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

Jens Dannull,1 Zhen Su,1 David Rizzieri,2 Benjamin K. Yang,1 Doris Coleman,1 Donna Yancey,1 Aijing Zhang,1 Philipp Dahm,1 Nelson Chao,2 Eli Gilboa,1 and Johannes Vieweg1

1Genitourinary Cancer Immunotherapy Program, Division of Urology, Department of Surgery, and 2Division of Cellular Therapy, Department of Medicine, Duke University Medical Center, Durham, North Carolina, USA.

In this study, we investigated whether elimination of CD4+CD25+ Tregs using the recombinant IL-2 diaphtheria 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 autoimmunity by regulating immune responses against 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 cells expressing the IL-2 receptor CD25 subunit.

In this study, we used the recombinant IL-2 diaphtheria 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 catalytical and membrane translocation domain of diaphtheria toxin (11). The binding domain for the diaphtheria 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-actinomycin D; CTLA-4, CTL-associated antigen-4; DAB389IL-2, recombinant IL-2 diaphtheria 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-diphenyl tetrazolium 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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Results

Phenotypic and functional characterization of Tregs. 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...
high T cells were isolated from RCC, and CD4 high Tregs exhibited profound neg, and all other T cell subsets exhibited only –, and CD4 high 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 forkhead 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.

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 responder 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 for forkhead 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.

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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 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 DAB389-IL-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 DAB389-IL-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 DAB389-IL-2 did not result in significant cell death except when these cells were exposed to DAB389-IL-2 concentrations higher than 10 nM (latter not shown). In another set of experiments, DAB389-IL-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 DAB389-IL-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 DAB389-IL-2 concentrations while DAB389-IL-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 DAB389-IL-2 on freshly activated lymphocytes was analyzed after stimulation with allogeneic DCs in MLR cultures. DAB389-IL-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 increase in Treg numbers in MLR cultures. Thus, DAB389-IL-2–mediated Treg depletion did not alter the overall stimulation response in MLR cultures.
approximately 80% reduction in T cell proliferation (1:1 Treg/responder ratio) (Figure 2C). Preincubation of Tregs with 5-nM DAB$_{389}$ 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 DAB$_{389}$ IL-2 two days after initiation of MLR (DC+DAB) completely abrogated the proliferation of responder cells, suggesting that DAB$_{389}$ IL-2 eliminated not only Tregs but also freshly activated naive T cells that acquired CD25 expression.

These experiments suggest that DAB$_{389}$ 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, DAB$_{389}$ IL-2 should only be applied prior to immunization but not during the vaccination phase, since activated effector T cells appear susceptible to DAB$_{389}$ IL-2-mediated toxicity.

Enhancement of T cell responses after Treg depletion in vitro. To provide evidence that DAB$_{389}$ 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 DAB$_{389}$ 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-1) mRNA. In addition, DCs pulsed with an HLA-A0201–restricted MART-1 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-1 mRNA than CTLs stimulated from nondepleted PBMCs. In contrast, only modest, statistically insignificant improvement of antigen-specific killing was observed when MART-1 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.
Depletion of Tregs in vivo. Having shown that Treg elimination is capable of enhancing T cell responses in vitro, a clinical study was initiated to test the Treg-depletion concept in a human vaccination setting. A total of 11 patients, including 10 with metastatic RCC and 1 with disseminated ovarian carcinoma (OVA), were treated in a study approved by the Duke University Institutional Review Board and the FDA. Seven subjects received a single intravenous dose of DAB389IL-2 (18 µg/kg) 4 days prior to vaccination with tumor RNA-transfected DCs while a second cohort of 4 subjects was treated with the vaccine alone (Table 1). Outside the study, PBMCs were obtained from 1 additional RCC subject who received a single dose of DAB389IL-2 (18 µg/kg) but no vaccine. Consistent with reports of others, DAB389IL-2–related toxicities included grade I constitutional symptoms such as low-grade fever and malaise (n = 4), grade I elevation of serum rheumatoid factor (n = 1), and transient grade II serum alanine aminotransferase (ALT) elevations (n = 1) (11). RNA-transfected DC injections were well tolerated without any major clinical toxicities or serologic/immunologic evidence of autoimmunity (2, 26).

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 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

Figure 4
Specificity of Treg depletion. (A) Calculated CD4⁺/CD25high Treg frequencies in 2 study subjects (01-RCC-DAB; 03-RCC-DAB) prior to, 4 days after, and 2 weeks after final vaccination (study week 8; d 56). (B) IFN-γ ELISPOT assays were performed on sorted CD4⁺/CD25neg, CD4⁺/CD25int, and CD4⁺/CD25high T cell subsets using tetanus toxoid and CMV lysate–pulsed DCs as stimulators. (C–E) In separate experiments, IFN-γ ELISPOT and antigen-specific proliferation analyses were performed on T cells isolated prior to and 4 and 28 days after DAB389IL-2 treatment (results from patient RCC-01-DAB). For ELISPOT assays, 1 × 10⁶ purified CD4⁺ T cells (C) or 1 × 10⁶ purified CD8⁺ T cells (D) were stimulated with 1 × 10⁵ DCs that were transfected with fluM1 mRNA, autologous PBMC RNA, CMV lysate (20 µg/ml), or tetanus toxoid (0.5 µg/ml). After 18 hours, visible spots were enumerated using an automated ELISPOT reader. The same stimulators and RCC RNA-transfected DCs were used in proliferation assays (E). For proliferation assays, isolated CD3⁺ T cells were used as responders. Assays were performed at a stimulator/responder ratio of 1:10. After 4 days, cells were pulsed with 1 µCi of ³H-thymidine, and incorporated radioactivity was determined after 16 hours by liquid scintillation counting.
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+/CD25neg, and CD4+/CD25high T cell subsets using tetanus toxoid or CMV lysate-pulsed DCs as stimulators. As shown in Figure 4B, only CD4+/CD25int memory T cells were capable of stimulating T cell responses against tetanus or CMV antigens while naive (CD4+/CD25neg) and CD4+/CD25high 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 reactivities against renal tumor antigens and unchanged reactivities 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 pre- and 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 cell responses 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 DAB389IL-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 DAB389IL-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 DAB389IL-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). DAB389IL-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 DAB389IL-2 and active immunotherapy (Figure 6, A and B). PBMCs were collected from 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 for 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 Th1 cytokine IFN-γ, TNF-α, and IL-2 as well as the Th2 type cytokines IL-4 and IL-10.

**Figure 6**
In vivo induction and cytokine profile of RCC-specific CD4+ T cell responses. (A) CD4+ T cells were isolated from pre- (white bars) and post-vaccination (black bars) PBMC samples of 3 study subjects (representative data from patient RCC-01-DAB are shown) who received DAB389IL-2 (18 µg/kg) followed by vaccination with RCC RNA-transfected DCs (2 cycles of 1 × 10^6 cells per treatment). Cells were stimulated for 18 hours with autologous PBMC RNA-, RE RNA-, or RCC RNA-transfected DCs. IFN-γ (left panel) or IL-4–expressing T cells (right panel) were enumerated using an automated ELISPOT reader, and antigen-specific T cell frequencies were expressed as the number of spot-forming cells per 1 × 10^6 CD4+ T cells. Staphylococcal enterotoxin B (SEB) at a concentration of 10 µg/ml was used as a positive control in the IL-4 ELISPOT assays (right panel). (B) The cytokine expression profile of CD4+ T cells prior to (gray) and after (white) vaccination was measured after overnight (18 hours) stimulation with RCC (DC+RCC) or RE RNA-transfected DCs (DC+RE) using human Th1/Th2 cytometric bead arrays. Culture supernatants were used to determine expression of the Th1 cytokines IFN-γ, TNF-α, and IL-2 and as well as the Th2 type cytokines IL-4 and IL-10.
Discussion

The objective of this study was to enhance the immunostimulatory efficacy of RNA-transfected DC vaccines by selectively eliminating CD4+/CD25^{high} Tregs in metastatic RCC patients. For Treg depletion, we used the recombinant fusion protein denileukin diftitox (DAB_{389}IL-2) (20) in a human vaccination setting. We show that human CD4+/CD25^{high} Tregs can be eliminated using a single dose of DAB_{389}IL-2 without apparent bystander toxicity and without having an impact on the function of other cells expressing CD25. However, DAB_{389}IL-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 DAB_{389}IL-2 was administered to RCC patients 4 days prior to DC-based vaccination. This time interval was chosen since, unlike antibodies, DAB_{389}IL-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, DAB_{389}IL-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 DAB_{389}IL-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 DAB_{389}IL-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 significantly higher frequencies of tumor-specific CD8^{+} T cells and vaccine-induced T cell response 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.

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 DAB_{389}IL-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 × 10^{7} 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.

DAB_{389}IL-2 and vaccine preparation. DAB_{389}IL-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, DAB_{389}IL-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 semiadherent 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 × 10^{7} cells/ml in ViaSpan (Barr Laboratories). Cells were then coincubated for 5 minutes with 5 µg RNA per 1 × 10^{6} 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-β, 150 ng/ml IL-6 (R&D Systems), and 1 µg/ml prostaglandin E_{2} (PGE_{2}) (Cayman 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^{high}, CD86^{high}, and CD83^{high}.
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 × 105 T cells and 1 × 104 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 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) and cytokine kit (Cytokine Bead Array; National Cancer Institute) and M01-RR-30 (National Center for Research Resources, Clinical Research Centers Program). We would like to thank Ligand Pharmaceuticals for provision of the fusion protein for the clinical study.

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Address correspondence to: Johannes Vieweg, Duke University Medical Center, MSRB, Suite 455, DUMC Box 2626, Durham, North Carolina 27710, USA. Phone: (919) 684-9949; Fax: (919) 681-7414; E-mail: j.vieweg@duke.edu.