/CD25high Tregs exhibited profound immunosuppressive activity in vitro, as evidenced by inhibition of allogeneic DC-stimulated mixed-lymphocyte reaction (MLR) cultures. The addition of increasing numbers of CD4+/CD25high cells (1:5 responder cells; 1:1 responder cells) to MLRs led to a dose-dependent inhibition of responders' T cell proliferation while CD4+/CD25high T cells did not proliferate significantly upon stimulation with DCs (DC+Treg). Tregs demonstrated strong cell surface expression of glucocorticoid-induced TNF receptor family gene (GITR) as well as intracellular CTL-associated antigen-4 (CTLA-4) and forhead box P3 transcriptional regulator (FoxP3) (Figure 1C). Stimulation of CD4+/CD25high T cells using anti-CD3/CD28 antibodies resulted in enhanced expression of GITR, CTLA-4, and FoxP3 while CD4+ T cells with negative or intermediate levels of CD25 exhibited significantly lower levels of these markers after nonspecific stimulation.

Since CD25 is not an optimal marker to enumerate Tregs in human subjects, as it is upregulated upon T cell activation, the number of FoxP3 transcripts was determined in various T cell populations using quantitative real-time PCR. FoxP3 is the most specific Treg marker currently available while other molecules expressed by Tregs (i.e., CD45RB, CD38, and CD62L) previously failed to demonstrate specificity for detecting Tregs with immunosuppressive activity.
of Fox3 transcripts were expressed by CD8+ T cells and CD3+ cells (B cells, monocytes/macrophages, and NK cells; data not shown). When assayed by FACS, CD4+/CD25high/Fox3+ Treg frequencies detected in the peripheral blood of metastatic RCC patients after tumor nephrectomy (n = 10) ranged from 2.5% to 4.6% and were significantly elevated (2.1 ± 1.2-fold increase) when compared with healthy volunteer controls (n = 10). Accordingly, there was a statistically significant increase in the number of Fox3 transcripts that could be amplified from isolated CD4+ T cells of RCC patients when compared with those of healthy volunteers (P = 0.009) (Figure 1D, right panel).

In summary, we show that CD4+/CD25high T cells isolated from the PBMCs of RCC patients exhibited suppressive activity while CD4+ cells with negative or intermediate CD25 levels represented either naive/resting or memory/effector T cells. Therefore, in clinical settings, it will be important to identify suitable reagents that allow selective elimination of CD25high Tregs while sparing other frequencies were measured in the peripheral blood of metastatic RCC patients when compared with healthy donor controls.

Selective elimination of Tregs in vitro. Human malignant cells overexpressing CD25 can be inactivated or eliminated using the recombinant IL-2 diphtheria toxin conjugate, denileukin diftitox (DAB389IL-2) (19). In order to determine whether DAB389IL-2 could serve as a suitable reagent to achieve Treg depletion under clinically relevant conditions, we analyzed Treg susceptibility to DAB389IL-2 in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) assays. In these experiments, we chose conditions that resembled the pharmacokinetics of a single intravenous dose of DAB389IL-2 (18 µg/kg) corresponding to 5 nM peak plasma concentrations. In previous clinical trials, this dose level demonstrated optimal clinical efficacy and acceptable toxicity profiles (20). Given a plasma half-life of approximately 60 minutes and a dissociation constant of 1 pM for DAB389IL-2 and the high-affinity IL-2 receptor, DAB389IL-2 plasma levels were projected to reach suboptimal concentrations after 6 hours. Therefore, in the experiments shown in Figure 2A, the viability of isolated CD4+/CD25high T cells was analyzed after a 6-hour exposure to increasing concentrations of DAB389IL-2 (range 0.05–5.0 nM) in vitro over 48 hours. For CD4+/CD25high Tregs, a significant reduction in cell viability was observed 24 hours after exposure to DAB389IL-2. Efficient killing of CD4+/CD25high cells was noted at 0.5 nM concentrations after 48 hours while complete depletion was achieved at a 5 nM concentration. In contrast, exposure of CD4+/CD25low and CD4+/CD25int cells to DAB389IL-2 did not result in significant cell death except when these cells were exposed to DAB389IL-2 concentrations higher than 10 nM (latter not shown). In another set of experiments, DAB389IL-2 used at a 5 nM concentration resulted in specific killing of Tregs but not of other bystander cells in vitro. As shown in Figure 2A, there were no significant differences in PBMC viability over time when DAB389IL-2 (5 nM) was added to the culture. In contrast, when PBMCs and Tregs were mixed (1:1 ratio), cell viability was reduced by more than 60% after 48 hours, suggesting selective, diphtheria toxin–mediated Treg killing without bystander toxicity. In order to corroborate these findings, 7-amino-actinomycin D (7-AAD) staining was performed on CD4+ T cell populations with increasing densities of CD25 (Figure 2B). Consistent with the experiments shown in Figure 2A, optimal killing of CD4+/CD25high Tregs was achieved using 5-nM DAB389IL-2 concentrations while DAB389IL-2 exposure to other CD4+ T cells with lower or no CD25 expression revealed only background levels of 7-AAD staining.

Next, the impact of DAB389IL-2 on freshly activated lymphocytes was analyzed after stimulation with allogeneic DCs in MLR cultures. DAB389IL-2–mediated Treg depletion prior to initiation of MLR culture resulted in a 2-fold increase in proliferation of responder cells (PBMC±DAB). Conversely, the addition of isolated Tregs (DC±Treg) resulted in an
approximately 80% reduction in T cell proliferation (1:1 Treg/responder ratio) (Figure 2C). Preincubation of Tregs with 5-nM DAB389 IL-2 (DC+Treg+DAB) significantly abrogated their inhibitory effect when added to MLR; however, responder cells did not proliferate as vigorously as in the absence of Tregs, suggesting potential contact inhibition by Tregs, as reported previously (21). Importantly, the addition of DAB389 IL-2 two days after initiation of MLR (DC+DAB) completely abrogated the proliferation of responder cells, suggesting that DAB389 IL-2 eliminated not only Tregs but also freshly activated naive T cells that acquired CD25 expression.

These experiments suggest that DAB389 IL-2 is a suitable reagent for selectively eliminating Tregs in vitro without affecting other lymphocytes, including naive and memory T cells with negative or intermediate expression levels of CD25, respectively. Our data further indicate that in a vaccination setting, DAB389 IL-2 should only be applied prior to immunization but not during the vaccination phase, since activated effector T cells appear susceptible to DAB389 IL-2–mediated toxicity.

Enhancement of T cell responses after Treg depletion in vitro. To provide evidence that DAB389 IL-2–mediated Treg depletion is capable of augmenting antigen-specific T cell responses in vitro, CTLs were stimulated from PBMCs that were pretreated with or without DAB389 IL-2 (5 nM) (Figure 2D). PBMCs were stimulated twice with autologous DCs transfected with human telomerase reverse transcriptase (hTERT) and melanocyte antigen recognized by T cells 1 (MART-I) mRNA. In addition, DCs pulsed with an HLA-A0201–restricted MART-I peptide were used as stimulators. RNA-transfected DCs were not only used as stimulators but also served as specific or control targets, as described previously (2, 22–24). The ability of the stimulated, antigen-specific CTLs to recognize their cognate but not control target cells was analyzed in standard cytotoxicity assays. As shown in Figure 2D, CTLs stimulated from Treg-depleted PBMCs exhibited significantly higher lytic activity against antigens encoded by hTERT or MART-I mRNA than CTLs stimulated from nondepleted PBMCs. In contrast, only modest, statistically insignificant improvement of antigen-specific killing was observed when MART-I peptide-pulsed DCs were used as stimulators.

These data suggest that depletion of Tregs from human PBMCs enhances the stimulation of antigen-specific CTLs in vitro. It appears that among other factors, the impact of this strategy is influenced by the strength of the antigenic signal (25), as evidenced by the fact that T cell responses against mRNA-encoded self antigens was significantly enhanced. In contrast, the Treg-depletion strategy was less effective in improving CTL responses when DCs presenting high densities of peptide-MHC complexes (peptide pulsing) were used for stimulation.
In order to quantify the presence of CD4+/CD25neg, CD4+/CD25int, and CD4+/CD25high T cells in PBMC samples collected prior to and after DAB389IL-2 treatment, flow cytometry was performed on patient-derived PBMC samples (Figure 3A). Gates were set on CD4+/CD25high T cells that constitutively expressed FoxP3 protein, as shown in Figure 1C. In all 7 subjects analyzed, DAB389IL-2 administration resulted in significant reduction (range 26% to 76%) of CD4+/CD25high Tregs 4 days following intravenous infusion. Additional evidence that Tregs were depleted and that CD25neg/int T cell subsets were unaffected was provided by the observation that the number of total CD25pos cells measured in each patient after DAB389IL-2 administration decreased correspondingly with the number of depleted CD4+/CD25high Tregs (data not shown). Moreover, as shown in 4 RCC patients from whom sufficient cells were available for analysis (02-RCC-DAB, 03-RCC-DAB, 05-RCC-DAB, and 06-RCC-DAB), DAB389IL-2 treatment resulted in a significant reduction of FoxP3 transcripts after DAB389IL-2 treatment, as determined by quantitative real-time PCR (Figure 3B). In these subjects, FoxP3 mRNA copy numbers (normalized to β-actin transcripts) were reduced by approximately 30% to 80% within purified blood CD4+ cells 4 days after DAB389IL-2 administration. Finally, CD4+/CD25+ T cells isolated from CD4+/CD25neg/int T cells prior to but not 4 days after DAB389IL-2 treatment consistently inhibited anti-CD3/CD28-mediated activation of CD4+/CD25+ indicator T cells in all RCC subjects analyzed (Figure 3C), suggesting abrogation of Treg-mediated immunosuppressive activity in vivo. Notably, DAB389IL-2–mediated Treg elimination was transient, since approximately 75% of Tregs were restored within 2 months in the patients' peripheral T cell pool (Figure 4A).

In 5 of 6 evaluable DAB389IL-2–treated subjects, minor reductions (averaging 10%) in absolute neutrophil counts were observed while 1 subject exhibited a decrease of 20%. Accordingly, among these 5 with only minor absolute neutrophil count reductions, there were no significant changes in the relative number of CD3+, CD4+, and CD8+ T cells, B cells, monocytes/macrophages (CD14), and NK cells after treatment. In order to address the concern that...
DAB385IL-2–mediated Treg depletion may induce toxicity within the memory T cell pool (CD4+/CD25int), a series of experiments was performed analyzing CD4+/CD25int T cell function prior to and 4 days after DAB385IL-2 administration. First, IFN-γ ELISPOT analyses were performed on sorted CD4+/CD25int, CD4+/CD25high, and CD4+/CD25low T cell subsets using tetanus toxoid or CMV lysate-pulsed DCs as stimulators. As shown in Figure 4B, only CD4+/CD25low memory T cells were capable of stimulating T cell responses against tetanus or CMV antigens while naive (CD4+/CD25high) and CD4+/CD25low T cells failed to stimulate T cell responses of a significant magnitude. In a second set of experiments, the frequency of IFN-γ secreting T cells was analyzed using CD4+ (Figure 4C) and CD8+ responder T cells isolated from human PBMCs (Figure 4D) prior to and 4 days after DAB385IL-2 administration (DAB) and 2 weeks after 3 vaccination cycles. Over the entire treatment cycle, no differences were observed in the absolute number of T cells with specificity against PBMC RNA-transfected DCs, influenza virus matrix protein 1 (fluM1) mRNA-transfected DCs, CMV lysate–loaded DCs, or tetanus toxoid–loaded DCs. Furthermore, antigen-specific proliferation assays (Figure 4E) revealed strong activities against renal tumor antigens and unchanged activities against the prototype recall antigens fluM1/tetanus toxoid.

Cumulatively, the experiments shown in Figures 3 and 4 demonstrate that administration of a single dose of DAB385IL-2 resulted in significantly reduced numbers of Tregs in the peripheral blood of RCC patients (Figure 3, A and B) and in significant abrogation of Treg-mediated suppressive activity (Figure 3C). Our data further suggest that DAB385IL-2–mediated toxicities against other hematopoietic cells expressing CD25 are unlikely and that lymphopenia-induced T cell proliferation (27) may not represent a significant issue in a vaccination setting.

In vivo stimulation of tumor-specific T cell responses. In order to determine whether DAB385IL-2–mediated Treg depletion is capable of augmenting vaccine-induced CD8+ and CD4+ T cell responses in cancer patients, IFN-γ ELISPOT analyses were performed to determine the frequencies of vaccine-induced, tumor-specific T cells from PBMC samples collected before and 2 weeks after the third vaccination. CD8+ and CD4+ T cells were isolated from preand post-vaccination PBMCs and cultured overnight with tumor RNA-transfected DC targets. As controls, autologous PBMC RNA and/or autologous benign RE-derived RNA-transfected DCs were used for short-term antigenic stimulation. As shown in Figure 5, A and B, only background levels of RE- or PBMC-specific T cells were observed prior to or after vaccination. Also, tumor-specific T cell responses did not significantly increase in independently evaluated subjects after treatment with DAB385IL-2 alone over a period of 28 days (data not shown). In contrast, all
but 1 (08-RCC) patient immunized with tumor RNA-transfected DCs exhibited significant increases in tumor-specific CD8+ and CD4+ T cell frequencies after vaccination (Figure 5B and Table 1). A 2.7-fold median increase (range 0 to 5.1) of tumor-specific CD8+ T cells and a 2.0-fold median increase in tumor-specific CD4+ T cells (range 1.0 to 4.5) was observed in the subjects receiving vaccination alone (Table 1). Although there was significant patient-to-patient variability in the magnitude of T cell responses measured in each patient, vaccination after Treg depletion stimulated significantly higher numbers of tumor-specific CD8+ T cells in RCC patients receiving DAB386IL-2 plus RCC RNA-transfected DCs, when compared with RCC patients receiving vaccination alone (P = 0.011). Moreover, there was a trend toward improved CD4+ T cell responses (P = 0.055) in RCC patients treated with combined therapy (Figure 5B). A 7.9-fold median increase in the number of tumor-specific CD8+ T cells (range 5.4 to 16.2) and a 7.2-fold median increase in CD4+ T cells (range 2.2 to 31.0) was detected in the 6 RCC patients receiving DAB386IL-2 plus vaccination with RCC RNA-transfected DCs. The absolute CD8+ T cell frequencies achieved with combined therapy were remarkably high with up to 0.90% of CD8+ T cells exhibiting tumor specificity after 3 vaccinations. In order to evaluate the temporal evolution of the T cell response stimulated by vaccination with or without DAB386IL-2 therapy, longitudinal monitoring of vaccine-induced CD8+ T cell responses was performed using IFN-γ ELISPOT in 2 subjects from whom sufficient numbers of cells were available for analysis (11-RCC and 05-RCC-DAB). DAB386IL-2–mediated Treg depletion followed by vaccination resulted in a significantly enhanced and also prolonged CD8+ T cell response when compared with the subject receiving vaccination alone. As demonstrated recently (26), the vaccine-induced and tumor-specific CTL response surged over the entire treatment course and peaked approximately 2 weeks after the third and final dose (Figure 5C).

In additional experiments, the cytokine secretion profiles by vaccine-induced and tumor-specific CD4+ T cells were analyzed after combined DAB386IL-2 and active immunotherapy (Figure 6, A and B). PBMCs were collected from 3 study subjects at baseline and 2 weeks after the final vaccination, and CD4+ T cells were isolated by magnetic bead sorting. CD4+ T cells were restimulated with 18 hours with renal tumor RNA-transfected DCs and analyzed for IFN-γ and IL-4 secretion using ELISPOT analysis. As control targets, GFP mRNA-, RE RNA-transfected DCs, and staphylococcal enterotoxin B (SEB) were used. As shown in Figure 6A, vaccination after Treg depletion resulted in significant stimulation of IFN-γ but not IL-4–secreting renal tumor–specific CD4+ T cells while no stimulation against GFP or RE-expressing targets was noted. In addition, human Th1/Th2 flow cytometry–based bead arrays (Figure 6B) confirmed secretion of the Th-1 cytokines IL-2 (increase from 680 to 1270 pg/ml), IFN-γ (160 to 270 pg/ml), and TNF-α (34 to 59 pg/ml) but not Th-2 type cytokines (IL-10 and IL-4) by vaccine-induced CD4+ T cells 18 hours after stimulation with RCC RNA-transfected DCs. This effect was not seen when RE RNA-transfected DCs were used as stimulators. These data further suggest that the vaccine-induced tumor-specific T cell response is directed against tumor-associated antigens but not gene products expressed by normal RE.

In conclusion, we show that Treg depletion using the diphtheria fusion protein DAB386IL-2 is capable of enhancing a vaccine-induced T cell response in patients with advanced RCC. Although only a limited number of patients were studied in this clinical trial, an up to 16-fold increase in tumor-specific CTL frequencies could be measured in subjects receiving combined treatment when compared with individuals receiving vaccination alone. The vaccine-induced T cell frequencies achieved without Treg depletion were similar to those observed in a prior study in which immature tumor RNA-transfected DCs were used for vaccination (1).
Discussion
The objective of this study was to enhance the immunostimulatory efficacy of RNA-transfected DC vaccines by selectively eliminating CD4+/CD25high Tregs in metastatic RCC patients. For Treg depletion, we used the recombinant fusion protein denileukin diftitox (DAB389IL-2) (20) in a human vaccination setting. We show that human CD4+/CD25high Tregs can be eliminated using a single dose of DAB389IL-2 without apparent bystander toxicity and without having an impact on the function of other cells expressing CD25. However, DAB389IL-2 also abrogated DC-mediated activation of T cells in vitro, suggesting that the applicability of this reagent should be restricted to a prevaccination setting (Figure 2C). These preclinical results provided important information regarding the design of our clinical study, in which DAB389IL-2 was administered to RCC patients 4 days prior to DC-based vaccination. This time interval was chosen since, unlike antibodies, DAB389IL-2 is characterized by a short duration of action, with a half-life of approximately 60 minutes, thereby minimizing the possibility of interfering with the ongoing vaccine-induced T cell response.

In this study, DAB389IL-2 profoundly reduced the number of Tregs present in the peripheral blood of RCC patients, reduced levels of peripheral blood–derived Foxp3 transcripts, and abrogated Treg-mediated immunosuppressive activity in vivo. Moreover, significantly higher frequencies of tumor-specific CD8+ T cells could be measured in patients treated with combined DAB389IL-2 and vaccination when compared with subjects receiving the vaccine alone. Also, there was a trend toward an improved CD4+ T cell response after combined therapy. Cumulatively, these data provide several independent lines of evidence that Tregs were depleted in the peripheral blood of RCC patients by using a single dose of the fusion protein DAB389IL-2.

The T cell frequencies achieved after Treg depletion and 3 vaccination cycles were remarkably high with up to 0.90% of all CD8+ T cells demonstrating tumor specificity. No clear correlation between the efficacy of Treg depletion and the magnitude of the vaccine-induced T cell response was observed. Also, serum diphtheria titers did not appear to have an impact on vaccine efficacy (11). The present study further suggests that the degree of Treg depletion achieved using a single dose of 18 µg/kg may be quite variable and that Treg depletion was transient, with several independent lines of evidence that Tregs were depleted in the peripheral blood of RCC patients, reduced levels of peripheral blood–derived Foxp3 transcripts, and abrogated Treg-mediated immunosuppressive activity in vivo. Moreover, significantly higher frequencies of tumor-specific CD8+ T cells could be measured in patients treated with combined DAB389IL-2 and vaccination when compared with subjects receiving the vaccine alone. Also, there was a trend toward an improved CD4+ T cell response after combined therapy. Cumulatively, these data provide several independent lines of evidence that Tregs were depleted in the peripheral blood of RCC patients by using a single dose of the fusion protein DAB389IL-2.

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Methods
Clinical trial design and patient eligibility. Treatment of patients was performed following written informed consent as part of a protocol approved by an Institutional Review Board and the FDA. Patients with histologically confirmed metastatic RCC were eligible for this study. One patient with disseminated OVA was included and treated on a compassionate basis. All patients were required to have adequate hepatic, renal, and neurological function, a life expectancy of more than 6 months, and a Karnofsky performance status of greater than or equal to 70%. Patients had to have recovered from all toxicities related to any prior therapy and not received any chemotherapy, radiation therapy, or immunotherapy for at least 6 weeks prior to study entry. Excluded from the study were patients with CNS metastases, with a history of autoimmune disease, and with serious intercurrent chronic or acute illnesses. Patients on immunosuppressive agents were also excluded. Eligible subjects were randomized with equal probability to receive either a single dose of DAB389IL-2 (18 µg/kg) followed by vaccination with tumor RNA-transfected DCs or vaccination alone. All subjects received 3 intradermal injections of tumor RNA-transfected DCs. The injections were given intradermally at biweekly intervals and consisted of 1 x 107 cells suspended in 200 µl 0.9% sodium chloride at each vaccination. Following treatment, subjects were evaluated for clinical toxicity and immunological and clinical responses. Due to regulatory restrictions and, in some subjects, limited access to tumor tissue, no tumor biopsies were performed.

DAB389IL-2 and vaccine preparation. DAB389IL-2 (ONTAK; Ligand Pharmaceuticals) was provided as a frozen, sterile solution formulated in citrate buffer in 2 ml single-use vials at a concentration of 150 µg/ml. After thawing, DAB389IL-2 was diluted with sterile normal saline to a final concentration of 15 µg/ml and delivered by intravenous infusion over a 30-minute period. Patients were permitted to receive acetaminophen (600 mg) and antihistamines 30 to 60 minutes prior to infusion. For DC culture, a concentrated leukocyte fraction was harvested by leukapheresis. PBMCs were isolated from the leukapheresis product by density gradient centrifugation (Histopaque; Sigma-Aldrich). The semiallogeneic cell fraction was used for DC culture in serum-free X-VIVO 15 medium (Cambrex Corp.) supplemented with recombinant human IL-4 (500 U/ml) (R&D Systems) and recombinant human GM-CSF (rhGM-CSF) (800 U/ml) (ImmuneX Corp.). After 7 days, immature DCs were harvested and transfected with total RNA extracted from tumor tissues histologically classified as clear cell carcinoma. Control RNA used for immunological monitoring studies was isolated from autologous benign renal tissues (RE) or from PBMCs. Transfection of immature DCs was carried out by electroporation. DCs were washed in PBS and resuspended at a concentration of 4 x 107 cells/ml in ViaSpan (Barr Laboratories). Cells were then coelectroporated for 5 minutes with 5 µg RNA per 1 x 107 cells and electroporated in 0.4 cm cuvettes via exponential decay delivery at 300 V and 150 µF (Gene Pulser II; Bio-Rad). After electroporation, cells were resuspended in X-VIVO 15 medium and matured for 20 hours in the presence of 10 ng/ml TNF-α, 10 ng/ml IL-1β, 150 ng/ml IL-6 (R&D Systems), and 1 µg/ml prostaglandin E2 (PGE2) (Cyramz Chemical Co.). Prior to administration, cells were characterized to ensure that they met the typical phenotype of fully mature DCs: Lin−/−, HLA class I and II−/−, CD86−/−, and CD83−/−.

Evaluation of immune status. IFN-γ and IL-4 ELISPOT analyses were performed using PBMCs obtained prior to, during, and after vaccination. PBMCs were cultured overnight in complete RPMI 1640 medium. CD4+ and CD8+ T-cells were isolated from PBMCs by negative depletion (Miltenyi Biotec). After blocking, 1 × 10^7 T-cells and 1 × 10^4 RNA-transfected DCs were added to each well of 96-well nitrocellulose plates (Multiscreen-IP; Millipore) precoated with 2 μg/ml IFN-γ capture antibody (Pierce Biotechnology Inc.) or with IL-4 capture antibody (BD Biosciences — Pharmingen). Plates were incubated for 20 hours at 37°C, and biotinylated IFN-γ detection antibody (Pierce Biotechnology Inc.) or biotinylated IL-4 detection antibody (BD Biosciences — Pharmingen) was added to each well. Cells were then incubated for an additional 2 hours at room temperature, then with streptavidin–alkaline phosphatase (1 μg/ml; Sigma-Aldrich), plates were developed with substrate (KPL). After washing, spots were counted using an automated ELISPOT reader (Zeiss). CTL assays were performed by coculturing RNA-transfected DCs with autologous PBMCs. Cells were restimulated once, and IL-2 (20 units/ml) was added after 5 days and every other day thereafter. After 12 days of culture, effector cells were harvested. Target cells were labeled with 100 μCi of Na^241CrO_4 (PerkinElmer) in 200 μl of complete RPMI 1640 for 1 hour at 37°C in 5% CO_2 and 10^4 labeled target cells were incubated in complete RPMI 1640 medium with effectors cells for 5 hours at 37°C. Then 30 μl of supernatant was harvested, and release of 51Cr was measured with a scintillation counter. For proliferation assays, purified CD3+ T cells were seeded into round-bottom microplates in the presence of mRNA-transfected DCs. T cells alone were used as the background control. After 4 days, 1 μCi of [methyl-H] thymidine (PerkinElmer) was added to each well for an additional 16 hours. Incorporation of thymidine was determined using a liquid scintillation counter. Cytotoxicity of DAB25IL-2 was determined in MTT assays. After 6 hours incubation with varying concentrations of DAB38IL-2, cells were seeded in 96-well plates at a density of 5 × 10^4 cells/well. After 48 hours of incubation, 20 μl MTT from a 5 mg/ml stock was added. After 4 hours, the formazan crystals were solubilized by adding 100 μl isopropanol/0.1 M hydrochloric acid. The absorbance of the formazan product was measured on an ELISA plate reader at 570 nm. Cytokine secretion by vaccine-induced CD4+ T-cells was measured using the human Th-1/Th-2 cytokine kit (Cytokine Bead Array; BD Biosciences — Pharmingen) according to the manufacturer’s instructions. Isolated CD4+ T cells were restimulated overnight with RNA-transfected DCs at a ratio of 10:1.

FACS analysis. Four-color FACS analyses were performed using the following antibodies: anti-CD4 FITC, anti-CD45RO, anti-CD45RA (CALTAG Laboratories), anti-CD25 PE (BD Biosciences — Pharmingen), and anti-GITR (R&D Systems) as well as isotypic controls (CALTAG Laboratories). Sorting of CD4+/CD25+ cells was performed using anti-CD4 (BD Biosciences — Pharmingen) followed by APC-streptavidin (BD Biosciences). Isolated CD4+ T cells were permeabilized with 30 μg/ml digitonin for 45 minutes at 4°C. Subsequently, cells were stained with anti-FoxP3 antibody (Abcam), and R-phycocerythrin anti-CD45RA (BD Biosciences) in the presence of 10 μg/ml digitonin for 30 minutes at 4°C. Following staining, cells were fixed and analyzed by FACS. For intracellular CTLA-4 detection, T cells were permeabilized, fixed, and stained with biotinylated anti-CD152 (BD Biosciences — Pharmingen) followed by APC-streptavidin (BD Biosciences — Pharmingen). A total of 1 × 10^6 cells were suspended in staining buffer (PBS with 1% FCS, 2 mM EDTA, and 0.1% sodium azide) and incubated for 20 minutes at 4°C with the antibody.

Treg functional evaluation. The suppressive activity of Treg isolated from PBMCs of study subjects prior to and 4 days after DAB25IL-2 administration was analyzed, as described previously (28). CD4+CD25+ T-cells were isolated from the PBMCs of study subjects using magnetic bead separation techniques. Cells were washed with PBS, resuspended in complete RPMI 1640 medium, and placed into 96-well round-bottom plates precoated with anti-CD3/CD28 antibodies (0.4 μg/well) (CALTAG Laboratories). CD4+CD25+ cells were plated at 2.0 × 10^3/well alone or in combination with CD4+CD25+ cells in triplicate wells at a ratio of 1:2 (CD4+/CD25+: CD4+CD25+). On day 5, 1 μCi of 3H thymidine was added for the final 16 hours of the cultures. Cells were then harvested on glass fiber filters and assessed for uptake of radiolabeled thymidine.

Detection of FoxP3 transcripts. Details of real-time PCR-based quantification of β-actin transcripts were previously provided by Heiser et al. (2). FoxP3 mRNA transcripts were quantified using the Hs00239585_m1 TaqMan gene expression assay (Applied Biosystems) according to the protocol provided by the manufacturer. A plasmid containing the full-length FoxP3 insert was used to generate standard curves.

Statistics. T cell analysis before and after treatment was performed by IFN-γ ELISPOT on all patients who completed immunotherapy. Increases of antigen-specific CD4+ and CD8+ T cells after vaccination were compared using the Wilcoxon matched-pairs signed rank test, analyzing the null hypothesis that the rates of change in T cell response were equivalent prior to and after therapy. A 2-sided P value of less than 0.05 was considered statistically significant.

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