A Novel Clinically Relevant Strategy to Abrogate Autoimmunity and Regulate Alloimmunity in NOD Mice

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OBJECTIVE—To investigate a new clinically relevant immunoregulatory strategy based on treatment with murine Thymoglobulin mATG Genzyme and CTLA4-Ig in NOD mice to prevent allo- and autoimmune activation using a stringent model of islet transplantation and diabetes reversal.

RESEARCH DESIGN AND METHODS—Using allogeneic islet transplantation models as well as NOD mice with recent onset type 1 diabetes, we addressed the therapeutic efficacy and immunomodulatory mechanisms associated with a new immunoregulatory protocol based on prolonged low-dose mATG plus CTLA4-Ig.

RESULTS—BALB/c islets transplanted into hyperglycemic NOD mice under prolonged mATG+CTLA4-Ig treatment showed a pronounced delay in allograft rejection compared with untreated mice (mean survival time: 54 vs. 8 days, $P < 0.0001$). Immunologic analysis of mice receiving transplants revealed a complete abrogation of autoimmune responses and severe downregulation of alloimmunity in response to treatment. The striking effect on autoimmunity was confirmed by 100% diabetes reversal in newly hyperglycemic NOD mice and 100% indefinite survival of syngeneic islet transplantation (NOD.SCID into NOD mice).

CONCLUSIONS—The capacity to regulate alloimmunity and to abrogate the autoimmune response in NOD mice in different settings confirmed that prolonged mATG+CTLA4-Ig treatment is a clinically relevant strategy to translate to humans with type 1 diabetes. ***Diabetes*** **59:2253–2264, 2010**

Successful islet transplantation can improve metabolic control and re-establish normoglycemia in subjects with type 1 diabetes (1). Unfortunately, transplanted islets are subject to both alloimmune responses and the recurrence of autoimmunity; either of which, when left uncontrolled, is capable of jeopardizing long-term islet function (1–4). Nonobese diabetic (NOD) mice represent the most commonly used animal model for human type 1 diabetes, and when used in settings of islet transplantation, the results obtained can be used to predict the results in type 1 diabetic patients (5–9). Indeed, several of the immunoregulatory defects observed in type 1 diabetic patients parallel those in NOD mice (10).

Thymoglobulin (ATG), a widely used induction therapy in solid organ transplantation (11), as well as in islet transplantation (12–14), depletes peripheral T cells and inhibits T-effector cell (Teff) expansion, but spares T regulatory cells (Treg) (15). ATG may be used for this purpose in immunoregulatory protocols. Moreover, murine ATG (mATG), similar to other depleting agents with immunoregulatory function (9), has been shown to be effective in downregulating the autoimmune response and in providing therapeutic efficacy in terms of preventing and reverting type 1 diabetes in NOD mice (16,17); mATG was recently shown to be particularly effective when combined with granulocyte colony-stimulating factor therapy (18). We have used a new mATG-based protocol in which low chronic doses were administered to continually promote Treg expansion and maintain low numbers of Teffs. A major issue when using T-cell–depleting strategies, such as ATG, in the clinical setting is the robust proliferation of residual or new emerging lymphocytes in the process of homeostatic proliferation (19), which can create a barrier to tolerance induction because re-emerging lymphocytes typically have an activated phenotype and are more resistant to regulation compared with naïve cells (19). In this regard, CD28, which is expressed constitutively on T cells and ligates either B7-1 (CD80) or B7-2 (CD86) on antigen-presenting cells (APC), thus resulting in delivery of signals that promote clonal expansion and the effector function of T cells (20,21), may play a pivotal role in regulating homeostatic proliferation (19). Targeting CD28 signaling may thus be an effective strategy to control homeostatic proliferation during T-cell depletion. The most widely used reagent to target CD28-CD80/CD86 costimulation is CTLA4-Ig, a fusion protein modeled on the structure of CTLA4 (22). CTLA4 is a coinhibitory

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molecule expressed on T cells after activation that inhibits the T-cell response after CD80/CD86 engagement (23,24). Moreover, because the affinity for CD80/CD86 is greater in CTLA4 than CD28, the use of CTLA4-Ig prevents CD28 engagement on T cells and thus prevents T-cell activation (20). CTLA4-Ig is currently used in clinical settings for the treatment of autoimmune disorders (25–27).

Based on this information, we hypothesized that combining short-term CTLA4-Ig treatment with prolonged low-dose mATG could prevent the homeostatic proliferation that follows mATG treatment, as well as synergizing with mATG to downregulate the allo- and autoimmune responses, thereby promoting both islet allograft survival and reversal of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Mice. Female NOD and NOD.SCID mice, as well as BALB/c mice, were obtained from Jackson Laboratory (Bar Harbor, ME). All mice were cared for in accordance with institutional guidelines under specific-pathogen–free conditions at the Harvard Medical School Facilities for Animal Care and Housing. Protocols were approved by the Institutional Animal Care and Use Committee.

Monitoring for diabetes. Blood glucose was measured using Accu-Check Advantage glucometers (Roche Diagnostics, Indianapolis, IN). The diabetes reversal protocol was tested in newly hyperglycemic mice after 2 consecutive days of blood glucose >250 mg/dl. Stable reversal of diabetes was assessed with blood glucose measurements twice weekly.

Insulitis score. Insulitis scoring was performed on hematoxylin and eosin (H&E)-stained pancreatic sections. A score of 0 to 4 was assigned based on islet infiltration by an experienced pathologist, as previously described (7). Insulitis scores were graded as follows: grade 0, normal islets; grade 1, mild mononuclear infiltration (<25%) at the periphery; grade 2, 25–50% of the islets infiltrated; grade 3, >50% of the islets infiltrated; grade 4, islets completely infiltrated with no residual parenchyma remaining. At least 30 islets per group were analyzed and pooled from sections obtained from different mice.

Islet infiltration score. A score from 0 to 4 was assigned to graft islets based on grade of infiltration and islet preservation. Grade 0 = no inflammation; grade 1 = peri-islet inflammation only; grade 2 = intra-islet inflammation occupying <50% of the islets; grade 3 = intra-islet inflammation occupying >50% of the islets; grade 4 = complete or near complete obliteration of islets by inflammation.

Isolation of pancreatic islets. Pancreatic islets were isolated by collagenase digestion followed by Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) density gradient separation and hand-picking, as described previously (28).

Islet transplantation. NOD mice were allowed to develop diabetes spontaneously, and 800 islets from BALB/c donors (fully allogeneic) or NOD.SCID donors (syngeneic) were transplanted under the renal capsule. Once normoglycemia was achieved, graft rejection was defined on the basis of stable blood glucose levels of >250 mg/dl.

Cardiac transplantation. Heart transplantation was performed in NOD mice as described previously (29) to compare the rejection of islet allografts with the rejection of nonautoimmune subjected grafts. Cardiac graft survival was assessed by palpation. Islet and cardiac grafting procedures were performed independently.

Skin transplantation. Full-thickness trunk skin grafts (1 cm^2) harvested from BALB/c donors were transplanted onto the flank of NOD.SCID recipient mice, sutured with 6.0 silk, and secured with dry gauze and a bandage for 7 days. Skin graft survival was monitored daily thereafter, and rejection was defined as complete graft necrosis.

Antibodies and treatment protocol. Murine ATG (mATG) or control Ig were administered as follows: *1*) induction mATG (500 μg on days 0 and 4); *2*) CTLA4-Ig (500 μg on day 0; 250 μg on days 2, 4, 6, 8, and 10); *3*) prolonged mATG (500 μg on days 0 and 4; 100 μg twice weekly starting on day 10); *4*) induction mATG+CTLA4-Ig (mATG: 500 μg on days 0 and 4 + CTLA4-Ig: 500 μg on day 0; 250 μg on days 2, 4, 6, 8, and 10); or *5*) prolonged mATG+CTLA4-Ig (mATG: 500 μg on days 0 and day 4, then twice weekly starting at day 10 + CTLA4-Ig: 500 μg on day 0; 250 μg at days 2, 4, 6, 8, and 10).

Islet pathology and immunohistochemistry. Transplanted mice were killed at various time points to obtain histology specimens. Kidney sections were stained with H&E. Immunohistochemistry was performed using 5-μ*m*–thick formalin-fixed, paraffin-embedded tissue sections as previously described (7). $FoxP3^+$ percentage on $CD3^+$ cells was assessed using a Aperio ScanScope. Briefly, stained slides were scanned at ×200 magnification using an Aperio ScanScope XT workstation (Aperio Technology, Vista, CA). Images were visualized and annotated using ImageScope software (version 10.0.35.1800, Aperio Technology) and were analyzed using a standard analysis algorithm (color deconvolution v9.0, Aperio Technology).

Flow cytometry (fluorescence-activated cell sorter). Rat anti-mouse CD19 PE, CD20 PE, CD22 PE, B220 PE, B220 PerCP, CD80 (B7-1) PE, CD86 (B7-2) PE, H-2^d FITC, CD4 FITC, CD23 FITC, CD25 PE, CD44 PE, CD45 FITC, CD62L APC, CD93 PE (C1qRp), and IgM PAPC were purchased from BD Biosciences (San Jose, CA) and eBiosciences (San Diego, CA). FoxP3 APC was purchased from eBiosciences. Cells recovered from spleen and peripheral blood were subjected to fluorescence-activated cell sorter analysis and run on a FACSCalibur (Becton Dickinson). Data were analyzed using FlowJo software version 6.3.2 (Treestar, Ashland, OR). FoxP3 analysis was performed after overnight permeabilization of cells extracted from spleen and peripheral lymphoid tissue using commercially available antibodies and gating on $CD4^+CD25^+$ cells.

Enzyme-linked immunosorbent spot. To measure cytokine production by splenocytes extracted from different groups of NOD mice, our enzyme-linked immunosorbent spot (ELISPOT) assay was used as previously described (4). Briefly, Millipore immunospot plates (Millipore Corporation, Bedford, MA) were coated with capture antibodies (BD Biosciences). Plates were blocked with 1% BSA to prevent nonspecific binding. NOD splenocytes (1×10^6) were challenged in the presence of 150 μg/ml BDC2.5 peptide (Ac-RTRPLWVRME amide, QCB, Hopkinton MA), 50 μg/ml IGRP $^{(206–214)}$ peptide (VYLKTNVFL) or 1×10^6 BALB/C irradiated splenocytes. After 2 days of culture in 10% FCS, 1% penicillin-streptomycin RPMI at 37°C and 5% CO_2, plates were washed and biotinylated antibodies specific for each cytokine were added to the wells, followed by incubation at 4°C for 12 h. Plates were then washed, incubated at room temperature with streptavidin-horseradish peroxidase (HRP) for 2 h, and developed using aminoethyl carbazole (AEC, Sigma Aldrich) diluted in N,N-dimethylformamide. Spots were counted on an immunospot analyzer (Cellular Technology, Cleveland, OH).

In vivo proliferation. A quantity of 50×10^6 Carboxyfluoroscein-succinimidyl-ester (CFSE)-labeled lymphocytes from NOD mice were injected intravenously into NOD.SCID mice in 0.5 ml of sterile PBS. Recipients were treated according to standard protocols. Spleens were harvested 3 days after adoptive transfer. The proliferation of CFSE-labeled donor cells in the recipient spleen was examined by flow cytometry based on CFSE dilution analysis. In particular, proliferation of $CD4^+$ (using anti-CD4 APC) and $CD8^+$ (using anti-CD8 APC) cells was assessed.

Ovalbumin immunization. Ovalbumin peptide (Sigma Aldrich) emulsified in complete Freund's adjuvant (Sigma Aldrich) was injected once (100 μg/mouse i.p.) in naïve or transplanted NOD mice. Splenocytes were collected and used in in vitro assays after rechallenge with 1 μmol/l ovalbumin peptide for 24 h.

Luminex serum cytokine determination. Treated or control NOD mice were bled via tail perforation on days 0, 7, 14, and 28 after islet transplantation, and serum samples were collected. The BeadLyte Mouse Multi-Cytokine Beadmaster Kit (Millipore) was used according to the manufacturer's protocol to determine cytokine levels of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-15, IL-17, IFN-γ, and TNF-α. Briefly, supernatant samples were incubated overnight at room temperature with beads conjugated to the aforementioned cytokines, matched biotinylated reporters were added and incubated for 1.5 h, and streptavidin-phycoerythrin solution was incubated with samples for 30 min. After the addition of stop solution, sample cytokine levels were calculated from a standard curve using a Luminex100 reader (Luminex Corporation, Austin, TX).

Adoptive transfer studies. Skin grafts from BALB/c donors were transplanted onto immunodeficient NOD.SCID hosts. Splenocytes (15×10^6) from transplanted NOD mice treated with prolonged mATG+CTLA4-Ig at day 56 or from untransplanted NOD mice were adoptively transferred into NOD.SCID that received skin transplantation. These NOD.SCID mice were monitored for the onset of hyperglycemia and skin graft rejection.

Statistical analyses. Data are expressed as mean ± SE. Kaplan-Meier analysis was used for survival analysis. ANOVA (for parametric data) and Kruskal-Walliss (for nonparametric data) were also used. When the two groups were compared cross-sectionally, the two-sided unpaired Student *t* test (for parametric data) or the Mann-Whitney test (for nonparametric data) was used according to value distribution. A P value < 0.05 (by two-tailed testing) was considered an indicator of statistical significance. Analyses of data were performed using a SPSS statistical package for Windows (SPSS, Chicago, IL).

RESULTS

mATG depletes T-lymphocytes in islet-transplanted NOD mice. We tested the depleting potential of mATG on peripheral blood in an allogeneic fully mismatched model

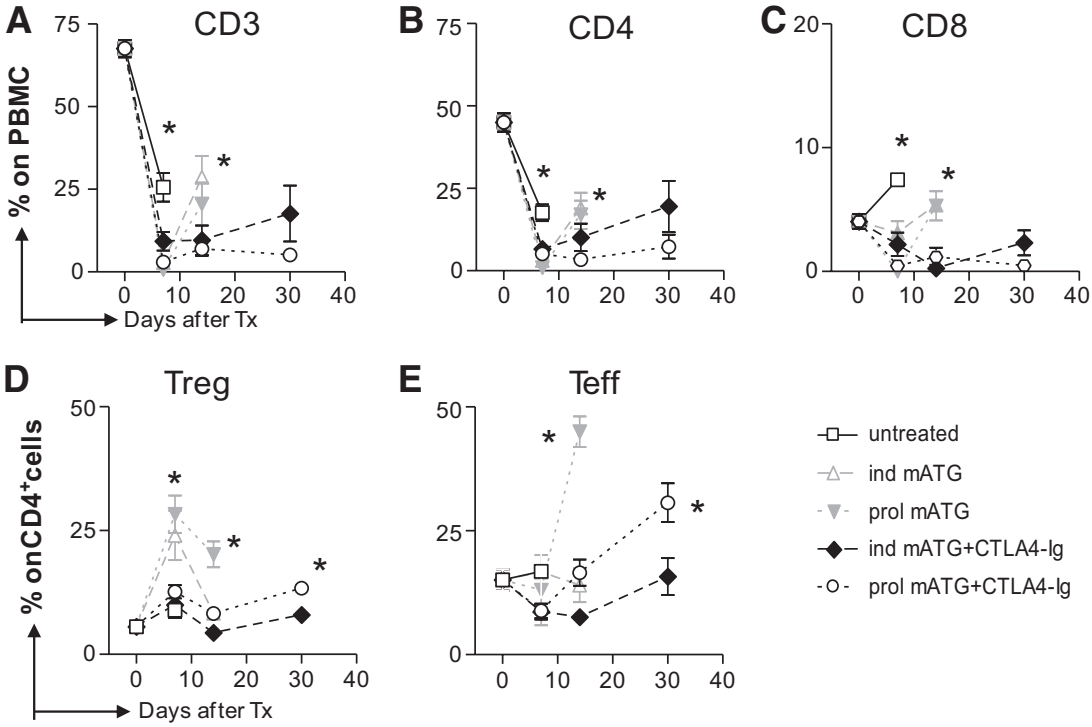

FIG. 1. Analysis of Peripheral Blood Mononuclear Cells (PBMC) was performed at various time points after transplantation in diabetic NOD mice. $CD4^+$ and $CD8^+$ T-cell depletion is detected on day 7 after mATG treatment (untreated vs. all treatment, $P < 0.05$) (*A–C*). Addition of CTLA4-Ig slowed reconstitution after mATG depletion on day 14 ($CD3^+$, $CD4^+$, and $CD8^+$ cells: prolonged mATG+CTLA4-Ig vs. prolonged mATG, $P < 0.05$). On day 28, peripheral T-cell percentage in animals treated with prolonged mATG+CTLA4-Ig was similar to that of animals treated with induction mATG+CTLA4-Ig (P = NS). On day 7, $FoxP3^+CD25^+CD4^+$ (Tregs) was increased by both induction and prolonged mATG treatment ($P < 0.05$ vs. day 7 untreated), and on day 14 by prolonged mATG treatment ($P < 0.05$ vs. day 7 untreated) (*D*). Addition of CTLA4-Ig abrogated this increase (day 7: induction mATG+CTLA4-Ig vs. induction mATG, $P = 0.04$; day 7 and day 14 prolonged mATG+CTLA4-Ig vs. prolonged mATG, $P = 0.002$ and $P = 0.008$, respectively) (*D*). Peripheral Treg frequency at day 28 was increased with prolonged mATG + CTLA4-Ig treatment compared with induction mATG+CTLA4-Ig treatment ($P < 0.05$) (*D*). On day 14, a relative increase in $CD44^{high}CD62^{low}CD4^+$ cells (Teffs) was evident with prolonged mATG treatment ($P < 0.05$ vs. day 7 untreated); this increase was prevented by the addition of CTLA4-Ig (prolonged mATG+CTLA4-Ig vs. prolonged mATG, $P = 0.0001$) (*E*). On day 28, Teffs were increased in the prolonged mATG+CTLA4-Ig group compared with the induction group ($P < 0.05$) (*E*). *$P < 0.05$.

of islet transplantation (i.e., BALB/c islets transplanted into NOD mice). Mice were followed until rejection, as after rejection mice became lymphopenic and immunocompromised. In untreated mice, the numbers of peripheral $CD3^+$ cells dropped soon after islet transplantation (Fig. 1*A*). An induction dose of mATG (500 μg at days 0 and 4) further depleted $CD3^+$ cells in the peripheral blood of NOD transplanted mice (Fig. 1*A*), and $CD4^+$ and $CD8^+$ cells appeared to be equally affected (Fig. 1*B* and *C*). Prolonged mATG treatment (mATG 500 μg at days 0 and 4, then 100 μg twice weekly) did not appear to result in further depletion.

Addition of CTLA4-Ig treatment (500 μg at day 0 and 250 μg at days 2, 4, 6, 8, and 10) slowed T-cell reconstitution (Fig. 1*A*). At day 28, the T-cell pool in the induction group treated with mATG+CTLA4-Ig had been replenished enough to appear similar to the T-cell pool in the prolonged mATG+CTLA4-Ig group (P = NS) (Fig. 1*A*).

We then analyzed the percentages of $CD4^+CD25^+$ $FoxP3^+$ (Treg) and $CD4^+CD44^+CD62^{low}$ (Teff) cells. Prolonged mATG treatment increased the peripheral Treg percentage at days 7 and 14 compared with untreated mice. At day 7 only, in the induction mATG group, the Treg percentage also increased (Fig. 1*D*). Combining CTLA4-Ig with mATG inhibited the increase in Treg percentage produced by the mATG treatment during both induction and prolonged mATG treatment (Fig. 1*D*). Notably, at day 28, the percentage of Tregs was higher in the prolonged mATG+CTLA4-Ig group compared with the induction mATG+CTLA4-Ig group (Fig. 1*D*).

Induction mATG treatment per se did not have any effect on Teff percentage; however, prolonged mATG treatment increased Teff cells at day 14 (Fig. 1*E*), whereas the combination of mATG and CTLA4-Ig reduced the percentage of Teff cells (Fig. 1*E*). At day 28, Teff percentages were higher in the prolonged mATG+CTLA4-Ig group compared with the induction mATG+CTLA4-Ig animals (Fig. 1*E*).

Prolonged mATG+CTLA4-Ig treatment expands splenic Tregs and controls Teff proliferation. We confirmed the effect of induction mATG, induction mATG+CTLA4-Ig, and prolonged mATG+CTLA4-Ig on Treg and Teff balance in the mouse spleen. At day 14, mATG induction increased the Treg percentage in the spleen compared with hyperglycemic untransplanted mice (i.e., hyper) (Fig. 2*A*). Teff percentage was also increased by induction mATG (Fig. 2*B*). The combination of mATG and CTLA4-Ig did not significantly affect the Treg percentage compared with induction mATG treatment alone (Fig. 2*A*), but significantly reduced Teffs (Fig. 2*B*). We then compared the effect of prolonged mATG treatment to induction mATG, both combined with CTLA4-Ig, at day 28. Prolonged mATG treatment significantly increased Tregs compared with induction mATG (Tregs, shown as percentages of the $CD4^+$ population, on day 28: prolonged mATG+CTLA4-Ig 19.5 ± 3.5% vs. induction mATG+

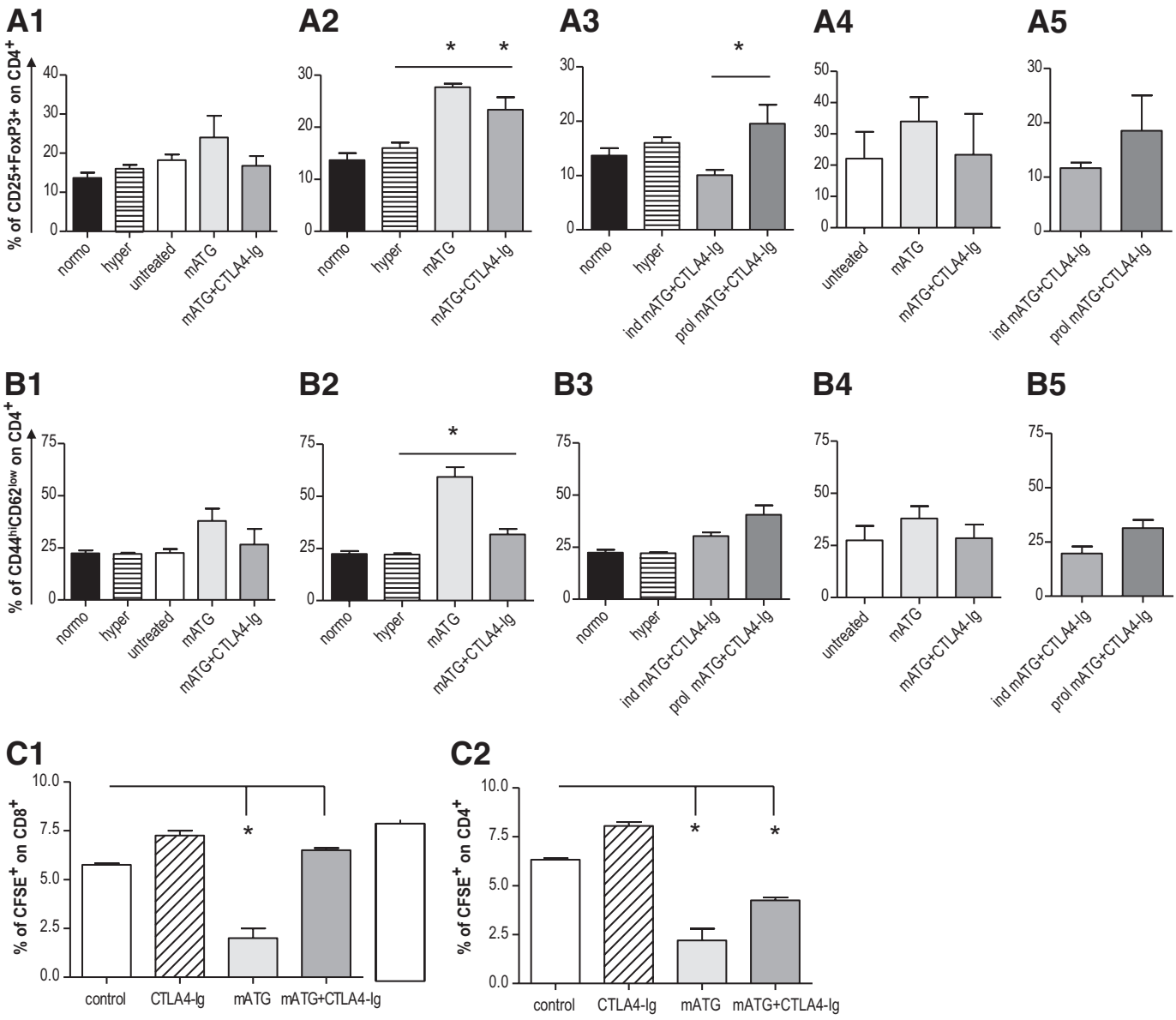

FIG. 2. Splenocytes and islet graft draining lymph nodes were analyzed at various time points in treated and control mice. Treg ($CD4^+CD25^+Foxp3^+$ cells) frequency was stable after mATG treatment on day 7 (*A*1) and increased at day 14 ($P < 0.05$ vs. hyper) (*A*2). The combination with CTLA4-Ig on day 14 appeared not to inhibit Treg percentage increase ($P < 0.05$ vs. hyper) (*A*2). On day 28, the Treg proportion was higher in the prolonged mATG+CTLA4-Ig group compared with the induction group ($P < 0.05$) (*A*3). A similar increased proportion, although the difference did not reach statistical significance, was seen in graft draining lymph nodes on days 7 (*A*4) and 28 (*A*5). Teff ($CD4^+CD44^{hi}CD62^{low}$) percentage in the spleen was shown to be stable on day 7 after transplantation (*B*1), but Teffs were increased after mATG treatment on day 14 ($P < 0.05$ vs. hyper) (*B*2); its combination with CTLA4-Ig completely prevented any Teff increase, (*B*2). On day 28, Teff cell proportions in prolonged and induction mATG+CTLA4-Ig-treated mice were similar (*B*3). A similar profile was observed in graft draining lymph nodes on days 7 (*B*4) and 28 (*B*5). Splenocytes (50×10^6) were CFSE-labeled and adoptively transferred into NOD.SCID mice treated using a standard protocol. mATG-treated mice showed a reduced percentage of $CFSE^{high}$-undividing $CD8^+$ and $CD4^+$ cells ($P < 0.05$ vs. untreated), revealing postmATG depletion homeostatic proliferation (*C*1 and 2). On the contrary, combination with CTLA4-Ig reverted (completely [$CD8^+$ cells] or partially [$CD4^+$ cells]) the reduction in undividing cells percentage after mATG treatment (*C*1 and 2). *$P < 0.05$.

CTLA4-Ig 10.1 ± 0.9%, $n = 3$, $P = 0.04$) (Fig. 2*A*); however, there was no significant difference in Teff cells (Fig. 2*B*). Tregs and Teffs were also analyzed in transplanted islet-draining lymph nodes, confirming the pattern observed in the spleen (Fig. 2*A* and *B*). These data together showed that prolonged low-dose mATG treatment, compared with induction mATG treatment, is capable of tipping the Treg/Teff balance to favor Treg.

CTLA4-Ig treatment reduces the homeostatic proliferation that follows mATG depletion. Homeostatic proliferation is considered a barrier to tolerance. To evaluate the effectiveness of our approach (mATG+CTLA4-Ig) to blunt homeostatic proliferation, we performed adoptive transfer experiments. NOD splenocytes (50×10^6) were labeled with CFSE and transferred into treated-NOD.SCID mice. Three days after adoptive transfer, splenocytes were harvested and the percentage of nondividing T cells ($CD4^+/CD8^+$ $CFSE^{high}$) was assessed as a parameter of homeostatic proliferation (19). The number of nondividing T cells was decreased in mATG-treated mice compared with untreated mice, confirming the existence of post-depletion homeostatic proliferation upon mATG treatment. However, the combination of mATG and CTLA4-Ig reduced homeostatic proliferation, with an increase in the

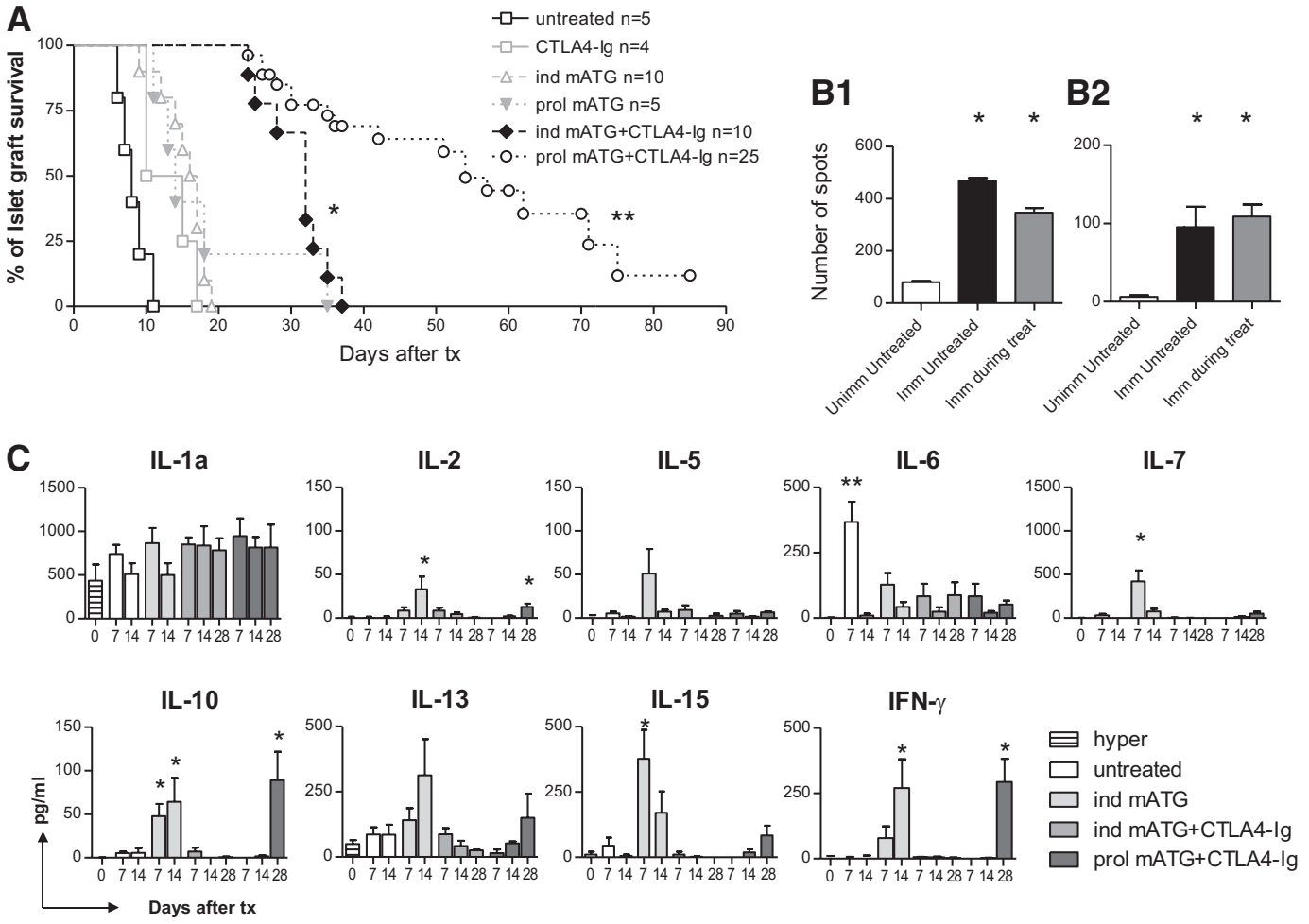

FIG. 3. BALB/c islets were transplanted into hyperglycemic NOD mice, and allograft survival was evaluated. All treatments significantly prolonged graft survival ($P < 0.05$ vs. untreated). The addition of CTLA4-Ig synergized with mATG treatment to delay rejection (graft survival: induction mATG vs. induction mATG+CTLA4-Ig, $P < 0.05$; prolonged mATG vs. prolonged mATG+CTLA4-Ig, *$P < 0.05$) (*A*). Prolonged mATG+CTLA4-Ig was shown to be more efficacious than induction mATG+CTLA4-Ig ($P < 0.05$) (*A*). Immunocompetence was examined in prolonged mATG+CTLA4-Ig treated mice via evaluation of the capacity to mount an ovalbumin-specific response. The IFN-γ (*B1*)- and IL-4 (*B2*)-specific responses were higher in mice immunized during treatment compared with unimmunized untreated mice (*$P < 0.05$). Serum samples were collected after islet transplantation on days 7, 14, and 28, and peripheral cytokine profile was evaluated. The Th1-related cytokines (IFN-γ, IL-2) were increased by mATG treatment ($P < 0.05$ vs. hyper), whereas this increase was prevented by mATG combination with CTLA4-Ig (*C*). A similar pattern was observed for the immunoregulatory cytokine IL-10 and for the cytokines related to homeostatic proliferation (IL-7, IL-15) (*C*). The proinflammatory cytokine IL-6 increased after transplantation ($P < 0.05$ vs. hyper), whereas treatment with mATG completely prevented this increase (*C*). IL-1α and the Th2 cytokine IL-5 were not significantly affected (*C*).**

number of nondividing cells (Fig. 2*C*). This blunting of homeostatic proliferation was more evident in $CD8^+$ T cells, although it was partially effective in $CD4^+$ T cells. Taken together, these data reveal the capacity of CTLA4-Ig for inhibiting mATG-induced homeostatic proliferation, providing an appealing combination therapy for a novel immunomodulatory approach. We also tested whether the activation of dendritic cells (DCs) is influenced by CTLA4-Ig treatment. Hyperglycemic mice were transplanted with allogeneic islets and either treated with anti-CTLA4-Ig or left untreated. No major differences were observed with regard to costimulatory molecule expression in DCs obtained from the two groups at day 7 after transplantation (data not shown).

Prolonged mATG treatment synergizes with CTLA4-Ig to prolong islet allograft survival in spontaneously diabetic NOD mice. We then compared the impact of mATG treatment alone or combined with CTLA4-Ig on islet allograft survival. Untreated NOD mice invariably rejected allografts (untreated: mean survival time [MST] of 8 days, $n = 5$). We then tested mATG as a single agent. Considering the potential of mATG treatment to cause reversal in newly hyperglycemic NOD mice (16), NOD mice were used as islet recipients after at least 14 consecutive days of glucose measurements >400 mg/dl. The mATG induction extended allograft survival (induction mATG: MST of 16.5 days, $n = 10$, $P = 0.0001$ vs. untreated) (Fig. 3*A*). Prolonged mATG treatment per se did not further increase graft survival (prolonged mATG: MST of 14 days, $n = 5$, NS vs. induction mATG) (Fig. 3*A*) with 1 of 5 mice reaching 40 days of survival. We therefore combined mATG with CTLA4-Ig, with the goal of reducing activation of the immune system in response to alloantigen and to counteract homeostatic proliferation, which follows T-cell depletion. CTLA4-Ig alone slightly prolonged graft survival (CTLA4-Ig: MST of 12.5 days, $n = 4$, $P = 0.04$ vs. control) (Fig. 3*A*) and synergized with mATG to further increase allograft survival (induction mATG+CTLA4-Ig: MST of 32 days, $n = 9$, $P < 0.0001$ vs. induction mATG) (Fig. 3*A*). Finally, the combination of prolonged mATG

treatment and CTLA4-Ig significantly increased graft survival (prolonged ATG+CTLA4-Ig: MST of 54 days, $n = 10$, $P = 0.007$ vs. mATG+CTLA4) (Fig. 3*A*). Notably, in this stringent model of enhanced allo-/auto-immune responses, 30% of these mice retained graft function for more then 70 days. We also tested whether depletion of Tregs through treatment with anti-CD25-Ig at the time of transplantation (500 μg at days −6 and −1) was able to abrogate the effect of prolonged mATG+CTLA4-Ig. Although CD25-depleted untreated NOD mice rejected islet grafts rapidly (MST of 8 days), CD25-depleted NOD mice treated with prolonged mATG+CTLA4 showed a prolongation of islet graft survival (MST of 53 days), suggesting the capacity of our approach to reconstitute the Treg pool (data not shown).

Prolonged mATG+CTLA4-Ig treatment has little effect on immunocompetence. We then evaluated whether immunocompetence was preserved during prolonged mATG+CTLA4-Ig treatment. We assessed the ability of treated mice to mount an antigen-specific response. Prolonged mATG+CTLA4-Ig-treated and untreated NOD mice were immunized at day 28 with ovalbumin. After 3 days, splenocytes were harvested and rechallenged in vitro with ovalbumin.

Modest IFN-γ and IL-4 production was seen after in vitro rechallenge in unimmunized mice independent of the treatment (unimmunized: IFN-γ = 79 ± 7, IL-4 = 6 ± 3, measured as cytokine-producing cells, $n = 3$). On the contrary, markedly high IFN-γ and IL-4 production was observed in immunized mice. No differences were seen between mice immunized during treatment and immunized untreated mice with regard to IL-4 production (immunized during treatment = 108 ± 15 vs. immunized untreated = 95 ± 26, $n = 3$, P = NS). Only a slight reduction was noted in IFN-γ production (immunized during treatment = 347 ± 17 vs. immunized untreated = 468 ± 11, $n = 3$, $P = 0.01$) (Fig. 3*B*2). These data suggest that the ability of mounting an antigen-specific response is only slightly affected during treatment.

Prolonged mATG and CTLA4-Ig combination treatment reshapes the peripheral cytokine profile. We then evaluated the effect of the treatments above on peripheral cytokine profiles using the Luminex assay. After transplantation, Th1 cytokines (IFN-γ and IL-2) appeared to be increased in the induction mATG group compared with hyperglycemic untransplanted mice (hyper) (Fig. 3*C*). Adding CTLA4-Ig to the induction mATG induction therapy completely prevented an increase in Th1 peripheral cytokines (Fig. 3*C*). CTLA4-Ig treatment also abrogated production of Th1 cytokines in the prolonged mATG+CTLA4-Ig group at early time points (days 7 and 14), whereas there was an increase in Th1 cytokines observed later (Fig. 3*C*). The Th2 cytokine IL-4 was found to be poorly expressed (data not shown), whereas IL-5 and IL-13 did not demonstrate clear Th2 activation after mATG treatment (Fig. 3*C*). The increase in the proinflammatory cytokine IL-6, evident after islet transplantation in untreated mice, seemed to be controlled in mATG-treated mice regardless of the use of CTLA4-Ig (Fig. 3*C*). After depletion, the remaining cells enter a proliferation phase, which tends to repopulate the T-cell niche during homeostatic proliferation. Although several cytokines are involved in this process, IL-7 and IL-15 are central (30,31). After mATG treatment, these two cytokines rapidly increased in their concentration (Fig. 3*C*), whereas combination treatment with CTLA4-Ig maintained levels of both cytokines (Fig. 3*C*), which may explain the moderate recovery curve in CTLA4-Ig-treated mice and may also cause a lower state of activation of proliferating T cells. IL-10, a Treg-associated cytokine, increased after induction mATG (Fig. 3*C*); however, concomitant treatment with CTLA4-Ig prevented the increase (Fig. 3*C*). At day 28 in prolonged mATG+CTLA4-Ig-treated mice, IL-10 levels were increased (Fig. 3*C*), whereas IL-17 was almost undetectable (Fig. 3*C*).

Prolonged mATG+CTLA4-Ig treatment preserves islet morphology. Grafts were analyzed at significant time points, and immune cells infiltration was blinded quantified by a pathologist (S.R.) with islet infiltration score (IIS) grading from 0 (no infiltration, preserved islets) to 4 (massive infiltration, no islet preservation, signs of fibrosis). H&E staining in untreated mice showed that islets were infiltrated by $CD3^+$ and $B220^+$ cells throughout the graft 7 days after transplantation; islet architecture was lost, with only a few remaining insulin-positive cells, IIS:three (Fig. 4*A*). The percentage of FoxP3 on $CD3^+$ cells was then assessed using a Aperio ScanScope; only a small proportion of $CD3^+$ cells appeared to be FoxP3-positive (11%). In the induction mATG group, islet morphology was preserved with positive insulin staining, whereas immunostaining showed a mild extra-islet $CD3^+/B220^+$ infiltrate, (IIS:1), with an increased proportion of Tregs ($FoxP3^+$ in $CD3^+$ cells: 25%) (Fig. 4*B*). This reduced islet infiltration was also detected in the mATG+CTLA4-Ig group at day 7, IIS:0; $FoxP3^+$ in $CD3^+$ cells: 28% (Fig. 4*C*). When mATG was discontinued, a massive infiltrate composed of $CD3^+$ and $B220^+$ cells was observed throughout the graft area by day 28, with few insulin-positive cells remaining (IIS:3–4) and a reduced proportion of Tregs ($FoxP3^+$ in $CD3^+$ cells: 17%) (Fig. 4*D*). In the prolonged mATG+CTLA4-Ig group, islet morphology was well preserved (with insulin staining still detectable), with lymphocyte infiltrate consisting of predominantly B cells surrounding, but not infiltrating, the islets (IIS:1), whereas Treg numbers were increased ($FoxP3^+$ in $CD3^+$ cells: 23%) (Fig. 4*E*).

Prolonged mATG+CTLA4-Ig treatment reduces responses to allo- and autoantigen ex vivo. To determine the relative contributions of auto- and alloimmune responses in the rejection process, we tested the potential for those responses in NOD mice treated with different regimens. Splenocytes isolated from mice receiving the various treatments were challenged in an ELISPOT assay with 1×10^6 irradiated BALB/c splenocytes to assess the veracity of alloimmune response or with 150 μg/ml BDC2.5 peptide (an islet-derived peptide) to assess the autoimmune response, and IFN-γ/IL-4 production was analyzed. At day 7 after islet transplantation, untreated mice showed considerable IFN-γ production in response to alloantigen; this response was highly suppressed in the mATG+CTLA4 group (IFN-γ day 7: untreated 367.3 ± 160.0 vs. mATG+CTLA4-Ig 14 ± 5, $n = 3$ vs. 4, $P = 0.04$; IL-4 day 7: 91 ± 8 vs. 18 ± 17, respectively, $n = 3$, $P = 0.02$). An intermediate response was seen in mATG-treated mice (Fig. 5*A*1). At day 14, mATG+CTLA4 treatment was clearly more effective than mATG, as shown by reduced IFN-γ production in the alloimmune assay (IFN-γ day 14: mATG+CTLA4-Ig 135 ± 44 vs. mATG 354 ± 27, $n = 3$, $P = 0.03$) (Fig. 5*A*2). IL-4 production did not differ between the two groups (data not shown). We next compared the effect of prolonged versus induction mATG treatment combined with CTLA4-Ig at day 28. An alloimmune assay showed that prolonged mATG treatment suppressed IFN-γ production compared with induction mATG (IFN-γ: prolonged

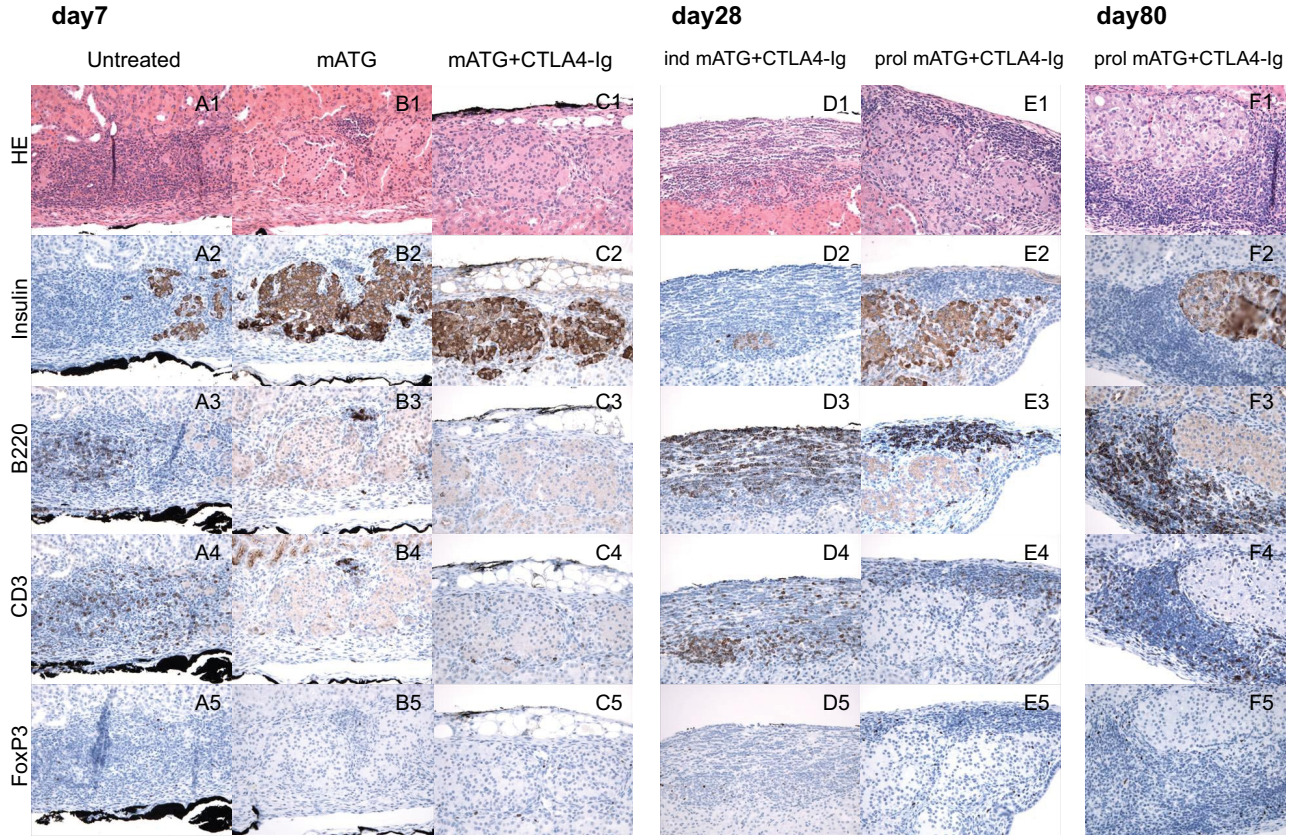

FIG. 4. Islets were transplanted under the kidney capsule of hyperglycemic NOD mice and retrieved for histologic analysis. At day 7, untreated mice showed massive infiltrate with completely disrupted islet architecture (*A*1). Few insulin-positive cells (*A*2) were found in the graft. B220$^+$ (*A*3) and CD3$^+$ (*A*4) cells were equally represented in the infiltrate, but FoxP3$^+$ cells were scarcely represented (*A*5), as confirmed by Aperio ScanScope quantification. A different pattern is apparent at day 7, both in mATG- and mATG+CTLA4-Ig-treated mice (*B* and *C*). Islet architecture was preserved with a mild infiltrate (*B*1 and *C*1), and insulin (*B* and *C*2) staining was clearly preserved. Few B220$^+$ (*B*3) and CD3$^+$ (*B*4) cells in equal proportions could be found in mATG-treated mice; few FoxP3$^+$ cells were present in the graft (*B*5). In the mATG+CTLA4-Ig-treated group, the infiltrate was almost absent (*C*3 and 4). Some of the CD3$^+$ cells also appeared to be FoxP3$^+$ (*C*5). A massive organized infiltrate in the graft area was found in the induction mATG+CTLA4-Ig-treated group at day 28 with no evidence of any islet allograft remaining (*D*1). Insulin staining appeared weak and limited to very few cells (*D*2). CD3$^+$ and B220$^+$ cells were equally represented (*D*3 and 4) with very few FoxP3$^+$ cells (*D*5). In mice treated with prolonged mATG+CTLA4-Ig, islet structure was preserved at day 28; infiltrate surrounds but does not infiltrate the islets (*E*1). Insulin-positive cells are well represented (*E*2). More B220$^+$ cells than CD3$^+$ were represented in the infiltrate (*E*4); notably, a higher proportion of CD3$^+$ cells appeared to be FoxP3$^+$ (*E*5). At day 80 after transplantation, in the prolonged mATG+CTLA4-Ig group, islet morphology remained preserved with massive infiltrate around but not infiltrating the islets (*F*1), and insulin staining was well preserved (*F*2). B220$^+$ cells appeared more abundant in the infiltrate than T cells (*F*3), as very few CD3$^+$ cells were evident (*F*4). Several FoxP3$^+$ cells were present in the infiltrate (*F*5). (A high-quality digital representation of this figure is available in the online issue.)

mATG+CTLA4-Ig 33 ± 12 vs. induction mATG+CTLA4-Ig, 314 ± 39, $n = 3$, $P = 0.01$) (Fig. 5*A*3).

The autoimmune response was suppressed at day 7 with regard to IFN-γ production in response to BDC2.5 peptide after mATG or mATG+CTLA4 treatment. The IFN-γ response was similar to that observed in normoglycemic untransplanted NOD (normo), whereas hyperglycemic untransplanted NOD (hyper) mice showed a higher response (IFN-γ day 7: mATG 39 ± 28, $n = 3$; mATG+CTLA4 6 ± 5, $n = 2$ vs. normo 16 ± 3, $n = 5$, P = NS; mATG and mATG+CTLA4-Ig vs. hyper 123 ± 23, $n = 4$, NS and $P = 0.015$, respectively) (Fig. 5*B*1). The IL-4 response was similarly reduced (data not shown). At day 14, the autoimmune response was suppressed in the mATG+CTLA4-Ig group, but not in the mATG group (IFN-γ: mATG+CTLA4-Ig 18 ± 8, $n = 3$ vs. normo, P = NS; mATG 78 ± 33, $n = 3$ vs. normo, $P = 0.02$) (Fig. 5*B*2).

The autoimmune response appeared to be attenuated by either prolonged or induction mATG+CTLA4-Ig treatment at day 28 (IFN-γ: prolonged mATG+CTLA4-Ig and induction mATG+CTLA4-Ig vs. normo, P = NS) (Fig. 5*B*3); in terms of the IL-4 response, no differences were observed between prolonged or induction mATG treatment+CTLA4-Ig in response to allo- or autoantigens (data not shown).

Collectively, these data demonstrate that mATG and mATG+CTLA4 treatment prevents alloimmune activation and reversion of autoimmune status at day 7. Activation of the alloimmune and autoimmune responses at days 7 and 14 may explain rejection in the untreated group and mATG-treated group, respectively. However, activation of alloimmunity seems to occur during islet rejection only in induction mATG+CTLA4-Ig treatment.

Interestingly, despite rejection, both allo- and autoimmune responses are controlled at day 56 in prolonged mATG+CTLA4-Ig-treated mice (Fig. 5*A*4–*B*4). To further challenge the allo- and autoimmune response potential in these animals, we performed crucial adoptive transfer experiments. BALB/c skin transplants were performed in NOD.SCID recipients, and 15×10^6 NOD splenocytes from prolonged mATG+CTLA4-Ig-treated mice at day 56 or from untransplanted mice were injected intraperitoneally the next day. To evaluate the transfer of alloimmunity, mice were followed for skin rejection; to evaluate the transfer of autoimmunity, the onset of diabetes was as-

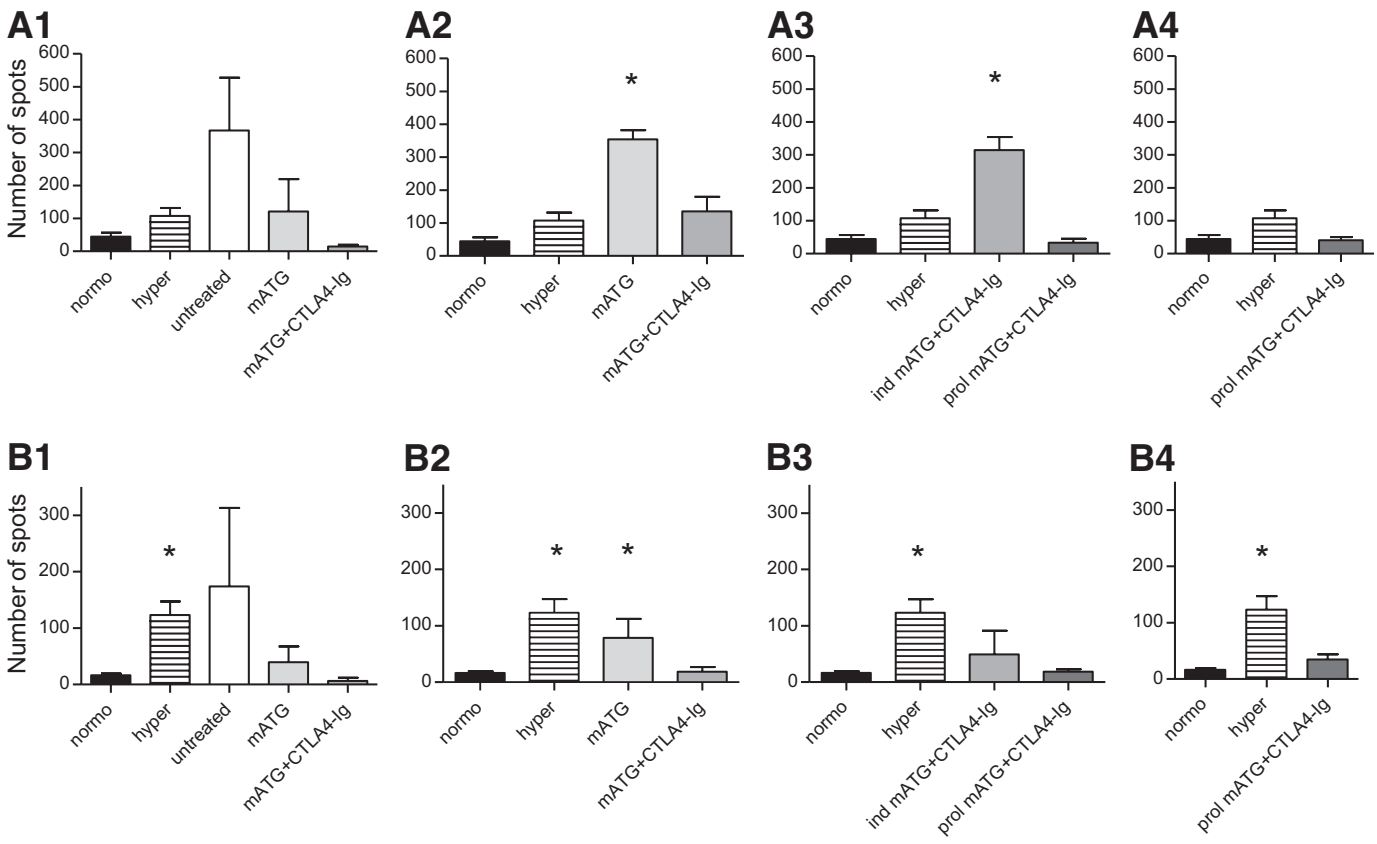

FIG. 5. IFN-γ production by splenocytes of transplanted mice was tested in response to donor-derived antigens (*A*) or BDC2.5 peptide (*B*), in an ELISPOT assay. After transplantation, a robust antidonor antigen response was observed in untreated mice. This response was completely prevented in the mATG+CTLA4-Ig group ($P < 0.05$ vs. day 7 untreated) (*A*1). At day 14, IFN-γ production in response to donor-derived antigens became significant in the induction mATG group, whereas it was still prevented in the mATG+CTLA4-Ig group ($P < 0.05$) (*A*2). Prolonged mATG+CTLA4-Ig prevented substantial IFN-γ production compared with induction mATG+CTLA4-Ig at day 28 ($P < 0.05$) (*A*3). At day 56, the IFN-γ response to alloantigen appeared to remain suppressed in the prolonged mATG+CTLA4-Ig group (*A*4). Induction mATG reduced autoimmune activation at day 7 (NS vs. normo) (*B*1), but not at day 14 ($P < 0.05$ vs. normo) (*B*2). The combination of mATG and CTLA4-Ig (either induction only or prolonged mATG) completely abrogated the immune response to the autoantigen BDC2.5 (NS vs. normo) (*B*2–4). *$P < 0.05$.

sessed. When splenocytes derived from untransplanted mice, either hyperglycemic or normoglycemic, were adoptively transferred, skin was promptly rejected (MST BALB/c skin: hyper, 13 days, and normo, 23 days, $n = 4$, $P = 0.0067$), and diabetes was transferred (diabetes onset: hyper 23 days; normo 49 days, $n = 5$, $P = 0.0067$) (Fig. 6*A* and *B*). When splenocytes obtained from prolonged mATG+CTLA4-Ig-treated mice (either hyperglycemic or normoglycemic) were adoptively transferred, skin rejection was significantly delayed; this was particularly evident when splenocytes were derived from normoglycemic mice (MST BALB/c skin: normoglycemic prolonged mATG+CTLA4 35 days, $n = 5$, $P = 0.0067$ vs. normo; hyperglycemic prolonged mATG+CTLA4-Ig 25 days, $n = 4$, $P = 0.0011$ vs. hyper) (Fig. 6*A*). Notably, when skin grafts from C57BL/6 mice, a 3rd party strain, were transplanted into NOD.SCID mice receiving adoptively transferred splenocytes from treated or untreated normoglycemic NOD mice, no significant differences were observed in graft survival (MST C57BL/6 skin: normo 23 days; normoglycemic prolonged mATG+CTLA4 29 days, $n = 5$, $P =$ NS). Moreover, the adoptive transfer of splenocytes from either hyper- or normoglycemic treated mice did not cause diabetes onset in NOD.SCID mice (Fig. 6*B*).

Prolonged mATG+CTLA4-Ig treatment completely abrogates the autoimmune response and downregulates the alloimmune response. Although it is clear that splenocytes obtained from prolonged mATG+CTLA4-Ig-treated mice retain the potential to generate an alloimmune response in the adoptive transfer experiment, it remained unclear whether this effect was related to the suspension of treatment after adoptive transfer. To determine whether the alloimmune response could develop during treatment, we established a model of allogeneic heart transplantation. BALB/c hearts were transplanted into the abdominal cavity of NOD mice, and rejection was assessed through heart pulsation monitoring. Control mice rapidly rejected the graft (MST of 11 days, $n = 2$), whereas a significant delay, but not indefinite survival, was elicited by prolonged mATG+CTLA4-Ig treatment (MST of 42 days, $n = 4$, $P = 0.02$) (Fig. 6*C*). We therefore tested our treatment in a severe inflammatory setting by performing cardiac transplantation in hyperglycemic NOD mice. Although control mice promptly rejected heart transplants (MST of 12 days), prolonged mATG+CTLA4-Ig treatment significantly delayed heart cardiac allograft rejection (MST of 21 days, $n = 4$, $P = 0.03$). Notably, in a purely autoimmune setting, we confirmed the ability of prolonged mATG+CTLA4-Ig treatment to strongly suppress autoimmunity. NOD.SCID islets were transplanted under the kidney capsule of diabetic NOD mice, and none of the treated mice rejected their islet grafts (MST of >100 days, $n = 5$, $P = 0.002$ vs. untreated) (Fig. 6*D*). The histopathologic analysis of the graft at 100 days after transplantation confirmed that islets were fully preserved by our treatment (IIS:1) (Fig. 6*E*).

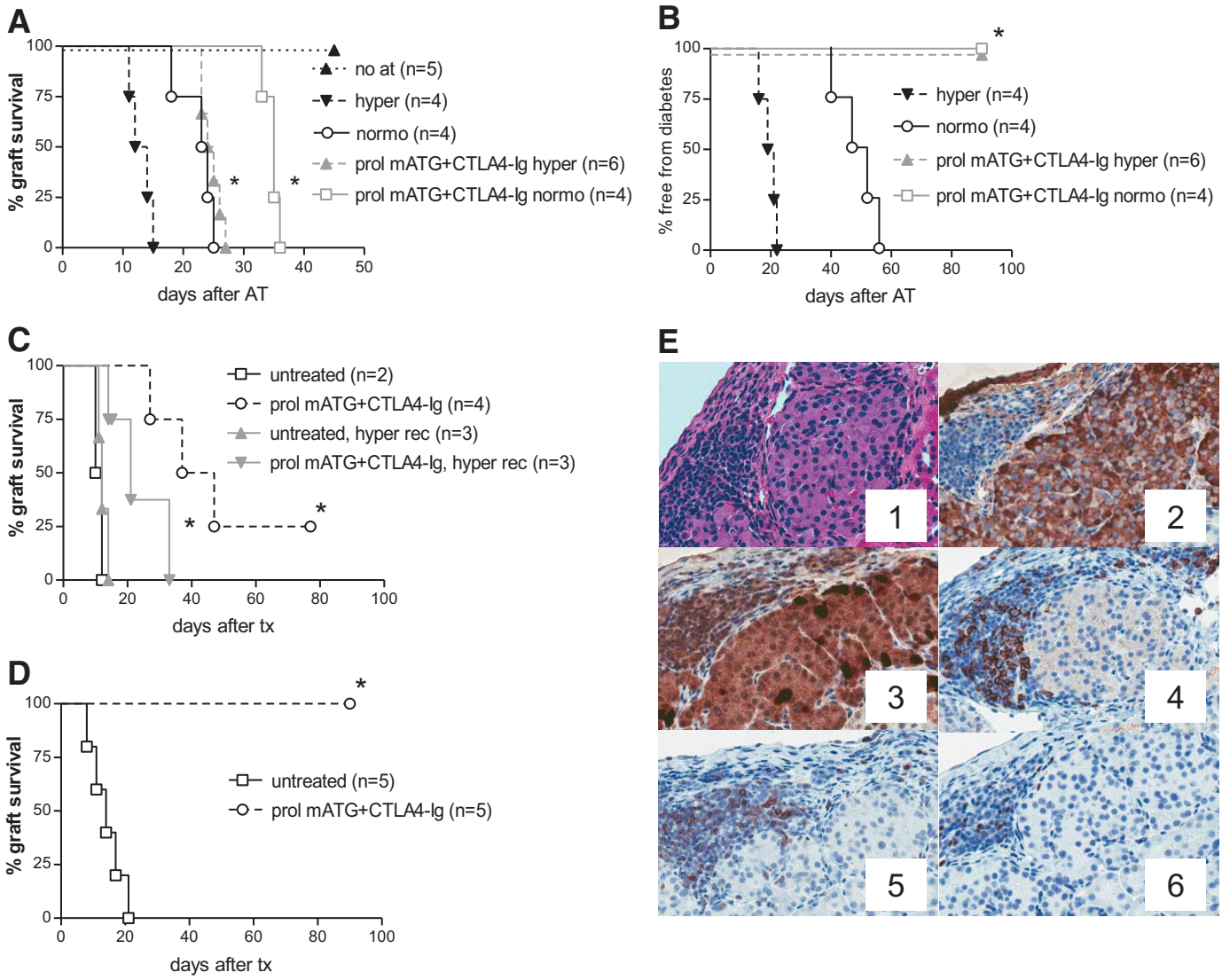

FIG. 6. BALB/c skin was transplanted into NOD.SCID mice, which subsequently received (via adoptive transfer) 15 × 10^6 splenocytes from normoglycemic or hyperglycemic untransplanted mice or from normoglycemic (not rejecting) or hyperglycemic (rejecting) transplanted mice treated with prolonged mATG+CTLA4-Ig at day 56 after transplantation. Skin rejection was slowed when adoptive transfer was performed with splenocytes from treated transplanted mice compared with untreated untransplanted mice (normoglycemic vs. normoglycemic prolonged mATG+CTLA4-Ig $P < 0.05$; hyperglycemic vs. hyperglycemic prolonged mATG+CTLA4-Ig; $P < 0.05$) (*A*). Therefore, in the same NOD.SCID mice, we evaluated the onset of diabetes. Mice that received splenocytes by adoptive transfer from normoglycemic or hyperglycemic NOD mice developed diabetes, as expected. However, the ability to transfer diabetes by splenocytes was completely abrogated in NOD mice treated with prolonged mATG and CTLA4-Ig, either rejecting or not (untreated vs. prolonged mATG+CTLA4-Ig; $P < 0.001$) (*B*). BALB/c hearts were transplanted in NOD mice in a purely alloimmune setting; a prolongation of graft survival was observed in treated mice (untreated vs. prolonged mATG+CTLA4-Ig; $P = 0.017$), similar results were obtained in hyperglycemic recipients ($P = 0.03$) (*C*). NOD.SCID islets were transplanted into hyperglycemic NOD mice; indefinite survival was achieved in treated mice (untreated vs. prolonged mATG+CTLA4-Ig; $P = 0.0018$) (*D*). NOD.SCID islet grafts harvested 100 days after transplantation in treated mice revealed completely preserved islet allografts with very few infiltrating cells surrounding the islets (*E*1, H&E; *E*2, insulin; *E*3, glucagon; *E*4, B220; *E*5, CD3; *E*6, FoxP3). *$P < 0.05$. (A high-quality digital representation of this figure is available in the online issue.)

Prolonged mATG+CTLA4-Ig fully reverses diabetes in newly hyperglycemic NOD mice. Considering the effectiveness of prolonged mATG+CTLA4-Ig in abrogating the autoimmune response in a stringent model of allogeneic and syngeneic islet transplantation, we tested prolonged mATG+CTLA4-Ig for its effect in reversing established diabetes. Hyperglycemic NOD mice were treated after 2 consecutive days of glucose measurements >240 mg/dl with prolonged mATG+CTLA4-Ig. Notably, normoglycemia was restored in 100% of treated mice (10 of 10 mice) (Fig. 7*A*). Reversal was, for the most part, achieved rapidly: 7 mice reverted within the first week after the initiation of treatment; the other 3 mice reverted in 2, 3, and 6 weeks, respectively. Mice that reverted at later time points generally exhibited higher glucose levels at the initiation of treatment. Normoglycemia was maintained for 60 days of follow-up (Fig. 7*A*). Mice in the control group and the group treated with CTLA4-Ig alone did not show any sign of restoration of normoglycemia (Fig. 7*B* and *C*). On the contrary, 3 of 6 in the group treated with prolonged mATG alone (Fig. 7*D*) and 8 of 12 in the induction mATG+CTLA4-Ig group stably reverted from hyperglycemia (Fig. 7*E*).

Histologic analysis of the pancreas of treated mice was performed 60 days after the onset of hyperglycemia. Islets were predominantly free from infiltrates or were only mildly infiltrated by lymphocytes, disposed around the islets, with no invasive pattern. A major proportion of T

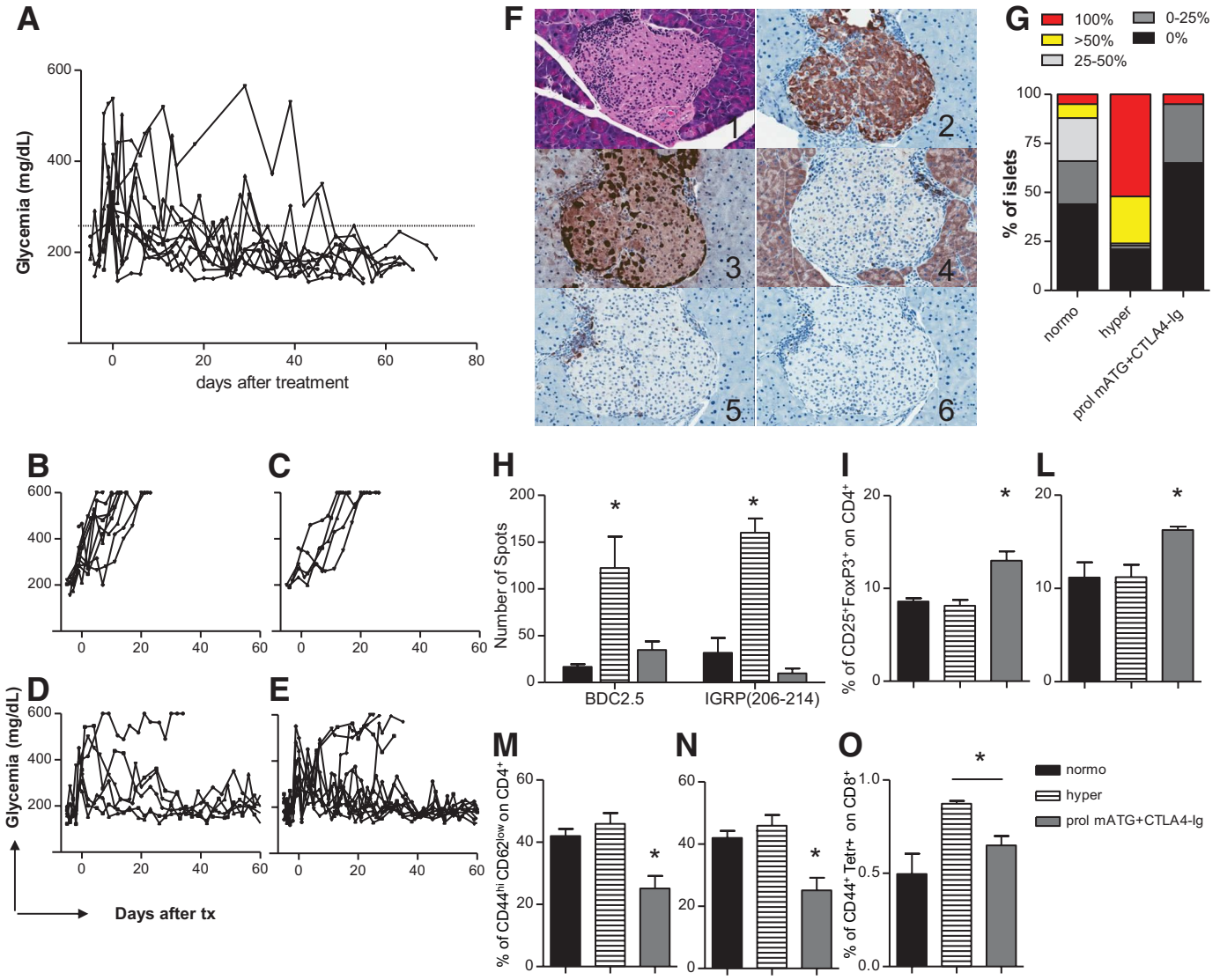

FIG. 7. Diabetic mice were treated with prolonged mATG+CTLA4-Ig the second day of glycemia >250 mg/dl. In 10 of 10 mice, diabetes was reversed, and 7 of 10 reverted in the first week. Although some fluctuation was observed, a stable reversal of diabetes was observed in treated mice (*A*). Untreated mice or CTLA4-Ig-treated mice never displayed reversal of hyperglycemia (*B* and *C*); 3 of 6 mice stably reverted in the prolonged mATG group (*D*), whereas 8 of 12 reverted in the induction mATG+CTLA4-Ig group. Histologic analysis showed a clear protection of islets in treated mice, with an almost complete clearance of the lymphocyte infiltrate, which was confined to the islet periphery. Notably, insulin staining was well represented (*F*1, H&E; *F*2, insulin; *F*3, glucagon; *F*4, B220; *F*5, CD3; *F*6, FoxP3). Insulitis score confirmed the reversal of the infiltrate by the treatment (*G*). IFN-γ production by splenocytes was tested in response to BDC2.5 peptide and to IGRP $^{(206–214)}$ peptide in reverted mice at day 60 after treatment; prolonged mATG+CTLA4-Ig completely suppressed the autoimmune response (NS vs. normo; $P < 0.05$ vs. hyper, $n = 3$) (*H*). The proportion of Tregs was increased in treated mice ($P < 0.05$ vs. normo or hyper, $n = 3$) (spleen: *I*; PLN: *L*), whereas the Teff proportion was reduced ($P < 0.05$ vs. normo or hyper, $n = 3$) (spleen: *M*; PLN: *N*); quantification of autoreactive T cells by tetramer staining confirmed the reduction of autoimmunity in prolonged mATG+CTLA4-Ig treated mice ($P < 0.05$ vs. hyper, $n = 3$) (*O*). *$P < 0.05$. (A high-quality digital representation of this figure is available in the online issue.)

cells were also positive for FoxP3. Insulin staining was clearly positive (Fig. 7*F*). Islets from control hyperglycemic mice appeared completely infiltrated with no appreciable insulin staining (data not shown). Insulitis score confirmed that treatment had elicited reversal of islet infiltration (Fig. 7*G*).

We then challenged splenocytes from treated mice 60 days after restoration of normoglycemia with BDC2.5 peptide and IGRP $^{(206–214)}$ peptide to test the CD4 and CD8 autoimmune specific responses (32). IGRP $^{(206–214)}$ peptide is an islet peptide specific for some diabetogenic $CD8^+$ cells. IFN-γ production in response to either BDC2.5 peptide or IGRP $^{(206–214)}$ peptide was similar in treated mice and normoglycemic NOD mice, although it was markedly increased in hyperglycemic mice (Fig. 7*H*). This was paralleled by a Treg increase, both in the spleen (Fig. 7*I*) and pancreatic draining lymph nodes (PLN) (Fig. 7*L*), as compared with either normo or hyperglycemic NOD mice. On the contrary, the Teff frequency was reduced in prolonged mATG+CTLA4-Ig treated mice, both in the spleen (Fig. 7*M*) and PLN (Fig. 7*N*). Quantification of autoreactive T cells by tetramers confirmed the reduction of autoimmunity obtained with the treatment (Fig. 7*O*).

DISCUSSION

Islet transplantation has been shown to be a viable alternative to chronic insulin injection; however, sustained graft function has yet to be achieved (1,13,33,34). We used a novel clinically relevant combination of mATG and

CTLA4-Ig to promote downregulation of allo- and autoimmune responses and to enhance long-term graft survival in a stringent model of allogeneic islet transplantation in hyperglycemic NOD mice. Notably, although long-term islet function was lost, there were no signs of recurrence of autoimmunity. This finding, together with the fact that both ATG and CTLA4-Ig are readily available for clinical use, indicates this combination is a novel candidate treatment for autoimmune type 1 diabetes (25,27,35).

The efficacy of the combination of ATG and CTLA4-Ig appears to be related to the immunoregulatory properties of each drug alone, which have a synergistic effect when administered together. The mATG is a depleting agent with the potential for immunomodulatory activity; indeed, we confirmed an increase in Tregs after mATG treatment. However, depleting agents are a double-edged sword; that is, although they reduce the number of alloreactive T cells, they can also activate the immune system through the process of homeostatic proliferation (19). In our model, mATG-mediated depletion is also followed by a rapid T-cell recovery associated with an increase in Teffs and a Th1 peripheral cytokine storm. The technique of using a combination of CTLA4-Ig and mATG rather than mATG alone has the potential to overcome this effect. Though CTLA4-Ig per se has little impact on allo- and autoimmunity, its effect in combination with mATG is striking. Although CTLA4-Ig likely exerts immunoregulatory effects, we propose that most of the impact on graft survival is related to inhibition of homeostatic proliferation, as the recovery of the T-cell curve is in fact slowed, which may be related to an inhibition of the release of proliferative cytokines such as IL-7 and IL-15. Conversely, CTLA4-Ig may also somehow reduce Treg percentage after mATG treatment, confirming the role of CD28 in Treg homeostasis (24,36). The net balance of these effects is most likely in favor of a Treg increase and a regulatory immune response profile. Prolonged mATG+CTLA4-Ig treatment has been shown to be superior to induction mATG+CTLA4-Ig treatment in terms of graft survival; although it is possible that some of the enhanced effect may be mediated by further T-cell depletion, data suggest an active modulation of the immune system during T-cell reconstitution. In fact, compared with induction mATG+CTLA4 treatment, prolonged mATG+CTLA4-Ig treatment elicited an increase in the proportion of Tregs, a reduction in IFN-γ, and a preservation of IL-4 production versus both allo- and autoimmune antigens.

Immunologic analysis also revealed a complete suppression of autoimmunity in prolonged mATG+CTLA4-Ig-treated mice. Specifically, IFN-γ production of splenocytes extracted from treated mice and challenged with the islet-derived BDC2.5 peptide was markedly if not completely abrogated. The adoptive transfer of splenocytes from nonrejecting- and rejecting-treated mice into NOD.SCID did not transfer diabetes. Syngeneic islet grafts survived indefinitely in 100% of transplanted mice, and newly hyperglycemic NOD mice, when treated with prolonged mATG+CTLA4-Ig, reverted to normoglycemia in 100% of the mice and showed a complete reversal of CD4 and CD8 autoimmune responses.

With regard to alloimmunity, prolonged mATG+CTLA4-Ig treatment appears to exert a profound immunosuppressive effect, although it may not be sufficient in the long term to prevent graft loss. In fact, although in vitro experiments demonstrated that the IFN-γ response is suppressed, in adoptive transfer experiments, splenocytes from treated animals retained the potential to transfer the ability to reject donor-derived skin transplants. In accordance with this observation, in a model that assesses the alloantigen response alone, cardiac allografts were eventually rejected.

These findings suggest that ATG can be used not only as an induction therapy, but also as a prolonged treatment. Interestingly, its combination with CTLA4-Ig is shown to reduce postdepletion homeostatic proliferation. Both allo- and autoimmune responses were affected; in particular, the autoimmune response was completely abrogated with 100% diabetes reversal and 100% indefinite survival of syngeneic islet grafts. We submit the use of prolonged mATG+CTLA4-Ig as a major breakthrough in the field of type 1 diabetes treatment.

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A.V. researched data and wrote the manuscript. F.D.A. and A.P. researched data. M.J. researched data and edited the manuscript. T.W. performed heart transplantation. K. Lui performed heart transplantation. K. Law produced histology. C.S., E.O., S.D., J.M.A., C.S., J.M., and M.A. contributed to discussion. M.C. performed islet transplantation. S.J.R. read and quantified histology and contributed to discussion. R.A. and M.H.S. contributed to discussion and reviewed data. J.W. reviewed data. P.F. reviewed data, edited the manuscript, and contributed to discussion.

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