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Partial Reduction of Human FOXP3+ CD4 T Cells In Vivo After CD25-directed Recombinant Immunotoxin Administration

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Summary

The regulation of tolerance to self-proteins and the suppression of T-cell responses have in part been attributed to the activity of $CD25^+CD4^+$ T regulatory (T_{reg}) cells. Further, T_{reg} cells can inhibit the antitumor effectiveness of adoptive immunotherapy and active immunization approaches in preclinical models. In an effort to selectively eliminate T_{reg} cells from human peripheral blood mononuclear cell to potentially bolster antitumor responses, we have evaluated the T_{reg}-cell depleting capacity of the CD25-directed immunotoxin, RFT5-SMPT-dgA. In preclinical studies, incubation of human peripheral blood mononuclear cell with RFT5-SMPT-dgA mediated a partial reduction in the levels of $CD25^+$, *Foxp3*- expressing $CD4^+$ T cells in vitro. Administration of RFT5-SMPT-dgA to 6 patients with metastatic melanoma induced a transient but robust reduction in the number of $CD25^{high}$ $CD4^+$ T cells in vivo (a 97.5% mean reduction at nadir; from 69.4±12.4 cells/μL to 1.7±0.3 cells/μL). The reduction in $FOXP3^+$ $CD4^+$ T-cell number was less comprehensive (a 71.3% mean reduction at nadir; from 66.6±16.5 cells/μL to 14.2±3.9 cells/μL). This resulted in the selective persistence of a stable number of $CD25^{low/neg}$ $FOXP3^+$ $CD4^+$ T cells in vivo. No objective antitumor responses were seen in any patient. Our results indicate that the CD25-directed, RFT5-SMPT-dgA immunotoxin can mediate a transient, partial reduction in T_{reg}-cell frequency and number in vitro and in vivo and suggest that comprehensive eradication of human T_{reg} cells in vivo may require the ability to target and eliminate $FOXP3^+$ $CD4^+$ T cells expressing both high and low levels of CD25.

Keywords

human; immunotoxin; RFT5-SMPT-dgA; CD25; regulatory T cell; depletion

Self/tumor antigens, such as cancer/testis antigens, differentiation antigens and overexpressed self-tissue proteins, are common targets of naturally occurring tumor infiltrating lymphocytes (TILs) and immunotherapeutic strategies. As such, the ability to shift the immune balance from self-tolerance toward anti–self-recognition holds substantial promise for the bolstering of antitumor responses in vivo. Immunosuppressive cellular elements such as T regulatory (T_{reg}) cells, which regulate responses to self-proteins, may thus represent a prohibitive barrier to effective tumor treatment. In human metastatic melanoma, tumor-reactive TIL isolated from excised, progressive lesions regularly recognize self/tumor antigens including the melanocyte differentiation antigens MART-1

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and gp100. Moreover, functional circulating T_{reg} cells and tumor infiltrating, tumor antigen-specific T_{reg} cells from patients with metastatic melanoma have been described.[1,2] Additionally, T_{reg} cells from metastatic melanoma reportedly inhibit the function of the infiltrating T-cell population.[3]

In humans, naturally developing T_{reg} cells comprise about 5% to 10% of the peripheral $CD4^+$ T-cell compartment and seem to preserve homeostatic peripheral self-tolerance via suppression of self-antigen–reactive T cells.[4] Their role as regulators of self-tolerance is evident in autoimmune individuals with immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), a recessive and frequently fatal genetic disorder resulting from a lack of functional T_{reg} cells.[5] Thus, the ability to selectively eliminate T_{reg} cells may strengthen preexistent, self/tumor antigen-specific TIL responses in vivo. However, the ability to comprehensively eliminate human T_{reg} cells in vivo has been limited by the lack of an exclusive cell surface marker for identification and depletion of the entire T_{reg}-cell population. T_{reg} cells constitutively express the high affinity α-chain of the IL-2 receptor (CD25), glucocorticoid- induced tumor necrosis factor receptor (GITR), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and Forkhead box P3 (*Foxp3*),[6] a transcription factor required for T_{reg}-cell development and function.[7] In the absence of clinically-approved strategies for targeted depletion of FOXP3-expressing cells in vivo,[8] clinical efforts to selectively deplete T_{reg} cells have relied heavily on targeting T_{reg}-cell associated surface molecules.

In murine models, administration of a CD25-specific antibody can effectively deplete T_{reg} cells to promote autoimmune induction[9] and improve antitumor responses in vivo.[10–15] The impact of clinical-grade, CD25-depleting reagents on circulating T_{reg}-cell number in vivo and tumor immunity in humans has been largely limited to in vitro study.[16] A clinical-grade CD25-specific antibody (anti-Tac, daclizumab) exists yet possesses a long half-life in vivo which may affect T_{reg} and activated effector T cells alike. Anti-Tac is known to block T-cell activation to reduce immune disorders[17] but its T_{reg}-cell depleting capacity in vivo is not well established.[18] Clinical reagents with short half-lives in vivo, such as immunotoxins (ITs), which combine the targeting specificity of a monoclonal antibody with a cellular toxin, have been used to selectively eliminate cell subpopulations in vivo.[19] RFT5-SMPT-dgA is an IT comprised of the IL-2Rα-specific murine IgG1 antibody RFT5 linked to deglycosylated ricin A chain (dgA) via the sterically hindered heterobifunctional disulfide linker SMPT [4-succinimidyl-oxycarbonyl-α-methyl-a-(2-pyridyldithio)-toluene]. RFT5-SMPT-dgA exhibited potent antitumor activity in severe combined immunodeficiency mice xenografted with L540 cells which express CD25[20] and has been administered in a phase 1 to 2 clinical trial in patients with Hodgkin lymphoma (HL),[21,22] a disease characterized by constitutive CD25 expression on malignant cells.[23] In the current study, RFT5-SMPT-dgA was evaluated for the ability to selectively eliminate $CD25^+$ T_{reg} cells from human peripheral blood mononuclear cells (PBMCs) in vitro and in vivo.

MATERIALS AND METHODS

Patient Samples and Culture Media

Patient PBMCs were isolated by Ficoll-Hypaque separation after obtaining an informed consent and were cryopreserved in heat-inactivated human AB serum (HSA, Gemini Bioproducts, Woodland, CA) with 10% dimethyl sulfoxide and stored at −180°C until the time of study. Serum samples were collected and stored at 4°C until study. Complete media (CM) consisted of RPMI 1640 (Invitrogen Corp, Carlsbad, CA) supplemented with 2mM glutamine (Biofluids, Rockville, MD), 25mM HEPES buffer (Biofluids), 100 U/mL penicillin (Bio-fluids), 100 μg/mL streptomycin (Biofluids), 50 μM 2-mercaptoethanol (Invitrogen), and 10% heat-inactivated fetal bovine sera (FBS, Gemini Bioproducts).

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Treatment Regimen

All patients in this study had metastatic melanoma and were entered on an institutional review board-approved protocol in the Surgery Branch of the National Cancer Institute. Informed consent was obtained from all subjects. Starting on day 0, patients received 3 mg/m^2 of RFT5-SMPT-dgA intravenously every other day for a total of 3 doses. Four to five weeks after the last dose of RFT5-SMPT-dgA, patients were evaluated for tumor response and toxicity. This constituted one treatment course.

Patient Eligibility

Patients who were 18 years of age or greater, with measurable metastatic melanoma which was not responsive to standard therapy, who met standard laboratory safety criteria, and who did not have concomitant major medical illnesses or require steroid therapy were eligible for enrollment. Patients had clinical Eastern Cooperative Oncology Group performance status of 0 or 1 or 2. Eligibility criteria required serum creatinine levels ≤1.6 mg/dL and bilirubin ≤2.0 mg/dL. Serum eligibility criteria included serum albumin >2.5 g/dL, aspartate aminotransferase/alanine aminotransferase <2.5 times normal, and serum human antimouse antibody (HAMA) levels ≤1 μg/mL. Blood eligibility criteria included white blood cell ≥3000/mm^3, and platelets ≥90,000/mm^3. Excluded from the protocol were patients who previously received RFT5-SMPT-dgA on another trial or monoclonal antibody therapy within 12 weeks of enrollment, had prior radiotherapy, had a resting left ventricular ejection fraction of <45%, or extensive lung disease and patients with autoimmune disease, immunodeficiency, HIV infection, or other concurrent malignancies.

IT

The RFT5-SMPT-dgA IT was prepared as described previously[24] by linking the IL-2Rα–specific murine IgG1 antibody RFT5 to deglycosylated ricin A chain (dgA; Inland Laboratories, Austin, TX) via the sterically hindered heterobifunctional disulfide linker SMPT [4-succinimidyl-oxycarbonyl-α-methyl-a-(2-pyridyldithio)- toluene; Pierce Endogen, Rockford, IL]. Vials of drug stored at −80°C were thawed and filtered through a 0.22 μm filter. RFT5-SMPT-dgA was formulated as a sterile solution at 0.5 mg/mL in 0.85% NaCl USP, containing 5mM lysine.

Antibodies and Flow Cytometry

Fluorescein isothiocyanate-conjugated antihuman CD4 and CD8 antibodies and antigen-presenting cell–-conjugated CD3 antibody were all obtained from BD Biosciences (San Jose, CA). For CD25 detection, PE-conjugated CD25 antibody (4E3 clone; Miltenyi Biotech, Auburn, CA) was used. CD25high expression was defined by comparison to isotype antibody staining. FOXP3 (clone PCH101) and control rat IgG2a antibodies were obtained from eBiosciences. Thawed PBMCs were resuspended in fluorescence-activated cell sorting buffer consisting of phosphate-buffered saline with 2% FBS (Gemini Bioproducts) at 10^7 cells/mL and blocked with 10%normal mouse Ig (Caltag Labs, Burlingame, CA) for 10 minutes on ice. Cells (10^6) in 100 μL were stained with fluorochrome-conjugated monoclonal antibody at 4°C for 40 minutes in the dark. In some cases, cells were briefly stained with propidium iodide for nonviable cell exclusion and analyzed in a FACSCalibur (BD Biosciences). FOXP3 staining was performed according to manufacturer's instructions (eBiosciences).

In Vitro Sensitization

The 10-day in vitro sensitization was carried out as previously described.[25] Briefly, after 48-hour culture in CM with or without RFT5-SMPT-dgA, PBMCs were washed and plated at 3 × 10^6/well in 24-well plates with 1 μM soluble peptide [$Flu_{58–66}$ GILGFVFTL or

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$gp100_{280–288(288\ V)}$ YLEPGPVTV] for 10 days. Cells were harvested, washed, and plated in 96-well plates with T2 cells alone or pulsed with either peptide at 1 μM. After 24 hours the supernatant from each well was harvested and interferon-γ was measured using enzyme-linked immunosorbent assay according to the manufacturer's instructions (Pierce Endogen).

Real-time Polymerase Chain Reaction

Levels of mRNA *Foxp3* were analyzed by quantitative real-time polymerase chain reaction using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA) as described previously.[26]

Lymphocyte Separation

$CD4^+$ cells were separated from whole PBMC by magnetic bead selection using negative isolation (Dynal Biotech; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. $CD4^+$ cells were further purified into $CD25^-$ and $CD25^+$ fractions using the Dynal T_{reg} kit according to the manufacturer's instructions. Separations were performed in phosphate-buffered saline with 0.1% bovine serum albumin.

Proliferation Assay

PBMCs treated with 0 or 100 ng/mL RFT5-SMPT-dgA for 48 hours were plated in 96-well plates coated with CD3 antibody (OKT3; 1 μg/mL) at a cell concentration of 50×10^3 PBMCs per well. On days 2 and 4 of cell culture, 1 μCi [^{3}H]-thymidine incorporation was added per well and further cultured for 18 hours before harvesting for measurement on days 3 and 5. Plates were harvested onto nylon filters using the Betaplate system and radioactivity quantified using a Betaplate counter. Results are expressed as the mean counts per minute of 24 cultures ±SEM per condition.

HAMA and Human Antiricin Chain Antibody Detection

HAMA and human antiricin chain antibody (HARA) were measured as described previously.[27]

RESULTS

Impact of RFT5-SMPT-dgA on $CD25^+CD4^+$ T Cells In Vitro

Resting PBMCs were incubated with doses of RFT5-SMPT-dgA ranging from 0 to 1000 ng/mL final concentration in vitro for 48 hours and assessed for CD25 and *Foxp3* expression by $CD4^+$ T cells in 2 independent experiments. At high concentrations, the percentage of $CD3^+$ $CD4^+$ lymphocytes expressing CD25 decreased from 14.9±1.5% to 0.4±0.2%, for a 97.6% mean reduction (Fig. 1A). This paralleled a decrease in *Foxp3* expression from 7.2±1.5 to 1.7±0.1 copies per 10^3 β-actin copies as quantified by real-time quantitative polymerase chain reaction, representing a 77.4% mean reduction (Fig. 1B). Sensitivity to RFT5-SMPT-dgA was detectable at 10 ng/mL, but a maximum impact was observed near 100 ng/mL. Serial harvesting of PBMC at 12, 24, 48, and 72 hours after a single administration of IT at 100 ng/mL suggested maximum reduction in CD25 and *Foxp3* expression by $CD4^+$ T cells occurred beginning 48 hour after exposure in vitro (data not shown).

To quantify the impact of RFT5-SMPT-dgA on resting T_{reg} cells, large numbers of freshly isolated PBMCs were treated with or without IT. After 48-hour incubation, PBMCs were mechanically sorted into $CD4^+$ fractions by negative isolation and then into $CD4^+CD25^-$ and $CD4^+CD25^+$ fractions and counted (Fig. 1C). In 2 independent experiments performed on separate patient PBMC samples (containing 1.5×10^9 and 3.0×10^8 cells, respectively), the impact of RFT5-SMPTdgA on the absolute number of $CD25^+CD4^+$ cells was profound,

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producing a 94.1% and 73.3% reduction compared with untreated controls (from 5.1×10^6 to 0.3×10^6 and from 0.15×10^6 to 0.04×10^6, respectively). In both experiments, the impact upon the absolute $CD4^+$ count (+1.3% and −18.8%) and the absolute $CD4^+CD25^-$ count (+8.5% and −11.1%, respectively) was minimal, suggesting a preferential cytotoxicity of RFT5-SMPT-dgA directed against cells expressing CD25. Together, these data demonstrate the capacity of the CD25-directed IT, RFT5-SMPT-dgA, to mediate a partial elimination of human regulatory T cells in vitro.

To determine the impact of RFT5-SMPT-dgA treatment on the surviving non-$CD25^+$ T-cell population, we evaluated their proliferation and reactivity in vitro. PBMC harvested after a 48-hour culture in CM alone or containing 100 ng/mL RFT5-SMPT-dgA were stimulated with plate-bound anti-CD3 antibody and measured for [^{3}H]-thymidine incorporation on days 3 and 5 of cell culture (Fig. 1D). No difference in proliferation was detected at either time point suggesting that the surviving non-$CD25^+$ cells were unharmed by treatment. To test the ability of IT-surviving effector cell precursors to respond to peptide stimulation, RFT5-SMPT-dgA treated and untreated PBMCs were sensitized in vitro with relevant ($Flu_{58–66}$), irrelevant [$gp100_{280–288\ (288\ V)}$], or no soluble peptide for 10 days. The cells were then washed, cultured overnight with T2 cells alone or pulsed with each peptide and assayed for interferon-γ secretion (Table 1). In 2 independent experiments, both the untreated and RFT5-SMPT-dgA-treated PBMC responded only to relevant $Flu_{58–66}$ peptide stimulation, however, the magnitude of response was greater in the treated group (4177 vs. 2517 pg/mL, Table 1; 4720 vs. 799 pg/mL, data not shown). Thus, the response to antigen stimulation among RFT5-SMPT-dgA-surviving cells was equivalent, if not better than, that of the untreated cells suggesting that the IT pretreatment had no deleterious effects on the remaining non-$CD25^+$ $CD4^+$ and $CD8^+$ cells.

As RFT5-SMPT-dgA could mediate a partial reduction in $CD25^+$ *Foxp3*-expressing $CD4^+$ T-cell numbers in vitro without impairing the function of the remaining lymphocytes, a phase 2 prospective trial was developed to determine whether objective clinical responses could be obtained in patients with metastatic melanoma after administration of RFT5-SMPT-dgA, with secondary objectives to determine whether administration of RFT5-SMPT-dgA could mediate changes in circulating $CD25^+$ $CD4^+$ T_{reg}-cell levels and evaluate the toxicity profile of patients treated.

Patient Characteristics and Treatment

Six patients with progressive metastatic melanoma (5M/1F) were enrolled to receive RFT5-SMPT-dgA therapy; 5 patients had visceral metastases. Patients age ranged from 39 to 64 years old (mean of 51.5±4.2). All patients had received prior surgery, chemotherapy, and immunotherapy. The dosing regimen consisted of RFT5-SMPT-dgA (3 mg/m^2) administered intravenously every other day for 3 total doses (9 mg/m^2 total) with patient evaluation occurring 4 to 5 weeks after the last dose of IT. All patients received a full course of RFT5-SMPT-dgA therapy, except 1 patient who was withdrawn from the protocol after experiencing abdominal pain and hypotension shortly after administration of the first IT dose.

Treatment-related Toxicities, Antibody Responses, and Clinical Outcome

As established in a previous phase 1 trial in patients with HL, RFT5-SMPT-dgA has a maximum-tolerated dose (MTD) of 15 mg/m^2 per course with moderate toxicities including vascular leak syndrome, dyspnea, fatigue, and malaise.[22] Although RFT5-SMPT-dgA has been administered to patients with hematologic cancers, the impact of RFT5-SMPT-dgA on patients with metastatic melanoma, who have comparatively smaller numbers of circulating $CD25^+$ lymphocytes, is unknown. A sub-MTD of 9 mg/m^2 per course was therefore chosen

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based upon the findings that grade III or IV toxicities were seldom observed among patients treated at doses of 5 to 10 mg/m^2 and the suggestion that T_{reg} cells might be sensitive to RFT5-SMPT-dgA at lower doses.[21,28] Patients with metastatic melanoma receiving IT at 9 mg/m^2 per course experienced a similar panel of toxicities (Table 2). Mild to moderate side effects were observed in the 6 patients receiving RFT5-SMPT-dgA, including 4 cases of grade II hypoalbuminemia and 3 cases of grade II hypocalcemia. Individual cases of grade I/II fatigue, fever, dyspnea, abdominal pain, leukopenia, and altered transaminase levels were also observed. Few patients experienced grade III events but individual cases of fatigue, transient VLS, hypoalbuminemia, and a line placement-related infection did develop (Table 2).

As an eligibility criterion, all enrolled patients were negative for HAMA response before treatment. To determine whether immune responses had been elicited against RFT5-SMPT-dgA after treatment, serum from treated patients was assayed for HAMA and HARA responses (Table 3). After RFT5-SMPT-DGA therapy, HAMA responses were detectable in assessed serum samples from 2/4 patients. In the testing of nearly 1000 patients from multiple trials, HARA were seldom detected (<1.3%) and were therefore not tested before treatment. After IT treatment, 2/4 patients, the same 2 patients who developed HAMA, also developed HARA responses. Of note, patient 1 developed a profound HARA response of >5 mg/mL, which was persistent more than 2 months after the start of treatment. HARA were not detected in a retrospective analysis of serum from this patient obtained before treatment. The development of HAMA and HARA responses in these patients is consistent with the HAMA and HARA induced in 69% of HL patients receiving RFT5-SMPT-dgA at the MTD.[22] No patient receiving RFT5-SMPT-dgA therapy experienced an objective cancer regression (0/5) or overt autoimmune disease, however, patient 1 has stable disease 14 months after the start of therapy.

IT-mediated Reduction in CD25$^+$ FOXP3+ CD4$^+$ T Cells In Vivo

At the start of RFT5-SMPT-dgA therapy, the mean patient lymphocyte cell count was 1614±191 cells/μL (Fig. 2A). After administration of the first dose of IT, a minor, transient reduction in mean lymphocyte count was observed on day 1 (a 17.5% reduction to 1346±194 cells/μL; $P \leq 0.009$, 2-tailed paired *t* test for means) and again on day 5 postinfusion (a 20.4% reduction to 1278±151 cells/μL; $P \leq 0.009$). Cell counts were similar to pretreatment levels at the intervening days. One month after the first dose of RFT5-SMPT-dgA, the mean cell count was restored to pretreatment levels.

To determine the impact of RFT5-SMPT-dgA on CD25$^+$ T_{reg} T cells in vivo, circulating CD4$^+$ T cells from patients receiving the entire IT dosing regimen were measured for expression of CD25. Representative kinetic analysis in Figure 2B shows that CD25 expression on CD4 T cells from the peripheral blood of patient 4 was substantially reduced for the first week after initial IT administration and was restored to pretreatment levels after 1 month. The mean frequency of CD25 expressing CD4$^+$ T cells from all patients was significantly reduced beginning on day 1 after the start of therapy from 10.3±1.4% to a 0.7±0.3% ($P<0.0001$; Fig. 2C). This reduction was maintained for the entire week after the first IT infusion. At 1 month after the first IT dose, the mean frequency of CD4 T cells expressing CD25 (9.0%±1.9%) was similar to pretreatment levels. Similarly, the mean frequency of CD25$^+$ CD8$^+$ T cells from all treated patients was reduced from 3.8%±1.2% to nearly 1% for the entire week after the first IT-dose and restored to normal levels after 1 month (4.3%±3.2%; data not shown).

To better determine the impact of RFT5-SMPT-dgA on T_{reg} cells in vivo, CD4$^+$ T cells were measured for expression of FOXP3 protein, a surrogate marker of T_{reg} cells whose intracellular expression cannot be directly affected by potential blocking effects of RFT5-

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SMPT-dgA (Figs. 2B, C). Before IT administration, the mean percentage of $CD4^+$ CD3+ cells expressing FOXP3 protein from all patients was 10.0%±2.8%. Figure 2B shows that FOXP3 expression was primarily restricted to $CD4^+$ T cells expressing high levels of CD25, although FOXP3 was also detected in a subset of $CD4^+$ T cells expressing lower levels of CD25. RFT5-SMPT-dgA administration induced a reduction in mean FOXP3+ $CD4^+$ T-cell frequency that was significant from 3 to 7 days after the first IT dose ($P \leq 0.05$) with a nadir of 2.2±1.3% FOXP3+ $CD4^+$ T cells occurring on day 4 (Fig. 2C). Thus, the mean frequency of FOXP3+ $CD4^+$ T cells was reduced by up to 78% after RFT5-SMPT-dgA administration. One month after the first dose of IT, the mean frequency of FOXP3+ $CD4^+$ T cells (8.5% ±1.9%) was restored to pretreatment levels. These results show that the reduction in circulating FOXP3+ CD4 T-cell frequency mediated by RFT5-SMPT-dgA infusion was significant, albeit transient and incomplete.

To quantify to impact of RFT5-SMPT-dgA-therapy on circulating lymphocyte numbers, $CD25^+$ or FOXP3+ $CD4^+$ T-cell counts were enumerated and compared with total T-cell and $CD8^+$ T-cell numbers (Fig. 2D). Compared with pretreatment numbers (1264±145 cells/μL), total $CD3^+$ T-cell counts were not significantly reduced after IT-administration, with the exception of day 5 of therapy (951±87 cells/μL; $P \leq 0.003$). A similar effect was observed for $CD4^+$ $CD3^+$ T cells, dropping from 789±118 cells/μL to 566±57 cells/μL on day 5 ($P \leq 0.01$). Despite the fact that few circulating $CD8^+$ T cells expressed CD25 before treatment (3.8%±1.2%; data not shown), $CD8^+$ T-cell counts, which began at 320±104 cells/μL, were significantly reduced on days 1, 2, 5, and 6 post-IT infusion with a maximum significant reduction of 29.5% on day 5 ($P < 0.02$). The mean frequency of $CD25^+$ $CD8^+$ T cells from all treated patients was reduced to nearly 1% for the entire week after the first IT-dose and restored to normal levels after 1 month (4.3%±3.2%). The greatest overall effect of IT-administration, however, was observed on $CD25^+$ $CD4^+$ T-cell counts which were significantly reduced from 69±12 cells/μL before therapy, for the entire 7 days after the first RFT5-SMPT-dgA dose ($P \leq 0.05$), with a nadir of 1.7±0.3 cells/μL on day 5 for a 97.5% mean reduction. Albeit to a lesser degree, FOXP3+ $CD4^+$ T-cell counts were similarly reduced after RFT5-SMPT-dgA infusion with statistically significant reductions occurring 4 and 5 days after the first dose of IT (from 66±16 cells/μL to 14±4 cells/μL and 36±14 cells/μL, respectively; $P \leq 0.004$), accounting for a 71.3% mean reduction at nadir on day 4.

Selective Survival of CD25int FOXP3+ CD4 T Cells In Vivo

Although CD25 is highly expressed on the surface of the majority of FOXP3+ CD4 T cells in vivo, a smaller number of FOXP3+ $CD4^+$ T cells do not express high levels of CD25 (CD25int, Fig. 2B). Accordingly, these latter cells may elude the cytotoxic effects of the CD25-directed IT in vivo and thereby account for the inability of RFT5-SMPT-dgA to mediate comprehensive elimination of FOXP3+ CD4 T cells in vivo. To determine the impact of CD25 expression on the susceptibility of FOXP3+ T cells to the cytotoxic effects of RFT5-SMPT-dgA, the frequency and number of FOXP3+ CD4 T cells expressing high or intermediate/low levels of CD25 was measured. Before treatment, the overall frequency of CD4 T cells that were FOXP3+ and expressed either high or intermediate/low levels of CD25 was similar (4.7±1.2% were CD25high; 5.2±1.7% were CD25int; Fig. 3A). CD25high FOXP3+ CD4 T-cell frequencies were significantly reduced for the first 6 days after first IT dose to 0.4%±1.7% on day 4 for a 98.7% reduction ($P < 0.02$; Fig. 3B). Alternatively, CD25int FOXP3+ CD4 T cells were not significantly reduced at any time point after IT administration, indicating their continued survival. It is possible that our inability to detect CD25high FOXP3+ cells after IT therapy results in part from blocking of CD25 by residual bound RFT5-SMPT-dgA. However, use of another CD25 detecting antibody specific for an alternative CD25 epitope yielded nearly identical results, suggesting that the CD25 high population had in fact been eliminated (data not shown). Isolation and

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assessment of the CD25int FOXP3+ CD4 T-cell population for in vitro suppressor function was technically inhibited by the inability to selectively enrich live FOXP3-expressing cells, coupled with the lack of high CD25 expression by CD4 T cells after IT administration. Nevertheless, our data show that the limitation of this CD25-directed IT therapy for comprehensive elimination of FOXP3+ T_{reg} cells in vivo seems to reflect the innate inability of RFT5-SMPT-dgA to selectively target and destroy FOXP3+ T cells that do not express high levels of CD25.

DISCUSSION

$CD25^{high}$ $CD4^+$ T_{reg} cells represent a good therapeutic target in the treatment of human cancers because these cells exhibit immune-suppressive function and are prevalent in the peripheral blood and tumor microenvironment of these individuals.[29] Indeed, T_{reg} cells reportedly contribute to the growth of human ovarian carcinomas in vivo by suppressing tumor-specific T-cell immunity and are associated with reduced survival of patients with ovarian cancer.[30] The noted association between objective tumor regression and autoimmunity in patients receiving nonspecific immunotherapeutic agents such as IL-2[31] and anti-CTLA4 antibody,[32,33] and after lymphodepleting preconditioning and autologous transfer of melanoma-reactive T cells[31,32,34] suggests that immune responses directed against self-proteins may induce cancer regression and supports the use of self-tolerance–modulating agents for cancer therapy. In mouse studies, immune-modulation via elimination of $CD25^+CD4^+$ T_{reg} cells can markedly improve cancer immunotherapy. [13,35] For example, cotransfer of CD25-depleted $CD4^+$ T helper cells with tumor/self-reactive $CD8^+$ T cells and vaccination into $CD4^+$ T-cell deficient recipient mice resulted in regression of established tumor and concomitant autoimmunity, whereas cotransfer of $CD25^+$ T_{reg} cells abolished this effect.[35] These findings suggest that T_{reg} cells may account, in part, for the poor clinical response rates reported in cancer patients receiving conventional immunotherapy and that elimination of T_{reg} cells via the selective depletion of CD25-expressing cells in vivo may improve cancer therapy.

As recombinant ITs can deliver a cytotoxic signal targeted by the specificity of a high affinity monoclonal antibody,[36] we evaluated whether a CD25-directed IT could serve as a clinical agent to target and eliminate $CD25^+$ T_{reg} cells in vivo. In preclinical testing, incubation of human PBMC in vitro with 1000 ng/mL of RFT5-SMPT-dgA induced a 98% reduction in $CD25^+$ CD4 T-cell frequency with a concomitant 77% reduction in *Foxp3* copy number. The capacity of RFT5-SMPT-dgA to reduce T_{reg} cells in vitro was similar to that noted in our previous evaluation of another CD25-directed IT, LMB-2.[16] There was no detectable negative impact on the CD25-negative T-cell population surviving RFT5-SMPT-dgA incubation. In fact, the reactivity of in vitro expanded Flu-specific T cells from IT-treated PBMC was increased relative to the untreated PBMC control in the 2 PBMC samples tested. On the basis of its ability to reduce T_{reg} cells in vitro while preserving the function of the remaining cell population, RFT5-SMPT-dgA was selected for clinical evaluation.

In a previous phase 1/2 trial to determine whether administration of RFT5-SMPT-dgA could reduce the risk of graft-versus-host disease after human leukocyte antigen-matched, unrelated marrow transplantation by targeting allo-activated T cells expressing CD25, acute graft-versus-host disease was exacerbated in patients who received small doses of RFT5-SMPT-dgA (1 to 3 mg/m^2) suggesting a susceptibility of $CD25^+$ T_{reg} cells to the toxic effects of the IT.[28] The impact of IT administration on $CD25^+$ T_{reg}-cell number and frequency in vivo was, however, not evaluated. In the current trial, patients with metastatic melanoma received a total of 9 mg/m^2 of RFT5-SMPT-dgA; less than the 15 mg/m^2 MTD established in patients with HL.[22] Treatment-related toxicities were largely mild to moderate and consistent with previously reported results[22] with few grade III toxicities observed

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suggesting that patients with melanoma are not at an increased risk for toxicities compared with patients with hematologic malignancies who harbor comparatively large numbers of circulating $CD25^+$ cells. Two of four evaluated patients developed positive HAMA and HARA responses. Importantly, administration of RFT5-SMPT-dgA induced a transient but robust 97.5% mean reduction in the number of $CD25^{high}$ $CD4^+$ T cells in vivo at nadir that was maintained for at least 1 week. The reduction in $FOXP3^+$ $CD4^+$ T-cell number was less comprehensive (a 71.3% mean reduction at nadir; from 66.6±16.5 cells/μL to 14.2±3.9 cells/μL) resulting in the selective persistence of a relatively stable number of $CD25^{int/low}$ $FOXP3^+$ $CD4^+$ T cells in vivo. Whether RFT5-SMPT-dgA administration mediated the elimination of activated self/tumor antigen-specific T cells in vivo is unknown. Despite the transient reduction in T_{reg}-cell numbers, no patient experienced an objective clinical response or autoimmunity suggesting a more comprehensive and/or durative elimination may be necessary to alter the balance in self-tolerance in vivo. Although our trial was designed to treat up to 41 patients to determine whether the RFT5-SMPT-dgA can produce a response rate targeted to be 20%, we chose to terminate the trial after laboratory data showed an incomplete elimination of T_{reg} cells in all 6 treated patients. Eligibility for retreatment required disease stabilization or a clinical response without the development of positive HAMA or HARA responses. Because no patient fulfilled this criterion, the impact of multiple course RFT5-SMPTdgA administration on T_{reg} cells reduction or cancer regression was not evaluated. Further clinical investigation will be required to resolve whether the level of T_{reg}-cell depletion achieved in these 6 heavily pretreated patients is sufficient to induce cancer regression in a subset of patients with melanoma or other cancers.

IT-mediated elimination of T_{reg} cells was not complete on the basis of the evaluation of the surrogate T_{reg}-cell marker, FOXP3. Our data and that of others[37] show that although CD25 is highly expressed on the surface of many FOXP3+ CD4 T cells in vivo, a second population of human FOXP3+ $CD4^+$ T cells expresses low to intermediate levels of CD25 and may thereby be resistant to the eliminatory effects of CD25-directed clinical agents. Accordingly, we found that although the frequency of FOXP3+ CD4 T cells that were $CD25^{high}$ or $CD25^{int/low}$ was similar before treatment, only $CD25^{high}$ FOXP3+ CD4 T-cell frequencies were significantly reduced after IT dosing. The mean frequency of $CD25^{int/low}$ FOXP3+ CD4 T cells was not significantly reduced at any time point. Our data indicate that the inability of RFT5-SMPT-dgA and other high affinity IL-2 receptor targeted agents to mediate comprehensive elimination of FOXP3+ CD4 T cells in vivo may result from an intrinsic inability to selectively target and destroy FOXP3+ T cells that do not express high levels of CD25. This may also help explain the observed disconnect between level of CD25 and *Foxp3* reduction seen after treatment of human PBMC with the CD25-directed IT LMB-2 in vitro.[38] The limitation of CD25-directed approaches for the total elimination of T_{reg} cells in vivo suggests that alternative strategies which target a combination of T_{reg}-cell–associated surface molecules or directly target and eliminate FOXP3+ cells[8] in vivo may be more effective. Elucidating the cell surface phenotype of the surviving $CD25^{int/low}$ FOXP3+ CD4 T cells in vivo may thus be important in developing successful combinatory T_{reg}-cell depleting clinical approaches.

In previous clinical trials designed to selectively eliminate T_{reg} cells in vivo through targeting of the IL-2 receptor, comprehensive elimination of T_{reg} cells has also remained elusive and the impact of T_{reg}-cell depletion on cancer treatment still remains unknown. Selective depletion of functional $CD25^+$ T_{reg} cells from large-scale patient cell samples has been performed ex vivo under clinical-grade conditions for use in adoptive immunotherapy. [26] However, in vivo transfer of autologous CD25-depleted mononuclear populations to lymphopenic patients in combination with high-dose IL-2 was not sufficient to mediate prolonged reduction of T_{reg} cells, and contrarily resulted in a transient elevation in T_{reg}-cell numbers in vivo.[39] Candidate T_{reg}-cell depleting agents, which are easily

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administered to patients, have been more commonly used in clinical studies. Indeed, administration of anti–CTLA-4 antibody is effective in inducing cancer regression and autoimmunity in treated patients,[32,33] although the antitumor effects of CTLA-4 blockade seem related to increased T-cell activation rather than inhibition or depletion of T_{reg} cells which express CTLA-4.[40] Alternatively, an incomplete, transient reduction in T_{reg} cells in vivo was seen after administration of LMB-2 IT to 8 patients with melanoma, however, LMB-2 therapy did not augment immune responses to cancer vaccination and no patient responses were observed.[41] In another approach, DAB_{389}IL-2 (denileukin diftitox, ONTAK), a cytotoxin comprised of the interleukin-2 cytokine enzymatically linked to the active portion of the diphtheria toxin,[42] has been clinically used to direct the toxic effects of diphtheria to malignant cells of hematologic origin expressing CD25.[43–48] In the treatment of 13 patients with metastatic melanoma with DAB_{389}IL-2, we found no significant impact on $CD25^+$ T_{reg} cells or cancer regression.[49] This was consistent with the inability of DAB_{389}IL-2 to eliminate $CD4^+CD25^+$ T_{reg} cells in vitro. In some patients, the frequency of $CD25^+CD4^+$ cells actually increased as did normalized levels of *Foxp3* expression. In 2 other clinical studies, DAB_{389}IL-2 mediated an incomplete reduction in T_{reg} cells in vivo that was reportedly capable of augmenting the potency of cancer vaccines.[50,51] The impact of these reductions on clinical response was not evaluated. The mechanisms which account for the inability of DAB_{389}IL-2 to comprehensibly eliminate T_{reg} cells in vivo are not known but may be attributable to the ability of DAB_{389}IL-2 to provide homeostatic IL-2 signals to T_{reg} cells,[52,53] a low binding affinity of IL-2 for its receptor relative to antibodies, or the selective survival of T_{reg} cells expressing the low affinity IL-2 receptor complex[37] as suggested by the current study.

The ability to surmount peripheral self-tolerance may hold significant promise to bolster in vivo antitumor responses in patients with cancer. Overcoming the immunoregulatory influences of T_{reg} cells in vivo will require clinical-grade reagents and novel approaches to specifically target and comprehensively eliminate or abrogate the function of T_{reg} cells in vivo.

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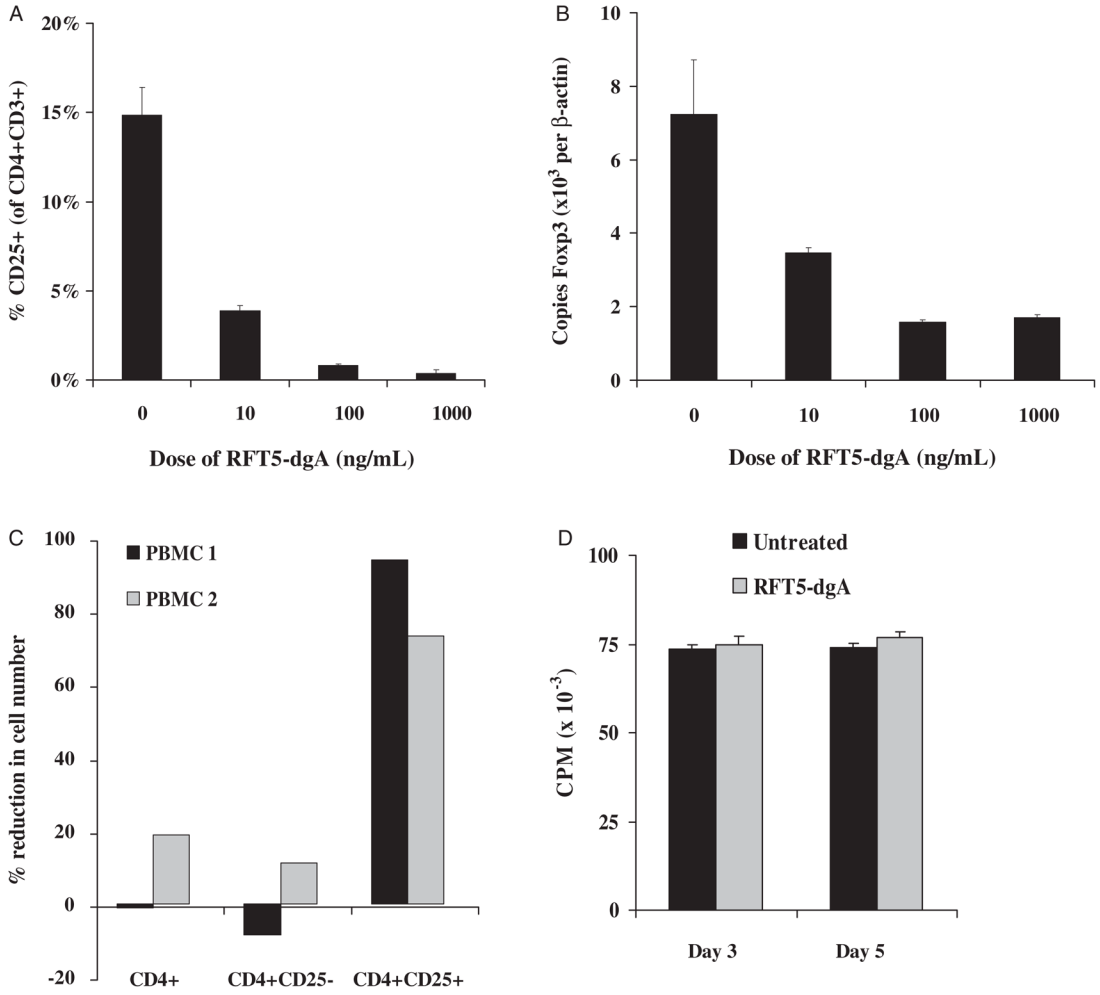

FIGURE 1.

Varying doses of RFT5-SMPT-dgA were incubated with resting human PBMC for 48 hours and the percent of residual $CD25^+$ $CD4^+$ cells (A) and the number of *Foxp3* mRNA copies per 104 copies of β-actin mRNA (B) evaluated. A dose-related reduction in these 2 surrogate markers of human T_{reg} cells was seen. This experiment was representative of 3 independent dose titrations performed. C, Whole $CD4^+$, $CD4^+$ $CD25^-$, or $CD4^+$ $CD25^+$ cell subsets were purified from resting human PBMC after 48-hour incubation in CM with or without RFT5-SMPT-dgA (100 ng/mL) and cell yield determined in 2 independent experiments. Percent reduction was calculated as cell number of the IT-treated PBMC subset relative to the cell count of the untreated PMBC subset. D, PBMCs were cultured for 48 hours in CM containing RFT5-SMPT-dgA (100 ng/mL) or CM alone (untreated), washed, stimulated with plate-bound anti-CD3 antibody and measured for $[^3H]$-thymidine incorporation on days 3 and 5 of cell culture. Results are expressed as the mean counts per minute of 24 independent well cultures ± SEM per condition.

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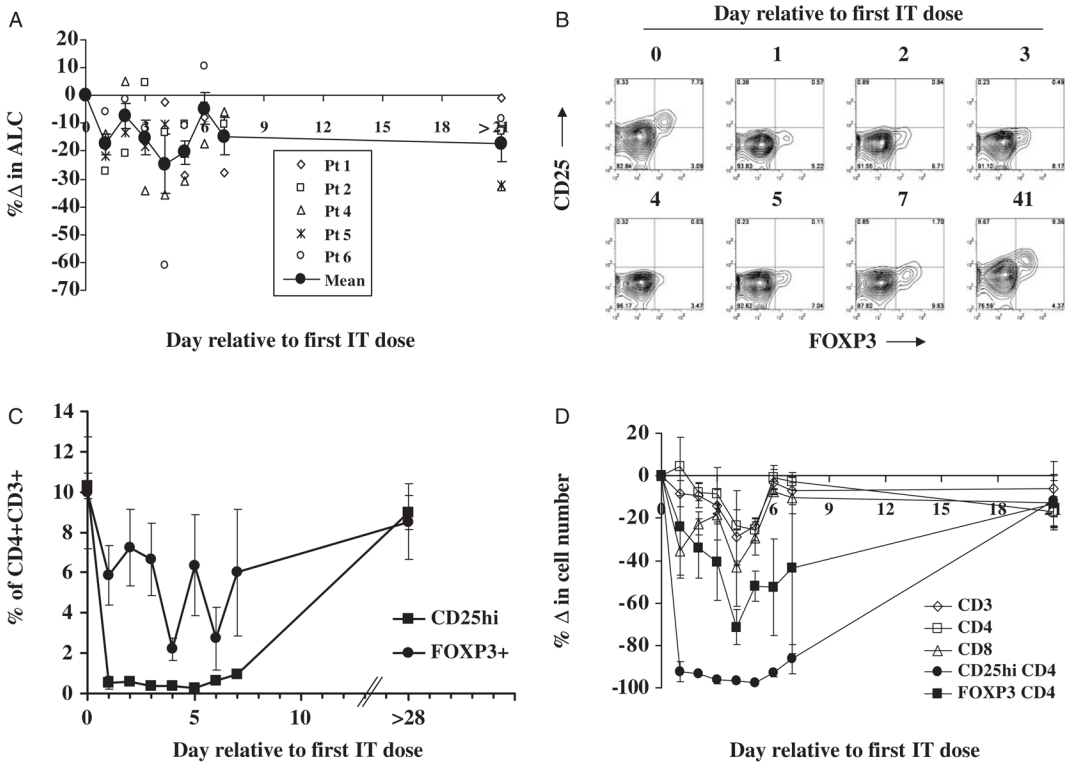

FIGURE 2.

A, The percent change in absolute lymphocyte count (ALC) was calculated relative to pretreatment ALC values on day 0. The mean percent change in ALC ± SEM is shown in filled circles. B, Representative dot plots show the expression of CD25 (*y*-axis) and FOXP3 (*x*-axis) on $CD4^+$ $CD3^+$-gated T cells before and on the indicated day after the start of IT-therapy in PBMC from patient 4. C, The mean frequency of $CD4^+$ $CD3^+$-gated T cells from all treated patients that expressed high levels of CD25 or FOXP3 ± SEM was longitudinally measured. D, The absolute number of T cells ($CD3^+$) that were $CD4^+$, $CD8^+$, $CD25^{high}$, or FOXP3+ was calculated and the percent change relative to pretreatment cell numbers determined.

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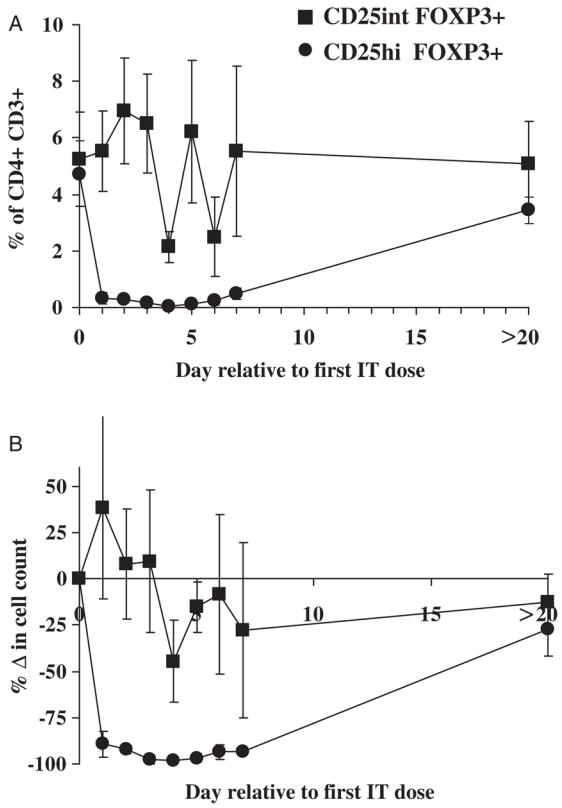

FIGURE 3.

FOXP3+ $CD4^+$ T cells expressing intermediate levels of CD25 persist after CD25-directed IT therapy. A, The frequency of FOXP3+$CD4^+CD3^+$-gated T cells expressing either intermediate (squares) or high (circles) levels of CD25 after administration of RFT5-SMPT-dgA therapy was calculated. B, The mean change in the absolute number of FOXP3+$CD4^+$ $CD3^+$ T cells was calculated relative to levels measured before the initiation of IT therapy.

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TABLE 1

Effector Cell Precursors are not Negatively Affected by RFT5-SMPT-dgA Pretreatment In Vitro

	Preconditioning					
	CM Alone			CM+RFT5-dgA		
Stimulation	**None**	**g280**	**Flu**	**None**	**g280**	**Flu**
Flu/T2	112	74	2517	46	54	4177
g280/T2	670	607	602	664	579	553
T2 alone	44	31	118	50	59	99
None	39	49	39	50	50	45

Patient PBMC were incubated with or without RFT5-SMPT-dgA at 100 ng/mL for 48 h, washed and cultured for 10 d at 3×10^6 cells/well in CM containing IL-2 (50CU/mL) alone (none) or with 1 μM of soluble peptide (Flu: $Flu_{58–66}$ GILGFVFTL; g280: $gp100_{280}$).

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TABLE 2

Toxicity Events After RFT5-SMPT-dgA Treatment

	Grade			
Toxicity	**I**	**II**	**III**	**IV**
Fatigue		1	1	
Fever	1			
Line infection			1	
Abdominal pain	1	1		
Dyspnea	1			
Back pain	1	1		
Joint pain	1			
aVLS			1	
Hypoalbuminemia		4	1	
Hypocalcemia		3		
Hypophosphatemia		1		
Hyperkalemia		1		
Hemoglobin		1		
Leukopenia		1		
Neutropenia		1		
SGPT ALT		1		
SOT AST		1		
Total	4	18	4	0

aVLS indicates acute vascular leakage syndrome; SGPT ALT, serum glutamate pyruvate transaminase or alanine transaminase; SOT AST, serum glutamic oxaloacetic transaminase or aspartate transaminase.

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TABLE 3

HAMA/HARA Responses in Patients Receiving Immunotoxin Therapy

Treated Patient	Day (Relative to First Dose)	HAMA (ng/mL)	HARA (ng/mL)
1	Pre	0	0
	28	NT	**5200678**
	35	**3931**	**4002919**
	69	**13076**	**500470**
2	Pre	0	NT
	35	0	0
3*	Pre	0	NT
4	Pre	0	NT
	33	0	0
5	Pre	0	NT
	27	**1718**	**148154**
6	Pre	0	NT
	Post	NT	NT

Values represent serum antibody concentrations in ng/mL.

Antibody concentrations >1 μg/mL are bolded.

*Patient treatment was terminated due to toxicities.

NT indicates samples not tested; Pre, pretreatment.