Selective Depletion of Foxp3+ Regulatory T Cells Improves Effective Therapeutic Vaccination against Established Melanoma

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Abstract

Tumor-bearing individuals have been reported to harbor increased numbers of Foxp3+ regulatory T cells (Treg), which prevent the development of efficient antitumor immune responses. Thus, Treg depletion has already been tested as a promising therapeutic approach in various animal models and entered clinical trials. However, the use of nonspecific Treg targeting agents such as CD25 depleting antibodies, which in addition to CD25+ Tregs also deplete recently activated CD25+ effector T cells, potentially masked the tremendous potential of this therapeutic strategy. To avoid such nonspecific effects, we used transgenic DEREG (depletion of regulatory T cells) mice, which express a diphtheria toxin receptor under control of the Foxp3 locus, allowing selective depletion of Foxp3+ Tregs even during ongoing immune responses. We showed that Foxp3+ Treg depletion induced partial regression of established ovalbumin (OVA)-expressing B16 melanoma, which was associated with an increased intratumoral accumulation of activated CD8+ cytotoxic T cells. The antitumor effect could be significantly enhanced when Treg depletion was combined with vaccination against OVA. To further assess whether this therapeutic approach would break self-tolerance, we crossed DEREG mice with RipOVAlow mice, expressing OVA as neo-self-antigen under control of the rat insulin promoter. In these mice, combined Treg depletion and vaccination also induced tumor regression without the onset of diabetes. Together, our data suggest that selective Treg targeting strategies combined with vaccinations against tumor-associated (self) antigens have the potential to evoke efficient antitumor responses without inducing overt autoimmunity. These findings might have implications for future therapeutic interventions in cancer patients. Cancer Res; 70(20); 7788–99. ©2010 AACR.

Introduction

Regulatory T cells (Treg) play an essential role in modulating host responses to tumors and infections, in preventing transplant rejection, and in inhibiting the development of autoimmunity and allergy (1–3). Originally, CD4+ Tregs were identified by the constitutive expression of CD25, however, both the existence of CD25− Tregs as well as the expression of CD25 on activated conventional T cells limits the use of this marker (3). Currently, the most widely used and reliable Treg marker is the forkhead box transcription factor Foxp3, which is expressed specifically in murine CD4+ Tregs and which is essential for their lineage identity and suppressive function (4–8).

Numerous studies have reported that tumor-bearing mice as well as cancer patients show elevated numbers of Tregs within peripheral blood, lymphoid tissues, and the tumor itself, which was often associated with poor prognosis (9, 10). Accordingly, the main obstacle tempering successful immunotherapies and active vaccination might be the migration of Treg into tumors and their suppression of effective antitumor immune responses in the tumor microenvironment (2, 9, 11, 12). Various groups have already investigated whether Treg removal using the depleting anti-CD25 antibody PC61 in mice might improve antitumor immunity, and it was shown that depletion of Tregs before the inoculation of tumor cells led to their efficient rejection.

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(13–18). In contrast, Treg depletion simultaneously with or after tumor inoculation resulted in no tumor regression (13, 19, 20), likely because the administered depleting antibodies also removed CD25-expressing effector T cells and CD25-Foxp3+ Tregs persisted. Thus, despite the great potential for Treg depletion in cancer therapies, the efficacy has been largely limited to prophylactic settings where depletion of Tregs occurs prior to the establishment of tumors.

Despite these limitations, clinical trials using denileukin diftitox (Ontak), a CD25-directed diphtheria toxin to eliminate Tregs, have already been initiated in patients suffering from renal cell carcinoma or melanoma (21–23). Although first results from these studies were promising, the genuine potential of Treg removal as a therapeutic strategy to treat cancer patients probably was masked by the unwanted effects of these depleting agents on tumor-reactive effector T cells (24).

To show the true therapeutic potential of Foxp3+ Treg depletion in established tumors, we used the recently generated transgenic DEREG mice (depletion of regulatory T cells), which express a fusion protein of the receptor for diphtheria toxin (DT) and enhanced green fluorescent protein under the control of the Foxp3 locus (25). These mice allow selective depletion of total Foxp3+ Tregs without affecting CD25+ effector T cells. Using DEREG mice and ovalbumin (OVA)-expressing B16 melanoma as a tumor model, we showed that selective removal of Foxp3+ Tregs induced partial regression of established melanoma, further enhanced by additional vaccination against OVA. When DEREG mice were crossed to mice expressing OVA as neo–self-antigen under control of the rat insulin promoter (DEREG × RipOVAlow mice), combined Treg depletion and vaccination resulted in efficient regression of tumor growth but only weak T-cell infiltration and partial destruction of pancreatic islets. Thus, our present data suggest that selective depletion of Foxp3+ Tregs constitutes a highly effective strategy to improve the outcome of vaccinations against tumor-associated antigens.

Materials and Methods

Mice

RipOVAlow mice (26) and DEREG mice (25) were bred at the Helmholtz Center for Infection Research (Braunschweig, Germany) or the Peter MacCallum Cancer Center (Melbourne, Australia). Animal experiments were performed in accordance with institutional, state, and federal guidelines.

Cell lines and B16 melanoma model

The B16-F1 melanoma cell line stably transfected with an OVA-expressing vector (B16-OVA, clone MO4) was described previously (27) and received from Natalio Garbi (German Cancer Research Center, Heidelberg, 2006). The same cell line was received from Mark Hulett (John Curtin School of Medical Research, Canberra, Australia, 2005) to perform experiments for Fig. 3. B16-OVA was passaged once in vivo by s.c. injection of 2 × 10^5 cells to ensure progressive growth. B16-OVA cells were re-isolated from pigmented tumors, confirming the melanoma identity, and were frozen at −150°C. B16-OVA was cultured under G418 selection (1 mg/mL; Invitrogen) and not passaged more than three times. Cells (2 × 10^5) were injected into the right flank and tumor growth was monitored by measurement of orthogonal tumor diameters. Mice were sacrificed if the mean tumor diameter exceeded 12 mm.

Treg depletion, antibody treatment, and vaccination

Tregs were depleted at the indicated time points after tumor cell inoculation by i.p. injection of 1 μg DT (Merck) on 2 consecutive days if not stated otherwise. In some experiments, CD8+ T cells were depleted by i.p. injection of 100 μg anti-CD8 (53-6.7, purified in-house) on days 6, 7, 14, and 21 after tumor cell inoculation. For vaccinations, mice were injected with 50 μg of OVA grade VI (Sigma) and 10 nmol of phosphothioate-stabilized CpG oligodeoxynucleotides (ODN-1826: 5′-TCCATGACGTTCCTGACGTT-3′) or 50 μg of agonistic anti-CD40 (IC10) in the tail base at indicated time points.

Tumor-infiltrating lymphocyte preparation, antibodies, and flow cytometry

For enrichment of tumor-infiltrating lymphocytes (TIL), tumors were cut into small pieces and digested with 0.2 mg/mL of collagenase/dispase and 0.02 mg/mL of DnaseI (both from Roche) followed by erythrocyte lysis and Percoll gradient. Lymphoid organs were prepared similarly without gradient. The following fluorochrome-conjugated antibodies were purchased from ebioscience: anti-CD4 (RM4-5), anti-CD8 (53-6.7), and anti-CD44 (IM7). The granzyme B–specific antibody GB12 was obtained from Invitrogen. Intracellular Foxp3 staining was performed with the anti-mouse Foxp3 staining set (ebioscience) according to the instructions of the manufacturer. Dead cells were excluded by ethidium monoazide (Sigma) or live/dead fixable dead cell stain kit (BD). Cytometric acquisition was performed using an LSRII analyser and FACSDiva software (BD). Data were processed using FlowJo software (Treestar).

Immunohistochemistry

Pancreata were fixed in formalin and embedded in paraffin. H&E, Foxp3, and CD3 stainings were performed as described previously (25). Insulin staining was performed using an anti-insulin antibody by Novocastra (clone 2D11-H5, 1:20) followed by staining with Streptavidin Alkaline Phosphatase Kit (K5005, Dako). Alkaline phosphatase was revealed by FastRed as chromogen.

Statistics

Statistical significance was determined by Mann Whitney test (bar graph analyses) or log-rank (Mantel Cox) test (Kaplan-Meier analysis) using GraphPad Prism software. *P < 0.05 values were considered statistically significant (*) and **P < 0.01 values were considered highly significant (**).

Results and Discussion

Selective depletion of Foxp3+ Tregs leads to the regression of established tumors

Several studies have shown that removal of CD25+ Tregs using depleting antibodies before the inoculation of tumor
cells leads to their efficient rejection (13–18, 28); however, no tumor regression was observed in the majority of studies where CD25+ Tregs were depleted after inoculation of tumor cells (13, 19, 20). This therapeutic failure is most likely due to a concomitant elimination of tumor-specific, CD25-expressing effector T cells (13, 19) or to the persistence of CD25− Tregs (12), which cannot be targeted by anti-CD25 antibodies.

To investigate the genuine therapeutic potential of selective Foxp3+ Treg depletion for the treatment of established tumors, we used transgenic DEREG mice, which express a DT receptor–enhanced green fluorescent protein fusion protein under the control of the Foxp3 locus (25). This allows selective depletion of total Foxp3+ Tregs (CD25+ and CD25−) without affecting CD25+ effector T cells proven critical for the attack of various tumors (18, 29, 30). DEREG mice have been used previously to investigate the functional importance of Foxp3+ Tregs to suppress immune responses against parasites (31), viruses (32), and self-antigens (33). Recently, the specificity of Foxp3 expression has been doubted (34), and the implication of Foxp3 as tumor suppressor gene (35) questions Foxp3-directed therapies. Importantly, we and others could not detect any evidence for Foxp3 expression in nonhematopoietic epithelial cells of mice (7) and humans (36), reinsuring the specificity of Foxp3 expression to the Treg lineage.

DEREG and wild-type (WT) control mice were subcutaneously challenged with the poorly immunogenic OVA-expressing B16 melanoma (B16-OVA), which displayed a rapid growth, reaching a mean diameter of 10 to 12 mm in less than 20 days in Figure 1. Efficient depletion of Foxp3+ Tregs within B16-OVA melanoma using DEREG mice. A, WT and DEREG mice were injected with 2 × 10^5 B16-OVA cells s.c. on day 0 and tumor growth was measured (n = 5). One out of two independent experiments is shown. B, after establishment of tumors, mice were injected with 1 μg of DT i.p. on days 14 and 15 and sacrificed on day 16. TILs, TdLNs, and spleens were stained for CD4 and Foxp3. Representative histograms display Foxp3 expression among live-gated CD4+ T cells and numbers indicate percentages within the respective Foxp3+ gate. The percentages of Foxp3+ cells among CD4+ T cells are summarized in the right graphs (n = 2–5). Results are representative for two independent experiments.
both DEREG and WT mice (Fig. 1A). In established tumors (day 16), we observed up to 40% of Foxp3+ cells among the CD4+ TILs, which was clearly higher than in tumor-draining lymph nodes (TdLN) and spleens of the very same WT mice (Fig. 1B), confirming results from previous studies (9, 10). Interestingly, DT treatment of tumor-bearing DEREG mice resulted not only in a highly efficient depletion of Foxp3+ Tregs in spleens and TdLNs (~90%), confirming our previous observations from naïve mice (25), but also efficiently eliminated the large population of Foxp3+ Tregs from the tumor site (depletion efficiency ~95%) when compared with DT-treated WT mice (Fig. 1B).

To investigate the consequences of Treg depletion on tumor development, we next treated DEREG and WT control mice twice with DT at different time points after s.c. tumor cell inoculation. We chose an early time point right after the inoculation (days 1 and 4), an intermediate time point (days 7 and 8), when the tumor was already established (mean...
Figure 3. The therapeutic effect of Foxp3+ Treg depletion is dose-dependent and largely requires CD8+ T cells. DEREG mice were inoculated s.c. with 2 x 10^5 B16-OVA cells at day 0 and tumor growth was measured. A, mice were treated with i.p. injections of PBS or DT (1, 10, 100, or 1,000 ng) on days 7 and 8 after tumor cell inoculation. B, two groups either injected with PBS or 1,000 ng of DT additionally received 100 μg of anti-CD8 monoclonal antibody on days 6, 7, 14, and 21 to deplete CD8+ T cells. Data are shown as tumor growth curves of individual mice from one experiment (n = 10).
Selective Foxp3+ Treg depletion results in increased CD8+ T cell activation and recruitment to the tumor. DEREG mice were inoculated s.c. with 2 × 10^5 B16-OVA cells at day 0. A control group of WT mice remained untreated. DEREG mice were treated with i.p. injections of 1 μg DT at days 7 and 8 or left untreated. Mice were sacrificed at day 14 post-tumor inoculation and TILs, TdLN, and spleens were stained and analyzed by fluorescence-activated cell sorting. A, the percentage of total CD8+ T cells, absolute CD8+ T-cell numbers per organ, and absolute numbers of CD8+ T cells per tumor volume is shown. B, representative plots display CD44 versus granzyme B expression among live-gated CD8+ T cells. Numbers indicate percentages within the respective quadrants. The percentage of CD44+ (C) and granzyme B+ (D) cells among live-gated CD8+ T cells as well as absolute cell numbers and absolute numbers per tumor volume are shown (mean ± SEM; n = 8, specimens of two mice each were pooled to obtain enough cells for multicolor cell analysis). Data are representative for two independent experiments (*, P < 0.05).
diameter ~3 mm) and a late time point (days 13 and 14), when the tumor was reaching a mean diameter >7 mm, and followed tumor development by measuring the tumor size over time. In WT control mice, we did not observe any effect of DT treatment on tumor growth at any time point tested (Fig. 2A–C). Interestingly, DEREG mice treated with DT at the early time point (days 1 and 4) showed an efficient regression of tumor growth (Fig. 2A). This effect of DT treatment on tumor growth was still obvious, although weaker, if Foxp3+ Tregs were depleted at the intermediate time point (days 7 and 8; Fig. 2B); however, no tumor regression could be observed if Foxp3+ Tregs were depleted at later time point (days 13 and 14; Fig. 2C). Similar results were obtained when nontransgenic B16 melanoma was used (data not shown).

Titrating the DT dose at the intermediate time point (days 7 and 8), we observed a better tumor regression at increased DT doses reaching a plateau between 100 ng and 1 μg, suggesting that the efficacy of Foxp3+ Treg depletion is critical for the outcome of tumor growth (Fig. 3A). It has previously been shown in the B16 melanoma model that tumor rejection is dependent on CD8+ CTLs (15). Because it is not obvious whether these cells are also involved in tumor regression in the absence of Foxp3+ Tregs, we injected depleting antibodies demonstrating that the regression of tumor growth, which was induced by selective depletion of Foxp3+ Tregs, was critically dependent on the presence of CD8+ CTLs (Fig. 3B). A similar Foxp3-DTR mouse model has been previously used to study the therapeutic effect of selective Foxp3+ Treg depletion in the B16 melanoma model, but surprisingly failed to induce regression of established tumors (20). This discrepancy might be attributed to different (intratumoral) Foxp3+ Treg depletion efficiencies. Nevertheless, our DT titration experiments clearly showed that even suboptimal Foxp3+ Treg depletion could have therapeutic potential as long as it occurs in a Foxp3+ Treg-specific manner.

Thus, using DEREG mice, we were able to show that specific and selective depletion of Foxp3+ Tregs at time points when tumors already are established, but not excessive in size, could result in the development of an efficient CD8+ T cell–mediated, tumor-specific immune response. This leads to a superior impairment of tumor growth as compared with CD25-targeting strategies, limiting the interpretation of previous data (13, 15, 19, 23).

Figure 5. Combined Foxp3+ Treg depletion and vaccination leads to regression of established melanoma. WT and DEREG mice were inoculated s.c. with 2 × 10^5 B16-OVA cells at day 0 and tumor growth was measured. A, WT mice were left untreated or injected with 1 μg of DT on days 7 and 8. B, similarly, DEREG mice were treated with 1 μg of DT on days 7 and 8 and WT mice were treated with tail base injection of 50 μg OVA and 10 nmol of CpG (Vac).
were already established, DEREG mice were treated with DT or left untreated. One week later (day 14), mice were sacrificed and frequencies, absolute numbers as well as the activation status of CD8+ T cells in various organs were determined by flow cytometry. In spleens of DT-treated and untreated DEREG mice, similar CD8+ T-cell frequencies and absolute numbers were observed, showing no difference with untreated, tumor-free control mice (Fig. 4A). Although TdLNs of DT-treated and untreated DEREG mice did not show any differences in the frequencies of CD8+ CTL, the absolute number of CD8+ T cells was higher in TdLNs of DT-treated DEREG mice, which could be explained by an increased LN cellularity upon Foxp3+ Treg depletion. Interestingly, the most striking effects of Foxp3+ Treg depletion on the CTL compartment were observed within the tumor itself. Comparing DT-treated and untreated DEREG mice, we found a doubling in the frequency of CD8+ TILs as well as a drastic increase in their absolute number (Fig. 4A). Bearing in mind that Foxp3+ Treg depletion at the intermediate time point results in an efficient regression of tumor growth (Fig. 2B), the depletion of Foxp3+ Tregs had an even larger effect on the number of CD8+ CTL per 1 mm3 tumor volume (Fig. 4A).

Next, we investigated the CTL activation status and observed a significantly increased frequency and absolute cell number of CD44+CD8+ or granzyme B+CD8+ T cells in spleens and TdLNs of DT-treated DEREG mice when compared with untreated mice (Fig. 4B–D). Again, the most striking effects of Foxp3+ Treg depletion were observed within the tumor itself. Here, essentially all CD8+ TILs became fully activated (CD44+) and most of them also acquired granzyme B expression when Foxp3+ Tregs were selectively depleted, resulting in a dramatic increase in the absolute number of

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**Figure 5. Continued.** C, DEREG mice were treated with CpG/OVA on day 6 and DT on days 7 and 8 (Vac + DT) as described above. Some of these groups received further vaccinations (combination therapy) with CpG/OVA (Vac 3x + DT) or CpG/αCD40 (Vac + DT + αCD40/OVA 2x) on days 12 and 19, or were injected with DT on days 20 and 21 (Vac + DT 2x). D, Kaplan-Meier analysis shows survival of mice treated as described in A to C (n = 6–8; **, P < 0.01, combination therapy compared with no treatment, DT treatment, or Vac treatment). Data are representative for two independent experiments.
activated CD44+ and granzyme B+ CTL, particularly if related to the tumor volume (Fig. 4B–D). Together, our data suggest that Foxp3+ Tregs very efficiently control the homeostasis of CD8+ T cells especially within the tumor site, whereas they suppress CTL activation not only within the tumor, but also within the TdLNs and the spleen.

**Combining selective Foxp3+ Treg depletion with vaccination improves the regression of established tumors**

Sole depletion of Foxp3+ Tregs at intermediate time points after tumor cell inoculation (days 7 and 8) was not sufficient for a full tumor regression (Fig. 2B), although this treatment already caused a massive increase in the number of activated CTL within the tumor site (Fig. 4). To more specifically boost the antitumor immune response, we next vaccinated tumor-bearing mice with OVA, which is expressed by the transgenic B16 melanoma, and CpG oligodeoxynucleotides. We have previously shown that selective Foxp3+ Treg depletion synergizes with vaccination strategies to elicit CTL responses to foreign (37) and tumor antigens (38), and CpG oligodeoxynucleotides are known to potently stimulate DC activation, cross-presentation and CTL responses in mice (39, 40). Tail-base vaccination of tumor-bearing WT mice using OVA protein plus CpG oligodeoxynucleotides at day 6 after tumor cell inoculation (Vac) resulted in a clear but minor

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**Figure 6.** Combination therapy leads to B16-OVA regression in DEREG × RipOVA\textsubscript{low} mice. DEREG × RipOVA\textsubscript{low} mice were inoculated s.c. with 2 × 10\textsuperscript{5} B16-OVA cells at day 0 and tumor growth was measured. A, mice were left untreated. B, DEREG × RipOVA\textsubscript{low} mice were either treated with 1 μg DT on days 7 and 8 or with tail base injection of 50 μg OVA and 10 nmol CpG on day 6. C, as combination therapy, DEREG × RipOVA\textsubscript{low} mice were treated with CpG/OVA on days 6, 12, and 19 and DT on days 7 and 8 (Vac 3x + DT). Data were pooled from two independent experiments (n = 8–13).
reduction of tumor growth compared with untreated control mice (Fig. 5A and B). A reduction of tumor growth was again seen in DEREG mice upon Foxp3+ Treg depletion at days 7 and 8 (Fig. 5B). In general, the vaccination scheme used in this study was powerful enough to generate effective tumor-specific immunity because, in accordance with previously published data (41), prophylactic vaccination 7 days before tumor challenge fully prevented tumor growth (data not shown).

Next, we asked whether the generation of antitumor immunity could be improved by combining OVA-specific vaccination and selective Foxp3+ Treg depletion. Tumor-bearing DEREG mice were first vaccinated using CpG/OVA on day 6 after tumor challenge, followed by DT treatment on days 7 and 8. This double treatment (Vac + DT) resulted in a significantly reduced tumor progression when compared with both single treatments (Vac and DT; Fig. 5B and C). Because repetitive vaccinations have been shown to induce superior CTL responses against EG7 tumors in contrast to single vaccinations (42), we next tested repetitive therapies. The reduction in tumor growth could be slightly improved when in addition to the double treatment, tumor-bearing DEREG mice received either one additional DT treatment at days 20 to 21 (Vac + DT 2×), two additional CpG/OVA vaccinations at days 12 and 19 (Vac 3× + DT) or two additional OVA vaccinations in the presence of anti-CD40 antibodies (Vac + DT + αCD40 2×; Fig. 5C). Repetitive DT treatments were less well tolerated (Fig. 5C) because active immunization induces DT toxicity in WT mice (43). However, in all three kinds of these multimodality treatments, one individual mouse had a complete and long-lasting tumor remission and the survival significantly improved compared with single treatments (Fig. 5C and D). We therefore showed that specific therapeutic Foxp3+ Treg depletion combined with vaccinations has the potential to eradicate established melanoma. This synergism is in gross contrast with CD25-directed therapies combined with vaccinations or other immunotherapies such as anti-GITR or anti-CTLA4 (15, 19, 20, 23, 44, 45), and suggests that CD25+ effector T cells are critical for the antitumor effect.

The combination of Foxp3+ Treg depletion and OVA-specific vaccinations does not result in the development of diabetes in RipOVAlow mice

The combination of selective Foxp3+ Treg depletion and vaccination against tumor-associated antigens is a promising therapeutic approach to treat cancer patients. However, most of the tumor-associated antigens are self-antigens and the immune system normally is self-tolerant against these antigens. To experimentally prove whether this state of self-tolerance could be broken by the combined Foxp3+ Treg depletion plus vaccination protocol, we crossed DEREG mice with RipOVA low mice (26) expressing OVA as neo–self-antigen under control of the rat insulin promoter. It has been previously shown that transfer of OVA-specific CTL in RipOVA low mice results in the destruction of pancreatic islets and diabetes onset (46). In untreated DEREG × ripOVA low mice, we observed a rapid growth of B16-OVA melanoma, reaching a mean tumor diameter of 10 to 12 mm within 2 weeks (Fig. 6A). As seen in DEREG mice (Fig. 5B), sole depletion of Foxp3+ Tregs at intermediate time points after tumor cell inoculation (days 7 and 8) or tail-based vaccination using CpG/OVA at day 6 (Vac) resulted in a mild regression of tumor growth in DEREG × RipOVA low mice (Fig. 6B). However, the combination of Foxp3+ Treg depletion with triple OVA vaccination (Vac 3× + DT) led to efficient B16-OVA melanoma regression with one mouse showing complete tumor remission ∼30 days after tumor cell inoculation (Fig. 6C). Thus, our data clearly show that self-tolerance can be broken by the combined Foxp3+ Treg depletion plus vaccination protocol. Although autoimmunity has been regarded as a somewhat positive indicator of successful immunotherapy against human cancers,

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**Figure 7.** Combination therapy does not induce severe autoimmunity in DEREG × RipOVAlow mice. Mice were treated as described in Fig. 6. Untreated and CpG/OVA-treated DEREG × RipOVAlow mice were sacrificed on day 14 and DEREG × RipOVAlow mice treated with DT or combination therapy (Vac 3× + DT) were sacrificed on day 25. Pancreas tissue was embedded in paraffin and sections were stained with H&E (top), anti-CD3 (middle), and anti-insulin (bottom). Images are displayed at ×200 magnification and are representative for two independent experiments (n = 3).
Untreated DEREG × RipOVAlow mice did not show any inflammatory infiltration of the pancreatic islets and displayed normal insulin production 14 days after tumor cell inoculation (Fig. 7). Insulin production was only slightly reduced in DEREG × RipOVAlow mice, in which Foxp3+ Tregs had been depleted by DT injections at days 7 and 8 or which were vaccinated with Cpg/OVA at day 6 after tumor cell inoculation. Under these conditions, we did observe only a very weak infiltration of T cells into the pancreatic islets. Interestingly, even the combined Foxp3+ Treg depletion plus triple OVA vaccination (Vac 3× + DT) only resulted in a mild infiltration of inflammatory cells and T cells into the islets 25 days after tumor cell inoculation (Fig. 7). Most importantly, although this therapeutic treatment regimen resulted in a significant reduction of tumor growth (Fig. 6C), a significant number of insulin-producing β-cells were still detectable 25 days after tumor cell inoculation (Fig. 7) and normal blood glucose levels were measured (data not shown). These results are in agreement with a previous study in which B16-LCMV(gp-33) tumor growth was inhibited in RipOVAlow mice via adoptive transfer of CTL with dual-specificity for gp-33 and OVA without the induction of severe diabetes, yet a transient increase in blood glucose levels and inflammatory infiltrates were noted (50). Interestingly, we also observed no signs of melanocyte antigen-specific autoimmunity such as skin depigmentation or uveitis in treated DEREG and DEREG × RipOVAlow mice, including those which completely rejected B16-OVA. This is in contrast to previous combination therapies of B16 melanoma where such reactions were positively correlated with therapeutic effects (15, 16). Together, our data suggest that the amplification of antitumor immunity by combined Foxp3+ Treg depletion and vaccination did not unleash overt autoimmune responses.

Importantly, using systemic DT application in the DEREG mouse model, we could very efficiently deplete Foxp3+ Tregs not only from lymphoid organs, but also from the tumor tissue itself. Because Foxp3+ Tregs have been found at increased numbers especially within tumors (10), it is assumed that the tumor microenvironment constitutes a preferential site of immune suppression (2, 9, 11, 12) and that systemically administered Treg-targeting agents also need to efficiently access the tumor tissue to allow a boost of the antitumor immune response at the effector site. Although it has not been experimentally proven that the observed limited therapeutic efficacy of CD25-targeting agents might have been caused by an inefficient Treg depletion within tumors, various groups have successfully used intratumoral Treg depletion as an alternative, clinically relevant, and appealing approach (10, 19), especially because certain tumors are not suitable for surgery. When anti-GITR was administered intratumorally into Meth A fibrosarcomas, no antiparietal cell autoantibodies were detectable despite an efficient tumor regression (19). Interestingly, moderate autoantibody levels were detected after systemic administration of the Treg-targeting agent, albeit no autoimmune gastritis developed in the mice (19). Therefore, a detailed evaluation of the administration route of both selective Treg-targeting agents and/or combinatorial agents will be of future interest with regard to therapeutic efficacy and autoimmune risk.

In conclusion, our findings have implications in the design of immune-based strategies and suggest that in contrast to previous attempts using nonselective Treg-targeting agents in combination with rather aggressive immunotherapies, the combination of simply composed vaccinations with selective Foxp3+ Treg depletion might be superior to achieve potent therapeutic antitumor immunity with reduced autoimmune side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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