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Immune Depletion with Cellular Mobilization Imparts Immunoregulation and Reverses Autoimmune Diabetes in NOD Mice

Running Title: ATG + G-CSF Reverses Overt Diabetes in NOD Mice

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Objective—The autoimmune destruction of β cells in type 1 diabetes (T1D) 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 for the immune depleting agent anti-thymocyte globulin (ATG), as well as the mobilization agent granulocyte-colony stimulating factor (G-CSF), to reverse overt hyperglycemia in the non-obese diabetic (NOD) mouse model of T1D.

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 beta cell area.

Results—Here, we show that combination therapy of murine ATG and G-CSF 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 post-onset) in animals having pronounced hyperglycemia (i.e., up to 500mg/dl). Additionally, glucose control improved over time in mice subject to remission from T1D. Mechanistically, this combination therapy resulted in both immunological (increases in CD4: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.

Type 1 diabetes (T1D) 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 NOD mouse model of T1D, the disorder's pathogenesis appears dependent on aberrant immune regulation (3-6). A reversal of T1D 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).

Anti-thymocyte 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 more than 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 questionsed 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 (G-CSF).

GCSF was initially developed as a means of mobilizing neutrophils (23, 24), but recent reports have also indicated a G-CSF-induced immunoregulatory impact (25). These studies indicated the ability of G-CSF 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 T1D, G-CSF 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 G-CSF, subject to clinical use in settings outside of T1D, for the purpose of testing their ability to reverse disease in NOD mice as well as to monitor their ability to re-instill 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 T1D in NOD mice.

RESEARCH DESIGN AND METHODS

Mice. Female NOD mice were purchased from Jackson Labs and housed in specific pathogen-free (SPF) facilities at the University of Florida. These studies received the approval of the Institution Animal Care and Use Committee (IACUC) at The University of Florida. Suboptimal studies were also performed using female NOD mice and were carried out at Genzyme's SPF facilities (Oklahoma City, OK) according to approved protocols.

T1D reversal studies. Mice used in reversal trials were monitored 3 times per week for hyperglycemia, defined as a blood glucose ≥ 240mg/dL, by tail bleed. Animals measuring above this threshold on two 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 (IP) injections of 500μg murine ATG or, as a control, 500μg rIgG (Jackson ImmunoResearch) given 72 hours apart for a total dose of 1mg. These animals also received a subcutaneous LinBit insulin

implant (LinShin Canada) providing sustained release of insulin for approximately 3 weeks. Failure of the therapy was defined as blood glucose levels above 400 mg/dl for two consecutive measurements. In the suboptimal dosing study, the dose of murine ATG was reduced to 290ug per animal, over two injections. Neupogen (Amgen, Inc.) was used for G-CSF therapy for both suboptimal and standard dosing studies. A dose of 6ug/animal was diluted in 100uL of 5% dextrose per manufacturer's recommendation and injected IP daily for a maximum of 8 weeks. Blood sugar was monitored 3 times per week until either failure occurred (as described above) or animals reached the endpoint post-onset (as indicated).

Pre-diabetic time course study. Combination therapy of standard dose murine ATG and G-CSF (as described above) was performed in pre-diabetic female NOD mice beginning at 12 weeks of age and lasted up to 8 weeks. Four groups were treated with control, murine ATG, G-CSF, or ATG+G-CSF. As with the T1D reversal studies, murine ATG was administered in two doses 72 h apart. G-CSF was administered for up to 8 weeks. Timed sacrifices were performed at weeks 0, 2, 4, and 8 post-initiation of therapy (n=5/group/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 prediabetic study were bled via tail perforation at predetermined time points (0, 2, 4, and 8 weeks) post-injection 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 timepoint or endpoint 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-PerCP and the corresponding isotype which were purchased from BD Biosciences. T cells were stained for CD8-FITC (clone 53-6.7), CD4-PerCP (clone RM4-5), Foxp3-PE (clone FJK-16a), and CD25-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 1ug per $1x10^6$ 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 hour food-restriction was implemented prior to testing. After 12 hours, 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 minutes blood glucose was sampled.

Immunoglobulin isotyping. Immunoglobulin isotyping was performed on sera obtained at each sacrifice 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-G-CSF antibody measurement. Sera was collected from mice at 0, 2, 4, and 8 week time points in the prediabetic study. To determine whether the immunoglobulin increases seen were G-CSFspecific, Nunc-Immuno 96-well plates were coated with 50uL/well of 2ug/mL GCSF (Amgen) overnight at 4°. Plates were blocked for 2 hours with 300uL/well 5%BSA/PBS and washed 5x with PBS/Tween. Sera was diluted 1:10000 and was incubated for 2 hours on a plate shaker. The plates were washed 5x as before, and were then coated with either 50uL/well 1:2500 Rat anti-mouse IgM-HRP or 1:5000 Donkey anti-mouse IgG-HRP (Southern Biotech) for 1 hour on a plate shaker. The plates were once again washed 5x followed by addition of 50uL TMB protected from light. After 5 minutes, the reaction was stopped with 50uL stop solution (Mercodia) and the plate read at 450nm 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 mean SD with P values <0.05 considered significant.

RESULTS

G-CSF 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 upon these blood glucose values, the administration of murine ATG alone to new-onset NOD mice resulted in durable (i.e., > 180 days post-onset) remissions from overt hyperglycemia in 33% (5/15) of treated animals, while neither control (0/16) nor G-CSF monotherapy (0/14 mice) provided such reversions (Fig. 2). However, the combination of murine ATG and G-CSF therapy resulted in a remission rate of 75%, a significantly greater rate of remission than was seen with murine ATG monotherapy (12/16; P = 0.0000006 versus control, P = 0.013 versus murine ATG). In order to provide further evidence for the notion that the therapeutic efficacy of murine ATG was enhanced by the addition of G-CSF, studies were undertaken wherein murine ATG was administered with sub-optimal dosing (i.e., 0.29 mg per mouse versus 1.0 mg per mouse used in the aforementioned efforts). Interestingly, G-CSF dramatically improved the therapeutic capacity for diabetes reversal even when in combination with this suboptimal dose of murine ATG (Supplemental Fig. 1 which is available in the online appendix at http://diabetes.diabetesjournal.org).

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

≤380mg/dL (mean 317.2mg/dL; range 256-398mg/dL), whereas combination therapy of murine ATG and G-CSF significantly increased the therapeutic ceiling to ~500 mg/dL (mean 401.8 mg/dL; range 264-500mg/dL).

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 (IPGTT) at 60, 90, and 120 days post-onset in reversed mice. An improvement in IPGTT as measured by the area under the curve from 0 to 120 minutes was observed from the 60 to 120 day time points (Fig. 4). This improvement in glucose control occurred in spite of the cessation of both murine ATG and G-CSF therapies prior to the 60-day time point.

Murine ATG + G-CSF combination therapy induces immunomodulation. To address the question of whether G-CSF-mediated enhancement of diabetes reversal was due to induction of immunoregulation, murine ATG and G-CSF (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 week (Fig. 5*A*), with movement back towards pre-treatment levels at 4 and 8 weeks post-induction. However, the addition of G-CSF to murine ATG afforded a significant increase in leukocytes at 2 weeks versus murine ATG alone. In particular, G-CSF increased the percentage of splenic macrophages (Fig. 5*B*) and neutrophils (Fig. 5*C*).

Both murine ATG as well as G-CSF 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 G-CSF. G-CSF 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 G-CSF 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 G-CSF, induced a significant increase in the splenic CD4+:CD8+ ratio (Fig. 6) compared with control and G-CSF treated mice, with the increase peaking at 2 weeks and remaining significant at 4 and 8 weeks.

Anti-G-CSF antibodies correlate with reduced action of G-CSF beyond two 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 G-CSF 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 G-CSF-induced upregulation of multiple isotypes versus control mice at 8 weeks, including IgM and IgG1

(Supplemental Fig. 2). To address whether these antibodies were G-CSF-specific, anti-G-CSF ELISAs were performed. This further analysis of sera revealed significant G-CSF-specific IgM, and IgG antibody responses (Supplemental Fig. 3). These responses became evident beginning 2 weeks after initiation of G-CSF administration and continued to increase out to 8 weeks of therapy.

Pancreatic islets are protected from further autoimmune destruction by murine ATG and G-CSF. The health of the islets at the endpoint of the prediabetic 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 prediabetic 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 T1D 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 T1D 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 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 T1D (14). Consequently, herein we have described an approach using two clinically relevant therapies, ATG and G-CSF, for the purpose of immunomodulation that would provide benefit in terms of reversing T1D, 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 G-CSF 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 previous studies using similar immune depleting agents as monotherapy (e.g., anti-CD3 monoclonal antibody) note a diminished ability to reverse T1D in NOD mice in this metabolic range (i.e., ≥350mg/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 G-CSF, 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 post-onset, the quality of glucose control significantly increases as measured by AUC analysis. The exact reason for this improvement is uncertain, but possibly due to the recovery of endogenous β cells (35). Previous reports have indicated that the efficacy of reversal therapies hinges upon the recovery of these cells rather than the generation of new β cells (8, 36). A time course analysis of the pancreas in future reversal studies may address this hypothesis.

In our prediabetic 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 G-CSF 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 co-transfer 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-G-CSF IgG1 and IgM antibodies may explain the reduction in macrophages and neutrophils after 2 weeks of G-CSF therapy. It is also possible that a reduction in G-CSFR mRNA (Supplemental Table 1) may also play a role (38-40). This response is not surprising given that the recombination G-CSF 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 G-CSF remained viable for both reversal of overt disease and for maintaining the health of islets when administered to prediabetic NOD mice. If this immune response against the G-CSF 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 prediabetic 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 G-CSF monotherapy group reflects the reversal study (Fig. 1*A*) in which G-CSF 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 G-CSF offers a highly effective means for reversal of T1D 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 T1D 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 man 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, rheumatoid arthritis (43). There has also been promise for the efficacy of ATG in the treatment of T1D. Early studies of equine ATG in combination with prednisone in new onset T1D 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, Germany), T1D participants aged 18-35 years received a total dose of 18 mg/kg of ATG which was administered in four infusions. Of the 17 study participants, 11 received 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 below 126 mg/dL). A pilot study is currently underway in humans with new-onset T1D, funded by the Immune Tolerance Network (ITN), 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 T1D. Since this study will also help establish safety data for the use of ATG in humans in T1D, 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 G-CSF. 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 T1D. 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 man.

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Figure Legends

Fig. 1. Blood glucose values were obtained for up to 180 days post-onset in NOD mice treated with A: control, B: G-CSF, C: ATG, or D: ATG + G-CSF.
Fig. 2. Long-term diabetes reversal was achieved in 75% (12/16; $P = 0.0000006$ versus control) of murine ATG + G-CSF treated mice, which was significantly improved versus murine ATG monotherapy ($P = 0.013$), which reversed 33% (5/15) mice. Long-term remission was observed in neither G-CSF (0/14) nor control-treated (0/15) mice.
Fig. 3. G-CSF enhances the ability of murine ATG to reverse mice with greater hyperglycemia at diabetes onset. Therapeutic success of murine ATG therapy (black circles) was largely limited to starting blood glucose values of 380mg/dL and below with an average of 317.2mg/dL. The addition of G-CSF to murine ATG treatment significantly raised the starting average blood glucose of successful therapy (black triangles) to 401.8/mg/dL ($P = 0.019$).
Fig. 4. IPGTT indicates improving glucose control from 60 to 120 days post-onset in combination therapy-treated NOD mice. NOD mice remitted from diabetes using combination therapy were administered an IPGTT at A: 60, B: 90, and C: 120 days post-onset. An area under the curve (AUC) analysis revealed a significant improvement at 120 (21940 ± 1250, n=7) days compared with 60 (26840 ± 1068, n=7) days ($p=0.0235$, unpaired t test).
Fig. 5. Murine ATG and G-CSF 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 + G-CSF 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. G-CSF and murine ATG + G-CSF treated mice exhibited significant increases in splenic B: macrophages ($P < 0.0001$, $P = 0.0027$, respectively; unpaired t test) and C: neutrophils ($P < 0.0001$, $P < 0.0001$, 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 G-CSF, but was significantly increased versus control at 4 weeks in G-CSF-treated mice ($P = 0.003$, unpaired t test) and at 8 weeks in murine ATG + G-CSF treated mice ($P < 0.0001$, unpaired t test).
Fig. 6. Administration of mATG induces an increase in the splenic CD4:CD8 ratio. ATG-treated mice exhibited a significantly higher CD4:CD8 ratio than both controls and G-CSF-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 post initiation. Combination-treated mice also exhibited significantly higher ratios versus controls and G-CSF-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 post-initiation.
Fig. 7. Murine ATG and G-CSF combination therapy in NOD mice protects pancreatic islets from autoimmune destruction. A: Insulitis scoring of B: islets 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).

Figure 1

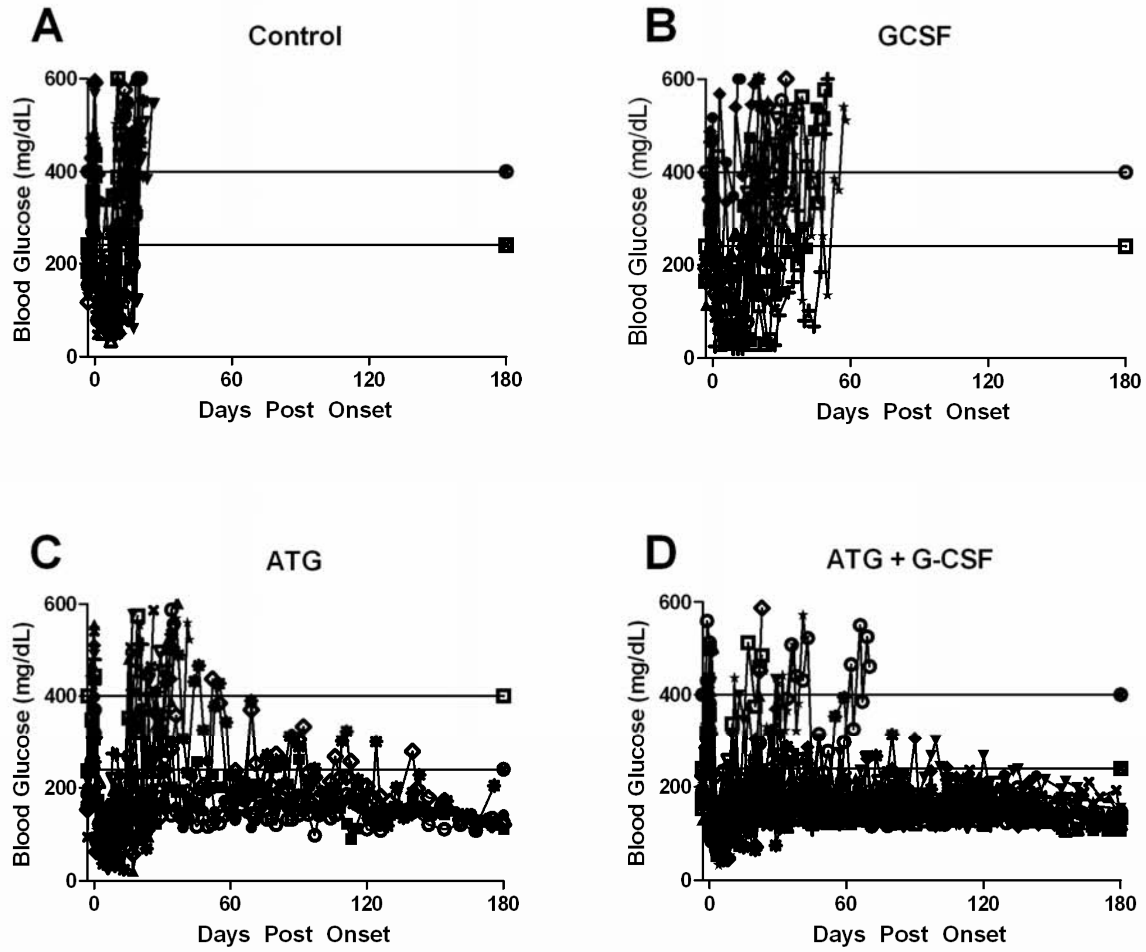
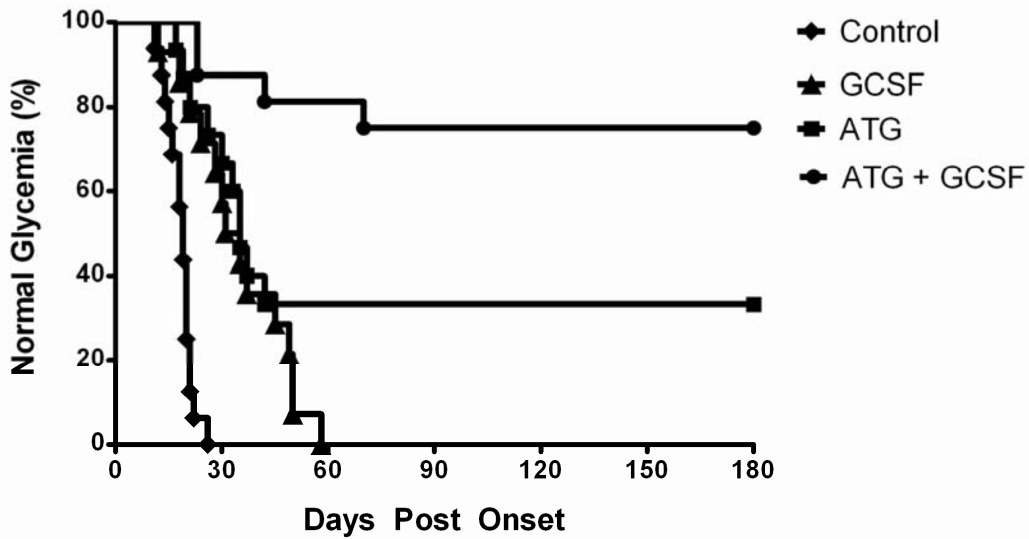


Figure 2



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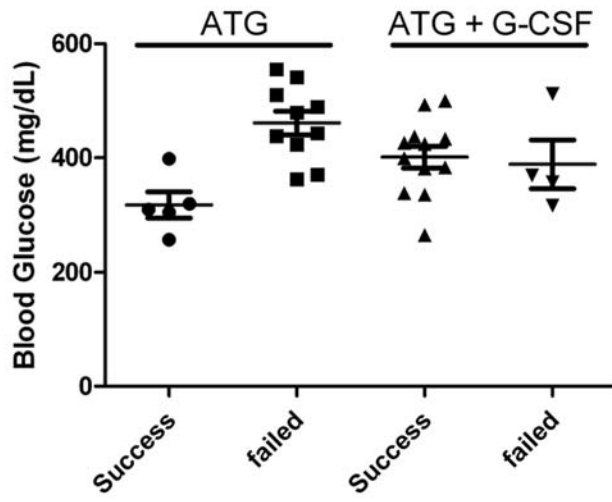
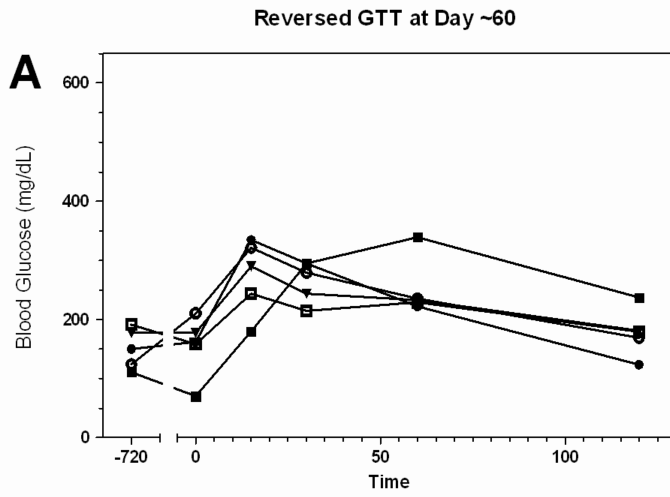
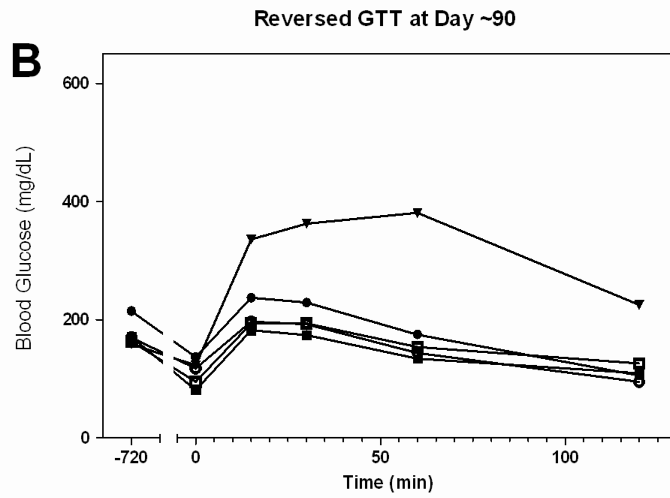


Figure 4





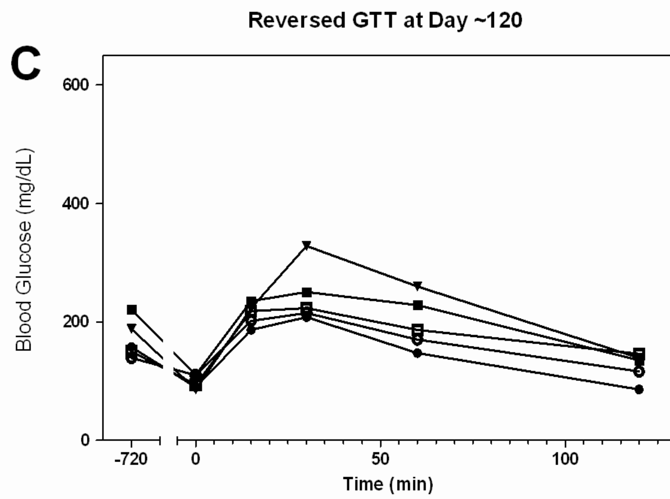


Figure 5

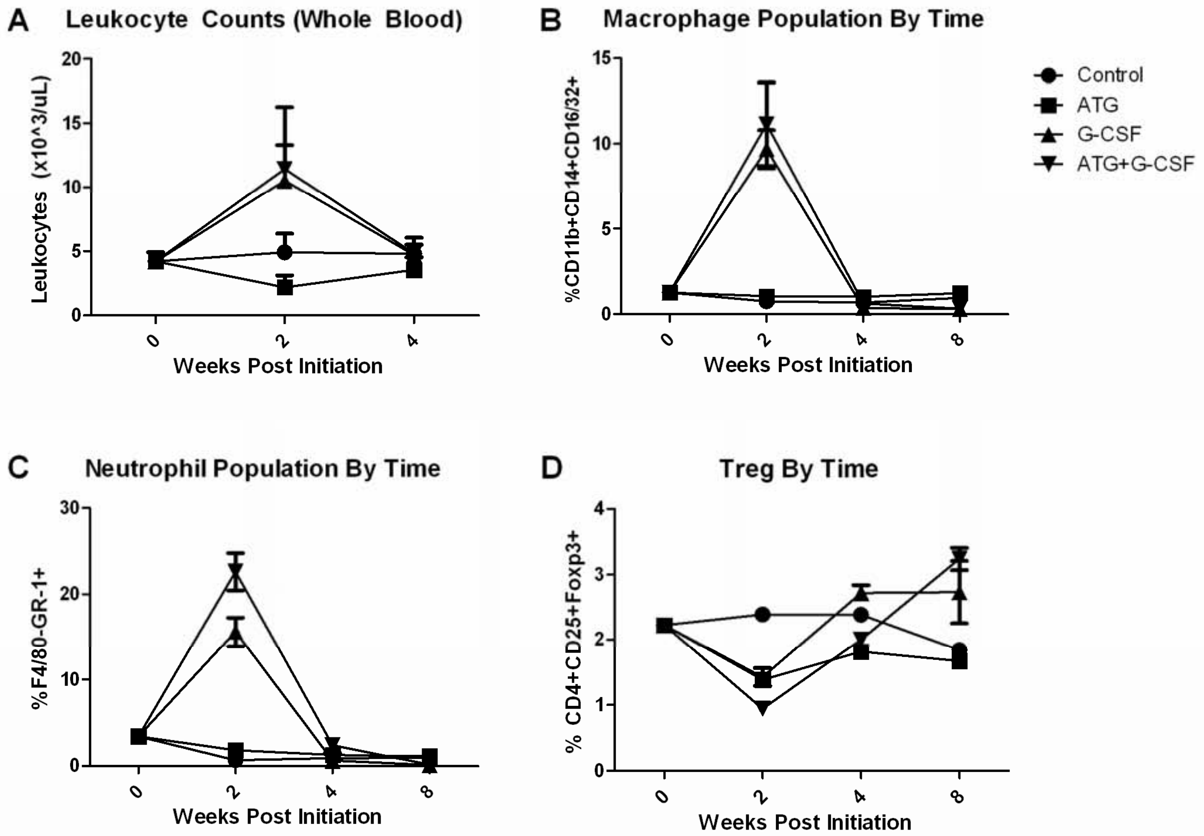


Figure 6

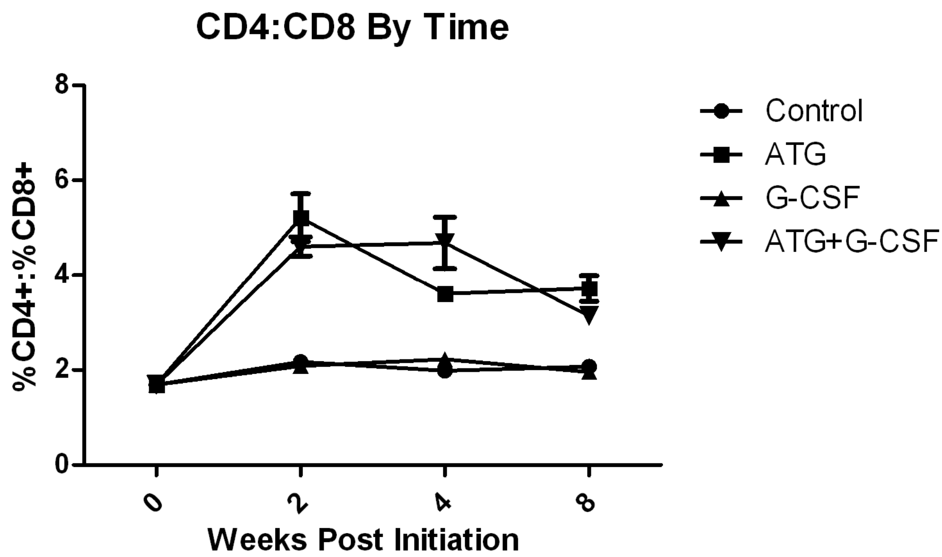


Figure 7