Interleukin-2 and Regulatory T Cells in Graft-versus-Host Disease

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ABSTRACT

BACKGROUND

Dysfunction of regulatory T (Treg) cells has been detected in diverse inflammatory disorders, including chronic graft-versus-host disease (GVHD). Interleukin-2 is critical for Treg cell growth, survival, and activity. We hypothesized that low-dose interleukin-2 could preferentially enhance Treg cells in vivo and suppress clinical manifestations of chronic GVHD.

METHODS

In this observational cohort study, patients with chronic GVHD that was refractory to glucocorticoid therapy received daily low-dose subcutaneous interleukin-2 (0.3×10^6, 1×10^6, or 3×10^6 IU per square meter of body-surface area) for 8 weeks. The end points were safety and clinical and immunologic response. After a 4-week hiatus, patients with a response could receive interleukin-2 for an extended period.

RESULTS

A total of 29 patients were enrolled. None had progression of chronic GVHD or relapse of a hematologic cancer. The maximum tolerated dose of interleukin-2 was 1×10^6 IU per square meter. The highest dose level induced unacceptable constitutional symptoms. Of the 23 patients who could be evaluated for response, 12 had major responses involving multiple sites. The numbers of CD4+ Treg cells were preferentially increased in all patients, with a peak median value, at 4 weeks, that was more than eight times the baseline value (P<0.001), without affecting CD4+ conventional T (Tcon) cells. The Treg:Tcon ratio increased to a median of more than five times the baseline value (P<0.001). The Treg cell count and Treg:Tcon ratio remained elevated at 8 weeks (P<0.001 for both comparisons with baseline values), then declined when the patients were not receiving interleukin-2. The increased numbers of Treg cells expressed the transcription factor forkhead box P3 (FOXP3) and could inhibit autologous Tcon cells. Immunologic and clinical responses were sustained in patients who received interleukin-2 for an extended period, permitting the glucocorticoid dose to be tapered by a mean of 60% (range, 25 to 100).

CONCLUSIONS

Daily low-dose interleukin-2 was safely administered in patients with active chronic GVHD that was refractory to glucocorticoid therapy. Administration was associated with preferential, sustained Treg cell expansion in vivo and amelioration of the manifestations of chronic GVHD in a substantial proportion of patients. (Funded by a Dana–Farber Dunkin' Donuts Rising Star award and others; ClinicalTrials.gov number, NCT00529035.)

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ALLOGENEIC HEMATOPOIETIC STEM-CELL transplantation (HSCT) invokes donor-derived immune responses that can result in therapeutic graft-versus-tumor activity and toxic graft-versus-host disease (GVHD). Chronic GVHD, a systemic inflammatory disorder with pleomorphic autoimmune manifestations that is associated with considerable morbidity and mortality, develops in more than half of patients who have undergone HSCT.[1-3] Treatment with systemic glucocorticoids has limited efficacy and substantial long-term toxicity. There is no established second-line therapy.

Regulatory T (Treg) cells — as defined by expression of CD4, CD25, and transcription factor forkhead box P3 (FOXP3) — account for approximately 5 to 10% of circulating CD4+ T cells, suppress autoreactive lymphocytes, and control innate and adaptive immune responses.[4-11] Treg-cell impairment is associated with loss of tolerance and autoimmunity and with chronic GVHD.[12-14] Treg-cell–mediated immunomodulation may be beneficial in patients with chronic GVHD, the pathogenesis of which involves effector T- and B-cell responses to both allogeneic (donor–recipient polymorphic) and autologous (donor–recipient nonpolymorphic) antigens.[15] In preclinical models, adoptive transfer of Treg cells has been shown to ameliorate GVHD, but the clinical application of this approach has been challenging.[16-19]

Interleukin-2 is critical for Treg-cell development, expansion, activity, and survival.[20,21] In patients who do not have GVHD after undergoing HSCT with T-cell depletion, treatment with low-dose intravenous interleukin-2 has been shown to be safe and to induce Treg-cell and natural killer–cell augmentation without inducing GVHD.[22,23] In patients with active chronic GVHD, it is uncertain whether low-dose interleukin-2 can enhance Treg cells without activating and expanding CD4+ conventional T (Tcon) cells. Moreover, immunosuppressive calcineurin inhibitors used in the treatment of chronic GVHD might impair Treg-cell expansion.[24] In this dose-escalation observational study, we investigated whether low-dose interleukin-2 would expand Treg-cell populations and whether it could induce meaningful clinical responses in patients with chronic GVHD.

METHODS

STUDY OVERSIGHT

Between September 2007 and June 2011, we conducted a phase 1 dose-escalation study to determine the maximum tolerated dose of daily low-dose subcutaneous interleukin-2 (Proleukin, Novartis and Prometheus Labs) in patients with active chronic GVHD that was refractory to glucocorticoid treatment. The protocol, available with the full text of this article at NEJM.org, was approved by the institutional review board of the Dana–Farber/Harvard Cancer Center. All participants provided written informed consent. The interleukin-2 was donated by Novartis and Prometheus Laboratories, which did not have any input into the manuscript content or the decision to submit the manuscript for publication. All authors vouch for the completeness and accuracy of the data presented, as well as the fidelity of the report to the study protocol.

STUDY DESIGN

This 12-week study assessed daily treatment with subcutaneous interleukin-2 at three dose levels (0.3×10^6, 1×10^6, or 3×10^6 IU per square meter of body-surface area) for 8 weeks, followed by a 4-week hiatus. Patients with a clinical benefit (a complete or partial response, or stable disease with a minor response not meeting the criteria for a partial response) could continue to receive interleukin-2 indefinitely thereafter.

Patients could be evaluated for toxic effects at any time during the study and for lack of response after 6 weeks of interleukin-2 treatment. Eligibility criteria included active chronic GVHD despite at least 4 weeks of treatment with prednisone at a dose of at least 0.25 mg per kilogram of body weight per day, no active infection or cancer, and stable immunosuppression for 4 weeks before the start of the study. Up to six patients could be treated at a dose level. If no more than one patient had a dose-limiting toxic effect (defined as progressive chronic GVHD, or toxic effects of a grade of 4 or higher according to the National Cancer Institute's Common Terminology Criteria for Adverse Events [CTCAE], version 3.0 [http://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcaev3.pdf], or recurrent CTCAE grade 3 toxic effects), the dose was increased. If two or more patients had a dose-limiting toxic effect, the previous dose level would be the maximum tolerated dose. Ten additional patients were enrolled, once the maximum dose level had been determined, to further assess safety and efficacy.

CLINICAL ASSESSMENTS

Assessments of chronic GVHD with the use of the National Institutes of Health (NIH) consensus cri-

teria[25] were undertaken at baseline, after 8 weeks of treatment with interleukin-2, and 4 weeks after discontinuation of interleukin-2 (see the form regarding assessment of chronic GVHD in the Supplementary Appendix, available at NEJM.org). A complete response was defined as resolution of all reversible chronic GVHD–associated manifestations, a partial response as an improvement of 50% or more on the organ-specific chronic GVHD scale (a decrease of 1 point or more on a 3-point categorical scale or 2 points or more on a 10-point scale is considered to indicate clinically significant improvement; see the Supplementary Appendix) without progression at other organs or sites, progressive disease as an increase of 25% or more on the organ-specific chronic GVHD scale, and stable disease as an improvement of less than 50% or increase of less than 25%.[26] Responses were not scored for sites of ocular or oral chronic GVHD, since topical therapy (and changes thereof) were permitted during the study. Stabilization of pulmonary chronic GVHD is a partial response according to the NIH criteria, but improvement was required in this study.

LABORATORY ANALYSIS

Flow Sorting of Immune Cells

Protocol-specified immunophenotypic analyses were performed at baseline; at 1, 2, 4, 6, and 8 weeks during treatment with interleukin-2; 4 weeks after discontinuation of interleukin-2; and every 4 weeks in patients receiving interleukin-2 for an extended period, as well as in samples obtained from 25 healthy controls. Treg cells were defined as $CD3+CD4+CD25^{med-high}CD127^{low}$, Tcon cells as $CD3+CD4+CD25^{neg-low}CD127^{med-high}$, natural killer cells as CD56+CD3−, natural killer T cells as CD56+CD3+, $\gamma\delta$ T cells as γ/δ-1-TCR+CD3+, and B cells as CD19+.

Fifty μl of whole blood (15% EDTA) was incubated with fluorophore-conjugated monoclonal antibodies: anti-CD3 V450 (clone UCHT1, BD Biosciences), anti-CD4 APC-H7 (clone RPA-T4, BD Biosciences), anti-CD8 Pacific Orange (clone 3B5, Invitrogen), anti-CD25 PE-Cy7 (clone M-A251, BD Biosciences), anti-CD127 PE-Cy5 (clone eBioRDR5, eBioscience), and anti-TCR-γ/δ-1 (clone 11F2, BD Biosciences) for T-cell subsets; anti-CD56 PE (clone B159, BD Biosciences) and anti-CD3 V450 (clone UCHT1, BD Biosciences) for natural killer cells and natural killer T cells; and anti-CD19 APC (clone HIB19, BD Biosciences) for B cells. Red-cell lysis with 500 μl of BD PharmLyse lysing solution followed. Cell analysis was performed with the use

of the FACSCanto II system (BD Biosciences) and FACSDiva software (BD Biosciences).

FOXP3 Staining

Peripheral-blood mononuclear cells (PBMCs) that had been isolated by density-gradient centrifugation (Ficoll-Hypaque, GE Healthcare), separated into aliquots, and cryopreserved were thawed and incubated for 20 minutes at 4°C with the following conjugated monoclonal antibodies: anti-CD4 Pacific Blue (clone RPA-T4, BD Biosciences), anti-CD25 PC7 (clone M-A251, BD Biosciences), anti-CD45RA FITC (clone M-A251, Beckman-Coulter), and anti–CD127-APC Alexa Fluor 750 (clone eBioRDR5, eBioscience). Stained PBMCs were processed with fixation buffer and permeabilization buffer (eBioscience) and were incubated with allophycocyanin-conjugated anti-FOXP3 (clone PCH101, eBioscience). Cell debris and doublets were excluded on the basis of side-scatter versus forward-scatter properties. Analysis was performed with the use of the FACSCanto II system (BD Biosciences) and FACSDiva software (BD Biosciences).

Treg Cell Ex Vivo Suppression Assay

Fresh PBMCs, density-gradient–centrifuged from 30 ml of whole blood, were sorted into more than 95% pure Tcon or Treg cells with the use of the FACSAria cell sorter (BD Biosciences). Tcon cells were labeled with 5 μM 5,6-carboxyfluorescein diacetate succinimidyl ester diacetate (CFSE) (Invitrogen) for 10 minutes at 37°C. Staining was stopped with RPMI-1640 medium (containing 10% fetal bovine serum) at 4°C, followed by a phosphate-buffered saline wash. A total of 1×10⁴ CFSE-labeled Tcon cells were cultured with an equal number of unlabeled autologous Treg cells in round-bottom 96-well plates with 0.1 μg of anti-CD3 antibody (clone OKT3, eBioscience) per milliliter and 1 μg of anti-CD28 antibody (clone L293, BD Biosciences) per milliliter. After 4 days, analysis of cell division was performed with the use of the FACSCanto II system (BD Biosciences). Treg cells plus Tcon cells activated by anti-CD3 and anti-CD28 antibodies (anti-CD3/CD28) Tcon cells were compared with controls (nonactivated Tcon cells and anti-CD3/CD28-activated Tcon cells alone).

STATISTICAL ANALYSIS

A two-sided Fisher's exact test was used for the comparison of baseline characteristics between patients with a response and those without a response, and for the comparison of the dichoto-

mized baseline Treg:Tcon ratio between patients with a response and those without a response (see the additional correlative analyses in the Supplementary Appendix). A two-sided exact Wilcoxon rank-sum test was performed for the group comparison of phenotype data per time point. A two-sided Wilcoxon signed-rank test was used for paired comparisons of differences in phenotype data be-

Table 1. Baseline Characteristics of the Patients.*	
Characteristic	Value
Patients enrolled (no.)	29
Patients who could be evaluated (no.)	28
Male sex (no./total no.)	20/28
Age (yr)	
Median	49.5
Range	22–68
Time since HSCT (days)	
Median	1123
Range	420–2766
Time since onset of chronic GVHD (days)	
Median	803
Range	117–2624
Concurrent agents for chronic GVHD (no. of agents per patient)	
Median	3
Range	1–3
Use of concomitant agents (no. of patients)	
Systemic glucocorticoids	27
Mycophenolate mofetil	16
Calcineurin inhibitors	14
Sirolimus	12
Imatinib	2
Previous therapies for chronic GVHD (no. of agents per patient)	
Median	2
Range	0–5
Discontinued therapies (no. of patients)	
Rituximab	17
Extracorporeal photopheresis	12
Mycophenolate mofetil	6
Imatinib	5
Calcineurin inhibitors	4
Alemtuzumab	3
Sirolimus	3
Systemic glucocorticoids	1
Thalidomide	1
Denileukin diftitox	1
Etanercept	1
Dasatinib	1
Bortezomib	1

Table 1. (Continued.)	
Characteristic	Value
Areas of chronic GVHD (no. of sites per patient)	
Median	3
Range	1–6
Site of chronic GVHD (no. of patients)	
Skin, subcutaneous tissue, or both	26
Joint, fascia, muscle, or all three	16
Liver	5
Eye	17
Mouth	11
Lung	6
Peripheral nerves	1
Gastrointestinal tract	1

* GVHD denotes graft-versus-host disease, and HSCT hematopoietic stem-cell transplantation.

tween each time point and baseline. Given their exploratory nature, multiple comparisons were not considered, and modeling was not explored.

RESULTS

PATIENTS

Twenty-nine patients were enrolled. One patient withdrew early, and 28 were evaluated for toxic effects (Table 1, and Table A and Table B in the Supplementary Appendix). The median age of the patients was 49.5 years (range, 22 to 68). The median time since HSCT was 1123 days (range, 420 to 2766), and the median time since the onset of chronic GVHD was 803 days (range, 117 to 2624). The median number of sites of chronic GVHD involvement per patient was 3 (range, 1 to 6), the median number of concurrent agents that the patients received for chronic GVHD was 3 (range, 1 to 3), and the median number of discontinued previous therapies was 2 (range, 0 to 5). The baseline median dose of prednisone was 20 mg (range, 0 to 80).

SAFETY

None of the patients had a relapse or progression of chronic GVHD. The maximum tolerated dose of interleukin-2 was 1×10^6 IU per square meter per day. A dose of 3×10^6 IU per square meter induced persistent CTCAE grade 1 constitutional symptoms (fever, malaise, and arthralgia) necessitating a 50% dose reduction. Other adverse events that were possibly or probably related to interleukin-2 included reversible CTCAE grade 3 injection-site induration (in three patients), grade 2 constitutional symptoms (fever, malaise, and fatigue; in one patient), grade 2 renal dysfunction (in one patient), and grade 2 thrombocytopenia (in one patient) (Table 2, and Table B in the Supplementary Appendix).

CTC grade 4 thrombotic microangiopathy–associated renal failure requiring dialysis developed in two patients (one patient who received interleukin-2 at a dose of 0.3×10^6 IU per square meter and one patient who received the dose of 1×10^6 IU per square meter). Both patients also received sirolimus plus tacrolimus. Thrombotic microangiopathy is a known toxic effect of these agents, but interleukin-2 might have contributed. Thrombotic microangiopathy did not recur after patients receiving concomitant sirolimus plus tacrolimus were excluded.

CTCAE grade 3 or higher infections developed in three patients. One patient who had a history of methicillin-resistant *Staphylococcus aureus* (MRSA) pneumonia had recurrent MRSA pneumonia after 7 weeks of interleukin-2 treatment. In the second patient, a MRSA furuncle on the buttock, not noted previously, preceded interleukin-2 treatment, which was withheld after 2 days. *Haemophilus influenzae* type-B bacteremia developed in the third patient after 4 weeks of interleukin-2 treatment.

One patient had a fatal myocardial infarction 70 days after discontinuing interleukin-2. Another

Table 2. Adverse Events and Outcome of Treatment at 8 Weeks.*

Variable	No. of Patients
Adverse events	
Patients who could be evaluated	28
Grade 4 thrombotic microangiopathy (dose-limiting toxicity)†	2
Grade 3 induration†	3
Grade 2 constitutional symptoms (fever, malaise, fatigue)†	1
Grade 2 increase in serum creatinine†	1
Grade 2 thrombocytopenia (with schistocytes)†	1
Grade 4 dyspnea	1
Grade 4 MRSA pneumonia	1
Grade 4 myocardial infarction	1
Grade 3 lower gastrointestinal bleeding	1
Grade 3 deep-vein thrombosis or left ventricular thrombus	1
Grade 3 *Haemophilus influenzae* type B bacteremia	1
Grade 3 MRSA abscess	1
Outcomes	
Patients who could be evaluated	23
Partial response	12
Stable disease‡	11
Progression of disease	0
Sentinel sites of response	
Skin, subcutaneous tissue, or both	11
Joint, fascia, muscle, or all three	8
Liver	1
Peripheral nerves	1

* MRSA denotes methicillin-resistant *Staphylococcus aureus*.
† The events were possibly or probably related to interleukin-2.
‡ Three of the patients had a minor response.

Figure 1 (facing page). Clinical and Regulatory T (Treg) Cell Responses to 8 Weeks of Daily Low-Dose Interleukin-2 Therapy in Individual Patients.
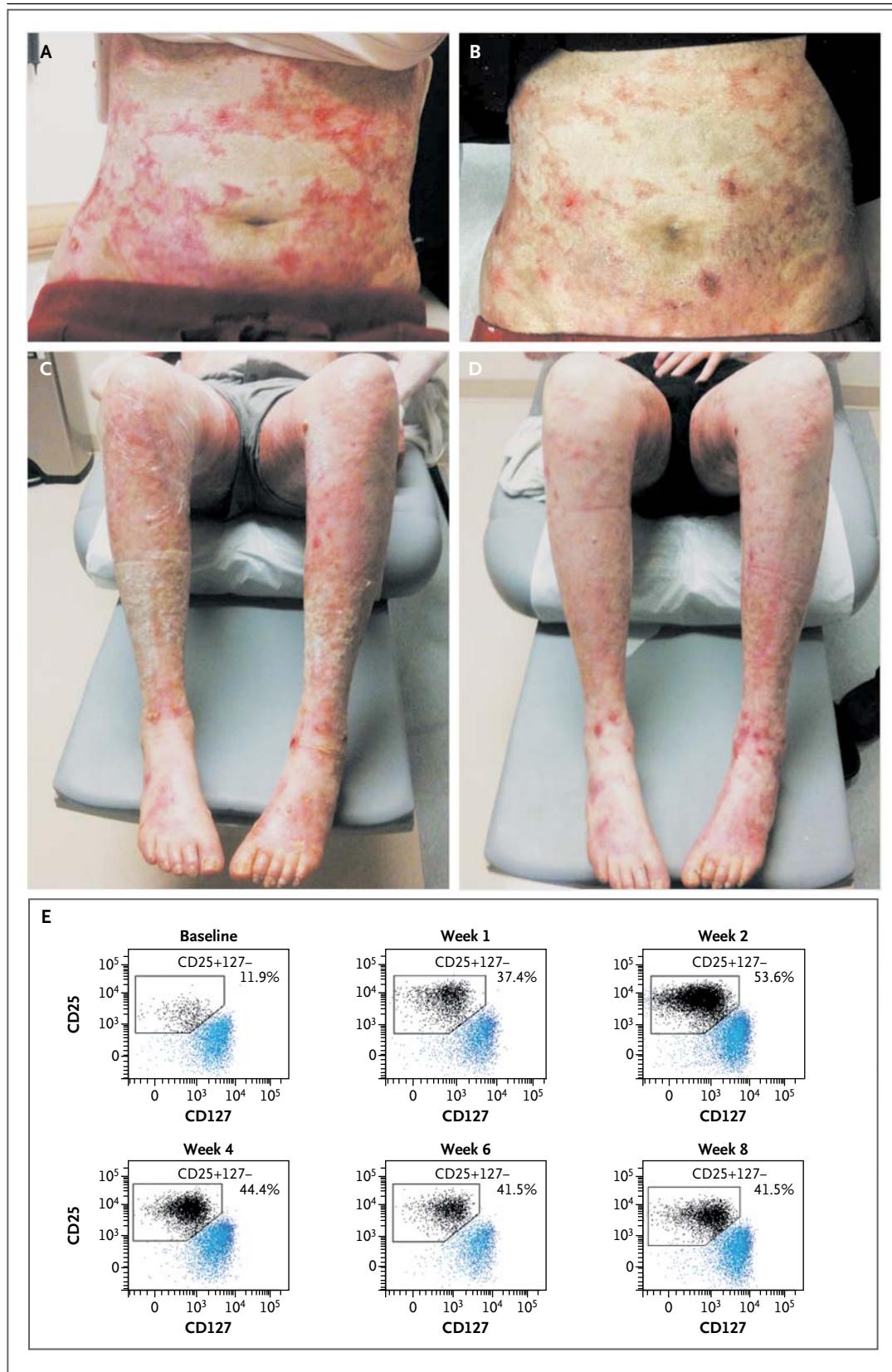
Panels A and C show manifestations of sclerodermatous chronic GVHD in two patients at baseline; Panels B and D show partial responses, with decreased erythema, after 8 weeks of treatment with low-dose interleukin-2. Softening of sclerodermatous hidebound skin, which cannot be seen in the photographs, was also prominent. Panel E shows flow-cytometric plots, gated on CD4, of Treg cells (CD4+CD25$^{med\text{-}high}$CD127^{low}) during 8 weeks of interleukin-2 therapy in one patient with chronic GVHD. The percentage of total CD4+ cells that are Treg cells (shown in the truncated rectangle) is indicated in each plot. The fluorescence intensity of fluorophore-conjugated CD127 and CD25 monoclonal antibody–bound cells is indicated on the x and y axes, respectively.

dermatous, hidebound skin; improved joint mobility and gait; improved liver function; and resolution of neuropathy. Two skin responses are shown in Figure 1. Eleven patients had stable disease (three with minor objective responses).

Of the 15 patients who had a response (12 who had a partial response and 3 who had stable disease with a minor response), 12 (9 who had a partial response and 3 who had stable disease with a minor response) received interleukin-2 for an extended period. Two patients who had stable disease with a minor response discontinued therapy after 1.5 and 4 months of extended treatment, without further improvement. The remainder had continued improvement during a median of 13 months (range, 2 to 36) of extended treatment with interleukin-2. One patient with extensive sclerodermatous chronic GVHD had a complete response after 14 months and discontinued both immunosuppressive agents and interleukin-2. Another patient, who had stabilization of chronic GVHD in the lung, discontinued immunosuppressive agents after 12 months. Two other patients discontinued glucocorticoids after 30 and 36 months. Overall, the glucocorticoid dose was tapered by a mean of 60% (range, 25 to 100).

CLINICAL LABORATORY MEASURES

In the entire cohort, 8 weeks of interleukin-2 treatment did not induce significant leukopenia, neutropenia, thrombocytopenia, or hepatic dysfunction. Asymptomatic peripheral-blood eosinophilia peaked at 10% (interquartile range, 2 to 19) after 4 weeks (P=0.002) and subsequently declined to 2% (interquartile range, 1 to 8) by 8 weeks.

patient, with known coronary artery disease, had a myocardial infarction after 2 weeks of interleukin-2 treatment, with subsequent in-stent thrombosis during extended treatment with interleukin-2. This patient continued to receive extended treatment with interleukin-2, with preserved cardiac function and further improvement in the clinical manifestations of chronic GVHD.

CLINICAL RESPONSE

A total of 23 patients could be evaluated for a response. Twelve had an objective partial response during 8 weeks of interleukin-2 treatment (Table 2, and Table B in the Supplementary Appendix). Responses included softened skin and subcutaneous tissue; decreased erythema and extent of sclero-

IMMUNOLOGIC RESPONSE

Treg and Tcon cell counts in one patient are shown in Figure 1E. In this patient, Treg cells expanded to 53.6% of total CD4+ T cells after 2 weeks, a level that was 20 times as high as the baseline level. Treg cells stabilized thereafter, accounting for approximately 40% of CD4+ T cells during the remainder of the treatment period.

Absolute immune-cell counts in the cohort are shown in Figure 2. The baseline median Treg-cell count was low as compared with the count in healthy donors, at 17 per cubic millimeter (interquartile range, 6 to 35) versus 43 per cubic millimeter (interquartile range, 27 to 57) (P=0.003) (Fig. 2A). Treg-cell counts increased rapidly while the patients were receiving interleukin-2, with a peak median count of 143 per cubic millimeter (interquartile range, 53 to 257) at 4 weeks — more than eight times as high as the baseline level (P<0.001). Treg-cell counts stabilized and remained elevated thereafter, with a median count of 101 per cubic millimeter (interquartile range, 43 to 236) at 8 weeks (P<0.001). When the patients were not receiving interleukin-2, Treg-cell counts declined but still remained above the baseline level after 4 weeks, with a median count of 32 per cubic millimeter (interquartile range, 11 to 92) (P=0.02).

The baseline median Tcon-cell count was low as compared with the count in healthy donors, at 206 per cubic millimeter (interquartile range, 131 to 412) versus 686 per cubic millimeter (interquartile range, 567 to 873) (P<0.001) (Fig. 2B). Tcon-cell counts did not change significantly from baseline, with or without the use of interleukin-2. The median Tcon-cell count was 270 per cubic millimeter (interquartile range, 110 to 678) (P=0.09) at 8 weeks of interleukin-2 treatment, and 209 per cubic millimeter (interquartile range, 153 to 550) (P=0.36) 4 weeks after discontinuation of interleukin-2.

The median Treg:Tcon ratio increased rapidly to a level that was more than five times as high as the baseline level — from 0.07 (interquartile range, 0.05 to 0.12) at baseline to 0.39 (interquartile range, 0.18 to 0.7) after 4 weeks (P<0.001) (Fig. 2C). Subsequently, the ratio remained stably elevated at 0.4 (interquartile range, 0.24 to 0.71) during treatment (P<0.001) and then declined when patients were not receiving interleukin-2, with a value that remained above baseline after 4 weeks (0.14; interquartile range, 0.09 to 0.22; P<0.001).

The median natural killer–cell count more than doubled, from 158 per cubic millimeter (interquartile range, 94 to 250) at baseline to 362 per cubic millimeter (interquartile range, 170 to 570) after 8 weeks (P<0.001), thereafter declining in patients who were not receiving interleukin-2 to 203 per cubic millimeter (interquartile range, 132 to 311) after 4 weeks (P=0.25) (Fig. 2D). There were no significant effects on CD3+CD8+ T cells (P=0.50), CD19+ B cells (P=0.23), or CD3+CD56+ natural killer T cells (P=0.35) after 8 weeks of interleukin-2 treatment. The counts of γ/δ T cells, evaluated in five patients, did not change significantly after 8 weeks of interleukin-2 treatment (P=0.81) (data not shown).

These immunologic changes (i.e., increases in immune-cell counts from baseline values) were sustained in patients receiving extended-duration interleukin-2. The median Treg-cell count was 60 per cubic millimeter (interquartile range, 38 to 97) at 4 months (P=0.02), 56 per cubic millimeter (interquartile range, 50 to 98) at 6 months (P=0.02), and 76 per cubic millimeter (interquartile range, 54 to 143) at 12 months (P=0.03) (Fig. 2E). The interleukin-2–induced increases in the Treg:Tcon ratio and natural killer cells were also sustained (data not shown).

The combined phenotype — CD4+CD25$^{med-high}$CD127^{low} — used in the study was shown to be selective for Treg cells,[27,28] but it could also indicate activated CD4+ T cells. We therefore conducted additional phenotypic studies to characterize these interleukin-2–expanded CD4+ T cells. Figure 3A shows the results in a representative patient. The CD4+CD25$^{med-high}$CD127^{low} population expressed relatively high FOXP3 levels. FOXP3 was not expressed above background levels in CD4+ Tcon cells during therapy. Similar results were obtained in all five patients evaluated.

We also assessed the functional ability of interleukin-2–expanded Treg cells to suppress autologous Tcon-cell activation ex vivo. In six independent experiments, these Treg cells efficiently inhibited proliferation of anti-CD3 plus anti-CD28–stimulated autologous Tcon cells, as compared with the proliferation of anti-CD3 and anti-CD28–stimulated Tcon cells without the addition of Treg cells in controls (P<0.001) (Fig. 3B and 3C).

CORRELATIVE ANALYSES

Counts of T-cells (CD3+, CD4+, CD8+, and Tcon), B cells, natural killer cells, and natural killer T cells were not associated with response, nor were the sex
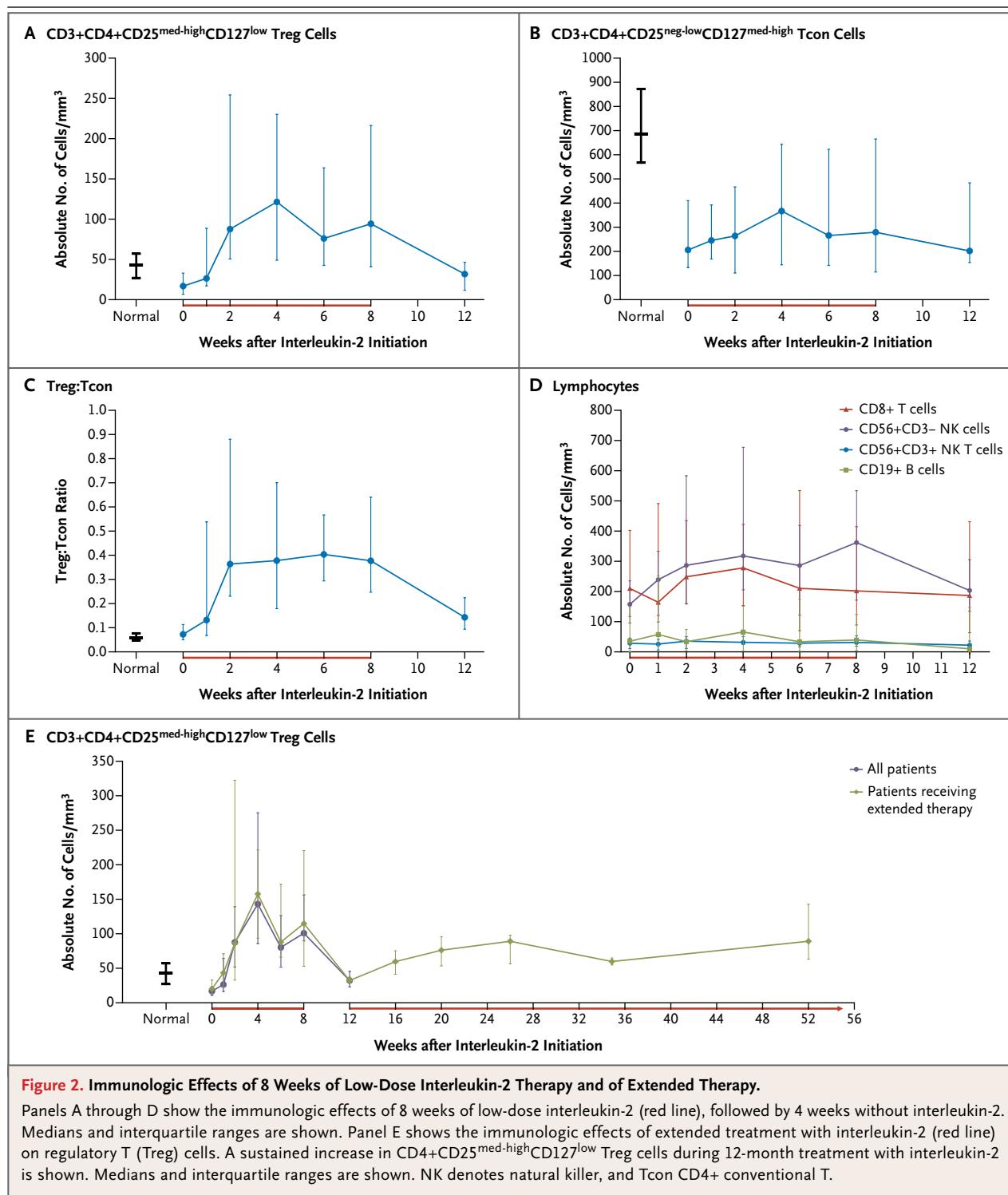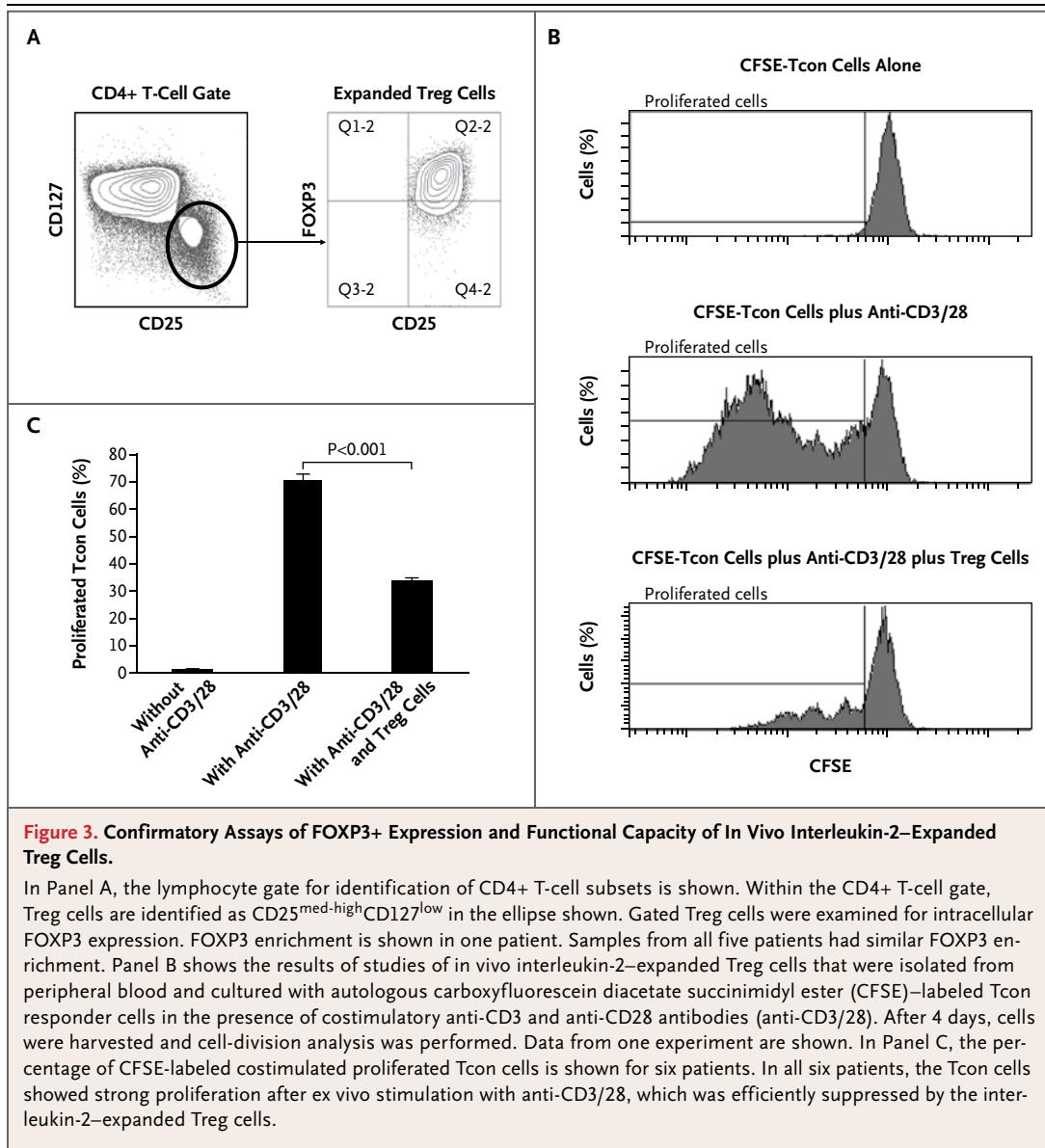
Figure 2. Immunologic Effects of 8 Weeks of Low-Dose Interleukin-2 Therapy and of Extended Therapy.

Panels A through D show the immunologic effects of 8 weeks of low-dose interleukin-2 (red line), followed by 4 weeks without interleukin-2. Medians and interquartile ranges are shown. Panel E shows the immunologic effects of extended treatment with interleukin-2 (red line) on regulatory T (Treg) cells. A sustained increase in CD4+CD25$^{med-high}$CD127^{low} Treg cells during 12-month treatment with interleukin-2 is shown. Medians and interquartile ranges are shown. NK denotes natural killer, and Tcon CD4+ conventional T.

of the patient or donor, the previous diagnosis of a hematologic cancer, the intensity of the conditioning regimen (either myeloablative or reduced intensity), the donor type (matched related, matched unrelated, or mismatched), the stem-cell source (bone marrow or peripheral blood), prior acute GVHD (grade 0 or 1 vs. 2 to 4), or the interleukin-2 dose level. Associations of the Treg-cell count and

A

CD4+ T-Cell Gate

Expanded Treg Cells

B

CFSE-Tcon Cells Alone

CFSE-Tcon Cells plus Anti-CD3/28

CFSE-Tcon Cells plus Anti-CD3/28 plus Treg Cells

C

Figure 3. Confirmatory Assays of FOXP3+ Expression and Functional Capacity of In Vivo Interleukin-2–Expanded Treg Cells.

In Panel A, the lymphocyte gate for identification of CD4+ T-cell subsets is shown. Within the CD4+ T-cell gate, Treg cells are identified as CD25$^{med-high}$CD127^{low} in the ellipse shown. Gated Treg cells were examined for intracellular FOXP3 expression. FOXP3 enrichment is shown in one patient. Samples from all five patients had similar FOXP3 enrichment. Panel B shows the results of studies of in vivo interleukin-2–expanded Treg cells that were isolated from peripheral blood and cultured with autologous carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled Tcon responder cells in the presence of costimulatory anti-CD3 and anti-CD28 antibodies (anti-CD3/28). After 4 days, cells were harvested and cell-division analysis was performed. Data from one experiment are shown. In Panel C, the percentage of CFSE-labeled costimulated proliferated Tcon cells is shown for six patients. In all six patients, the Tcon cells showed strong proliferation after ex vivo stimulation with anti-CD3/28, which was efficiently suppressed by the interleukin-2–expanded Treg cells.

the Treg:Tcon ratio with response are described in the Supplementary Appendix.

DISCUSSION

Chronic GVHD is a serious complication of HSCT. Besides conferring an increased risk of death, moderate or severe chronic GVHD impairs the quality of life. The impairment is similar to that with systemic sclerosis, systemic lupus erythematosus, or multiple sclerosis and is greater than that with diabetes, hypertension, or chronic lung disease.[3] Improved treatment for chronic GVHD is a major unmet need.

Treg cells play a central role in immune tolerance and prevention of aberrant immune responses. Treg-cell dysfunction occurs in systemic autoimmune disorders as well as in chronic GVHD.[12-14] There is considerable interest in strategies to restore Treg cells in these disorders; these strategies are mostly focused on the adoptive transfer of Treg cells that have been purified and expanded ex vivo.[29,30] In animal models of autoimmunity, Treg-cell enhancement can reverse target-organ damage.[31-33] In animal models of GVHD, adoptive transfer of Treg cells can prevent disease, but reversal of established inflammation remains uncertain.[19] Unfortunately, Treg-cell isolation and large-

scale expansion have been difficult to achieve in humans, and measurable immune effects have been limited and brief.[34,35]

We evaluated the alternative approach of preferential Treg-cell stimulation in vivo with the use of daily subcutaneous low-dose interleukin-2, documenting its feasibility and acceptable side-effect profile in patients with active chronic GVHD. Although the number of patients was small, we did not observe a flare of GVHD. We did identify thrombotic microangiopathy as a potential risk that was not previously reported with low-dose interleukin-2.[36-39] This condition occurred in patients receiving concomitant sirolimus plus tacrolimus and is a known toxic effect of these agents.

The 8-week interleukin-2 regimen induced objective partial responses in about half the patients who could be evaluated. Responses coincided with markedly increased Treg-cell counts and Treg:Tcon ratios. Expanded Treg cells were biologically active ex vivo. Responses were further enhanced in patients who received interleukin-2 for an extended duration, with improvement in advanced fibrotic and sclerotic manifestations of chronic GVHD that were previously considered to be irreversible.[26]

Improvement of sclerodermatous chronic GVHD has also been described in studies of therapies such as extracorporeal photopheresis and imatinib.[40,41] These studies differed from our study with respect to the criteria for patient eligibility and the response criteria (the previous studies did not use the NIH response criteria), making a retrospective comparison difficult. Such alternative therapies should be compared with interleukin-2 in future studies of treatment for chronic GVHD. We observed responses (partial responses) to interleukin-2 in 5 of the 8 patients who could be evaluated (of 13 enrolled) who had disease that was refractory to extracorporeal photopheresis and in 4 of the 6 patients who could be evaluated (of 7 enrolled) who had disease that was refractory to imatinib.

Increases in Treg-cell counts are not a universal feature of therapies that improve chronic GVHD. We found no increase in Treg-cell counts in pa-tients with chronic GVHD in another study evaluating treatment with rituximab (see the Supplementary Appendix).

Low-dose interleukin-2 did not result in obvious immune impairment. Although three major infections were documented, they were probably not related to interleukin-2, since several patients had infections before the initiation of interleukin-2 therapy, and chronic GVHD and concomitant immunosuppression both increase the risk of infection. Furthermore, increased opportunistic fungal and viral infections were not observed during extended-duration therapy; these findings are similar to those in other trials of low-dose interleukin-2 in immunocompromised patients.[36,37] We also observed no relapses of an underlying hematologic cancer in this small sample, suggesting that graft-versus-tumor responses were not abrogated by expanded Treg cells. Interleukin-2–mediated natural killer–cell augmentation may also be relevant in this regard.

Despite Treg-cell expansion, not all patients had a clinical benefit. Although patient characteristics, underlying disease, concomitant immunosuppressive medications, and the extent of tissue injury may all influence the clinical response, Treg-cell activity and homing to sites of tissue damage may also vary from one patient to another.

In summary, we found that the administration of daily subcutaneous low-dose interleukin-2 was safe in patients with active chronic GVHD, rapidly induced preferential and sustained Treg-cell expansion, could reverse advanced manifestations of chronic GVHD, and permitted a substantial reduction in the glucocorticoid dose.

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