Editor's Summary

Regulatory T Cells Interfere with Graft-Versus-Tumor Effects

Patients search for organ donors who are close genetic matches to avoid immune reactions. But sometimes a little immune activation is a good thing. Hematopoietic stem cells in the form of bone marrow are often used to treat blood cancers, and the donated cells not only engraft in bone to provide a source of healthy blood cells but also contain T cells that attack and destroy any remaining cancerous cells. This graft-versus-tumor effect is also harnessed when such patients suffer a relapse and donor T cells are infused into the patient. These infusions often fail to quell the malignancy, however. The reason for failure, Maury et al. have now shown, is that the donor cells can often include regulatory T cells (Tregs), a class of T cells that dampens the immune response. Removing these cells before the infusion markedly improved the graft-versus-tumor effect and the patients survival.

The beneficial graft-versus-tumor effect of transplantation can be accompanied by the not-so-desirable graft-versus-host disease. Like the transplanted T cells that perceive cancer cells as foreign, T cells can also attack the hosts skin, liver, intestinal lining, and other internal organs a condition that is serious but can be treated. The authors of this study used the presence of graft-versus-host disease as a sign that there were active, functioning T cells that also provided graft-versus-tumor effects. They treated 17 patients with relapsed blood cancer who had previously received an infusion of lymphocytes and had neither clinical manifestations of graft-versus-host disease nor control of their malignancy. After receiving a new infusion of lymphocytes from which the Tregs had been removed, two of the patients developed graft-versus-host disease for the first time in their transplant history. Hypothesizing that this low rate of response was a result of Treg cells present in the recipient, they treated four of the patients who needed more infusions with the same Treg-depleted cells but now infused them immediately after recipient Tregs were eliminated with lymphodepletive chemotherapy. These four patients, all of whom had Hodgkins lymphoma, reacted to the infused cells by developing graft-versus-host disease, a sign that the infused cells were likely attacking the tumor cells as well. When the whole group was assessed 1 year after treatment, the patients who had experienced a graft-versus-host reaction after cell infusion were found to have survived longer, likely a result of successful immune control of the cancer cells by the infused Treg-depleted lymphocytes.

This preliminary study shows that depleting donor lymphocytes of inhibitory regulatory T cells can be a safe and effective way to free active T cells from inhibition so that they can fight cancer cells in the recipient. Further studies are needed, but this seemingly inappropriate encouragement of immune reactions in transplant recipients may prove a boon to patients with blood cancers.

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CD4⁺CD25⁺ Regulatory T Cell Depletion Improves the Graft-Versus-Tumor Effect of Donor Lymphocytes After Allogeneic Hematopoietic Stem Cell Transplantation

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Donor T cells play a pivotal role in the graft-versus-tumor effect after allogeneic hematopoietic stem cell transplantation. Regulatory T cells (Tregs) may reduce alloreactivity, the major component of the graft-versus-tumor effect. In the setting of donor lymphocyte infusion after hematopoietic stem cell transplantation, we postulated that Treg depletion could improve alloreactivity and likewise the graft-versus-tumor effect of donor T cells. The safety and efficacy of Treg-depleted donor lymphocyte infusion was studied in 17 adult patients with malignancy relapse after hematopoietic stem cell transplantation. All but one had previously failed to respond to at least one standard donor lymphocyte infusion, and none had experienced graft-versus-host disease. Two of the 17 patients developed graft-versus-host disease after their first Treg-depleted donor lymphocyte infusion and experienced a long-term remission of their malignancy. Four of the 15 patients who did not respond after a first Treg-depleted donor lymphocyte infusion received a second Treg-depleted donor lymphocyte infusion combined with lymphodepleting chemotherapy aimed to also eliminate recipient Tregs. All four developed acute-like graft-versus-host disease that was associated with a partial or complete and durable remission. In the whole cohort, graft-versus-host disease induction through Treg depletion was associated with improved survival. These results suggest that Treg-depleted donor lymphocyte infusion is a safe, feasible approach that induces graft-versus-host or graft-versus-tumor effects in alloreactivity-resistant patients. In patients not responding to this approach, the combination of chemotherapy-induced lymphodepletion of the recipient synergizes with the effect of Treg-depleted donor lymphocyte infusion. These findings offer a rational therapeutic approach for cancer cellular immunotherapy.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for several types of severe hematological malignancy. Donor transplants contain HSCs that mediate engraftment and T cells that favor engraftment (1), promote peripheral T cell reconstitution (2), and, moreover, provide the graft-versus-tumor (GVT) therapeutic effect of HSCT (3). The GVT effect is due to the alloreactivity of transplanted T cells, which has been demonstrated by the increased risk of malignancy relapse after syngeneic twin graft or T cell–depleted allogeneic HSCT (4). Alloreactivity results from the stimulation of donor T cells by host dendritic cells presenting both human leukocyte antigens (HLAs) and minor histocompatibility antigens (mHAs) (5). The immunotherapeutic effect of donor T cells is further illustrated by the successful application of donor lymphocyte infusion (DLI) in the treatment of malignancy relapses after HSCT and DLI (3, 6). However, the beneficial effects of donor T cells must be counterbalanced by their implication in the pathogenesis of graft-versus-host disease (GVHD), a major cause of transplant-related morbidity and mortality after HSCT and DLI (7).

Although routinely used for relapse after HSCT, DLI is often not successful in controlling malignancy (3). In DLI failure, as after HSCT (4), the absence of the GVT effect correlates with lack of GVHD. Indeed, despite disparities that may exist between donor and recipient in HLAs and/or mHAs, some grafted patients never display any clinical manifestation of donor T cell alloreactivity after HSCT and DLI. This illustrates that histo-incompatibility in HLAs and/or mHAs is not the only determinant of alloreactivity. For instance, the subpopulation of CD4⁺CD25⁺FoxP3⁺ immunoregulatory T cells (Tregs), in addition to controlling autoimmune, has been shown to play a key role in the control of alloreactive immune responses (8). They physiologically regulate tolerance to paternal alloantigens during gestation in mice (9, 10). Their capacity to modulate allogeneic immune responses has
also been demonstrated in experimental solid organs (11) and HSC transplantation models (12). In the HSCT setting, we and others observed that the addition of donor-derived T_{reg} to the transplant could control GVHD in mice while preserving the GVT effect (13–16). Conversely, T_{reg} depletion from the transplant led to significantly accelerated GVHD in different murine models of allogeneic HSCT (13, 17).

Therefore, on the basis of these observations, we hypothesized that HSCT recipients who did not respond to DLI treatment for a relapsing hematological malignancy and who never experienced GVHD could benefit from T_{reg}-depleted DLI (d-DLI) with the potential to improve alloreactivity and induce a GVT effect.

RESULTS

d-DLI facilitates GVH or GVT effects in alloreactivity-resistant patients

Seventeen patients were included between December 2005 and December 2008 in a phase I/II clinical trial (18). One patient was excluded from the analysis owing to protocol violation in DLI cell dosing. Our patients experienced relapse of their hematological malignancy at a mean interval of 18 months (range, 3 to 90 months) after HSCT. This regimen involved bone marrow infusion preceded by a myeloablative conditioning regimen (n = 12) or peripheral blood stem cells after a nonmyeloablative regimen (n = 5) from a sibling (n = 13) or a matched unrelated (n = 4) donor (Table 1 and Fig. 1). Only one patient received d-DLI as first-line DLI because of a persisting excess of blasts in marrow, is still alive with persisting transfusion-dependent myelodysplasia at 37 months after d-DLI. Overall, despite the low rate of alloreactivity induced by d-DLI in this cohort, the induction of GVHD in those two patients indicated that d-DLI had the capacity to break alloreactivity resistance in some patients.

Improved alloreactivity of d-DLI after lymphodepleting chemotherapy

We hypothesized that the low rate of alloreactivity induced by d-DLI (2 of 17 patients with GVHD induction) could be due to endogenous T_{reg} of donor origin present in the recipient at the time of injection. Indeed, in the cohort, the mean (±SE) CD3+ T cell content in peripheral blood at the time of d-DLI was 994 ± 222 per microliter, with T_{reg} constituting 4.3 ± 0.65% of CD4+ T cells. We hypothesized that T_{reg} depletion in the recipient before d-DLI may favor the induction of GVH and GVT effects in such alloreactivity-refractory patients. We therefore used a cyclophosphamide and fludarabine lymphodepleting treatment adapted from Dudley et al. (19) in four patients who previously received d-DLI and did not develop GVHD manifestations (Table 1). This regimen always led to profound lymphopenia and to a significant decrease in T_{reg} counts at the time of d-DLI (fig. S2). It was associated with a mean 3-day period of neutropenia with values below 500 polymorphonuclear leukocytes per microliter.

In one patient (patient 6) who had stable Hodgkin’s lymphoma at 2 months after d-DLI, a second d-DLI was injected at a lower dose (1.25 × 10^7 versus 2.0 × 10^7 CD3+ T cells/kg for the previous d-DLI) after cyclophosphamide-fludarabine chemotherapy. The patient developed an acute-like grade 3 dermal and hepatic GVHD 28 days after the infusion (Fig. 4). Dermal immunohistological analyses showed CD8+ T cell infiltration in GVHD lesions, whereas no CD4+ cells expressing CD25 or FoxP3 were detected (Fig. 4). The patient was treated with methylprednisolone (1 mg/kg per day) to quickly control for GVHD. The lymphoma remained stable for 6 months but progressed thereafter, and the patient died 9 months after the second d-DLI.

Because of the strong and early GVHD that occurred in this patient, we aimed to discriminate the effects of (i) the lymphodepleting chemotherapy and (ii) d-DLI on the induction of clinically detectable alloreactivity. To this aim, the following three patients who were eligible for a second infusion (patients 8, 13, and 15) received the cyclophosphamide-fludarabine lymphodepleting regimen followed by std-DLI instead of...
d-DLI. With a mean follow-up of 5 months, none developed clinical manifestations of GVHD and one (patient 8) required chemotherapy for progressive disease. Given this lack of GVHD induction after lymphodepletion and std-DLI, the same three patients received a combination of the same lymphodepletion followed by d-DLI. All three patients developed an acute-like dermal GVHD associated with digestive (n = 2) or liver involvement (n = 1) at a mean interval of 26 days after the infusion. One patient with a grade 2 disease was not treated to preserve a potential GVT effect. However, lymphoma progressed 1 month later and the patient died of uncontrolled malignancy. Two patients had grade 3 GVHD that required treatment with methylprednisolone (2 mg/kg per day) and cyclosporine. Immuno-

Table 1. Patient characteristics and clinical outcome after d-DLI. Patients are ordered by increasing d-DLI cell dose received. Donor or recipient cytomegalovirus serostatus was as follows: −/− (n = 6), −/+ (n = 5), +/+ (n = 4), and +/− (n = 2). Nine patients needed to receive chemotherapy between relapse and their first std-DLI. Patient 2 relapsed at 6 weeks after d-DLI and further received a second allogeneic HSCT from an HLA-matched unrelated donor at 7 months after HSCT. D/R, donor/recipient; M, male; F, female; HD, Hodgkin's disease; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; CR, complete remission; PR, partial remission; MUD, matched unrelated donor; NA, not available.

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<th>Maximal cell dose of std-DLI (×10^7 CD3/kg)</th>
<th>Interval between relapse and d-DLI (months)</th>
<th>Disease status at d-DLI</th>
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<th>% CD3CD25 depletion</th>
<th>% CD4FoxP3 depletion</th>
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suppression was discontinued after 4 and 6 months of treatment, and the patients remained in complete remission at 13 and 11 months after their last d-DLI, respectively (Fig. 5). One patient had detectable disease by positron emission tomography (PET) imaging before treatment infusion, which resolved with the development of GVHD (Fig. 5B). Overall, in terms of alloreactivity, the addition of a lymphodepleting treatment in four patients significantly increased the capacity of d-DLI to induce GVHD compared to d-DLI alone (P = 0.003).

Among the 11 patients who received only one d-DLI and did not develop GVHD, two remain in complete remission of their underlying disease at 11 and 15 months after infusion (Fig. 1). Overall, 7 of the initial 17 patients are alive, 5 of them disease-free, with a mean follow-up for surviving patients of 24 months after their first d-DLI. Among the entire cohort, GVHD induction was found to be significantly associated with improved survival (1-year survival = 83 ± 15% in the 6 patients with GVHD versus 27 ± 13% in the 11 others with no GVHD induction, P = 0.035; Fig. 5C).

**DISCUSSION**

Since the discovery that Treg cells suppress antitumor immune responses, inhibiting their function has become a major challenge for the development of efficient immunotherapies for cancer (20, 21).

DLI is routinely used for patients in relapse after HSCT but is often unsuccessful in controlling malignancy (3, 6). Previous attempts to increase response rates, with adjuvant interleukin-2 (IL-2) administration (22, 23), interferon-α combined with granulocyte-macrophage colony-stimulating factor (3), or ex vivo–expanded and ex vivo–activated T cells (24), have provided encouraging preliminary results. However, because of their complex biotechnology (24) or the lack of large-scale trials (3, 22, 23), these strategies have not been incorporated into wide clinical use. Improving the efficacy of DLI for patients with few therapeutic options remains a major challenge. Given that GVT and GVH are linked effects of DLI, improving alloreactivity is a potential avenue for improving the GVT effect.

Several factors such as histo-incompatibility or sex mismatch between donor and recipient, as well as the CD3⁺ T cell dose infused, are classical determinants of alloreactivity (25, 26). In clinical practice, some patients exposed to such factors appear resistant to alloreactivity because they never display any sign of GVHD after HSCT and DLI. We specifically chose this target population of patients in the present trial for safety reasons. Indeed, Treg depletion in an allogeneic setting was associated with an unknown risk of severe GVHD. In this context, we show that complete Treg depletion from DLI can induce alloreactivity, as assessed by GVHD occurrence in 2 of the 17 patients included for the first time in their transplant history. This finding is in agreement with previous results suggesting that Treg cells present in infused cells may inhibit the GVH or GVT effect after DLI (27) and also HSCT (12, 28). In target organs of patients developing GVHD, immunohistological studies revealed CD8⁺ T cell infiltration and the absence of Treg, which may reflect the specific recruitment of alloreactivity-resistant patients.

In such patients, we hypothesized that donor-derived Treg present in the recipient at the time of injection may limit the ability of d-DLI to induce alloreactivity. We thus administered d-DLI in the context of chemotherapy-induced lymphodepletion, which would not only eliminate Treg from the recipient but also drive the homeostatic expansion or activation of the transfused T cells. Such lymphopenia-induced proliferation...
has been reported to be distinct from antigen-induced proliferation (30). In four patients who previously received d-DLI with no effect, the addition of lymphodepletion with cyclophosphamide and fludarabine immediately before d-DLI led to GVHD induction. This phenomenon required the synergistic effect of Treg depletion from both (i) the DLI and (ii) the recipient. Indeed, in three of those patients, previous unmanipulated DLI administered after the same lymphodepletion was not sufficient to induce alloreactivity. This observation contrasts with a previous study reporting that lymphodepletion before DLI was associated with a significantly higher incidence of acute GVHD than after DLI alone (31). It is likely that this discrepancy reflects our specific recruitment of alloreactivity-resistant patients. Overall, our results indicate that in such patients the infusion of Treg-depleted effector T cells in a Treg-depleted environment is necessary for alloreactivity induction. This strategy appears to be safe in our study population because all induced GVHDs were controlled by standard immunosuppressive regimens.

Treg depletion may also render more efficient autologous cell-based immunotherapies of cancer (19, 32). A previous investigation of autologous Treg-depleted lymphocytes in patients with metastatic melanoma has revealed disappointing results (33). However, the administration of high-dose IL-2 after the adoptive transfer, which is now known to result in Treg expansion (34–36), may have hampered the potential beneficial effect of Treg depletion. Other strategies with Treg depletion to enhance vaccine-mediated (37–39) or NK cell antitumor immunity (40, 41) have recently provided encouraging preliminary results.

Several patients in the present study have benefited from the induced donor T cell alloreactivity because they remain in long-term complete remission of their underlying disease, despite repeated failure of previous treatments. Notably, the four patients who benefited from the successive steps of our strategy all had Hodgkin’s lymphoma, a disease that might have a distinctive pattern of susceptibility to immunological effects mediated by donor cells. Alternatively, it is also possible that the efficacy of Treg depletion is in part mediated by either nonalloreactive antitumor effector T cells that may not be present in all patients or other mediators such as NK, NKT, or myeloid-derived suppressor cells. Because our Treg depletion strategy has been well tolerated, even when combined with lymphodepletion, we intend to study its use in less advanced diseases after allogeneic HSCT.

MATERIALS AND METHODS

Study design

This phase I/II study included patients who had experienced relapse of their hematological malignancy after HSCT and never displayed any clinical manifestations of donor alloreactivity (GVHD or GVT effect). Thirteen patients were included from two centers (Henri Mondor and Pitié-Salpêtrière hospitals), whereas five others were referred from three French centers to the Henri Mondor hospital for treatment. They were recruited to receive d-DLI either after failure of one or several previous std-DLI or also possibly as first-line DLI when their probability to respond to std-DLI was low (that is, with detectable disease at the time of inclusion). Failure of std-DLI was defined after a minimal 2-month follow-up as (i) persisting or relapsing malignancy and (ii) lack of GVHD. A minimal cell dose of 10^7 CD3^+ cells/kg was also required to define failure of std-DLI. To evidence the potential effect of Treg depletion independently of a cell dose effect, we adjusted the d-DLI cell dose to be inferior or equal to the maximal cell dose previously received in one std-DLI. Additionally, to avoid a putative excess of severe GVHD related to d-DLI, the six first patients could not receive >2 × 10^7 CD3^+ cells/kg. Patients could receive a chemotherapeutic treatment of their relapse before d-DLI. All were required to have >95% donor chimerism from peripheral blood mononuclear cells (PBMCs) and CD3^+ cells at the time of inclusion, as determined by real-time quantitative polymerase chain reaction (RT-qPCR) amplification of specific polymorphic sequences with a TaqMan technology (Applied Biosystems).

For patients who failed to respond after d-DLI, the protocol was secondarily amended so that they could receive a second

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**Fig. 3.** Chronic GVHD after d-DLI. (A) Skin lesions of patchy hyperpigmentation observed in the back and in the front side of the trunk were associated with lichenoid reactions in the tongue. (B) Immunohistological analyses of kidney biopsy with antibodies to CD8, CD4, CD25, and FoxP3 showed CD8^+ T cell infiltration, whereas no CD4^+ cells expressing CD25 or FoxP3 were detected. (C) Positive controls for Treg immunostaining were obtained from digestive biopsies in patient 9 who had transient nausea after d-DLI, finally not related to GVHD. Treg^+ were detected in the mucosa upon the colocalization of the CD4 membranous staining with either CD25 membranous staining or intracytoplasmic FoxP3 staining. (D) Proteinuria disappeared with the introduction of mycophenolate mofetil (MMF) associated with tacrolimus after failure of steroids, cyclosporine, and rituximab.
Written informed consent was obtained from all patients and donors before enrollment and again before secondary infusions. The study was approved in October 2005 by the French Drug Evaluation Agency (Agence Française de Sécurité Sanitaire des Produits de Santé) and the institutional ethics committee of Hôpital Pitié-Salpêtrière (Paris, France). It was conducted in accordance with the Declaration of Helsinki. Consensual published criteria were used to define (i) acute and chronic GVHD (42, 43) and (ii) antitumoral responses (44, 45).

**Preparation of CD25-depleted leukapheresis products**

Leukapheresis products (two to three blood masses, ≤12 liters) were freshly collected from donors according to institutional guidelines. Up to $2 \times 10^{10}$ PBMCs were collected in standard leukapheresis collection bags on acid citrate dextrose anticoagulant. All donors were negative for HIV, human T cell leukemia virus, hepatitis C virus, and hepatitis B virus. Leukapheresis products were first washed to remove contaminating platelets: Briefly, PBMCs were transferred from the collection bag into 500-ml transfer pack (TP) bags (Macopharma) under sterile conditions and with tubing connector (Terumo Sterile Connection Device). The 500-ml TP bags were previously prepared with phosphate-buffered saline (PBS)-EDTA buffer supplemented with human serum albumin (HSA) at a final concentration of 0.5% (w/v) (LFB), this buffer being named as CliniMACS buffer. PBMCs were diluted with CliniMACS buffer, and cells were centrifuged at 20°C at 3000 g for 3 min, without brake (KR4i centrifuge, Thermo Fisher Scientific). Then, the supernatant was removed with a plasma extractor. The PBMC pellet was recovered in a second 500-ml TP bag and resuspended in 400 ml of CliniMACS buffer. Cells were washed again, and the supernatant was discarded and resuspended in CliniMACS buffer at $5 \times 10^{7}$ to $5 \times 10^{8}$ cells/ml. They were further labeled with CD25 microbeads (CliniMACS CD25 reagent, Miltenyi Biotec) at 20°C on a rotator at 25 rpm for 30 min. Then, cells were transferred into a 600-ml TP bag (Macopharma), washed once at 300g for 15 min, and resuspended at a concentration of no more than $1 \times 10^{8}$ cells/ml in CliniMACS buffer. The 600-ml TP bag was connected to the CliniMACS device (Miltenyi Biotec), which was used following the manufacturer’s standard operating procedure with the depletion 1.2 program. After depletion, cells were harvested in a cell collection bag in 180 ml of CliniMACS buffer, washed once, and finally resuspended at $10^{6}$ cells/ml in a 4% HSA buffer before infusion into patients. At different steps of the procedure, aliquots were taken for various quality controls.

**Immunological analyses**

Cell counts and viability (trypan blue dye exclusion assay) were performed from (i) initial leukapheresis samples, (ii) after removing contaminating platelets, and (iii) after CD25+ T cell depletion. For flow cytometry analysis, $5 \times 10^{7}$ to $5 \times 10^{8}$ cells were incubated in 100 μl with the appropriate mAbs at 4°C for 20 min, washed twice in PBS–2% fetal calf serum (FCS), and immediately analyzed.

For patient monitoring, CD3+, CD4+, and CD8+ T cells were counted from fresh blood samples with CYTO-STAT tetraCHROME kits according to the manufacturer’s instructions (Beckman Coulter). PBMCs were isolated from blood sample with Ficoll-Hypaque density gradient centrifugation, washed twice in PBS containing 2% FCS, and numerated before labeling. The flow cytometry analysis of lymphocyte subpopulations was performed with a panel of mAbs. Briefly, cells were incubated in PBS containing 2% FCS with the appropriate antibodies at 4°C for 20

d-DLI combined with previous lymphodepleting chemotherapy (Fig. 1). We used cyclophosphamide (1 g/m² at day −5 before infusion) and fludarabine (30 mg/m² per day at days −5, −4, −3, and −2) given intravenously with granulocyte colony-stimulating factor support [filgrastim (5 μg/kg per day) from day +2 until neutrophil recovery]. The CD3+ T cell dose administered for this d-DLI was adjusted to be equal to that of the previous std-DLI.

**Fig. 4.** Induction of GVHD upon d-DLI after chemotherapy. (A) Skin papular lesions were observed on the face, members, and trunk. (B) Dermal immunohistological analyses with antibodies to CD8, CD4, CD25, and FoxP3 evidenced a CD8+ T cell infiltrate under the epithelioma (red membrane staining in the left upper panel). No colocalization with the green CD25+ membrane staining could be found (right upper panel). A lower number of CD4+ T cells were observed under the epithelioma (green membrane staining in left middle and lower panels) with no colocalization with the green membranous CD25 or intracytoplasmic FoxP3 staining. A diffuse nonspecific staining of collagen fibers can be observed.
min, washed in PBS containing 2% FCS, and then fixed in PBS containing 1% paraformaldehyde before analysis.

The following mAbs directly conjugated to phycoerythrin (PE), PE–Texas red (ECD), allophycocyanin (APC), or PE–cyanine 7 were used for leukapheresis and patient immunomonitoring analyses: CD3–ECD, CD4–PC7, CD8–PC7, and CD8–APC (all from Beckman Coulter). CD25–PE was from BD Biosciences. Matched mouse isotype control antibodies were used.

FoxP3 labeling was performed after CD3, CD4, and CD25 membrane staining with APC anti-human FoxP3 kit (PCH101 clone, eBioscience) according to the manufacturer’s instructions. Rat immunoglobulin G2a (IgG2a) APC was used as isotypic control (eBioscience). Flow cytometry was performed on a FC500 cytometer, and data were analyzed with CXP software (Beckman Coulter).

FoxP3 mRNA levels were quantified by RT-qPCR with the ABI Prism 7700 System (PE Applied Biosystems). FoxP3-specific primers and an internal fluorescent TaqMan probe were designed as follows:

- FoxP3 primers: 5′-CAGCACATTCCAGAGTTCCTC-3′ and 5′-GCGTGTGACCAGTGGTAGATC-3′;
- FoxP3 probe: 5′-FAM-TCCAGAGAAGCAGCGGACACTCAATG-TAMRA-3′.

Predeveloped TaqMan Assay Reagent for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (PE Applied Biosystems), 20× premixture of GAPDH-specific primers and an internal fluorescent TaqMan probe, was used for measurement of GAPDH mRNA levels as an internal control. Each PCR sample contained 0.3 μM primers and 0.2 μM TaqMan probe in a final volume of 50 μl, and amplification was carried out via 15 min at 95°C denaturation step followed by 45 cycles of 15 s at 94°C and 60 s at 60°C. All samples were run in duplicate (ΔC_T within replicates <0.5). The mean values for duplicates were used for calculations. Relative FoxP3 expression was calculated by dividing the relative quantity of FoxP3 by the relative quantity of GAPDH in each sample and normalized with calibrator cells (B cells purified with CD19 microbeads).

**Detection of T cells in tissues by double immunostaining**

Paraffin-embedded tissues (skin, renal, and digestive) were sectioned at 4 to 6 μm. For double immunostaining (CD4CD25, CD4FoxP3, CD8CD25), tissues were
stained with various primary and secondary antibodies, such as polyclonal goat antibody to human CD4 (AF379NA, 100 μg/ml, IgG, 1:10 dilution; R&D Systems), mouse antibody to human CD25 (NCL-CD25-305, IgG2b, 1:200 dilution; Novocastra), polyclonal rabbit antibody to human CD8 (ab4055, 3 μg/ml, IgG, 1:100 dilution; Abcam), or mouse antibody to human FoxP3 (ab29034, IgG1, 450 μg/ml, 1:50 dilution; Abcam), followed by fluorescein isothiocyanate–conjugated donkey antibody to goat (705-095-147, IgG, 1 mg/ml, 1:100 dilution; Jackson ImmunoResearch), cyanine 3 donkey antibody to rabbit (711-165-152, IgG, 1 mg/ml, 1:500 dilution; Jackson ImmunoResearch), or biotinylated donkey antibody to mouse (715-066-150, IgG, 1 mg/ml, 1:200 dilution; Amersham Biotech). Positive controls for CD4CD25 and CD4FoxP3 were obtained from digestive biopsies (Fig. S2), as previously reported before pretreatment at PH9 was needed. Fluorescent images of mounted sections were obtained from digestive biopsies (Fig. 3C), as previously reported.

Statistics
Overall survival were calculated from the date of first d-DLI with the Kaplan-Meier method and then compared by the log-rank test. Type 1 error was fixed at the 5% level.

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18. ClinicalTrials.gov NCT00987987.


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