Combination therapy reverses hyperglycemia in NOD mice with established type 1 diabetes

Song Xue¹, Amanda Posgai¹, Clive Wasserfall¹, Courtney Myhr¹, Martha Campbell-Thompson¹, Clayton E. Mathews¹, Todd Brusko¹, Alex Rabinovitch², Alexei Savinov², Manuela Battaglia³, Desmond Schatz⁴, Michael Haller⁴, and Mark Atkinson¹

¹Department of Pathology, Immunology, and Laboratory Medicine, College of Medicine, University of Florida, Gainesville, FL 32610, USA. ²Sanford Research, University of South Dakota, Sioux Falls, SD 57104, USA. ³San Raffaele Diabetes Research Institute, Milan, Italy. ⁴Department of Pediatrics, College of Medicine, University of Florida, Gainesville, FL 32610, USA.

Address correspondence to: Mark A. Atkinson, University of Florida, College of Medicine, Department of Pathology, Box 100275, 1275 Center Dr., BMSB Room J593, Gainesville, FL 32610. TEL (352) 273-8276; FAX (352) 273-9339. E-mail: atkinson@ufl.edu
An increasing number of therapies have proven effective at reversing hyperglycemia in the non-obese diabetic (NOD) mouse model of type 1 diabetes (T1D), yet situations of successful translation to human T1D are limited. This may be due, in part, to evaluating the effect of treating immediately at diagnosis in mice, which may not be reflective of the advanced disease state in humans at disease onset. In this study, we treated NOD mice with new onset as well as established disease using various combinations of four drugs: anti-thymocyte globulin (ATG), granulocyte-colony stimulating factor (G-CSF), a dipeptidyl peptidase IV inhibitor (DPP-4i), and a proton pump inhibitor (PPI). Therapy with all four drugs induced remission in 83% of new onset mice and remarkably, in 50% of NOD mice with established disease. Also noteworthy, disease remission occurred irrespective of initial blood glucose values and mechanistically, was characterized by enhanced immunoregulation involving alterations in CD4+ T cells, CD8+ T cells, and NK cells. This combination therapy also allowed for effective treatment at reduced drug doses (in comparison to effective monotherapy), thereby minimizing potential side effects while retaining efficacy. This combination of approved drugs demonstrates a novel ability to reverse T1D, thereby warranting translational consideration.
Type 1 diabetes (T1D) is an autoimmune disorder long thought amenable to disease prevention and perhaps even reversal (1). Indeed, a rich history of agents have shown the capacity to prevent T1D in non-obese diabetic (NOD) mice (2), with an increasing number of reports positing the capability to reverse hyperglycemia in animals at disease diagnosis (3). Though numerous human trials have utilized therapies shown effective in NOD mice, few translational success stories exist (e.g., anti-CD3, CTLA4 Ig, anti-CD20), and even those have proven heterogeneous in their efficacy in new onset populations (4).

To improve on this situation, we considered the regulatory approval status of potential drugs, their mechanism of action, as well as the notion that any such effort would most likely require a combination approach in order to maximize efficacy. With this, we selected drugs having prior FDA approval as well as proven effectiveness in settings of autoimmunity, transplantation, and/or beta cell dysfunction in NOD mice or related clinical settings. Specifically, we previously noted the synergism of anti-thymocyte globulin (ATG) with granulocyte-colony stimulating factor (G-CSF) in reversing T1D in NOD mice (5). This was mechanistically tied to enhanced immunoregulation involving an increase in Treg activities as well as alterations in dendritic cell (DC) phenotype and function (6). Translation of this therapy from studies of mice to man also proved remarkably successful. Specifically, we recently reported the results from a pilot clinical trial assessing the effectiveness of this combination therapy (i.e., ATG plus G-CSF) in humans. That effort, which utilized not recent-onset patients but those with somewhat “established” disease (6 to 24 months post-onset) noted the ability for this combination of agents to preserve C-peptide (7).

We have long been interested in combination therapies for T1D, and as part of this, we also demonstrated that a combination of a dipeptidyl peptidase IV inhibitor (DPP-4i) along with
a proton pump inhibitor (PPI) synergize to reverse T1D in NOD mice (8). The DPP-4i allows for a longer half-life of glucagon like peptide-1 (GLP-1) that has been shown to have favorable effects on beta cell regeneration and survival (8-10). The PPI class of drugs raises circulating levels of gastrin in vivo, which also allows for enhanced beta cell function and survival (11-14).

Based on these observations, we hypothesized that a combination of all four drugs (ATG, G-CSF, DPP-4i and PPI; AGDP) would not only allow for reversal of new onset T1D in NOD mice, but perhaps animals with established disease. This approach represented quite a challenge to existing dogma regarding reversal of T1D in NOD mice, where essentially all agents noted for efficacy require administration at the earliest signs of hyperglycemia or glycosuria (i.e., within hours to 1 or 2 days), as well as utilization when blood glucose levels are modestly elevated (e.g., as low as 180mg/dl) (3). Indeed, reports to date suggest reversal of established disease in NOD mice requires administration of an alternate source of insulin production (i.e., transplanted islet cells).
Research Design and Methods

Mice. The studies described here were approved by the institutional animal care and use committee at the University of Florida. Eight-week old female NOD/LtJ mice were purchased from The Jackson Laboratory and monitored twice weekly for diabetes by tail bleed. Diabetes was defined as blood glucose $\geq 240$ mg/dL on two consecutive days, measured by a hand-held glucometer. Diabetic mice were randomized into treatment arms in the new onset or established treatment groups. Non-diabetic mice analyzed in this study were age-matched female NOD/LtJ mice that had neither developed diabetes nor received treatment. These control mice were sacrificed in parallel with treated euglycemic mice at the study end point (120 d post treatment).

Reversal studies. Upon diabetes determination, all mice were implanted with a subcutaneous insulin pellet (LinBit; LinShin, Canada). Mice in the new onset groups began therapy that day (new onset day 0), while mice in the established groups began therapy 15 days later (established day 0). Therapy groups consisted of insulin pellet alone, ATG+G-CSF therapy, DPP-4i+PPI therapy, four agent (AGDP) therapy, or control treatment. Murine ATG (Genzyme; Cambridge, MA) was administered intraperitoneally (IP) at 250 µg/dose on days 0 and 3. Human G-CSF (Neulasta; Amgen) at 120 µg/dose was administered IP on days 0 and 15. The selective DPP-4i 1-\{(3-hydroxy-1-adamantyl)amino\}acetyl\}-2-cyano-(S)-pyrrolidine (Dalton Chemical Laboratories, Toronto, ON, Canada) was administered orally at 200 µg daily for 84 d. PPI (pantoprazole; Nycomed, Oakville, ON, Canada) was given SQ at 600 µg twice daily for 84 days. Control mice received control treatments for all four drugs on their respective schedules: rIgG (Jackson Immunoresearch) for ATG, 5% dextrose for G-CSF, 5 mg/mL methylcellulose for DPP-4i, and saline for PPI. A group given the insulin pellet alone, without further manipulation, was also analyzed.
Mechanistic studies. Mechanistic studies were conducted similarly to reversal studies (described above) with the exception that treatment was initiated 10 days post-T1D onset in established groups, and tissues were harvested on day 30. The batch of insulin pellets (LinBit; LinShin, Canada) being used was observed to be less effective resulting than earlier return to hyperglycemia than expected; thus, the decision was made to initiate treatment at day 10 (rather than day 14) which theoretically still allows for appreciable continued beta cell loss.

Histology. For reversal studies, necropsy was performed at day 120, the pancreas fixed in 10% neutral buffered formalin overnight, and subsequently embedded in paraffin. Sections were stained with Hematoxylin and Eosin and scanned to create digital images using an Aperio GS Scanscope (Aperio Technologies, CA). Each section was reviewed for islets and insulitis scored according to the following: 0, no insulitis; 1, peri-islet only; 2, <50% infiltrates within islet; 3, >50% infiltrates within islets. Total numbers of islets within each scoring category were tabulated by animal, and the average islet number per score within each treatment group compared to age-matched female non-diabetic NOD mice. For mechanistic studies, necropsy was performed at day 30. For four mice per treatment group, the pancreas was fixed and embedded as described above. Sections were stained for insulin and Ki-67 via immunohistochemistry and scanned to create digital images using an Aperio GS Scanscope (Aperio Technologies, CA). Each section was reviewed for the number of insulin –positive and –negative islets. Fractional insulin area was determined using cytonuclear IHC quantification software (Indica Laboratories, Albuquerque, NM). An ImageScope plug-in tool was used to calibrate the RGB optical density values of the underlying tissues and set the input parameters. From mice sacrificed at day 30, serial pancreatic sections were also stained for CD4 and CD8 via
IHC, scanned, and analyzed using the Aperio with cytonuclear IHC quantification software for absolute number and percentage of islet cells staining positive for CD4 and CD8.

*Enzyme Linked Immunosorbent Assay-Serum.* For mechanistic studies, necropsy was performed at day 30. Maximum attainable blood volume was collected, and serum c-peptide levels were measured via ELISA (ALPCO Diagnostics, Salem, NH).

*Enzyme Linked Immunosorbent Assay-Pancreas.* For mechanistic studies, necropsy was performed at day 30. For 2-3 mice per treatment group, the pancreas was processed for total protein via acid ethanol extraction. Briefly, pancreata were weighed and incubated overnight at -20°C in 5ml 1.5% HCl in 70% EtOH prior to homogenization. Homogenates were again incubated overnight at -20°C and pelleted by centrifugation. The supernatant solution was brought to neutral pH with 1M Tris pH 7.5 and tested via ELISA for insulin, proinsulin, and c-peptide (ALPCO Diagnostics, Salem, NH). Results were normalized against pancreatic total protein as quantified via Bradford assay.

*Flow cytometry.* Spleens were harvested and passed through a 100 µm filter to obtain a cell suspension, and 1x10^6 cells were stained for flow cytometric analysis. For reversal studies, Tregs were stained with anti-mouse FoxP3-PE (clone FJK-16s), CD8a-FITC (53-6.7), CD25-APC (PC61.5), and CD4-PE-Cy7 (RM4-5) using standard procedures. Naïve and memory T cells were determined by staining with anti-mouse CD44-FITC (IM7), CD8b-PE (H35-17.2), CD62L-APC (MEL-14), and CD4-PE-Cy7 (RM4-5). Samples were run on an Accuri flow cytometer (BD Accuri Cytometers, Ann Arbor, MI). For mechanistic studies, Tregs were stained with anti-mouse FoxP3-PE (clone FJK-16s), CD8a-eFluor450 (53-6.7), CD25-APC (PC61.5), CD4-PE-Cy7 (GK1.5), CD62L-FITC (MEL-14) and CD44-PerCP-Cy5.5 (IM7). NK cells were stained
with anti-mouse CD49b-FITC (HMa2), CD122-PE (TM-b1), CD11b-PE-Cy7 (M1/70), and CD3-APC (17A2). Samples were run on a LSR Fortessa flow cytometer (BD Biosciences, San Jose, CA). Briefly, cells underwent surface staining for 30 m, followed by fixation for 2 h. Samples were washed in permeabilization buffer and blocked (anti-mouse CD16/CD32; eBioscience) for 15 m, followed by intracellular staining for 30 m (all antibodies and buffers from eBioscience, San Diego, CA). Results were analyzed in FCS Express v4 (De Novo Software, Los Angeles, CA).

Statistics. All data were analyzed on GraphPad Prism 5.1 (GraphPad, San Diego, CA). Survival curves were analyzed using a Kaplan-Meier test. Comparisons between multiple data sets were analyzed by ANOVA (non-parametric Kruskal-Wallis test) with Dunn’s multiple comparison post-test. Students T test was used in for comparison of remitted versus failed mice.
Results

*AGDP combination therapy induces durable diabetes remission in NOD mice with new onset as well as established diabetes.* On the day of diabetes onset, NOD mice received a slow-release subcutaneous insulin pellet and were randomized into the following treatment groups: murine ATG and human G-CSF, DPP-4i and PPI, the combination of all four drugs (AGDP therapy), or control treatments. Treatment was initiated in these cohorts at either disease onset (new onset groups) or after a 2-week delay (established groups). In new onset mice, ATG+G-CSF therapy resulted in durable remission (i.e., euglycemic for 120 days) in 6 of 12 mice (50%, \( P = 0.0010 \) vs. control), while 7 of 12 mice (54%, \( P < 0.0001 \)) treated with DPP-4i+PPI therapy at onset showed durable remission (Fig. 1A). Treating new onset mice with all four drugs increased remission rate to 11 of 13 mice (83%, \( P < 0.0001 \) vs. control). However, compared to ATG+G-CSF or DPP-4i+PPI, AGDP therapy did not significantly increase remission rates in mice with established diabetes. In established mice, 4 of 12 (33%, \( P = 0.0223 \) vs. control) treated with ATG+G-CSF, 5 of 12 mice (42%, \( P = 0.0962 \)) treated with DPP-4i+PPI, and strikingly, 6 of 12 mice (50%, \( P = 0.0013 \)) treated with AGDP therapy exhibited durable remission (Fig. 1B). Non-fasting morning blood glucose curves for each of these treatment groups (Fig. 1C-K) demonstrate that remitted mice maintained stable blood glucose levels for 120 days post-T1D onset.

In new onset mice, histological scoring of remitted animals showed fewer islets with insulitis with DPP-4i+PPI therapy, as well as fewer islets with less than 50% insulitis (Fig. 1L); however, remitted mice with ATG+G-CSF and AGDP therapy had similar scoring. In mice with established diabetes, insulitis was similar between all three therapies, with ATG-G-CSF therapy showing a minor decrease in islets with insulitis compared to the other therapies.
AGDP therapy results in disease remission despite insignificant increases in beta cell mass in a cross-sectional study of mechanism. Similar to the reversal studies described above, female NOD mice received a slow-release subcutaneous insulin pellet and were randomized into treatment groups at diabetes onset. However, endpoints were day 30 or treatment failure. In new onset mice, AGDP and ATG+G-CSF groups trended higher than controls regarding circulating C-peptide levels, pancreatic fractional insulin area, and number of insulin+ islets in the pancreas, but the difference was not significant. In mice with established disease, one outlier from the DDP-4i+PPI group demonstrated elevated fractional insulin area, but overall, the number of insulin+ islets and fractional insulin area did not differ between treatment groups (Fig. 2A-C, \( P = 0.1115 \), \( P = 0.7776 \), and \( P = 0.7237 \), respectively). We were unable to evaluate the effect of therapy on Ki67+ beta cells as a measure of beta cell replication because many animals had no detectable insulin+ cells, rendering the N too low for statistical analysis.

Pancreata from \( N=2-3 \) mice per group, as well as two non-diabetic control NOD mice, were analyzed for total proinsulin, C-peptide, and insulin content via ELISA. Results were normalized against total protein extracted from the pancreas. As expected, pancreata from non-diabetic NOD mice contained significantly higher levels of proinsulin, C-peptide, and insulin. Remarkably, despite disease reversal, AGDP therapy did not rescue pancreatic proinsulin, C-peptide, or insulin content to levels comparable with non-diabetic animals. In new onset mice, treatment with ATG+G-CSF and AGDP was associated with an insignificant increase in total pancreatic proinsulin, C-peptide, and insulin (Fig. 2C-E, \( P < 0.0001 \)). These data suggest that only a fraction of beta cell mass, undetectable by current methods, must be preserved for T1D reversal and insulin independence.
Initial blood glucose remission rate bias is ameliorated in AGDP therapy. Previous NOD reversal studies have shown a correlation between higher initial blood glucose levels and failure rate in some therapies (3), including ATG alone (5). Accordingly, we determined if a similar correlation exists under these therapy conditions. Initial blood glucose levels were similar in mice randomized to all treatment groups (Fig. 3A, \(P = \text{NS}\)) implying no bias occurred during the randomization to treatment. However, in mice treated with ATG+G-CSF, failure rate correlated with higher initial blood glucose levels in both the new onset group as well as the established group (Fig. 3B-C, \(P < 0.05\)). This was not surprising given that suboptimal ATG dosing was used. Initial blood glucose bias in DPP-4i+PPI treated mice was not significant (\(P = \text{NS}\)), while the addition of these two drugs in AGDP therapy ameliorated the initial blood glucose bias present with ATG+G-CSF therapy. Therefore, AGDP therapy was more effective than ATG+G-CSF therapy in mice with elevated initial blood glucose while in this study, the influence of DPP-4i+PPI was inconclusive.

AGDP therapy induces T cell and NK cell immunomodulation. We next determined the effects of therapy on T cell subsets. To determine the ratio of CD4\(^+\) to CD8\(^+\) T cells, splenocytes were isolated and analyzed by flow cytometry at the study end point (i.e., at reversal failure or 120 d). In mice with both new onset and established disease, treatment with AGDP significantly increased the CD4\(^+\):CD8\(^+\) T cell ratio (Fig. 4A-B, \(P < 0.05\)). Treatment with ATG+G-CSF trended higher in both new onset and established disease, but did not reach statistical significance, while DPP-4i+PPI did not have an effect on this phenotype (\(P = \text{NS}\)). These effects were even more robust in cross-sectional analysis. In both new onset and established disease groups, AGDP and ATG+G-CSF therapy significantly increased the splenocyte CD4\(^+\):CD8\(^+\) T
cell ratio compared to controls, and AGDP significantly increased the CD4\(^+\):CD8\(^+\) T cell ratio compared to those treated with DPP-4i+PPI (Fig. 4C-D, \(P < 0.001\) and \(P = 0.0016\), respectively). These effects were largely due to CD8\(^+\) T cell depletion which was evident in all ATG+G-CSF and AGDP treated animals with new onset and established disease (Fig. 4E). Meanwhile, splenic CD4\(^+\) T cell frequencies were only modestly modulated in response to therapy— increased in new onset mice with AGDP treatment, decreased in ATG+G-CSF treated animals with established disease and unchanged in all other groups, relative to control (Fig. 4F). Furthermore, mechanistic studies revealed that NK cell frequency in the spleen was significantly reduced in new onset mice treated with AGDP and in established disease mice treated with AGDP or ATG+G-CSF (Fig. 4G-H, \(P = 0.0015\) and \(P = 0.005\), respectively). Interestingly, mice that received DPP-4i+PPI treatment also trended toward reduced NK cell frequencies in new onset and established disease groups, but these differences were not statistically significant (Fig. 4E-F).

Pancreata harvested for cross-sectional studies were stained via IHC for CD4 and CD8, and digitally scanned images were manually annotated to exclude exocrine tissues from analysis. Within the pancreatic islet area, the CD4\(^+\):CD8\(^+\) T cell ratios did not differ across treatment groups (Fig. 5). Moreover, absolute numbers of CD4\(^+\) and CD8\(^+\) T cells within the insulitic lesion were not significantly different (data not shown). However, these data do not consider potential modulation of T cell phenotype or function within the pancreas or draining lymph node, and with small sample size (n=3-4 per group), possible immunomodulatory effects of therapy within the target organ cannot be excluded providing an important topic of investigation for future studies.
To analyze CD4+ and CD8+ naïve, memory, and regulatory T cells (Treg), remitted mice were compared to both therapy failures as well as age-matched non-diabetic female NOD mice which were used to control for natural shifts in cell populations with age. With new onset animals, remitted mice from all three therapy groups had an elevated percentage of splenic CD4+CD25+FoxP3+ Treg compared to non-diabetic control mice (Fig. 6A, P < 0.05). Within the ATG+G-CSF and DPP-4i+PPI groups, remitted mice showed significantly higher levels of Treg than failures (P < 0.05); remitted mice in the AGDP group appear to have higher Treg than the one failed mouse, however, the low N prohibits statistical analysis. Similarly, with established diabetes, remitted mice in all three-therapy groups had elevated Treg compared to failures (Fig. 6B, P < 0.05). Remitted mice in the DPP-4i+PPI and AGDP groups also had elevated Treg compared to non-diabetic controls.

CD8+CD25+FoxP3+ Treg were elevated in remitted mice in the new onset AGDP therapy group, versus the non-diabetic controls. In this same group, DPP4i+PPI successfully treated mice had higher CD8+CD25+FoxP3+ Treg than failed mice (Fig. 6C, P < 0.05). In the established disease group, only the successfully treated AGDP animals had elevated CD8+CD25+FoxP3+ relative to failed animals (Fig. 6D, P < 0.05). Collectively, we interpret this as evidence of immunoregulation by any of these combinations as measured by either CD4 or CD8 Treg cells and that the successful treatment yielded higher immunoregulatory cell frequency over those that failed. In established mice, this trended lower implying that endocrine or metabolic issues may underlie failure as opposed to failed immunoregulation. Additional studies will be needed to further investigate these mechanisms. DPP4i+PPI therapy also revealed a potential mechanistic basis in both CD4 and CD8 Treg modulation perhaps adding further evidence of
immunomodulatory effects beyond those favoring incretin hormone and beta cell survival mechanisms.

We next determined CD4$^+$ naïve (CD4$^+$CD62L$^+$CD44$^+$) and memory (CD4$^+$CD62L$^-$CD44$^+$) T cell populations in the therapy groups. In all new onset treatment groups, and in AGDP therapy in the established treatment group, treated mice had a higher percentage of naïve CD4 T cells than age-matched non-diabetic controls. The only difference in terms of failure versus success was in the DPP4i+PPI group wherein failed mice were left with more naïve CD4 T cells (Fig. 7A-B, $P < 0.05$). Memory CD4 T cells, however, did associate with successfully treated mice tending to have more of these cells than failed animals. This held true for all groups, in both new onset and established disease, except for the new onset AGDP group where small sample size in the failed group did not allow for statistical significance to be achieved (Fig. 7C-D). No significance was observed for naïve CD8 T cell frequency in any group with respect to successful reversal versus failure. There was an increase in naïve CD8$^+$ T cells in the successfully treated new onset ATG+G-CSF therapy relative to non-diabetic controls (Fig. 8A-B). In remitted mice from the new onset treatment group, but not the established treatment group, ATG+G-CSF and DPP-4i+PPI therapies had a lower percentage of memory CD8$^+$ T cells than non-diabetic age-matched controls, but higher memory CD8 T cell frequency associated with success compared to failed animals in both new onset and established disease groups, except again for the new onset AGDP group which had too few failures to evaluate statistically (Fig. 8C-D, $P < 0.05$).
**Discussion**

Despite instances of success in preventing and even reversing hyperglycemia in the NOD mouse model of T1D, efforts at clinical translation have, for the most part, been disappointing in terms of meeting their desired endpoints (2-4; 15). At the same time, this statement is not intended to convey that promising therapeutic efforts, either preclinical or clinical (e.g., Alefacept, Imatinib) do not exist (reviewed in (16)) as efforts are, thankfully, moving forward.

In the studies presented here, we tested combinations of four drugs that have in past settings demonstrated potential utility in either T1D, or other relevant autoimmune diseases with the notion that ATG+G-CSF treatment would promote immunoregulation while DPP-4i+PPI therapy might preserve or even increase beta cell mass and function (5; 6; 8-13). We therefore, hypothesized that AGDP therapy may result in synergy for the reversal of T1D. Indeed, our previous studies showing the efficacy of ATG+G-CSF therapy required higher doses of ATG to obtain similar reversal rates in new onset mice (3; 5). In this regard, AGDP therapy may be more readily translatable to human clinical trials, as lower doses of ATG would potentially be safer and cause fewer side effects. The ability of AGDP therapy to induce disease remission in mice with established diabetes is striking, considering the paucity of beta cells remaining after such an extended period of autoimmune destruction (17). Taken together, AGDP with a lower dose of ATG is superior to either ATG+G-CSF or DPP-4i+PPI. However, in established disease a very similar profile was observed with all therapeutic combinations, alluding to potential additional beta cell loss being responsible as opposed to failure to immunomodulate. In fact, similar immunomodulatory effects (increased Treg and memory T cell frequencies) were observed across cured animals from ATG+G-CSF, DPP-4i+PPI, and AGDP -treated groups when treatment was initiated either at onset or in mice with established disease. While the benefits of
increased Tregs in situations of autoimmunity are well recognized (18-20), a potential role for memory T cells is of particular interest. We would hypothesize that these observed memory T cells may possess a regulatory phenotype given previous reports of tolerance-inducing memory T cells preventing hyperglycemia in NOD mice (21-23); however, further \textit{ex vivo} evaluation of the function of these cells would be needed to definitively establish their role in controlling disease.

It is curious that despite long-term remission from hyperglycemia, only modest increases in serum C-peptide and beta cell mass are evident in AGDP-treated animals versus controls. Furthermore, pancreatic insulin, proinsulin, and C-peptide content were dramatically lower in animals that received any of the tested combinations compared to non-diabetic NOD controls. Thus, it is possible that surprisingly small amounts of insulin were sufficient for blood glucose control; however, glucose tolerance in the mice was not assessed and should be a subject of further investigation. Also, we did not investigate for possible effects of AGDP treatment on insulin sensitivity and glucose counter-regulatory hormones that might have contributed to glucose control. Future efforts should also monitor for pancreatic alterations as a function of region (head, body, tail) as it may correlate to observations of heterogeneous beta cell loss in humans (24; 25).

To date, one of the most promising forms of therapy to show long-term efficacy in humans (including subjects discontinuing insulin therapy) with T1D is one in which ATG, G-CSF, cyclophosphamide, and autologous stem cell therapy were combined (26; 27). However, this particular therapy remains controversial, particularly in terms of safety. Restoration of glycemic control in patients with T1D will likely require a combination therapy that confers both immunoregulation as well as beta cell benefits, yet is safe for use in humans (28). With this in mind, our use of low dose ATG (as a milder form of immunosuppression/immune modulation)
with the standard dose of G-CSF was taken alongside drugs that are FDA approved. AGDP treatment was well tolerated in NOD mice with no noted adverse effects. However, with combinatorial therapy, there is always a potential for unexpected off-target effects, and future translational efforts in humans should take this into account.

We (A.R.) also recently reported our first experience in utilizing a DPP-4i alongside a PPI in humans with T1D (29), specifically sitagliptin and lansoprazole. This double-blind, placebo-controlled, phase 2 trial (i.e., REPAIR-T1D) involved participants (11 - 36 years old) within six months of their T1D diagnosis. While this study failed to meet its primary endpoint (i.e., C-peptide response to a mixed meal challenge at 12 months measured as 2 h area under curve in treated subjects versus controls), no adverse or serious adverse events were related to the drug combination. Interestingly, although the primary endpoint was not achieved, not all participants had increases in glucagon-like peptide-1 and gastrin concentrations expected for this treatment. Hence, further studies (with greater statistical power) and monitored for glucagon-like peptide-1 and gastrin concentrations, along with the combination proposed here (AGDP) likely form an attractive future therapeutic possibility.

In combining the four drugs for this preclinical study, we have created a regimen that minimizes immunosuppression, yet remains effective at reversing established T1D in NOD mice with new onset as well as established disease. These data demonstrate the efficacy of AGDP to reverse murine T1D and the potential of this therapy in a translational context. Indeed, we believe future efforts exploring this combination in humans with T1D would appear warranted.
Acknowledgements

The authors wish to thank John Williams and Scott Eisenbeiss from Genzyme for their provision of murine ATG. These studies were funded through support from Sanford Research, Juvenile Diabetes Research Foundation, National Institutes of Health, and the Keene Family Professorship.

S.X. conceived of the study, wrote the manuscript, and researched data. A.P. conceived of the study, researched data, and wrote the manuscript. C.W. contributed to discussion and reviewed/edited the manuscript. C.M researched data. M.C. contributed to the research and contributed to discussion. C.E.M. contributed to the discussion and reviewed/edited the manuscript. A.R., A.S., T.B., M.B., and D.S. contributed to the study design and data interpretation, discussion and reviewed/edited the manuscript. M.H. contributed to discussion and reviewed/edited the manuscript. M.A.A. conceived of the study, evaluated the data, contributed to discussion, and reviewed/edited the manuscript.

One of the authors (M.A.A.) has filed an invention disclosure related to the use of ATG in T1D. The remaining authors have no conflict of interest to disclose.

M.A.A. is the guarantor of his work and, as such, takes responsibility for the integrity of the contents herein.
Figure Legends

**Figure 1.** AGDP combination therapy induces long-term euglycemia in NOD mice with new onset and established T1D.  
(A) At diabetes onset, mice were implanted with an insulin pellet and treatment initiated (N = 11-13 per group). AGDP therapy induced durable remission in 83% of mice (P < 0.0001 vs. control, Kaplan-Meier survival test), compared to 54% of mice treated with DPP-4i+PPI and 50% of mice treated with ATG+G-CSF.  
(B) Mice were implanted with an insulin pellet at onset, with treatment delayed 2 weeks (N = 11-13 per group). AGDP therapy induced remission in 50% of mice (P < 0.0013 vs. control), compared to 42% of mice treated with DPP-4i+PPI and 33% of mice treated with ATG+G-CSF.  
(C-K) Non-fasting blood glucose curves are shown for individual mice in each treatment group. The day of T1D onset was set to (C-G) day 0 or (H-K) day -14 for the new onset and established (delay) treatment groups, respectively, so that curves reflect blood glucose values following the initiation of treatment.  
(L) Insulitis scoring of remitted mice showed that despite euglycemia at 120 d, the numbers of remaining islets with no to minimal inflammation (scores 0-2) were significantly reduced in all treatment groups, as compared to age matched control non-diabetic NOD mice (P < 0.0001).

**Figure 2.** AGDP combination therapy and pancreatic insulin content. In a cross-sectional study, NOD mice with both new onset and established disease were implanted with a subcutaneous insulin pellet and treated with ATG+G-CSF, DPP-4i+PPI, AGDP, or no treatment control, and tissues were harvested 30 days after the initiation of treatment.  
(A) Serum C-peptide (N = 6-7 per group), as measured via ELISA, trended higher in new onset animals that received ATG+G-CSF and AGDP therapy compared to insulin only controls (P = 0.1115, Kruskal-Wallis test). Pancreata (N = 4 per group) were stained for insulin via IHC, and analyzed for the number of (B) insulin positive islets.
and (C) fractional insulin area. There was no significant difference between treatment groups, but there was a trend toward increased insulin positive islets and insulin fractional area in new onset mice treated with ATG+G-CSF and AGDP therapy ($P = 0.7776$, and $P = 0.7237$, respectively; one-way ANOVA). Pancreata ($N = 2-3$ per treatment group and age-matched non-diabetic NOD controls) were processed for total protein via acid-ethanol extraction and analyzed for total (D) proinsulin, (E) C-peptide, and (F) insulin via ELISA. Compared to insulin treated animals, there was a trend toward increased proinsulin in new onset mice treated with ATG+G-CSF and AGDP therapies, but all treatment groups demonstrated significantly reduced proinsulin, C-peptide, and insulin compared to non-diabetic controls ($P < 0.0001$, all; one-way ANOVA). Data presented as mean ± SEM.

**Figure 3.** AGDP combination therapy and initial blood glucose. (A) Initial blood glucose values were similar between mice enrolled in all therapy groups ($N = 11-13$ / group; $P = NS$, one-way ANOVA). In (B) new onset mice and (C) established T1D mice, ATG+G-CSF therapy was less effective at reversing diabetes in those with higher initial blood glucose (*$P < 0.05$, Student’s $t$ test). Data presented as scatter with mean ± SD.

**Figure 4.** Combination therapy induces T cell and NK cell immunomodulation. T cell subsets were analyzed by flow cytometry on treated mice ($N = 11-13$ per treatment group) or unmanipulated non-diabetic NOD mice ($N = 9$) at the study end point (therapy failure or 120 d). In (A) new onset and (B) established T1D mice, AGDP therapy increased the CD4$^+$: CD8$^+$ T cell ratio compared to control mice and mice treated with DPP-4i+PPI therapy, while ATG+G-CSF therapy had an increased ratio compared to DPP-4i+PPI therapy. These data were validated in a
cross-sectional study of NOD mice with both (C) new onset and (D) established disease where tissues were harvested 30 days after the initiation of treatment (\(N = 6\)-11 per group). ATG+G-CSF and AGDP therapies increased the CD4\(^+\):CD8\(^+\) T cell ratio compared to control mice and mice treated with DPP-4i+PPI therapy (\(P < 0.0001\) and \(P = 0.0016\), respectively; one-way ANOVA). (E) Splenocyte CD8\(^+\) T cell frequency within the lymphocyte population was significantly reduced by ATG + G-CSF and AGDP therapy in mice with new onset as well as established T1D, relative to insulin pellet controls (\(P < 0.0001\), one-way ANOVA). (F) Compared to control, splenic CD4\(^+\) T cell frequency was increased in new onset AGDP-treated animals but decreased with ATG+G-CSF treatment in mice with established disease (\(P < 0.05\)).

In NOD mice with (G) new onset T1D, AGDP therapy decreased the splenic NK cell frequency compared to insulin-treated control mice (\(P = 0.0015\); one-way ANOVA), while in NOD mice with (H) established disease, ATG+G-CSF and AGDP therapies decreased the NK cell frequency (\(P = 0.0050\)). Data presented as scatter with mean ± SD (*\(P<0.05\), **\(P<0.01\), ***\(P<0.001\), ****\(P<0.0001\)).

**Figure 5.** At day 30 post-initiation of treatment, pancreata were fixed and paraffin-embedded. Serial sections were stained via IHC for (A) CD4 and (B) CD8. The scanned images were annotated for quantification of percentage of cells staining positive for (C) CD4 and (D) CD8 within the islet area using cytonuclear IHC quantification software. (E) The CD4\(^+\):CD8\(^+\) T cell ratio within the pancreatic islets was not significantly different across treatment groups.

**Figure 6.** Combination therapy induces T cell immunoregulation. In (A) new onset and (B) established T1D mice, remitted mice receiving AGDP, ATG+G-CSF, and DPP-4i+PPI therapies
had elevated Treg compared to failures. With the exception of ATG+G-CSF therapy in established disease (B), remitted mice from all three therapies had elevated Treg compared to control non-diabetic mice. In (C) new onset and (D) established T1D mice, remitted AGDP animals exhibited increased CD8+ Treg at 120 d compared to (C) non-diabetic controls or (D) failed AGDP mice, respectively (*P < 0.05; one-way ANOVA).

Figure 7. Naïve and memory CD4+ T cell subsets modulation by combination therapy. T cell subsets were analyzed by flow cytometry in treated mice (N = 11-13 per treatment group) or unmanipulated non-diabetic NOD mice (N = 9) at the study end point. (A) At 120 d, mice in all new onset therapy groups, regardless of ability to reverse, had higher CD4+ naïve cell frequency than controls. (B) In established disease mice, only remitted AGDP animals corresponded to higher naïve T cells compared to non-diabetic controls while naïve T cells were higher in failed versus reversed animals treated with DPP-4i+PPI. CD4+ memory T cells were not different between control and treated animals with (C) new onset or (D) established T1D. However, successfully treated mice did show differences as indicated versus mice that failed to remit. Data presented as scatter with mean ± SD (*P < 0.05). Remitted vs. control mice analyzed by one-way ANOVA; remitted mice vs. failures by Student’s t test.

Figure 8. Memory and naïve CD8+ T cell analysis of treatment groups. Naïve CD8+ T cells were elevated at 120 d with ATG+GSCF therapy in (A) new onset mice but not (B) established T1D mice versus non-diabetic mice (one-way ANOVA). Successfully treated ATG+G-CSF and DPP-4i+PPI treated mice had lower levels of memory CD8+ cells than non-diabetic NOD mice at 120 d (*P < 0.05; one-way ANOVA), (C) but higher memory CD8 T cells than failed animals.
as indicated. (*D) Memory CD8+ cells were elevated in remitted mice compared to failures for all treatment conditions in the established disease group. (*P < 0.05; Student’s t test). Data presented as mean ± SD. Differences between remitted and failed mice within treatment groups were analyzed by Student’s t test (*P < 0.05).
References

4. Herold KC, Bluestone JA: Type 1 diabetes immunotherapy: is the glass half empty or half full? Sci Transl Med 2011;3:95fs91
28. 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
29. Griffin KJ, Thompson PA, Gottschalk M, Kyllo JH, Rabinovitch A: Combination therapy with sitagliptin and lansoprazole in patients with recent-onset type 1 diabetes (REPAIR-T1D):
12-month results of a multicentre, randomised, placebo-controlled, phase 2 trial. Lancet Diabetes Endocrinol 2014;2:710-718
Figure 1
Figure 1

Diabetes

- Insulin (C)
- Control (D)
- ATG + G-CSF (E)
- DPP-4i + PPI (F)
- AGDP (G)
- Control (Delay) (H)
- ATG + G-CSF (Delay) (I)
- DPP-4i + PPI (Delay) (J)
- AGDP (Delay) (K)
Figure 2

(A) C-peptide levels (pM) for different treatments in New Onset and Established groups. Bars represent mean with error bars indicating standard deviation.

(B) Number of Insulin Islets for different treatments in New Onset and Established groups. Bars represent mean with error bars indicating standard deviation.

(C) Fractional Insulin Area (%) for different treatments in New Onset and Established groups. Bars represent mean with error bars indicating standard deviation.

(D) Proinsulin levels (ng/mg Protein) for different treatments in New Onset and Established groups. Bars represent mean with error bars indicating standard deviation.

(E) Insulin levels (ng/mg Protein) for different treatments in New Onset and Established groups. Bars represent mean with error bars indicating standard deviation.

(F) C-peptide levels (ng/mg Protein) for different treatments in New Onset and Established groups. Bars represent mean with error bars indicating standard deviation.
Figure 3

Diabetes

A. Initial Blood Glucose (mg/dL) for New Onset and Established diabetes.

B. Initial Blood Glucose (mg/dL) for ATG+GCSF, DPPIV+PPI, and AGDP groups.

C. Initial Blood Glucose (mg/dL) for ATG+GCSF, DPPIV+PPI, and AGDP groups, with symbols indicating Failure and Euglycemic states.
Figure 4
Figure 5
Figure 7