Monoclonal antibody blockade of IL-2 receptor α during lymphopenia selectively depletes regulatory T cells in mice and humans

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Lymphodepletion augments adoptive cell transfer during antitumor immunotherapy, producing dramatic clinical responses in patients with malignant melanoma. We report that the lymphopenia induced by the chemotherapeutic agent temozolomide (TMZ) enhances vaccine-driven immune responses and significantly reduces malignant growth in an established model of murine tumorigenesis. Unexpectedly, despite the improved antitumor efficacy engendered by TMZ-induced lymphopenia, there was a treatment-related increase in the frequency of immunosuppressive regulatory T cells (TRegs; P = .0006). Monoclonal antibody (mAb)–mediated inhibition of the high-affinity IL-2 receptor α (IL-2Rα/CD25) during immunotherapy in normal mice depleted TRegs (73% reduction; P = .0154) but also abolished vaccine-induced immune responses. However, during lymphodepletion, IL-2Rα blockade decreased TRegs (93% reduction; P = .0001) without impairing effector T-cell responses, to augment therapeutic antitumor efficacy (66% reduction in tumor growth; P = .0024). Of clinical relevance, we also demonstrate that anti–IL-2Rα mAbs function differentially in nonlymphopenic versus lymphopenic contexts. (Blood. 2011;118(11):3003-3012)

Introduction

The alkylating chemotherapeutic agent temozolomide (TMZ) has been shown to prolong survival in patients with glioblastoma (GBM) and metastatic melanoma; however, patients with these diseases treated with TMZ possess a median survival of <15 months.1,2 Novel approaches are required to treat these devastating malignancies, and the exquisite specificity inherent to immunotherapy makes it an appealing option. Despite the potential of cancer immunotherapy, limited success has been achieved within this field due in large part to difficulties in generating appropriate numbers of high-avidity and persistent antitumor T cells.3-5 A recent and profound advance in immunotherapy is the use of lymphopenia to augment antitumor immunity through adoptive cellular therapy.6-8 Lymphodepletion induces homeostatic proliferation, enabling adoptively transferred activated T cells to become disproportionately overrepresented in the regenerating population and persist for months at high precursor frequencies.9-12 Recent studies have primarily examined the lymphodepletive properties of total body irradiation (TBI), and although informative, TBI is not routinely used therapeutically and has a limited clinical context. In contrast, lymphopenia resulting from various standard-of-care chemotherapies, although generally considered an undesirable but inevitable side effect of treatment, could provide a clinically significant means to augment immunotherapy.

TMZ is generally considered an immunosuppressive agent that induces lymphopenia in humans and patients receiving TMZ are routinely given prophylaxis to prevent the development of opportunistic infections.13-16 Low-dose TMZ has been shown to enhance “cross-priming” against tumor-derived antigens in experimental mice17; however, the direct effects of lymphodepletive doses of TMZ on vaccine-induced immunologic responses and regulatory T cells (TRegs) has not been examined.

Here, we report that lymphodepletive TMZ strongly augments vaccine-induced immune responses in a dose-dependent manner and that combinatorial vaccination and lymphopenia in mice bearing established B16/F10.9-OVA tumors significantly impaired malignant growth despite an increase in the frequency of CD4+CD25+Foxp3+ TRegs. This TMZ-induced enhancement of immunity is dramatically augmented when combined with anti–IL-2Rα monoclonal antibody (mAb)–mediated depletion of CD4+CD25+Foxp3+ TRegs, whereas identical treatment in normal mice impaired vaccine-induced effector responses. Anti–IL-2 receptor α (IL-2Rα) mAb treatment can suppress activated T cells in normal mice18 and can suppress vaccine-induced immune responses in patients with metastatic melanoma.19 However, to our knowledge, this is the first demonstration that the lymphopenic environment differentially impacts whether vaccination can be successfully combined with systemic antibody-mediated TReg depletion in the treatment of established...
tumors. To determine whether these findings could be translated in humans, TMZ-treated patients with GBM received combinatorial IL-2Rα blockade using daclizumab (Hoffman-La Roche) and DC vaccination targeting the human cytomegalovirus (CMV) antigen pp65 that we and others have shown to be expressed in a high proportion of GBM tumors.20–24 TREG frequencies in GBM patients were also significantly depleted and vaccine-induced antitumor immune responses were simultaneously enhanced. Cumulative preclinical and clinical results indicate that synergistic lymphopenia with concomitant IL-2Rα blockade selectively depletes TREGs and potentiates antitumor immunotherapy in both mice and humans.

### Methods

#### Mice and tumor cell lines

C57BL/6 and OT-I transgenic mice were from The Jackson Laboratory and were bred under pathogen-free conditions at Duke University Medical Center. All animal experiments were performed according to Duke University Institutional Animal Care and Use Committee–approved protocols. B16F10.9-OVA23 was a gift from Dr Smita Nair (Duke University Medical Center).

#### Peripheral blood draws and complete blood counts

Fifty to 100 μL of blood was collected into heparinized tubes by retro-orbital bleeding for complete blood counts (CBCs) and flow cytometric analysis. CBCs were performed on a VetScan HM5 hematology analyzer (Abaxis).

#### Isolation of murine T cells and flow cytometry

Murine T cells were isolated and stained for FACS analysis using protocols established previously in our laboratory.26 For analysis of peripheral blood, whole blood was incubated with antibodies in the dark for 15 minutes at room temperature before lysing red blood cells (RBCs) with 1× ammonium chloride lysis solution (BD Biosciences), cells were washed and resuspended in 2% paraformaldehyde. All samples were analyzed on a FACScan flow cytometer (BD Biosciences).

#### Antibodies and CFSE staining

Antibodies to CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD16/32 (2.4G2), CD25 (7D4), and isotype controls were from BD Biosciences. Antibodies to CD3 (145-2C11), CD4 (L3T4), CD8 (53-6.7), CD16/32 (2.4G2), CD25 (7D4), and isotype controls were from BD Biosciences. Antibodies and CFSE staining

B16F10.9-OVA cells were grown in DMEM, 10% FCS, and 2 mM L-glutamine. For tumor implantation, 1 × 10² cells in 100 μL of PBS were injected subcutaneously in the flank of C57BL/6 mice 3 days before treatment with TMZ. After 7 days, site of implantation was monitored daily for tumor growth, and tumor size was measured every 2-3 days. The volume of the tumor (cubic millimeters) was calculated by the formula: volume = length × width² × 0.52. Mice were killed when the tumor size reached 2 cm in any direction.

#### Preclinical temozolomide treatment, OT-I transfer, vaccination, and PC61 administration

C57BL/6 mice were injected intraperitoneally with TMZ (Temodar; Schering Plough) dissolved in a fresh solution of 85% saline and 15% DMSO; mice were weighed and injected intraperitoneally with a calculated single dose (milligrams per kilogram) or consecutive daily doses for 5 days (milligrams per kilogram per day) as indicated. Standard-of-care TMZ (200 mg/m²) for a 65-kg, 60-inch human is equivalent to a murine dose of 66.31 mg/kg. Twenty-four to 48 hours after TMZ treatment, lymphodepleted mice received spleenocytes from CD8⁺ OVA-specific TCR transgenic mice (OT-I mice) and normal C57BL/6 donor mice mixed at a 1:1 ratio (2 × 10⁷ cells in total). Concomitant with lymphocyte infusion, mice were vaccinated intradermally with 5 × 10² OVA-loaded DCs or intradermally with 100 μg of OVA protein (Sigma-Aldrich) and 100 μg of OVA class I peptide (sequence Ser-Ile-Ile-ser-Sta-Phe-Glu-Lys-Leu [SIINFEKL]; American Peptide in 10% DMSO with an equal volume of complete Freund adjuvant (100 μL/mouse; Difco); for repeat peptide vaccinations incomplete Freund adjuvant was used. For mAb-mediated blockade of IL-2Rα, mice received 300 μL (0.62 mg/mL) PC61 intraperitoneally simultaneously with vaccination.

#### Patient selection and clinical protocol

Adults with a newly diagnosed, single lesion WHO grade 4 GBM who had gross total resection, Karnovsky Performance Scale score ≥ 80, and a Curran Group status of I-IV at the time of enrollment were eligible for enrollment. Postresection, patients received 6-7 weeks of conformal external beam radiotherapy with concurrent TMZ at 75 mg/m². Patients with radiographic progression after external beam radiotherapy (> 20% increase in enhancement in comparison to postsurgical magnetic resonance imaging) did not receive vaccine. The trial design and informed consent were approved by the US Food and Drug Administration and the Duke University Medical Center Institutional Review Board.

Patients received adjuvant TMZ at 200 mg/m² for 5 days of a 28-day cycle and 3 biweekly DC vaccines (2 × 10⁷ DCs administered intradermally within 10 cm of the inguinal ligament) starting on day 21 of the first TMZ cycle. At vaccine 1, daclizumab was administered intravenously at a dose of 1 mg/kg. After daclizumab administration, patients received an autologous lymphocyte transfer intravenously at a dose of 3 × 10⁷ cells/kg.
The fourth and final vaccine was given on day 21 of the second TMZ cycle. Patients without tumor progression continued to receive monthly TMZ cycles for target of 6 to 12 cycles as tolerated. Patients were monitored bimonthly by magnetic resonance imaging. Progressive disease was defined radiographically according to the Macdonald criteria or by the development of a new contrast-enhancing lesion more than 1 cm in diameter. Adverse events were defined according to the National Cancer Institute’s Common Toxicity Criteria Version 2.0.

Production of pp65-LAMP/A64 mRNA

The 1.932-kb pp65 full-length cDNA insert was obtained from Dr Bill Britt (University of Alabama–Birmingham). The lysosome-associated membrane protein (LAMP)–targeting sequence ligated to pSP73/gp96ss/A64/Not was obtained from Dr Bill Britt (University of Alabama–Birmingham). The lysosome-associated membrane protein (LAMP)–targeting sequence ligated to pSP73/gp96ss/A64/Not was obtained from Dr Bill Britt (University of Alabama–Birmingham). The lysosome-associated membrane protein (LAMP)–targeting sequence ligated to pSP73/gp96ss/A64/Not was obtained from Dr Bill Britt (University of Alabama–Birmingham). The lysosome-associated membrane protein (LAMP)–targeting sequence ligated to pSP73/gp96ss/A64/Not was obtained from Dr Bill Britt (University of Alabama–Birmingham). The lysosome-associated membrane protein (LAMP)–targeting sequence ligated to pSP73/gp96ss/A64/Not was obtained from Dr Bill Britt (University of Alabama–Birmingham). 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In vitro generation of DCs

DCs were generated using the method of Romani et al and frozen in 80% human AB serum (HABS; Valley Biomedical) 10% DMSO (Bioniche...
Pharma), and 10% dextrose (Hospira). The cells were tested thawed and checked for endotoxin, Mycoplasma, and bacterial and fungal contamination. DCs were assayed by FACS for presence of the DC markers CD11c and HLA-DR and the absence of the lineage markers CD3, CD14, CD19, and CD56. All fluorochrome antibodies were from BD Biosciences.

Human samples were obtained from individuals who had given written and informed consent, and peripheral blood mononuclear cells (PBMCs) were obtained and stained for FACS analysis as described previously. To determine the absolute number of T<sub>reg</sub> and CD4<sup>+</sup> T cells, the frequency of these populations as established by FACS analysis was multiplied by the absolute number lymphocytes as determined by CBC, white blood cell frequencies, and lymphocyte frequencies from the Duke University Clinical Laboratory.

Statistical analysis

Unless otherwise stated, an unpaired 2-tailed Student t test was used to determine statistical significance. Two-way and 3-way ANOVA with interaction was used to assess the effects of temozolomide, anti–IL-2R α mAb, and vaccine on various immune measures and tumor volume. Parsimonious models were created by backward elimination. Pearson correlation was used to assess the association between TMZ dose and the levels of OVA-specific T cells. P values <.05 were considered significant. All figures shown are of representative experiments.

Results

Temozolomide as a lymphodepletive regimen

To determine a lymphodepletive dose (< 2000 cells/µL of peripheral blood) of TMZ in our murine model, mice were injected with increasing single doses (50-400 mg/kg) or multi-day doses (20-80 mg/kg/5 days) of TMZ along with a separate cohort receiving lymphodepletive TBI (5 Gy; supplemental Table 1). Both 200 mg/kg TMZ single dose and 60 mg/kg/day TMZ for 5 days induced lymphopenia similarly to TBI (Figure 1A), significantly reduced the absolute numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (supplemental Figure 1), and were considered lymphodepletive regimens.

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T-cell frequencies are elevated after lymphodepletive TMZ

To investigate the recovery from TMZ-induced lymphodepletion and to establish a model for evaluation of antigen-specific T cells, TMZ-treated mice were given an adoptive lymphocyte transfer of 2 × 10<sup>7</sup> naïve splenocytes consisting of an equal proportion of OVA TCR transgenic splenocytes (OT-I) and wild-type C57BL/6.
Figure 3. Inhibition of high-affinity IL-2Rα during lymphopenia depletes T_{reg}. (A-C) Untreated or TMZ-lymphodepleted C57BL/6 mice received OVA vaccination with or without concomitant αIL-2Rα mAb treatment (n = 5/group). One week after mAb administration and vaccination, mice were bled and CD4^+CD25^+Foxp3^− T_{reg} levels in the peripheral blood were assessed by FACS analysis. Experiments performed in at least triplicate with similar results. (A-B) For frequency T_{reg}: *P = .0154, **P = .0027, and ***P = .0001. By 2-way ANOVA with interaction, the magnitude of effect of anti-IL-2Rα mAb was greater among TMZ-treated mice as opposed to mice not treated with TMZ (P = .0024). For absolute number of T_{reg}, *P = .0025. (C) In the untreated and TMZ-treated cohorts, the percentage depletion of CD4^+CD25^+Foxp3^− T_{reg} after αIL-2Rα mAb administration was determined from the absolute number of T_{reg} after αIL-2Rα mAb treatment in comparison with the absolute number of T_{reg} in the cohort that did not receive IL-2Rα blockade.

splenocytes. The absolute numbers of all assessed T-cell compartments in TMZ-treated mice were not altered by the addition of OT-I cells (Figure 1B-C), with mice exhibiting similar counts of CD4^+ T cells. Importantly, T_{reg} isolated after TMZ treatment were equivalently immunosuppressive in vitro, because T_{reg} have been shown to suppress lymphodepletion-induced homeostatic proliferation and vaccine-stimulated immunity.34,35 We next assessed the impact of TMZ treatment on vaccine-induced immunologic responses. C57BL/6 mice were given escalating doses of TMZ before OVA immunization. We found that the frequency and absolute number of vaccine-induced immunologic responses were increased in a dose-dependent manner in mice previously treated with TMZ (Figure 2A, Pearson correlation = 0.69, P = .0008; Figure 2B, Pearson correlation = 0.54, P = .014) and that this increase in T-cell expansion was observed in mice after receiving a single dose of TMZ or a multiday regimen of TMZ (supplemental Figure 4). These results indicate that TMZ-induced lymphopenia leads to increased vaccine-induced immunologic responses despite increased T_{reg} proportions. To determine whether TMZ induces homeostatic proliferative responses in mice and to evaluate the relative kinetics of lymphopenia-induced homeostatic proliferation versus vaccine-induced proliferation, mice received CFSE-labeled OT-I T cells after lymphodepletive TMZ or 5 Gy of TBI and before vaccination. Proliferation was assessed by flow cytometric analysis of CFSE dilution 1 week after vaccination (Figure 2C). In both TMZ- and TBI-treated animals, proliferation was observed in the overall CD8^+ and CD4^+ compartments, with greater cell division (CFSE loss) seen in CD8^+ T cells, demonstrating that the lymphodepletive environments induced by 5 Gy of TBI and TMZ induce similar levels of lymphopenia-induced homeostatic proliferation. To examine the proliferation of vaccine-induced T cells, CFSE dilution was examined within the OVA tetramer-positive CD8^+ T-cell population. Vaccine-induced OVA-specific T cells, however, proliferated extensively, with > 94% of the cells exhibiting almost a complete loss of CFSE label (Figure 2C bottom panel). The vaccine-induced T cells proliferated to a similar extent in lymphopenic and normal

TMZ-mediated lymphodepletion enhances vaccine-induced immune responses despite increased frequencies of T_{reg}

Because TMZ quickly increases the proportion of T_{reg}, and because T_{reg} have been shown to suppress lymphodepletion-induced homeostatic proliferation and vaccine-stimulated immunity,34,35 we next assessed the impact of TMZ treatment on vaccine-induced immunologic responses. C57BL/6 mice were given escalating doses of TMZ before OVA immunization. We found that the frequency and absolute number of vaccine-induced immunologic responses were increased in a dose-dependent manner in mice previously treated with TMZ (Figure 2A, Pearson correlation = 0.69, P = .0008; Figure 2B, Pearson correlation = 0.54, P = .014) and that this increase in T-cell expansion was observed in mice after receiving a single dose of TMZ or a multiday regimen of TMZ (supplemental Figure 4). These results indicate that TMZ-induced lymphopenia leads to increased vaccine-induced immunologic responses despite increased T_{reg} proportions. To determine whether TMZ induces homeostatic proliferative responses in mice and to evaluate the relative kinetics of lymphopenia-induced homeostatic proliferation versus vaccine-induced proliferation, mice received CFSE-labeled OT-I T cells after lymphodepletive TMZ or 5 Gy of TBI and before vaccination. Proliferation was assessed by flow cytometric analysis of CFSE dilution 1 week after vaccination (Figure 2C). In both TMZ- and TBI-treated animals, proliferation was observed in the overall CD8^+ and CD4^+ compartments, with greater cell division (CFSE loss) seen in CD8^+ T cells, demonstrating that the lymphodepletive environments induced by 5 Gy of TBI and TMZ induce similar levels of lymphopenia-induced homeostatic proliferation. To examine the proliferation of vaccine-induced T cells, CFSE dilution was examined within the OVA tetramer-positive CD8^+ T-cell population. Vaccine-induced OVA-specific T cells, however, proliferated extensively, with > 94% of the cells exhibiting almost a complete loss of CFSE label (Figure 2C bottom panel). The vaccine-induced T cells proliferated to a similar extent in lymphopenic and normal
hosts, demonstrating that the lymphopenic environment, while inducing homeostatic proliferation within the overall T-cell compartments, did not convey a proliferative advantage to vaccine-induced T cells. Cumulatively, the data demonstrate that increased TReg frequencies, TMZ-induced lymphopenia stimulates homeostatic proliferation and enhances vaccine-mediated immune responses.

**Anti–IL-2Rα mAb administration synergizes with lymphopenia to deplete TRegs and potentiate immune responses**

Although it has been proposed that lymphopenia augments immunotherapeutic protocols in part via depletion of inhibitory TReg, our results demonstrate that immune responses are strongly potentiated after lymphodepletive TMZ despite an early increase in the frequency of functional CD4⁺CD25⁺Foxp3⁺ regulatory T cells and a decreased Teff:TReg ratio (Figure 1C-E). These data suggest that complementary TReg blockade strategies may synergize well with TMZ-induced lymphodepletion to further enhance immunotherapeutic antitumor immunity. To assess whether depletion or inhibition of TRegs in the context of TMZ treatment would further amplify vaccine-induced immunity, we examined the effect of anti–IL-2Rα mAb administration on TRegs in normal hosts and during TMZ-mediated lymphopenia. Untreated or lymphopenic mice were injected intraperitoneally with 10 mg/kg of the anti–IL-2Rα mAb PC61 concomitant with vaccination; peripheral blood TReg levels were assessed 1 week later. In both untreated (P = .0154) and TMZ-treated (P = .0001) mice, anti–IL-2Rα mAb administration caused a significant decrease in the frequency of remaining CD4⁺CD25⁺Foxp3⁺ TRegs (Figure 3A), and the absolute number was significantly depleted by anti–IL-2Rα blockade in TMZ-treated mice (P = .0025; Figure 3B). In addition, the percentage of depletion induced by anti–IL-2Rα mAb administration was not significantly different between TMZ-treated mice and controls (Figure 3C). Kohm et al.36 have alternatively shown that in vivo injection of anti–IL-2Rα mAb causes down-regulation or shedding of IL-2Rα on TRegs compared to that of the untreated mice, thus reducing the functional activity of TRegs.

To confirm that our anti–IL-2Rα mAb-induced reduction of CD4⁺CD25⁺Foxp3⁺ TRegs was not from IL-2Rα expression loss, we examined the impact of anti–IL-2Rα mAb administration on CD4⁺Foxp3⁺ TRegs. Similar to our data on CD4⁺CD25⁺Foxp3⁺ cells, the frequency and absolute number of CD4⁺Foxp3⁺ TRegs 1 week after PC61 and vaccination were reduced in both untreated and TMZ-treated cohorts, and the percentage of depletion induced by IL-2Rα blockade was not significantly different between untreated and
TMZ-treated mice (supplemental Figure 5A-C). However, because work from our laboratory has shown that CD25+ TRegs isolated after anti–IL-2Rα mAb administration are no longer functionally suppressive, we have continued to examine CD4+CD25+Foxp3+ TRegs as our primary population of interest.

Although TRegs have been shown to be particularly dependent on the expression of IL-2Rs and consequent IL-2 signaling, it is important to note that activated T cells also transiently express the high-affinity IL-2R. Therefore, it is possible that PC61 administration may impair vaccine-induced antigen-driven effector responses in addition to depleting TRegs. To investigate this possibility, we analyzed the kinetics of OT-I expansion in untreated and TMZ-lymphodepleted hosts after simultaneous OVA vaccination and PC61 administration (Figure 4A-B). The vaccine-driven expansion of OT-I cells in untreated animals was effectively abrogated in the presence of IL-2Rα blockade. This corroborates the work of others showing the dependence of activated T cells on IL-2 signaling for fully realized immunologic responses and the impairment of these responses by anti–IL-2Rα blockade. In sharp contrast, the dramatic expansion of OT-I cells engendered by the lymphopenic environment was not abolished during anti–IL-2Rα mAb administration but was increased in both magnitude and duration over the observed period of time. Four weeks after immunization, the percentage of OT-I cells increased from 0.3% among untreated animals to 21% among TMZ only–treated animals (P = .0004). TMZ-treated animals receiving PC61 and vaccine had a 2-fold increase in the percentage of OT-I cells in comparison with vaccinated mice receiving TMZ only (P = .036) and greater than a 100-fold increase over untreated and vaccinated animals (P = .0009).

Importantly, this led to increases in the Teff:TReg ratio for both CD4+CD25+Foxp3+ and CD4+Foxp3+ TReg populations in TMZ-treated animals (Figure 4C; supplemental Figure 6). These animals did not exhibit any signs of autoimmune toxicity during the observation period of 60 days (data not shown). Therefore, in the context of a lymphopenic setting, but not in a normal environment, administration of anti–IL-2Rα antibodies allows for the selective depletion of TRegs and enhancement of vaccine-stimulated effector T cells.

To investigate the impact of TMZ treatment on lymphocyte homeostatic cytokines in our model of lymphodepleting TMZ, we examined the levels of IL-2, IL-7, and IL-15 in the plasma of untreated or TMZ-lymphodepleted mice treated with or without anti–IL-2Rα mAb (Figure 4D). Plasma levels of IL-15 were unchanged; however, IL-7 levels were increased after TMZ treatment (analysis of variance, P = .0001) and IL-2 levels were increased after the combination of TMZ and anti–IL-2Rα mAb administration (analysis of variance with interaction, P = .0005). In vitro anti–IL-2Rα mAb administration has been shown previously to liberate IL-2, which may account for the up-regulation of IL-2 after lymphodepletion and IL-2Rα blockade. Therefore, the data indicate that in the context of TMZ-induced lymphopenia, the selective depletion of TRegs via IL-2Rα blockade simultaneous with vaccine-induced effector T-cell expansion may be mediated by an early surge in IL-7.

Previous studies also have demonstrated that although IL-2 signaling is dispensable during primary responses, it is essential for normal immunologic memory responses, thus prompting the possibility that immunologic memory responses may be significantly impaired in mice receiving PC61 treatment during vaccination. To investigate this possibility, the cytokine recall responses (IFNγ) of OVA-specific T cells were evaluated 5 weeks after vaccination in untreated and TMZ-treated mice. IFNγ responses were diminished in normal mice receiving IL-2Rα blockade but enhanced in mice receiving IL-2Rα mAb treatment during recovery from TMZ (Figure 4E), suggesting that the selective depletion of TRegs in TMZ-treated mice led to enhanced primary and memory T-cell responses.

IL-2Rα mAb blockade during lymphopenia effectively enhances antitumor efficacy in an established model of tumorigenesis

We and others have observed that TReg depletion via antibody-mediated blockade of IL-2Rα enhances antitumor efficacy most effectively in prophylactic rather than therapeutic settings, potentially because of the simultaneous inhibition of physiologic or vaccine-induced antitumor immune responses by anti–IL-2Rα mAbs when used against established tumors. To determine whether PC61 administration in the context of recovery from TMZ-induced lymphopenia could mediate significant improvements in therapeutic antitumor immunity, we assessed the efficacy of OVA vaccination on established B16/F10.9-OVA tumors (Figure 5; supplemental Figure 7). Mice were subcutaneously inoculated with tumor cells 3 days before lymphodepletive TMZ treatment and 1 week before initial immunization and PC61 administration, with additional vaccinations occurring on days 12 and 17 after tumor implantation. Mice were assessed for tumor burden throughout the course of the experiment. On day 22 after implantation, tumors reached maximal size in untreated mice, and the experiment was terminated for end point evaluation.

TMZ in combination with vaccine significantly reduced the tumor volume (P = .0015), and the addition of PC61 only in the context of TMZ and vaccine provided a further significant reduction in tumor volume (P = .0035; Figure 5; supplemental Figure 7). TMZ treatment alone or vaccine alone did not impair tumor growth in comparison with untreated tumor-bearing mice (P = .1922 and P = .3343, respectively; supplemental Figure 7). Administration of PC61 alone in untreated or TMZ-treated mice had no effect on tumorigenesis (P = .3343 and P = .5515, respectively; supplemental Figure 7) in comparison with controls, indicating that the systemic depletion of TRegs in normal or
lymphopenic mice with established tumor did not impair tumor growth. These results offer a striking parallel to our immune response data and indicate that IL-2Rx blockade during lymphopenia uniquely enables the selective depletion of TReg, to augment vaccine-induced effector T-cell function and potentiate antitumor immunotherapy in an established model of tumorigenesis.

Impact of anti–IL-2Rx mAb administration in TMZ-treated patients with GBM receiving targeted immunotherapy

Because of these preclinical findings, we assessed the potential of a single intravenous dose of a humanized anti–IL-2Rx mAb (daclizumab) to reduce or eliminate TReg and augment vaccine-induced antitumor immunotherapy in a pilot study of patients with newly diagnosed GBM receiving adjuvant TMZ therapy (“REGULATE” Protocol, Food and Drug Administration IND-BB-12839; Duke Institutional Review Board Protocol 0581). Six patients were treated in this pilot study (demographic data shown in Table 1). On day 21±2 of the first TMZ cycle (during lymphocyte nadirs), patients received 1 mg/kg daclizumab, adoptive transfer of 3×10⁷/kg naive lymphocytes harvested during leukapheresis to serve as a responder population to vaccination, and a vaccine consisting of 2×10⁷ DCs electroporated with CMV pp65 RNA. Patients also received 2 biweekly RNA-pulsed DC vaccinations and a fourth vaccine on day 21±2 after their second TMZ cycle. In parallel to our findings in mice, patients displayed a significantly increased frequency of CD4⁺CD25⁺Foxp3⁺ TReg after TMZ treatment (Figure 6A; P = .0236). After daclizumab administration, the frequency of TReg was significantly reduced (P = .0061) in treated patients, with absolute numbers decreasing by ~34% (P = .1494). Examination of CD4⁺Foxp3⁺ TReg revealed the same trends, with TReg frequency increased after TMZ (P = .0239) and decreased after daclizumab (P = .0160) and with the absolute number reduced after daclizumab by 25% (P = .3461; supplemental Figure 8). The kinetics of TReg depletion revealed that TReg levels spiked after the first cycle of TMZ but dropped rapidly after anti–IL-2Rx treatment (day 0) without returning to baseline over a 50-day period of observation (Figure 6B; supplemental Figure 9). Importantly, overall CD4⁺ and CD4⁺Foxp3⁺ T cells experienced no reduction in absolute number or frequency after daclizumab administration in comparison with post-TMZ levels, indicating that anti–IL-2Rx mAb administration during TMZ-induced lymphopenia selectively eliminates TReg, as was observed in our murine studies (supplemental Figure 10). IL-2Rx mAb blockade did not prevent our capacity to enhance immunologic responses, because

Figure 6. Patients with GBM possess an increased frequency of TReg after TMZ treatment that can be reduced by a single administration of an anti–IL-2Rx mAb. (A-B) Percentages and absolute numbers of TReg (CD4⁺CD25⁺Foxp3⁺) from leukapheresis (pre-TMZ and post-TMZ and daclizumab) and peripheral blood (post-TMZ) samples were determined by CBC counts and FACS analysis. TReg levels from preoperative samples (pre-TMZ, day −100), before initial vaccination and daclizumab (post-TMZ, day 0) and −7 weeks after vaccination and daclizumab (post-TMZ and daclizumab, day −50) were assessed. (A) Frequency of TReg by paired t test, *P = .0236 and **P = .0061. (B) Total CD4⁺, CD4⁺Foxp3⁺, and CD4⁺CD25⁺Foxp3⁺ T-cell frequencies from leukapheresis and peripheral blood samples from a representative patient were determined before and after daclizumab administration (day 0). Gating strategies for flow cytometric analyses were as follows and as shown in supplemental Figure 12: (1) For CD4⁺ T cells, all cells were displayed using forward and side scatter, and the lymphocyte population was selected. Lymphocytes were then displayed by side scatter and CD4 on a dot plot. The CD4⁺ population was then selected out of this lymphocyte population. (2) For Foxp3⁺ of CD4⁺, an identical gating strategy as described in 1 was used to select CD4⁺ T cells. The CD4⁺ T cells were then displayed by dot plot against Foxp3, and all Foxp3⁺ cells were selected. (3) For CD25⁺Foxp3⁺ of CD4⁺, an identical gating strategy as described in (1) was used to select the CD4⁺ population. The selected CD4⁺ population was then displayed by dot plot as CD25 versus Foxp3. CD25 and Foxp3 double-positive cells were then selected out of the CD24⁺ population.
Table 1. Characteristics, progression-free survival, and overall survival of patients in the REGULATE Trial

<table>
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<th>Patient</th>
<th>Age (y)</th>
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<th>Recursive partitioning analysis class</th>
<th>Progression-free survival, mo (surgery)</th>
<th>Overall survival, mo (surgery)</th>
<th>Progression-free survival, mo (vaccine 1)</th>
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<tr>
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<td></td>
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</table>

we found that 4 of 6 patients displayed an increase in pp65-specific T cells after vaccination with a mean 4-fold increase in tetramer-positive CD8+ T cells (Figure 7). The administration of daclizumab in combination with DC vaccination was well tolerated without any adverse events associated with immunotherapy, and although clearly not powered for a clinical end point, 4 of 6 patients treated have experienced progression-free survival times exceeding 24 months, warranting further exploration of the safety and efficacy of this treatment strategy in larger clinical trials (Table 1). The cumulative clinical data parallel our preclinical work and strongly suggests that anti–IL-2Rα mAb treatment in TMZ-treated patients with GBM may enhance vaccine-driven antitumor immunity through a selective reduction in immunosuppressive regulatory T cells.

Discussion

We report that TMZ has lymphopenic side effects that can be potently leveraged to improve immunotherapy as determined by both immune responses and therapeutic antitumor efficacy. We also demonstrate that improved antitumor immunity occurs despite an increased proportion of immunosuppressive TRegs and that the dramatic potentiation of vaccination by TMZ-induced lymphopenia can itself be significantly heightened through the concomitant depletion of TRegs by anti–IL-2Rα mAb treatment. High-affinity IL-2R antibodies administered during lymphodepletion selectively deplete TRegs to promote vaccine-stimulated immune responses and inhibit malignant growth. Although these results need confirmation in larger patient studies, we demonstrate that anti–IL-2Rα mAb administration in TMZ-treated patients also selectively depletes regulatory T cells while permitting the expansion of vaccine-induced effector T cells.

The significance of these observations are substantial, suggesting that the treatment of GBM and metastatic melanoma with TMZ provides a clinically relevant therapeutic window that can be manipulated to promote antitumor immunity. Of additional import is that TMZ-induced lymphopenia actually increases regulatory T cells in mice and humans, prompting us to examine the efficacy of tumor vaccination during combinatorial lymphopenia and systemic TReg depletion. In normal animals, our work and the work of others demonstrates that IL-2Rα blockade will impair immune activation, probably because of the transitory expression of IL-2Rα on activated T cells during priming. However, our data demonstrate that although TRegs are depleted or functionally inhibited by anti–IL-2Rα mAbs during both normal and lymphopenic conditions, it is only during lymphodepletion that anti–IL-2Rα mAb treatment does not impair immune responses but in fact strongly accentuates them while simultaneously inhibiting suppressive TRegs.

Several mechanisms may account for the selective depletion of TRegs by anti–IL-2Rα mAbs during lymphopenia. In our model of lymphodepletive TMZ, early elevation of IL-7 cytokine levels (Figure 4D) may permit effector T cells to expand in the absence of IL-2–mediated signaling, whereas TRegs will remain dependent on IL-2 for survival and proliferation. Alternatively, an indirect mechanism of daclizumab suppression mediated through CD56bright regulatory natural killer cells may be bypassed through the depletion of these cells during lymphodepletive regimens. Importantly, the above-mentioned hypotheses are not dependent on any unique properties of TMZ, and theoretically, the differential effects of anti–IL-2Rα mAbs during lymphopenia should hold true for other methods of lymphodepletion as well.

The anti–IL-2Rα mAbs basiliximab and daclizumab are already used clinically as immunosuppressants. Although the adoptive transfer of T cells depleted of TRegs possess enhanced antitumor efficacy in lymphopenic animals, TRegs may quickly regenerate, and a method to systemically deplete TRegs during successive rounds of chemotherapy, such as IL-2Rα–specific mAbs, would have high clinical applicability. Thus, our cumulative preclinical and clinical data demonstrate that the administration of anti–IL-2Rα mAbs during lymphopenia is not only a novel theoretical concept but also a broadly applicable and clinically relevant therapeutic modality for the potent enhancement of antitumor immunotherapy.

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Authorship

Conflict-of-interest disclosure: D.A.M. and J.H.S. have patents pending related to technology disclosed in this manuscript. D.A.M. has served as a paid member of the Schering Plough North American Investigators Advisory Board. D.A.R. has served as paid speaker for Schering/Merck and Genentech/Roche. The remaining authors declare no competing financial interests.

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Monoclonal antibody blockade of IL-2 receptor α during lymphopenia selectively depletes regulatory T cells in mice and humans

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