Immune Depletion With Cellular Mobilization Imparts Immunoregulation and Reverses Autoimmune Diabetes in Nonobese Diabetic Mice

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OBJECTIVE—The autoimmune destruction of β-cells in type 1 diabetes results in a loss of insulin production and glucose homeostasis. As such, an immense interest exists for the development of therapies capable of attenuating this destructive process through restoration of proper immune recognition. Therefore, we investigated the ability of the immune-depleting agent antithymocyte globulin (ATG), as well as the mobilization agent granulocyte colony–stimulating factor (GCSF), to reverse overt hyperglycemia in the nonobese diabetic (NOD) mouse model of type 1 diabetes.

RESEARCH DESIGN AND METHODS—Effects of each therapy were tested in pre-diabetic and diabetic female NOD mice using measurements of glycemia, regulatory T-cell (CD4+CD25+Foxp3+) frequency, insulitis, and/or β-cell area.

RESULTS—Here, we show that combination therapy of murine ATG and GCSF was remarkably effective at reversing new-onset diabetes in NOD mice and more efficacious than either agent alone. This combination also afforded durable reversal from disease (>180 days postonset) in animals having pronounced hyperglycemia (i.e., up to 500 mg/dl). Additionally, glucose control improved over time in mice subject to remission from type 1 diabetes. Mechanistically, this combination therapy resulted in both immunological (increases in CD4-to-CD8 ratios and splenic regulatory T-cell frequencies) and physiological (increase in the pancreatic β-cell area, attenuation of pancreatic inflammation) benefits.

CONCLUSIONS—In addition to lending further credence to the notion that combination therapies can enhance efficacy in addressing autoimmune disease, these studies also support the concept for utilizing agents designed for other clinical applications as a means to expedite efforts involving therapeutic translation. ***Diabetes*** **58:2277–2284, 2009**

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Type 1 diabetes is characterized by the autoimmune destruction of β-cells, resulting in a loss of insulin production and glucose control (1,2). In both humans and the nonobese diabetic (NOD) mouse model of type 1 diabetes, the disorder's pathogenesis appears dependent on aberrant immune regulation (3–6). A reversal of type 1 diabetes in NOD mice has been achieved, with varying levels of success, through administration of a limited number of immunosuppressive and immunomodulatory agents, some of which are controversial with respect to their translational capabilities (7–19).

Antithymocyte globulin (ATG) is currently in clinical use for a variety of purposes, including the treatment of acute rejection, graft versus host disease, and conditioning for stem-cell transplantation (20–22). It has been shown to target >40 epitopes and serves to induce lymphocyte depletion, the extent of which depends upon the dose administered. Previously, we have shown that murine ATG is capable of late prevention of diabetes in NOD mice and, importantly, that this agent was capable of inducing a regulatory T-cell population (16). With this, we questioned whether the efficacy of this therapy could be improved through the use of a second immunomodulatory agent differing in its presumed mechanism of therapeutic activity. To that regard, we elected to evaluate granulocyte colony–stimulating factor (GCSF).

GCSF was initially developed as a means of mobilizing neutrophils (23,24), but recent reports (25) have also indicated a GCSF-induced immunoregulatory impact. These studies indicated the ability of GCSF to induce an immunoregulatory shift from a T_H1 to a T_H2 cytokine phenotype (26), the induction of tolerogenic dendritic cells (27), and the mobilization of regulatory T-cells. In regards to type 1 diabetes, GCSF has successfully prevented the onset of disease in the NOD mouse via the induction of both tolerogenic dendritic and regulatory T-cells (28) and prevented the cyclophosphamide-mediated acceleration of diabetes (29).

Hence, in this report, we examined the therapeutic efficacy of these two agents, ATG and GCSF, subject to clinical use in settings outside of type 1 diabetes, for the purpose of testing their ability to reverse disease in NOD mice as well as to monitor their ability to reinstill self tolerance. In this study, we also tested the hypothesis that combination therapy will be more effective than either monotherapy for the purposes of treating type 1 diabetes in NOD mice.

RESEARCH DESIGN AND METHODS

Female NOD mice were purchased from The Jackson Laboratory and housed in specific pathogen-free facilities at the University of Florida. These studies received the approval of the institution animal care and use committee at the University of Florida. Suboptimal studies were also performed using female NOD mice and were carried out at Genzyme's specific pathogen-free facilities (Oklahoma City, OK) according to approved protocols.

Type 1 diabetes reversal studies. Mice used in reversal trials were monitored three times per week for hyperglycemia, defined as a blood glucose ≥240 mg/dl, by tail bleed. Animals measuring above this threshold on 2 consecutive days were considered diabetic. Murine ATG was prepared by immunizing rabbits with pooled lymph-node cells as previously described (Genzyme Corporation). In standard dosing studies, murine ATG was administered via two intraperitoneal injections of 500 μg murine ATG or, as a control, 500 μg rIgG (Jackson ImmunoResearch) given 72 h apart for a total dose of 1 mg. These animals also received a subcutaneous LinBit insulin implant (LinShin Canada), providing sustained release of insulin for ~3 weeks. Failure of the therapy was defined as blood glucose levels >400 mg/dl for two consecutive measurements. In the suboptimal dosing study, the dose of murine ATG was reduced to 290 μg per animal, over two injections. Neupogen (Amgen) was used for GCSF therapy for both suboptimal-and standard-dosing studies. A dose of 6 μg/animal was diluted in 100 ul of 5% dextrose per manufacturer's recommendation and injected intraperitoneally daily for a maximum of 8 weeks. Blood glucose was monitored three times per week until either failure occurred (as described above) or animals reached the end point postonset (as indicated).

Pre-diabetic time course study. Combination therapy of standard-dose murine ATG and GCSF (as described above) was performed in pre-diabetic female NOD mice beginning at 12 weeks of age and lasting up to 8 weeks. Four groups were treated with control, murine ATG, GCSF, or ATG+GCSF. As with the type 1 diabetes reversal studies, murine ATG was administered in two doses 72 h apart. GCSF was administered for up to 8 weeks. Timed killings were performed at weeks 0, 2, 4, and 8 postinitiation of therapy ($n = 5$ per group per time point), and various analyses were performed.

Histology. The β-cell area was calculated using MetaMorph software (Molecular Devices) analysis with insulin stained with fast red on pancreatic sections. The insulin-positive area was divided by the total acinar area to yield a final percentage. Insulitis scoring was performed on hematoxylin and eosin–stained pancreatic sections as described previously.

Leukocyte quantification in peripheral blood. Mice in the pre-diabetes study were bled via tail perforation at predetermined time points (0, 2, 4, and 8 weeks) postinjection for determination of leukocyte counts. Blood samples were collected in EDTA tubes (Fisher Scientific) and analyzed using a Coulter ACT diff-Tainer Hematology analyzer (Beckman Coulter).

Flow cytometry. Splenocytes and/or peripheral blood were collected as indicated at each time point or end point and stained for flow cytometric analysis using a FACScalibur (Becton Dickinson) flow cytometer. All antibodies were purchased from eBioscience, with the single exception of CD4–peridinin chlorophyll protein complex (PerCP) and the corresponding isotype, which were purchased from BD Biosciences. T-cells were stained for CD8–fluorescein isothiocyanate (FITC) (clone 53-6.7), CD4-PerCP (clone RM4–5), Foxp3-phycoerythrin (PE) (clone FJK-16a), and CD25-allophycocyanin (APC) (clone PC61). Macrophages were stained with CD11b-FITC (clone M1/70), CD14-PE (Sa2–8), and CD16/CD32-APC (clone 93). Neutrophils were stained with F4/80-PE (clone BM8), as a negative marker, and Gr-1-APC (clone RB6-8C5). All were added at a concentration of 1μg per 1×10^6 cells per tube.

Quantitative real-time PCR. Pancreatic lymph nodes and sections of spleen were collected in RNAlater (Ambion) and frozen at −80°C until subsequent RNA extraction. mRNA was extracted from the tissues using RNAqueous kits (Ambion). cDNA was produced from the mRNA using SuperScript III Reverse Transcriptase (Invitrogen). cDNA samples were analyzed with a 384-panel mouse immunology 384 StellArray qPCR array (Bar Harbor Biotechnology).

Intraperitoneal glucose tolerance test. A 12-h food restriction was implemented prior to testing. After 12 h, a blood glucose value was obtained and glucose tolerance testing was initiated immediately. Blood glucose levels were collected in the following manner: the tail was pricked with a lancet and blood glucose (mg/dl) was measured by an ACCU-CHEK Compact Plus Blood Glucose Meter. For glucose tolerance testing, each mouse was weighed and 2 g/kg of 20% D-glucose was drawn up via a 29-gauge ½ insulin syringe. The glucose solution was then injected into the intraperitoneal cavity at time 0. At 15, 30, 60, and 120 min, blood glucose was sampled.

Immunoglobulin isotyping. Immunoglobulin isotyping was performed on sera obtained at each killing time point using a mouse immunoglobulin isotyping kit (Millipore) in order to measure IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3. Mouse isotyping serum diluent and mouse immunoglobulin isotyping standard were ordered separately (Millipore).

Anti-GCSF antibody measurement. Sera was collected from mice at 0-, 2-, 4-, and 8-week time points in the pre-diabetic study. To determine whether the immunoglobulin increases seen were GCSFspecific, Nunc-Immuno 96-well plates were coated with 50 μl/well of 2 μg/ml GCSF (Amgen) overnight at 4°. Plates were blocked for 2 h with 300 μL/well 5% BSA/PBS and washed five times with PBS/Tween. Sera was diluted 1:10,000 and was incubated for 2 h on a plate shaker. The plates were washed five times as before and were then coated with either 50 μL/well 1:2,500 rat anti-mouse IgM–horse radish peroxidase (HRP) or 1:5,000 donkey anti-mouse IgG-HRP (Southern Biotech) for 1 h on a plate shaker. The plates were once again washed five times followed by addition of 50 μl TMB (tetramethylbenzidine) protected from light. After 5 min, the reaction was stopped with 50 μl stop solution (Mercodia) and the plate read at a 450-nm wavelength on a Spectramax Plate Reader (Molecular Devices).

Statistical analyses. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software). One-way ANOVA, unpaired t test two-tailed testing, and Kaplan-Meier life table analysis were used. All data are presented as means ± SD, with P values <0.05 considered significant.

RESULTS

GCSF enhances the long-term reversal of diabetes afforded by murine ATG. Blood glucose levels were monitored in all mice treated at diabetes onset and throughout the studies duration (Fig. 1). Based on these blood glucose values, the administration of murine ATG alone to new-onset NOD mice resulted in durable (i.e., >180 days postonset) remissions from overt hyperglycemia in 33% (5 of 15 mice) of treated animals, while neither control (0 of 16 mice) nor GCSF monotherapy (0 of 14 mice) provided such reversions (Fig. 2). However, the combination of murine ATG and GCSF therapy resulted in a remission rate of 75%, a significantly greater rate of remission than was seen with murine ATG monotherapy (12 of 16 mice; $P = 0.0000006$ vs. control, $P = 0.013$ vs. murine ATG). To provide further evidence for the notion that the therapeutic efficacy of murine ATG was enhanced by the addition of GCSF, studies were undertaken wherein murine ATG was administered with suboptimal dosing (i.e., 0.29 mg per mouse vs. 1.0 mg per mouse used in the aforementioned efforts). Interestingly, GCSF dramatically improved the therapeutic capacity for diabetes reversal even when in combination with this suboptimal dose of murine ATG (supplemental Fig. 1 [available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0557/DC1]).

Combination therapy enables reversal of higher new-onset glycemia than murine ATG monotherapy. Given the higher rate of reversal observed with combination therapy, it was necessary to examine how this higher rate correlated with the blood glucose at the time of diabetes onset. Indeed, a time course analysis following diabetes onset revealed pronounced differences in the ability of these therapies to remit based upon starting blood glucose values (Fig. 3). Successful treatment with murine ATG was largely limited to values of ≤380 mg/dl (mean 317.2 mg/dl [range 256–398]), whereas combination therapy of murine ATG and GCSF significantly increased the therapeutic ceiling to ~500 mg/dl (401.8 mg/dl [264–500]).

Glucose control improves with time in reversed NOD mice. Having observed substantial rates of diabetes remission, we sought to determine whether this return to euglycemia would be durable in the face of a glucose challenge. To do so, during the course of the reversal trial using the suboptimal dose of murine ATG, we measured glucose control via intraperitoneal glucose tolerance tests (IPGTTs) at 60, 90, and 120 days postonset in reversed mice. An improvement in IPGTT, as measured by the area under the curve from 0 to 120 min, was observed from the 60- to 120-day time points (Fig. 4). This improvement in

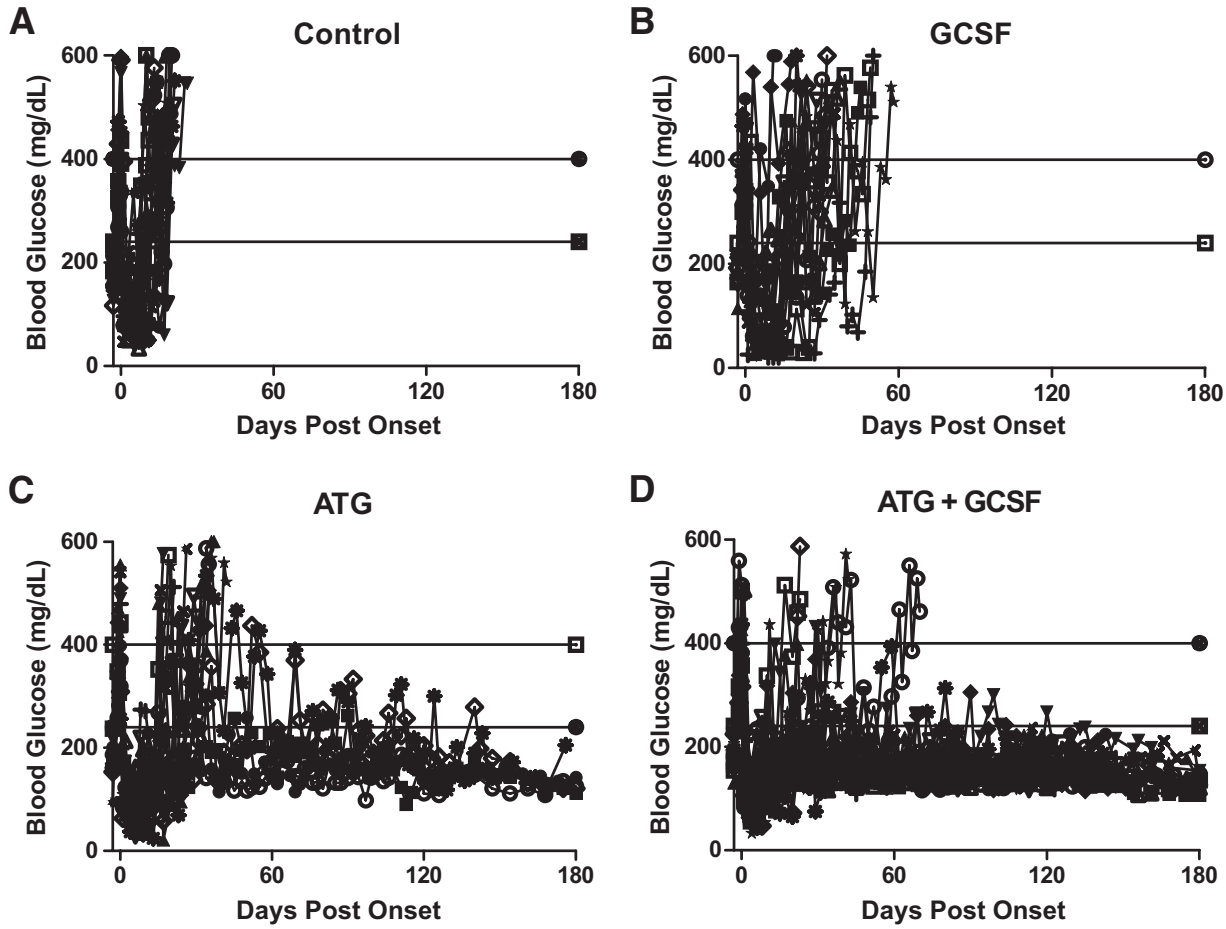

FIG. 1. Blood glucose values were obtained for up to 180 days postonset in NOD mice treated with control (*A*), GCSF (*B*), ATG (*C*), or ATG + GCSF (*D*).

glucose control occurred in spite of the cessation of both murine ATG and GCSF therapies prior to the 60-day time point.

Murine ATG plus GCSF combination therapy induces immunomodulation. To address the question of whether GCSF-mediated enhancement of diabetes reversal was due to induction of immunoregulation, murine ATG and GCSF (as both mono- and combination therapy) were administered to pre-diabetic 12-week-old female NOD mice. Analysis of peripheral blood revealed marked leukocyte depletion in murine ATG-treated mice versus all other groups at 2 weeks (Fig. 5*A*), with movement back toward pretreatment levels at 4 and 8 weeks postinduction. How-

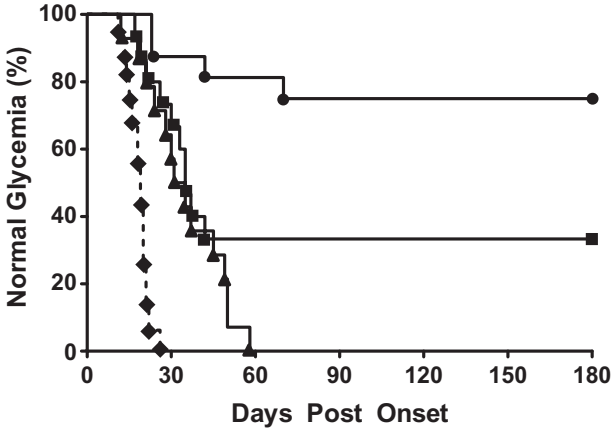

FIG. 2. Long-term diabetes reversal was achieved in 75% (12 of 16; P = 0.0000006 vs. control) of murine ATG + GCSF–treated mice, which was significantly improved versus murine ATG monotherapy (P = 0.013), which reversed 33% (5 of 15) mice. Long-term remission was observed in neither GCSF (0 of 14) nor control-treated (0 of 15) mice. ♦, control; ▲, GCSF; ■, ATG; ●, ATG + GCSF.

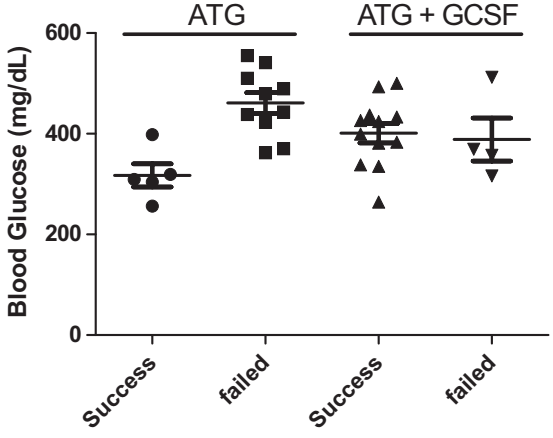

FIG. 3. GCSF enhances the ability of murine ATG to reverse mice with greater hyperglycemia at diabetes onset. Therapeutic success of murine ATG therapy (●) was largely limited to starting blood glucose values of 380 mg/dl and below with an average of 317.2 mg/dl. The addition of GCSF to murine ATG treatment significantly raised the starting average blood glucose of successful therapy (▲) to 401.8/mg/dl (P = 0.019).

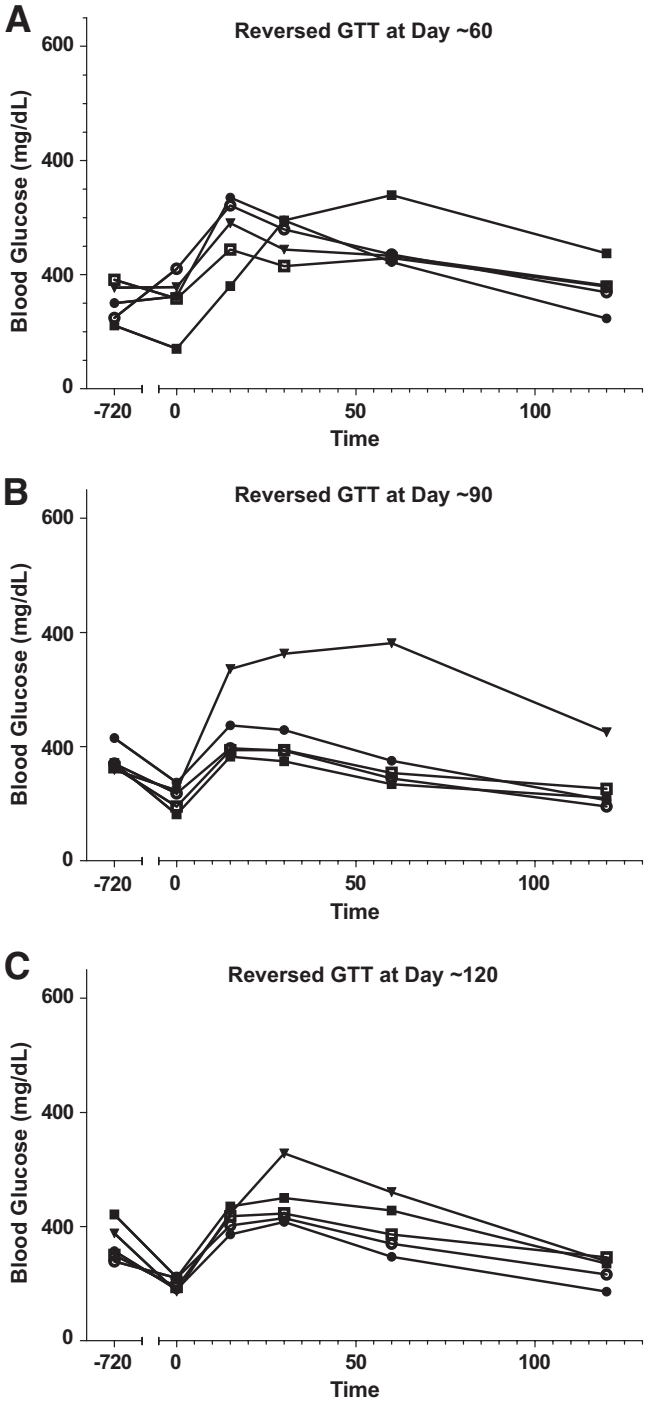

FIG. 4. IPGTT indicates improving glucose control from 60 to 120 days postonset in combination therapy-treated NOD mice. NOD mice remitted from diabetes using combination therapy were administered an IPGTT a 60 (*A*), 90 (*B*), and 120 (*C*) days postonset. An area under the curve analysis revealed a significant improvement at 120 (21,940 ± 1,250, $n = 7$) days compared with 60 (26,840 ± 1,068, $n = 7$) days ($P = 0.0235$, unpaired t test).

ever, the addition of GCSF to murine ATG afforded a significant increase in leukocytes at 2 weeks versus murine ATG alone. In particular, GCSF increased the percentage of splenic macrophages (Fig. 5*B*) and neutrophils (Fig. 5*C*).

Both murine ATG as well as GCSF have been reported to induce a population of regulatory T-cells in vivo, with regulatory T-cells being conventionally defined as CD4+CD25+Foxp3+ cells. Predictably, all treatments utilized in these efforts herein demonstrated a reduced percentage of regulatory T-cells at 2 weeks versus control animals (Fig. 5*D*), due to either short-term depletion by murine ATG or mobilization of macrophages and neutrophils by GCSF. GCSF therapy led to an increase in regulatory T-cells versus control as early as 4 weeks, while combination therapy had the greatest increase in regulatory T-cells versus all other treatments at 8 weeks. As indicated by the increase in regulatory T-cells, the immunomodulatory alteration afforded by GCSF continued through 8 weeks, despite the lack of mobilization of macrophages (Fig. 5*B*) and neutrophils (Fig. 5*C*) beyond 2 weeks. In addition, murine ATG, both alone and in combination with GCSF, induced a significant increase in the splenic CD4+-to-CD8+ ratio (Fig. 6) compared with control and GCSF-treated mice, with the increase peaking at 2 weeks and remaining significant at 4 and 8 weeks.

Anti-GCSF antibodies correlate with reduced action of GCSF beyond 2 weeks of therapy. Of interest was the short duration of this mobilization. To address this, RT-PCR analysis was performed on pancreatic lymph nodes (supplemental Table 1) and sections of spleen (supplemental Table 2) obtained at the 8-week time point from all treatment groups in the pre-diabetic study. These analyses revealed significant GCSF-induced alterations but overwhelmingly those involving B-cell activation. This included a 10.6-fold increase in IgM and a 9.7-fold increase in IgG1 versus control mice. This raised the possibility of an antibody response in the mice against the human protein.

Consistent with this hypothesis, immunoglobulin isotyping revealed significant GCSF-induced upregulation of multiple isotypes versus control mice at 8 weeks, including IgM and IgG1 (supplemental Fig. 2). To address whether these antibodies were GCSF specific, anti-GCSF enzyme-linked immunosorbent assays were performed. This further analysis of sera revealed significant GCSF-specific IgM and IgG antibody responses (supplemental Fig. 3). These responses became evident beginning 2 weeks after initiation of GCSF administration and continued to increase out to 8 weeks of therapy.

Pancreatic islets are protected from further autoimmune destruction by murine ATG and GCSF. The health of the islets at the end point of the pre-diabetic study was also an important consideration. As such, insulitis scoring (Fig. 7*A*) was performed to determine the degree of lymphocytic infiltration over the 8 weeks of therapy in pre-diabetic NOD mice. Combination therapy resulted in markedly lower insulitis intensity scores when compared with islets from control animals after 8 weeks (Fig. 7*B*). In addition, insulin staining revealed improved β-cell area in animals receiving combination therapy versus murine ATG monotherapy, while control animals demonstrated a decline in β-cell area over the 8-week period (Fig. 7*C*).

DISCUSSION

The pathogenesis of type 1 diabetes in both humans and NOD mice appears dependent upon an aberrant immune response that results in the destruction of insulin-producing β-cells. While prevention of type 1 diabetes in NOD mice can be accomplished through a wide variety of monotherapies, reversal of overt disease has considerably fewer reported efficacious therapies (30,31). Of those that

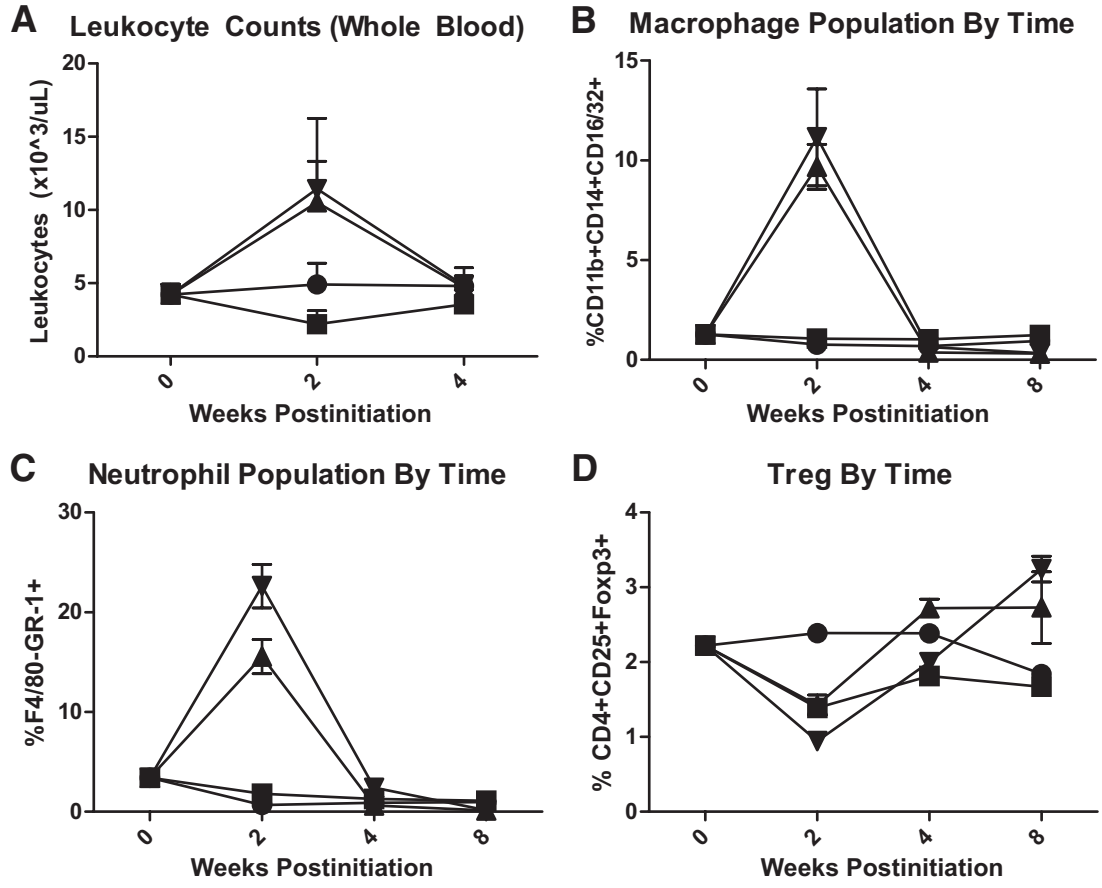

FIG. 5. Murine ATG and GCSF combination therapy in NOD mice induces immunomodulation. *A*: An alteration in peripheral blood leukocyte counts was observed only 2 weeks after initiation of therapy, with murine ATG + GCSF–treated mice exhibiting a significantly greater number ($P = 0.0129$, unpaired t test) than murine ATG monotherapy. No differences were seen at later time points. GCSF and murine ATG + GCSF–treated mice exhibited significant increases in splenic macrophages (*B*) ($P < 0.0001$, $P = 0.0027$, respectively; unpaired t test) and neutrophils (*C*) ($P < 0.0001$, $P < 0.0001$, respectively; unpaired t test) 2 weeks after initiation of therapy with no differences observed thereafter. *D*: As expressed out of total splenocytes, the percentage of splenic T_{reg} was reduced in all treatments versus control at 2 weeks due to murine ATG–mediated depletion and/or the mobilization effect of GCSF but was significantly increased versus control at 4 weeks in GCSF-treated mice ($P = 0.003$, unpaired t test) and at 8 weeks in murine ATG + GCSF–treated mice ($P < 0.0001$, unpaired t test). ●, control; ■, ATG; ▲, GCSF; ▼, ATG + GCSF.

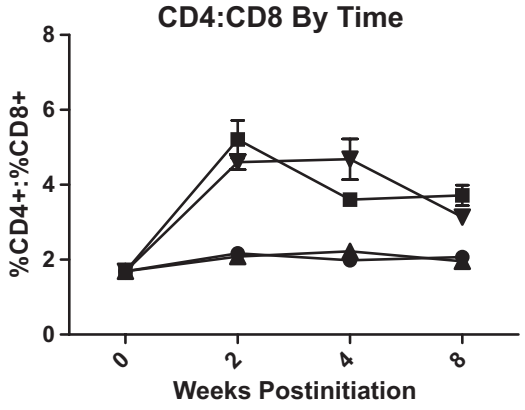

FIG. 6. Administration of mATG induces an increase in the splenic CD4-to-CD8 ratio. ATG-treated mice exhibited a significantly higher CD4-to-CD8 ratio than both controls and GCSF-treated mice at 2 ($P = 0.003$, $P = 0.0003$, respectively, unpaired t test), 4 ($P < 0.0001$, $P < 0.0001$, respectively, unpaired t test), and 8 ($P = 0.0004$, $P = 0.0003$, respectively, unpaired t test) weeks postinitiation. Combination-treated mice also exhibited significantly higher ratios versus controls and GCSF-treated mice at 2 ($P < 0.0001$, $P < 0.0001$, respectively, unpaired t test), 4 ($P = 0.0012$, $P = 0.0056$, respectively, unpaired t test), and 8 ($P = 0.0002$, $P = 0.0001$, respectively, unpaired t test) weeks postinitiation. ●, control; ■, ATG; ▲, GCSF; ▼, ATG + GCSF.

do show success, many combine two or more therapeutic agents to achieve this reversal (32,33). Indeed, one of the earliest demonstrations for the ability of combination therapy to reverse hyperglycemia in NOD mice utilized a somewhat similar form of murine ATG, anti-lymphocyte serum, in combination with exendin-4, to effectively reverse disease in this animal model of type 1 diabetes (14). Consequently, herein we have described an approach using two clinically relevant therapies, ATG and GCSF, for the purpose of immunomodulation that would provide benefit in terms of reversing type 1 diabetes, as demonstrated in the NOD mouse. Aside from the ability for combination therapy to provide improved reversal rates, we also questioned whether this combination would improve disease reversal in animals that would not be subject to disease remission were they provided monotherapy.

The observed enhancement of murine ATG's ability to reverse new-onset NOD mice with greater starting blood glucose levels when used in combination with GCSF not only demonstrated this latter notion, but it also likely reflects the ability of combination therapy to induce remissions in mice with greater loss in β-cells than possible with monotherapy; although this hypothesis is subject to debate (34). The finding is especially important as

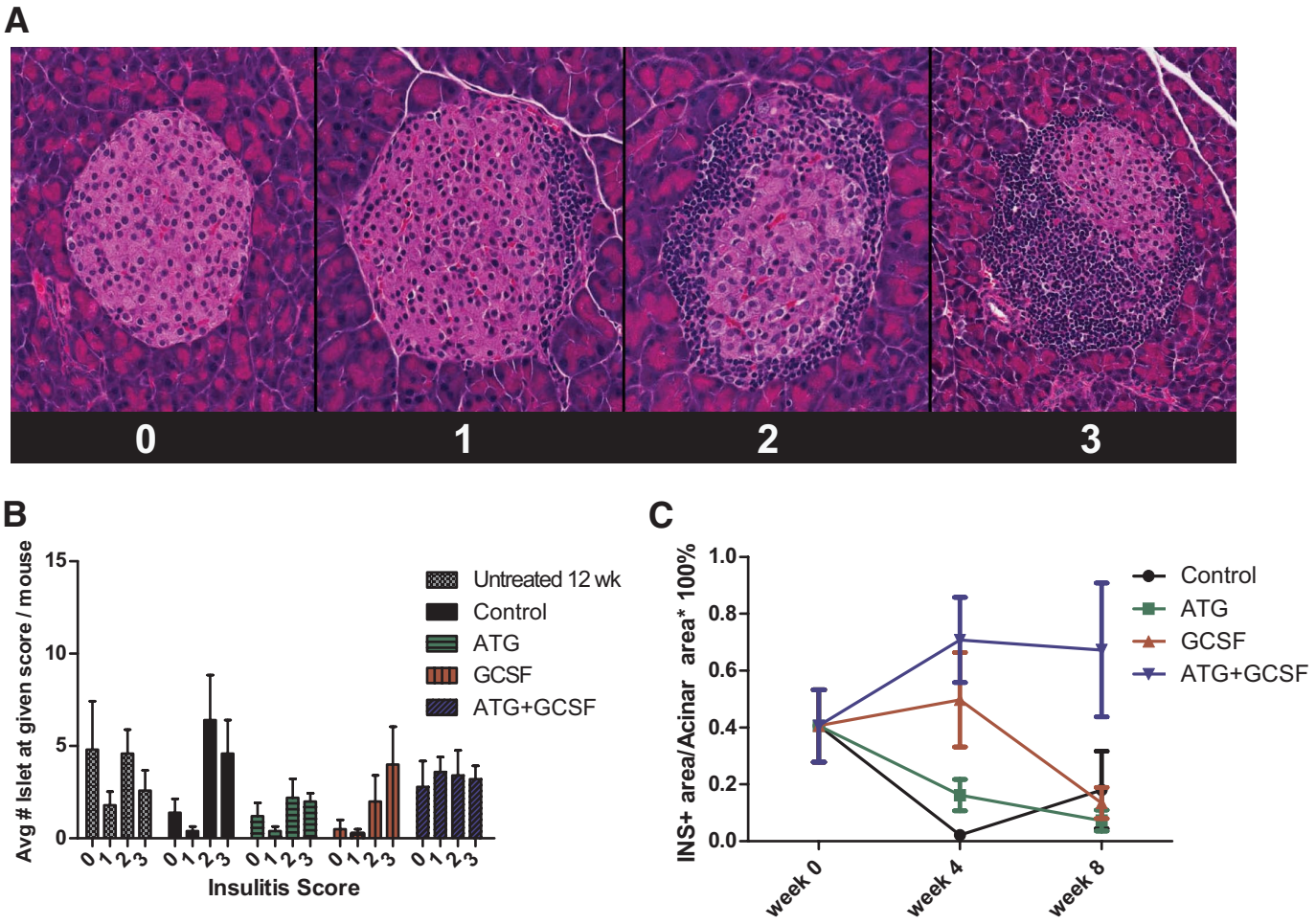

FIG. 7. Murine ATG and GCSF combination therapy in NOD mice protects pancreatic islets from autoimmune destruction. Insulitis scoring (*A*) of islets (*B*) 8 weeks after initiation of therapy indicated that combination therapy skewed scoring toward healthy islets, with significant improvement versus control in the number of islets with a score of 1 ($P = 0.0055$, unpaired t test). *C*: Insulin staining at the 8-week time point revealed that combination therapy afforded the greatest β-cell/acinar area and was significantly improved versus murine ATG monotherapy ($P = 0.0359$, unpaired t test). (A high-quality digital representation of this figure is available in the online issue.)

previous studies using similar immune-depleting agents as monotherapy (e.g., anti-CD3 monoclonal antibody) note a diminished ability to reverse type 1 diabetes in NOD mice in this metabolic range (i.e., ≥350 mg/dl) (8). It is conceivable that monotherapies such as murine ATG or anti-CD3 monoclonal antibody may induce immunoregulation yet still fail to remit diabetes due to a profound loss of β-cell mass prior to the induction of the therapeutic regimen. Future studies would be well served to measure C-peptide in response to glucose challenge at the onset of therapy, as well as to transplant islets into mice that fail to respond to therapy. This will help to address the impact of starting β-cell mass upon the efficacy of these therapies.

While several reports demonstrate an ability to induce euglycemia in new-onset NOD mice, there often remains some doubt regarding the long-term robustness of these therapies. In our reversal trial using suboptimal murine ATG in combination with GCSF, we attempted to alleviate this concern by performing an IPGTT time course study. We demonstrated that beginning at 60 days, by which time all therapy has ceased, and continuing out to 120 days postonset, the quality of glucose control significantly increases as measured by area-under-the-curve analysis. The exact reason for this improvement is uncertain but possibly due to the recovery of endogenous β-cells (35). Previous reports (8,36) have indicated that the efficacy of reversal therapies hinges upon the recovery of these cells rather than the generation of new β-cells. A time course analysis of the pancreas in future reversal studies may address this hypothesis.

In our pre-diabetic study, the greatest percentage of regulatory T-cells was observed in mice receiving combination therapy. This is not surprising given that both murine ATG and GCSF individually have been shown to induce a population of regulatory T-cells (16,37). The fact that by combining the two therapies results in a greater percentage of regulatory T-cells after 8 weeks of therapy than either monotherapy lends additional support to the use of combination therapy. Further studies, such as the adoptive cotransfer of these regulatory T-cell populations with effector T-cells into NOD.SCID mice, may be warranted to more explicitly demonstrate their suppressive potential. In addition, future efforts must expand on the effects of this therapy on regulatory T-cell populations in anatomic compartments beyond the spleen, such as the pancreatic lymph nodes and the islet infiltrate.

The presence of anti-GCSF IgG1 and IgM antibodies may explain the reduction in macrophages and neutrophils after 2 weeks of GCSF therapy. It is also possible that a reduction in GCSFR mRNA (supplemental Table 1) may

also play a role (38–40). This response is not surprising given that the recombination GCSF is a human protein and, consequently, is recognized as foreign in the treated mice (41). In spite of this apparent neutralization, the combination therapy of murine ATG and GCSF remained viable for both reversal of overt disease and for maintaining the health of islets when administered to pre-diabetic NOD mice. If this immune response against the GCSF could be overcome, it is conceivable that the efficacy of this treatment would be enhanced.

The apparent lack of β-cell durability in the murine ATG-treated pre-diabetic mice reflects a similar finding in a previous report in which 12-week-old pre-diabetic NOD mice exhibited only transient protection following anti-CD3 monoclonal antibody therapy (17). The transient protection seen with GCSF monotherapy group reflects the reversal study (Fig. 1*A*) in which GCSF only led to a delayed return to hyperglycemia compared with control-treated mice. By combining these two monotherapies, however, the health of the islets was maintained relative to control as measured by insulitis scoring and β-cell area.

These results indicate that combined treatment of murine ATG with GCSF offers a highly effective means for reversal of type 1 diabetes in NOD mice. This combination therapy provides for a series of beneficial mechanistic actions (e.g., increased regulatory T-cell frequency, reduced islet inflammation, improved β-cell area, etc.) and dramatically extends the range of β-cell dysfunction allowable for effective and durable disease remission. These studies also provide support for the performance of human type 1 diabetes trials with this combination of agents and suggest that this form of therapy may be amenable to treatment of other autoimmune disorders.

With that notion, what has been attempted with ATG in humans that might provide support for this potential application? Studies involving human transplantation and treatment of autoimmunity do, in fact, suggest that that ATG provides therapeutic benefit that may involve tolerance. Transplant recipients have seen successful management with ATG induction therapy followed only by limited maintenance immunosuppression by tacrolimus (42), while ATG has also been used successfully in the treatment of refractory systemic autoimmune diseases such as systemic lupus erythematosus, progressive systemic sclerosis, and rheumatoid arthritis (43). There has also been promise for the efficacy of ATG in the treatment of type 1 diabetes. Early studies of equine ATG in combination with prednisone in new-onset type 1 diabetic patients indicated a prolongation of the honeymoon phase (44). As far as more contemporary efforts, in a randomized, placebo-controlled, single-blinded trial with RATG (ATG-Fresenius; Hoechst Marion Roussel, Frankfurt, Germany), type 1 diabetic participants aged 18–35 years received a total dose of 18 mg/kg of ATG, which was administered in four infusions. Of 17 study participants, 11 received the drug and 6 received placebo. Increased glucagon-stimulated C-peptide levels, a lower insulin requirement, and lower glycosylated hemoglobin levels were observed in the ATG group, but not in the placebo group, 12 months into the study (45). Perhaps most promising were two ATG-treated subjects that achieved disease remission (i.e., no exogenous insulin for at least 1 month and a fasting glycemia $<$126 mg/dl). A pilot study is currently underway in humans with new-onset type 1 diabetes, funded by the Immune Tolerance Network, that seeks to determine whether ATG will preserve C-peptide. This study will test the notion that selective depletion of lymphocytes will reset the immunologic rheostat, induce dynamic immune regulation, and potentially induce and maintain tolerance in type 1 diabetes. Since this study will also help establish safety data for the use of ATG in humans in type 1 diabetes, the background adverse-event rate will be established in this population, allowing for the study of combination therapies including ATG and additional tolerance-inducing agents such as GCSF. With time, the equipoise for utilizing agents having the potential for imparting deleterious side effects must be carefully weighed against the benefits of preservation of C-peptide and/or insulin independence for those with type 1 diabetes. The answer to this equation is not simple to address. Clearly, additional research is required with this particular application, as well as others, to establish the parameters for safe and efficacious translation of therapies from mouse to humans.

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REFERENCES

1. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001;358:221–229
2. Gepts W. Islet changes suggesting a possible immune aetiology of human diabetes mellitus. Acta Endocrinol Suppl (Copenh) 1976;205:95–106
3. Schneider A, Rieck M, Sanda S, Pihoker C, Greenbaum C, Buckner JH. The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells. J Immunol 2008;181:7350–7355
4. D'Alise AM, Auyeung V, Feuerer M, Nishio J, Fontenot J, Benoist C, Mathis D. The defect in T-cell regulation in NOD mice is an effect on the T-cell effectors. Proc Natl Acad Sci U S A 2008;105:19857–19862
5. Morran MP, Omenn GS, Pietropaolo M. Immunology and genetics of type 1 diabetes. Mt Sinai J Med 2008;75:314–327
6. Honkanen J, Skarsvik S, Knip M, Vaarala O. Poor in vitro induction of FOXP3 and ICOS in type 1 cytokine environment activated T-cells from children with type 1 diabetes. Diabetes Metab Res Rev 2008;24:635–641
7. Maki T, Ichikawa T, Blanco R, Porter J. Long-term abrogation of autoimmune diabetes in nonobese diabetic mice by immunotherapy with anti-lymphocyte serum. Proc Natl Acad Sci U S A 1992;89:3434–3438
8. Sherry NA, Chen W, Kushner JA, Glandt M, Tang Q, Tsai S, Santamaria P, Bluestone JA, Brillantes AM, Herold KC. Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of beta-cells. Endocrinology 2007;148:5136–5144
9. Tian C, Ansari MJ, Paez-Cortez J, Bagley J, Godwin J, Donnarumma M, Sayegh MH, Iacomini J. Induction of robust diabetes resistance and prevention of recurrent type 1 diabetes following islet transplantation by gene therapy. J Immunol 2007;179:6762–6769
10. Zhang C, Todorov I, Lin CL, Atkinson M, Kandeel F, Forman S, Zeng D. Elimination of insulitis and augmentation of islet beta cell regeneration via induction of chimerism in overtly diabetic NOD mice. Proc Natl Acad Sci U S A 2007;104:2337–2342
11. Yang Z, Chen M, Carter JD, Nunemaker CS, Garmey JC, Kimble SD, Nadler JL. Combined treatment with lisofylline and exendin-4 reverses autoimmune diabetes. Biochem Biophys Res Commun 2006;344:1017–1022
12. Suarez-Pinzon WL, Yan Y, Power R, Brand SJ, Rabinovitch A. Combination therapy with epidermal growth factor and gastrin increases β-cell mass

and reverses hyperglycemia in diabetic NOD mice. Diabetes 2005;54:2596–2601
13. Suri A, Calderon B, Esparza TJ, Frederick K, Bittner P, Unanue ER. Immunological reversal of autoimmune diabetes without hematopoietic replacement of beta cells. Science 2006;311:1778–1780
14. Ogawa N, List JF, Habener JF, Maki T. Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4. Diabetes 2004;53:1700–1705
15. Phillips B, Nylander K, Harnaha J, Machen J, Lakomy R, Styche A, Gillis K, Brown L, Lafreniere D, Gallo M, Knox J, Hogeland K, Trucco M, Giannoukakis N. A microsphere-based vaccine prevents and reverses new-onset autoimmune diabetes. Diabetes 2008;57:1544–1555
16. Simon G, Parker M, Ramiya V, Wasserfall C, Huang Y, Bresson D, Schwartz RF, Campbell-Thompson M, Tenace L, Brusko T, Xue S, Scaria A, Lukason M, Eisenbeis S, Williams J, Clare-Salzler M, Schatz D, Kaplan B, Von Herrath M, Womer K, Atkinson MA. Murine antithymocyte globulin therapy alters disease progression in NOD mice by a time-dependent induction of immunoregulation. Diabetes 2008;57:405–414
17. Chatenoud L, Primo J, Bach JF. CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice. J Immunol 1997;158:2947–2954
18. Maki T, Gottschalk R, Ogawa N, Monaco AP. Prevention and cure of autoimmune diabetes in nonobese diabetic mice by continuous administration of FTY720. Transplantation 2005;79:1051–1055
19. Bertry-Coussot L, Lucas B, Danel C, Halbwachs-Mecarelli L, Bach JF, Chatenoud L, Lemarchand P. Long-term reversal of established autoimmunity upon transient blockade of the LFA-1/intercellular adhesion molecule-1 pathway. J Immunol 2002;168:3641–3648
20. Esposito L, Kamar N, Tkaczuk J, Abbal M, Durand D, Rostaing L. Long-term evolution of lymphocytes subsets after induction therapy based on continuous versus discontinuous administration of anti-thymocyte globulins in renal-transplant patients. Transplant Proc 2005;37:785–787
21. Seidel MG, Fritsch G, Matthes-Martin S, Lawitschka A, Lion T, Pötschger U, Rosenmayr A, Fischer G, Gadner H, Peters C. In vitro and in vivo T-cell depletion with myeloablative or reduced-intensity conditioning in pediatric hematopoietic stem cell transplantation. Haematologica 2005;90:1405–1414
22. Toor A, Rodriguez T, Bauml M, Mathews H, Shanti S, Senitzer D, Kini A, Norton J, Parthasarathy M, Mohideen N, Petrowsky C, Bonilla B, Smith S, Stiff P. Feasibility of conditioning with thymoglobulin and reduced intensity TBI to reduce acute GVHD in recipients of allogeneic SCT. Bone Marrow Transplant 2008;42:723–731
23. Abdallah FK. Single dose filgastrim in cytotoxic-induced neutropaenia in children. East Afr Med J 2008;85:30–35
24. Morishita M, Leonard RC. Pegfilgrastim: a neutrophil mediated granulocyte colony stimulating factor-expanding uses in cancer chemotherapy. Expert Opin Biol Ther 2008;8:993–1001
25. Rutella S. Granulocyte colony-stimulating factor for the induction of T-cell tolerance. *Transplantation* 2007;84(Suppl. 1):S26–S30
26. Hartung T, Doecke WD, Bundschuh D, Foote MA, Gantner F, Hermann C, Lenz A, Milwee S, Rich B, Simon B, Volk HD, von Aulock S, Wendel A. Effect of granulocyte colony-stimulating factor treatment on ex vivo blood cytokine response in human volunteers. Blood 1995;85:2482–2489
27. Rutella S, Bonanno G, Pierelli L, Mariotti A, Capoluongo E, Contemi AM, Ameglio F, Curti A, De Ritis DG, Voso MT, Perillo A, Mancuso S, Scambia G, Lemoli RM, Leone G. Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN-alpha. Eur J Immunol 2004;34:1291–1302
28. Kared H, Masson A, Adle-Biassette H, Bach JF, Chatenoud L, Zavala F. Treatment with granulocyte colony-stimulating factor prevents diabetes in NOD mice by recruiting plasmacytoid dendritic cells and functional CD4(+)CD25(+) regulatory T-cells. Diabetes 2005;54:78–84
29. Hadaya K, Kared H, Masson A, Chatenoud L, Zavala F. GCSF treatment prevents cyclophosphamide acceleration of autoimmune diabetes in the NOD mouse. J Autoimmun 2005;24:125–134
30. Roep BO, Atkinson M, von Herrath M. Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. Nat Rev Immunol 2004;4:989–997
31. Roep BO. Are insights gained from NOD mice sufficient to guide clinical translation? Another inconvenient truth. Ann N Y Acad Sci 2007;1103:1–10
32. Goudy KS, Tisch R. Immunotherapy for the prevention and treatment of type 1 diabetes. Int Rev Immunol 2005;24:307–326
33. Schatz D, Gale EA, Atkinson MA. Why can't we prevent type 1 diabetes? Maybe it's time to try a different combination. Diabetes Care 2003;26:3326–3328
34. Matveyenko AV, Butler PC. Relationship between beta-cell mass and diabetes onset. Diabetes Obes Metab 2008;10(Suppl. 4):23–31
35. Phillips JM, O'Reilly L, Bland C, Foulis AK, Cooke A. Patients with chronic pancreatitis have islet progenitor cells in their ducts, but reversal of overt diabetes in NOD mice by anti-CD3 shows no evidence for islet regeneration. Diabetes 2007;56:634–640
36. Sherry NA, Kushner JA, Glandt M, Kitamura T, Brillantes AM, Herold KC. Effects of autoimmunity and immune therapy on β-cell turnover in type 1 diabetes. Diabetes 2006;55:3238–3245
37. Liang HL, Yi DH, Zheng QJ, Du JF, Cao YX, Yu SQ, Zhu HL. Improvement of heart allograft acceptability associated with recruitment of CD4+CD25+ T cells in peripheral blood by recipient treatment with granulocyte colony-stimulating factor. Transplant Proc 2008;40:1604–1611
38. Jilma B, Hergovich N, Homoncik M, Jilma-Stohlawetz P, Kreuzer C, Eichler HG, Zellner M, Pugin J. Granulocyte colony-stimulating factor (GCSF) downregulates its receptor (CD114) on neutrophils and induces gelatinase B release in humans. Br J Haematol 2000;111:314–320
39. Hermans MH, Ward AC, Antonissen C, Karis A, Löwenberg B, Touw IP. Perturbed granulopoiesis in mice with a targeted mutation in the granulocyte colony-stimulating factor receptor gene associated with severe chronic neutropenia. Blood 1998;92:32–39
40. Ward AC, van Aesch YM, Gits J, Schelen AM, de Koning JP, van Leeuwen D, Freedman MH, Touw IP. Novel point mutation in the extracellular domain of the granulocyte colony-stimulating factor (GCSF) receptor in a case of severe congenital neutropenia hyporesponsive to GCSF treatment. J Exp Med 1999;190:497–507
41. Lu Y, Parker M, Pileggi A, Zhang B, Choi YK, Molano RD, Wasserfall C, Ricordi C, Inverardi L, Brantly M, Schatz D, Atkinson M, Song S. Human alpha 1-antitrypsin therapy induces fatal anaphylaxis in non-obese diabetic mice. Clin Exp Immunol 2008;154:15–21
42. Starzl TE, Murase N, Abu-Elmagd K, Gray EA, Shapiro R, Eghtesad B, Corry RJ, Jordan ML, Fontes P, Gayowski T, Bond G, Scantlebury VP, Potdar S, Randhawa P, Wu T, Zeevi A, Nalesnik MA, Woodward J, Marcos A, Trucco M, Demetris AJ, Fung JJ. Toleragenic immunosuppression for organ transplantation. Lancet 2003;361:1502–1510
43. Tarkowski A, Andersson-Gare B, Aurell M. Use of anti-thymocyte globulin in the management of refractory systemic autoimmune diseases. Scand J Rheumatol 1993;22:261–266
44. Eisenbarth GS, Srikanta S, Jackson R, Rabinowe S, Dolinar R, Aoki T, Morris MA. Anti-thymocyte globulin and prednisone immunotherapy of recent onset type 1 diabetes mellitus. Diabetes Res 1985;2:271–6
45. Saudek F, Havrdova T, Boucek P, Karasova L, Novota P, Skibova J. Polyclonal anti-T-cell therapy for type 1 diabetes mellitus of recent onset. Rev Diabetic Studies 2004;1:80–88