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Eukaryotic translation initiation factor 5A inhibition alters physiopathology and immune responses in a "humanized" transgenic mouse model of type 1 diabetes

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Imam S, Mirmira RG, Jaume JC. Eukaryotic translation initiation factor 5A inhibition alters physiopathology and immune responses in a "humanized" transgenic mouse model of type 1 diabetes. *Am J Physiol Endocrinol Metab* 306: E791–E798, 2014. First published February 4, 2014; doi:10.1152/ajpendo.00537.2013.—Therapeutic options for treatment of type 1 diabetes (T1D) are still missing. New avenues for immune modulation need to be developed. Here we attempted at altering the diabetes outcome of our humanized model of T1D by inhibiting translation-initiation factor eIF5A hypusination in vivo. Double-transgenic (DQ8-GAD65) mice were immunized with adenoviral vectors carrying GAD65 for diabetes induction. Animals were subsequently treated with deoxyhypusine synthase (DHS) inhibitor GC7 and monitored for diabetes development over time. On one hand, helper $CD4^+$ T cells were clearly affected by the downregulation of the eIF5A not just at the pancreas level but overall. On the other hand, the T regulatory cell component of CD4 responded with activation and proliferation significantly higher than in the non-GC7-treated controls. Female mice seemed to be more susceptible to these effects. All together, our results show for the first time that downregulation of eIF5A through inhibition of DHS altered the physiopathology and observed immune outcome of diabetes in an animal model that closely resembles human T1D. Although the development of diabetes could not be abrogated by DHS inhibition, the immunomodulatory capacity of this approach may supplement other interventions directed at increasing regulation of autoreactive T cells in T1D.

mouse model; type 1 diabetes physiopathology; deoxyhypusine synthase

IN TYPE 1 DIABETES (T1D) a complex interplay of immune cells makes pancreatic β-cells targets of destruction (9). T1D is a chronic autoimmune disease where autoantigen-activated $CD4^+$ T cells help $CD8^+$ T cells become mediators of selective β-cell destruction (16). Recent observations of human islets from diabetic patients have established a prominent role for $CD8^+$ T cells (3, 15). Therefore, facilitating $CD4^+$ T cell regulation of $CD8^+$ T cells appears to be a promising therapeutic strategy.

A T1D mouse model where antigen-specific diabetes after immunization with a clinically relevant human autoantigen, in the context of human MHC-class II diabetes-susceptibility transgenes occurs, was recently developed (5, 6, 8). In this transgenic model, human glutamic acid decarboxylase (GAD65) is expressed in pancreatic β-cells, and human MHC II (DQ8) is expressed in antigen-presenting cells (APCs). Upon a triggering event by which tolerance to GAD65 is broken in the periphery (1), APCs present antigen, activate T cells, and initiate the downstream events that lead to diabetes (5, 6, 8).

Eukaryotic translation initiation factor 5A (eIF5A) is a small (17-kDa), highly conserved protein identified as a translation initiation/elongation factor (11). Studies in mammalian cells have showed that only 5% of protein translation in a quiescent cell is dependent on eIF5A (18) and that, in actively dividing mammalian cells, it is necessary for proteins involved in cell cycle progression (12). eIF5A is the only known protein to contain the atypical amino acid hypusine (2). In a rate-limiting step, deoxyhypusine synthase (DHS) transfers an aminobutyl moiety from the polyamine spermidine to the epsilon-amino group of lysine 50 in eIF5A to form deoxyhypusine eIF5A. Subsequently, deoxyhypusine hydroxylase hydroxylates deoxyhypusine to form the final hypusine residue. The hypusinated form of eIF5A (eIF5AHyp) is considered the active form, and to date most known functions of eIF5A are dependent upon hypusination (2). Thus, targeting of hypusination serves as a specific means to hinder eIF5A action. In vivo inhibition of eIF5A hypusination by the DHS inhibitor GC7 conferred resistance to islet dysfunction and hyperglycemia in the nonobese diabetic mouse model (14).

Here we attempted at altering the diabetes outcome of our humanized model of T1D by inhibiting eIF5A hypusination in vivo.

MATERIALS AND METHODS

Mice. Murine MHC-class II molecule-deficient (mII^-), HLA-DQA1*0301/DQB1*0302 (DQ8) (20), and hGAD65 (19) transgenic mice (5) in BTBR background (6) were used in this study. DQ8 and hGAD65 homozygosity was determined as previously described (5, 6). All animal protocols were approved by the University of Wisconsin and the Veterans Affairs animal research committees.

Adenoviral constructs for diabetes induction. Adenoviral constructs were made using the Gateway system (Invitrogen, Carlsbad, CA) as previously described (6). hGAD65 was excised from pCI-hGAD65 with *Eco*RI and *Not*I (Invitrogen) and subcloned into the same sites in pENTR for pAD-CMV cloning, and the sequence was confirmed. Immunizations were performed two times at 2-wk intervals (Fig. 1*A*). Mice were intraperitoneally injected with 100 μl of PBS containing 10^{11} particles of pAD-CMVhGAD65 (7, 10).

eIF5A in vivo inhibition. Intraperitoneal GC7 or placebo (saline) injections were given to 16-wk-old (treated and nontreated groups, respectively) double-transgenic (DQ8-GAD65) mice, 4 wk postimmunization with hGAD65 adenoviral construct, at the dose rate of 4 mg/kg body wt 5 days in a week for 4 wk (Fig. 1).

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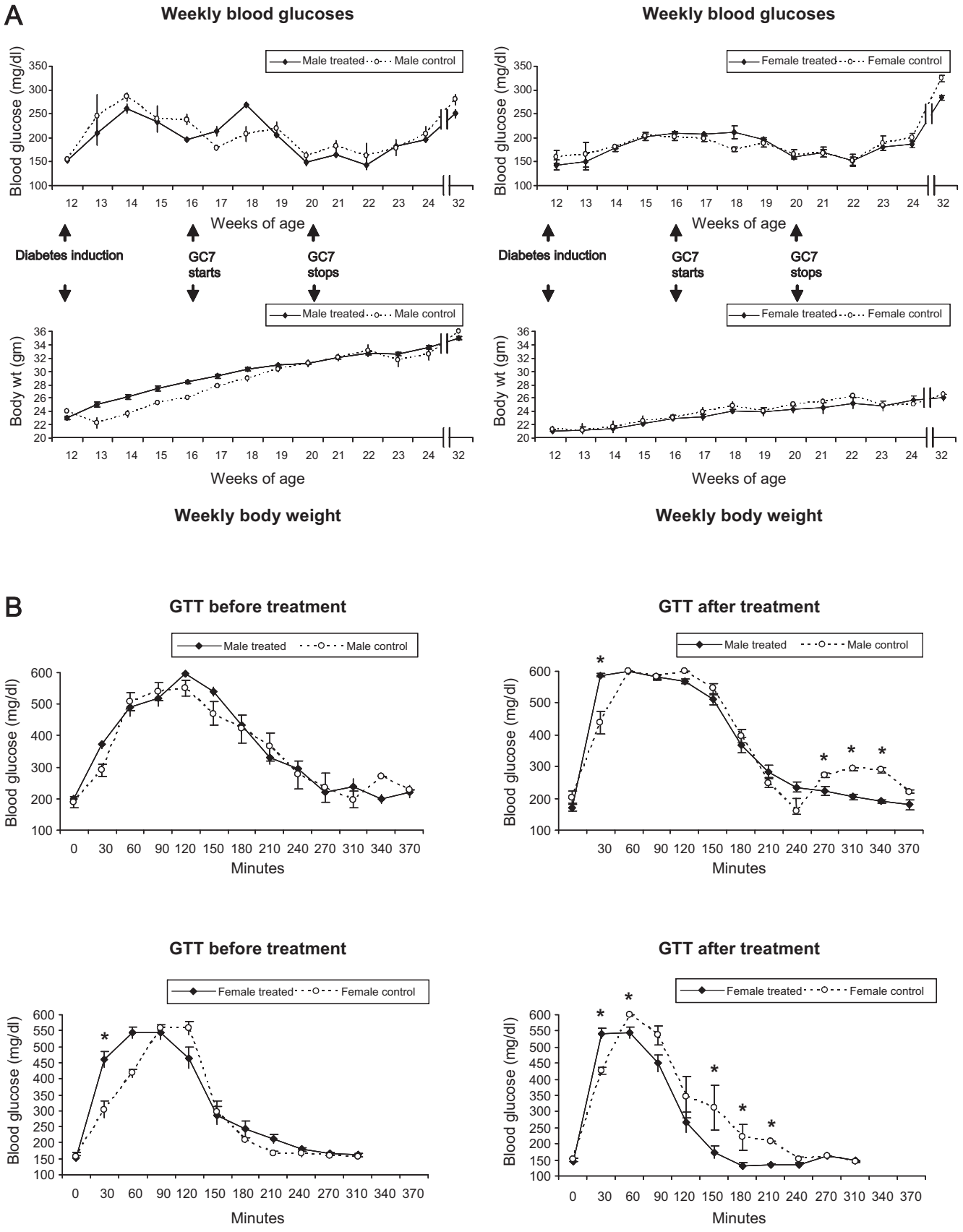


Fig. 1. *A*: fasting glycemia and weight monitoring after diabetes induction. Wide glucose fluctuations were noted right after GC7 treatment was started with an abrupt drop posttreatment. *B*: glucose tolerance test (GTT). *Left*, GTT at 4 wk postimmunization pre-GC7 treatment. *Right*, GTT at 4 wk post-GC7 treatment. There were some statistically significant (*) point differences between placebo (nontreated controls) and GC7-treated animals more noticeable in females.

Glucose tolerance test and fasting insulin measurement. All experimental and control animals were subjected to a glucose tolerance test (GTT) in at least two time points, 3–4 wk postimmunization (before GC7 treatment) and 10 wk postimmunization (3–4 wk after GC7 treatment; Fig. 1*A*). Mice were fasted overnight for 10 h before given an intraperitoneal injection of 2 g of glucose. Blood samples were obtained from a tail vein at 0, 20, 40, 60, 80, 120, and 180 min after injection, and glucose level was assessed using a glucometer (Fig. 1*B*).

Animals also underwent fasting insulin measurements before GC7 treatment (Fig. 2, BT), 2 (Fig. 2, 2M) and 4 (Fig. 2, 4M) mo after GC7 treatment initiation. Blood samples were obtained from 10-h-fasted mice, and insulin content was measured by ELISA using mouse insulin as a standard (Chrystal Chem, Chicago, IL).

Flow cytometry. In all flow cytometry experiments (Figs. 3 and 4), cells were stained with fluorochrome-conjugated antibodies against mice CD3, CD4, CD8, CD25, GDT, NKT, IL-17, interferon-γ (IFN-γ), and Foxp3 (BD Biosciences, San Jose, CA) or isotype controls. For cell phenotyping, spleen- and pancreas-infiltrating lymphocytes were obtained. Freshly isolated single cells were incubated with antibodies for 20 min on ice for cell surface staining, washed, and fixed in 1% paraformaldehyde. A subset of cells was permeabilized with cytofix/cytosperm fixation and permeabilization solution (BD Biosciences) and stained with fluorochrome-conjugated antibodies against mice intracellular proteins. Cells were also stained with Hoechst 33342 (10 μg/ml) to gate live cells containing 2n-4n cellular DNA. Cells were acquired in a BD LSR II flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (Treestar).

Quantitative RT-PCR analysis. Total mRNA was isolated by the TRIzol method (Invitrogen) from frozen pancreatic tissue. RNA (1 μg) was converted into cDNA using random hexamer/oligo(dT) primer cocktail and Moloney murine leukemia virus reverse transcriptase (Invitrogen). eIF5A mRNA expression was quantified by SYBR green chemistry (ABI) with specific primers using the $\Delta\Delta C_t$ method. Relative values were normalized to the corresponding 18S rRNA values. Minus-reverse transcriptase samples were used as negative controls to test for DNA contamination. The whole experiment was repeated three times (Fig. 5). Primers used were as follows: eIF5A F1 CCCAACATCAAACGGAATGAC and eIF5A R1 GCAGACGAAGGTCCTCTCGTA.

Statistical analysis. Two-tailed probability of the chi square distribution was used to compare results. Flow cytometry data on cells in various gated populations were statistically analyzed using SAS.

RESULTS

Glucose intolerance and diabetes after eIF5A inhibition. Homozygous double-transgenic mice carrying DQ8 and hGAD65 (6) were intraperitoneally injected with 10^{11} hGAD65-adenoviral particles (diabetes induction) and monitored for hyperglycemia weekly (Fig. 1*A*). GC7 or placebo was intraperitoneally administered to each treatment group 4 wk post-diabetes induction for 4 wk. Glycemic control was more erratic in GC7-treated animals compared with nontreated (placebo-given) controls. Wide fluctuations of fasting glucoses were noted right after the commencement of GC7 treatment that resembled the immediate post-diabetes induction period. Furthermore, higher blood glucoses were noted while on GC7 treatment with an abrupt drop post-treatment (Fig. 1*A*). GTT results showed that there were some differences between treated male and female groups before and after GC7 administration (Fig. 1*B*). GTT results post-GC7 treatment showed that there were significant point differences between treated and nontreated control. At 30 min postglucose administration, higher glucoses in treated males were observed (Fig. 1*B*, males). However, at 270, 310, and 340 min, lower glucoses were noted. Similarly, for the female groups, most of the significant point differences were for lower glucoses in the treated groups except at 30 min (Fig. 1*B*, females). Moreover, fasting insulin collected at three time points showed the effect of GC7 treatment on disease progression/regression. Fasting insulin was measured before GC7 treatment (Fig. 2, BT), 2 (Fig. 2, 2M) and 4 (Fig. 2, 4M) mo after GC7 treatment initiation. GC7-treated males had fasting blood insulin trending up at 2 mo and significantly higher at 4 mo when compared with nontreated controls. Female differences were not so impressive although the fasting insulin level over time was significantly lower for nontreated females than for treated ones (all in Fig. 2).

$CD4^+$ T cells and regulatory T cell responses. Animals were euthanized at the time of diabetes development (blood glucose ≥250 mg/dl for two consecutive days), and their lymphocytes were studied. An example of the flow cytometry analysis of $CD4^+/CD8^+$ T cells and their IFN-γ production is shown for GC7-treated pancreatic infiltrating and splenic lymphocytes (Fig. 3*A*). Flow cytometry analysis of $CD4^+$ T cells revealed that GC7 treatment significantly decreased the pancreatic $CD4^+$ T cell populations in the treated groups compared with nontreated controls (Fig. 3*B*, *top left*). Similar trend was observed for spleen-derived $CD4^+$ lymphocytes (Fig. 3*B*, *bottom left*). In females, GC7 treatment significantly reduced the $CD4^+$ T cell population in both pancreas and spleen (Fig. 3*B*). The pancreatic and splenic $CD4^+$ T cell count in the GC7-treated female group was 1.37 ± 0.78 and 20.22 ± 5.59,

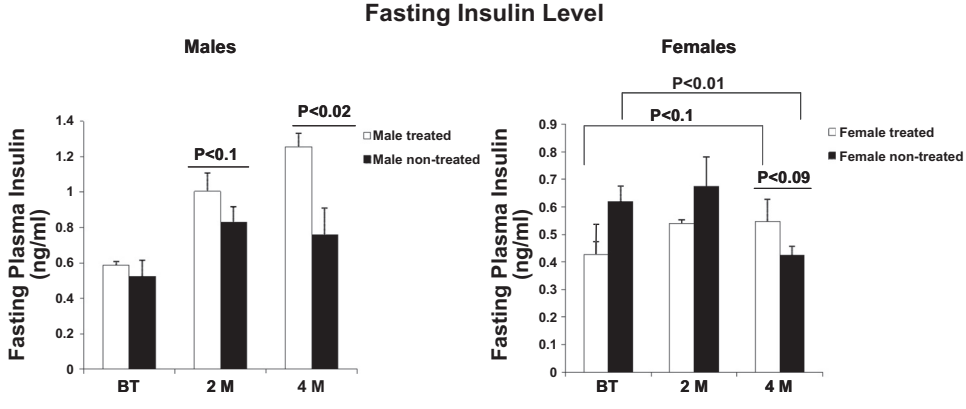
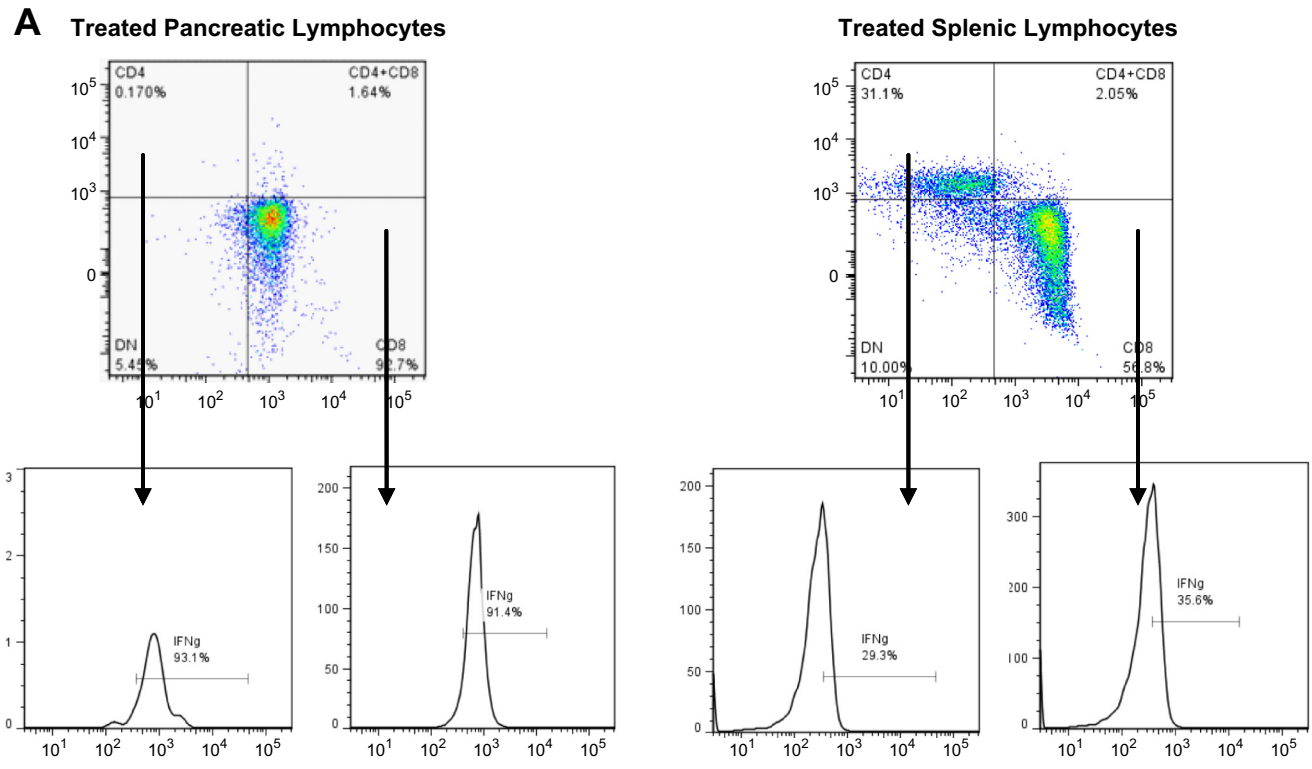
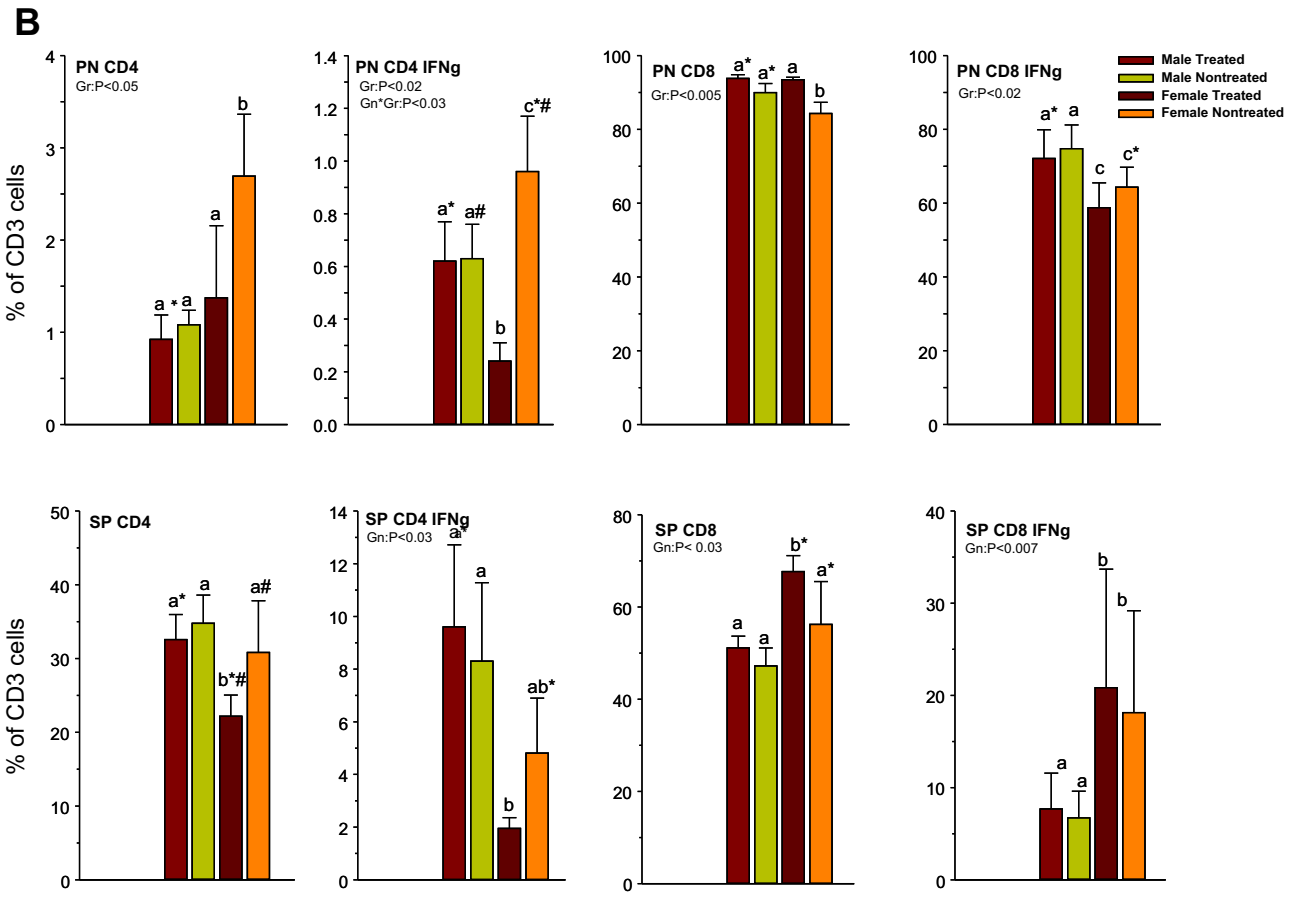


Fig. 2. Fasting insulin levels at three time points, before GC7 treatment (BT) and 2 (2M) and 4 (4M) mo after GC7 treatment initiation. Blood samples were obtained from 10-h-fasted mice, and insulin content was measured by ELISA using mouse insulin as a standard. Statistically significant differences were reached at 4 mo between GC7-treated (open bars) and nontreated (closed bars) in males (*left*). Female differences (*right*) were not significant at 4 mo. However, within the nontreated group, a statistically significant drop in fasting insulin was present 4 mo after treatment initiation (*top*, $P < 0.01$).





respectively, and in nontreated controls was 2.69 ± 0.67 and 24.58 ± 0.89, respectively, both significantly different (males trended along). $CD4^+$ IFN-γ production was significantly decreased in both organs in females. Pancreatic and splenic $CD8^+$ lymphocytes, however, did not seem to be affected by treatment at any anatomical site (Fig. 3*B*, *right*). As previously observed (6), pancreatic infiltrating lymphocytes were overrepresented by $CD8^+$ T cells in all groups.

We next analyzed the regulatory T cell (Treg) profile. An example of the analysis is provided (Fig. 4*A*). $CD4^+$ lymphocytes were tested for coexpression of CD25 (as a surrogate marker for activation) and Foxp3 (a transcription factor present in Tregs). Lymphocytes from pancreas and spleen are shown (Fig. 4). Treatment with GC7 significantly increased the "stable" pancreatic Treg population ($CD3^+CD4^+CD25^+Foxp3^+$) in the male group (mean 18.20 ± 6.31) compared with nontreated controls (mean 9.35 ± 1.8) (Fig. 4*B*, *top middle*, and Fig. 4*A*, *left*). Also, in spleen, a significant increase in the Treg population was observed (Fig. 4*B*, *bottom middle*, and Fig. 4*A*, *right*). In treated females pancreas Tregs also increased (Fig. 4*B*, *top middle*). The "transient" Treg ($CD3^+CD4^+CD25^-Foxp3^+$) population was clearly increased in pancreas of treated males (Fig. 4*B*, *top right*). The transient Treg population was significantly higher (mean 30.47 ± 4.94) compared with nontreated male controls (mean 10.57 ± 5.17) at the pancreas level, whereas in spleen no significant difference was observed (Fig. 4*B*, *bottom right*).

Male vs. female differences and eIF5A expression. eIF5A is widely expressed in different tissues (BioGPS). Differential levels of expression of pancreatic eIF5A based on gender have never been shown. eIF5A pattern of pancreas mRNA expression was significantly higher in males than in females before GC7 treatment (Fig. 5). The drop in expression after treatment was significant in both males and females (Fig. 5). However, although the drop was higher in magnitude for males, the absolute level of expression was much lower for females. This gender-biased differential expression may have influenced the described outcomes to some extent.

DISCUSSION

Therapeutic interventions for T1D have been overall disappointing. Although blunt immune suppression is capable of abrogating the autoimmune process and cure diabetes, the side effects are worse than the benefits provided (13). If immune suppression of the specific antigen responders were to be accomplished, the benefits would likely outweigh the risks. Short of antigen-specific immune suppression, suppression of individual immune components as opposed to blunt immune suppression may either delay onset or decrease severity of target destruction. Furthermore, combination of partial immune suppression and anti-inflammatory interventions might relieve autoimmune target destruction and/or allow for β-cell recovery.

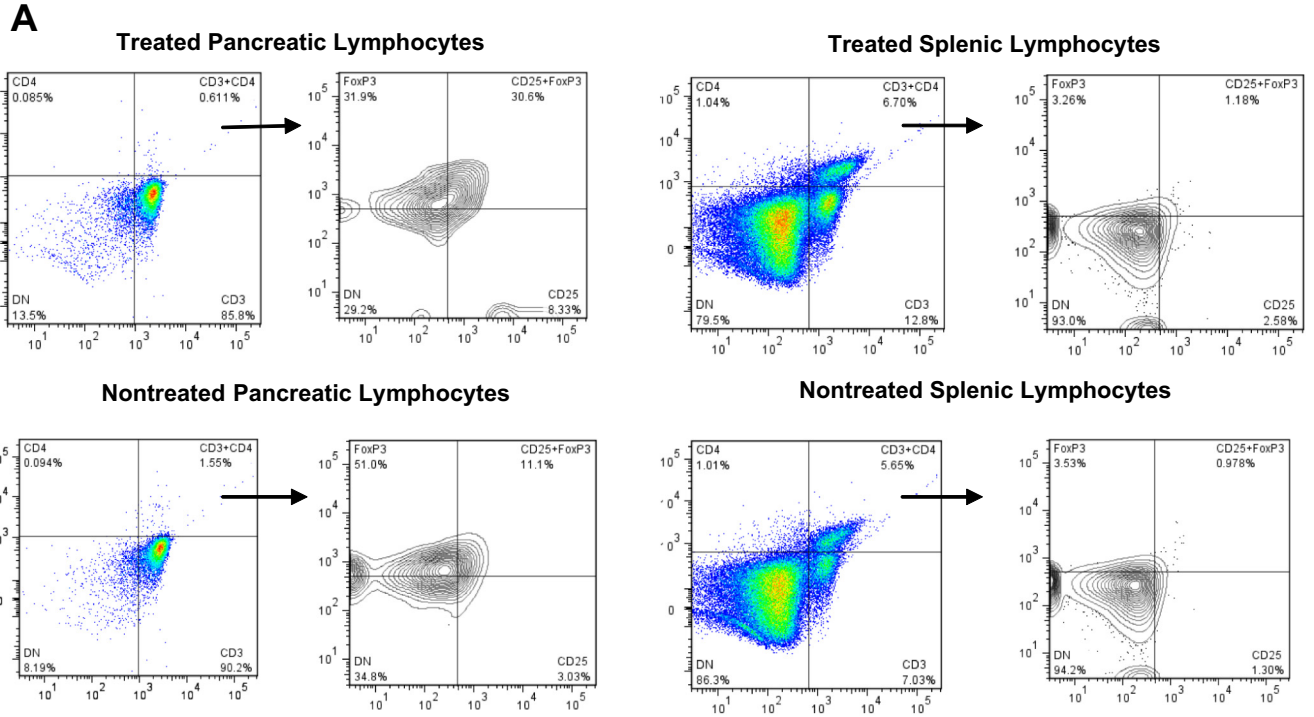
The peculiar translation initiation/elongation factor 5A seems to be linking immunity and inflammation in pancreatic islets. Furthermore, because of its need of hypusination for functionality, it becomes an obvious target for therapeutic intervention. Specifically, the enzyme responsible for its hypusination, DHS, is druggable by the specific inhibitor GC7 (14). Moreover, eIF5A seems to be localized to few cell types.

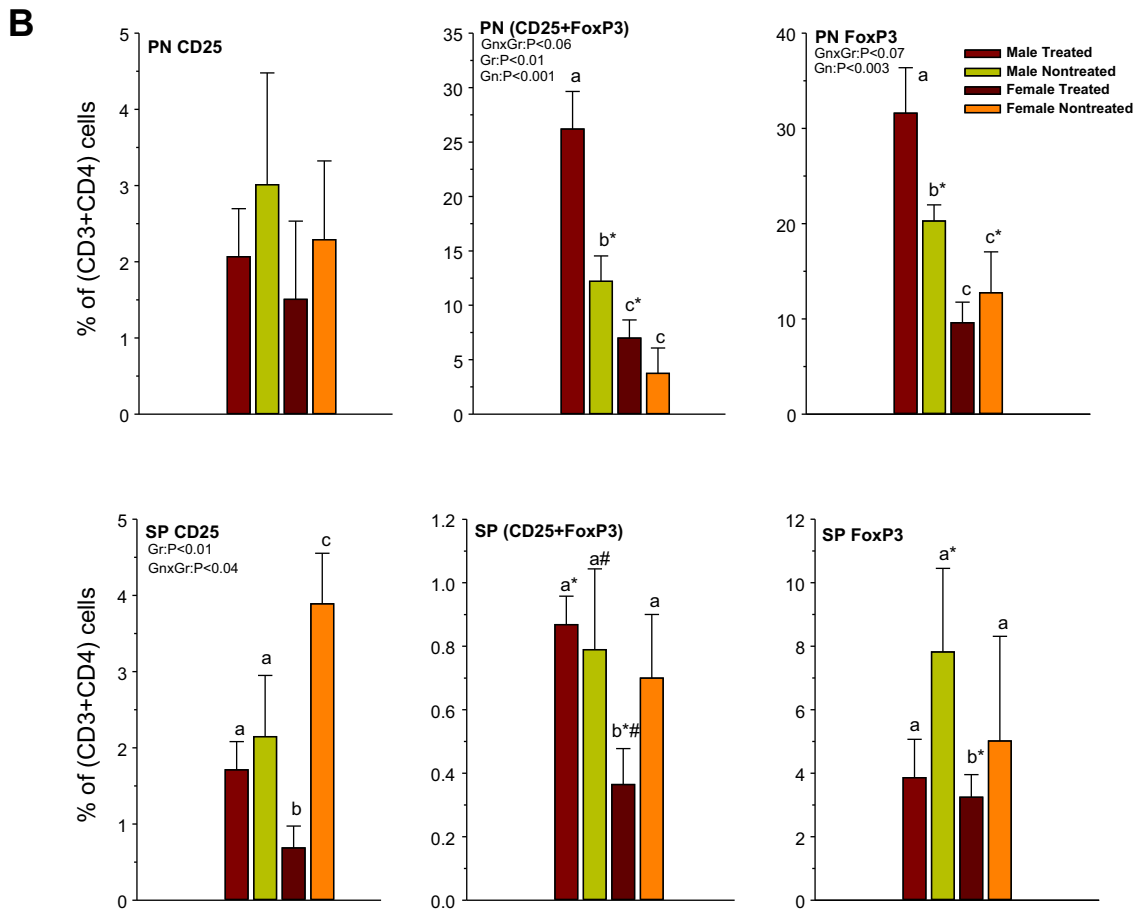
We have generated transgenic mice that express high levels of human GAD65 in β-cells and at the same time have their endogenous mouse MHC-class II replaced by the human HLA-DQ8 diabetes-susceptibility gene (5, 6, 8). Our double-transgenic mice develop impaired fasting blood glucose, glucose intolerance, and diabetes when immunized with adenoviral hGAD65.

We strategically introduced DHS inhibition with GC7 treatment to animals programmed to develop diabetes. Although we were unable to abrogate the diabetes outcome, DHS inhibition altered immune responses in a particular way. On one hand, $CD4^+$ T cells were clearly affected by the downregulation of eIF5A mostly at the pancreas level. In our study, female mice seemed to be more responsive to this effect over $CD4^+$ T count and function (Fig. 3). On the other hand, the T regulatory cell component of $CD4^+$ T cells responded with activation and proliferation significantly higher than in the non-GC7-treated male controls (Fig. 4). $CD8^+$ T cells, however, seem to overcome the regulatory suppression allowing diabetes to progress albeit in a delayed manner (onset of diabetes lagged behind by 2 wk average in the GC7-treated groups, Fig. 1). Moreover, fasting insulin monitored through the study seemed to hold normal levels longer in GC7-treated mice (Fig. 2).

eIF5A has cell context-dependent function, with some cell types (e.g., more proliferative ones like activated immune cells) requiring active eIF5A action and others (e.g., more quiescent ones like pancreatic β-cells) requiring eIF5A only under specific stress conditions (2). We can only speculate on where impaired translation might have taken place to account for the observed outcomes in our animals. If at the β-cell level, one would have expected to see more of a unified response, that is, if protein synthesis impairment would have affected β-cell antigen expression, the immune response might have been uniformly downregulated. The fact that T regulatory cells rise while conventional $CD4^+$ T cells decrease when animals are treated with the DHS inhibitor favors a direct effect on active immune effector cells. As in mice treated with anti-CD3 (17), GC7 treatment induced an increase in the number of $CD4^+Foxp3^+$ Tregs maybe because of selective depletion of $CD4^+Foxp3^-$ conventional T cells. Moreover, since transient ($CD25^-Foxp3^+$) and stable ($CD25^+Foxp3^+$) Tregs were not just increased but coexisted in GC7-treated animals, the possibility of downregulation of expression of CD25 needs to be considered. As opposed to stable, transient Tregs are described as unstable and short lived. They can effectively recover the CD25 expression after IL-2 treatment and become stable Tregs

Fig. 3. *A*: representative flow cytometry analysis (dot plots and histograms) of pancreatic and splenic lymphocytes of GC7-treated animals. The differences in CD4 as opposed to CD8 lymphocyte counts were also functional [interferon-γ (IFN-γ)]. *B*: statistical differences (bar graphs) of lymphocyte populations in the CD4 and CD8 compartment of all and IFN-γ-producing cells. Lymphocytes infiltrating the pancreas (PN, *top*) and splenocytes (SP, *bottom*) are shown. GC7 treatment appears to affect almost exclusively the CD4 compartment. Gr, group; Gn, gender. Statistical significance was determined at $P < 0.05$. Lowercase letters (a, b, c, and d) identify significant differences among the groups. Means with different superscript (* or #) have an approaching to significant difference ($P = 0.06$ to $P < 0.1$).





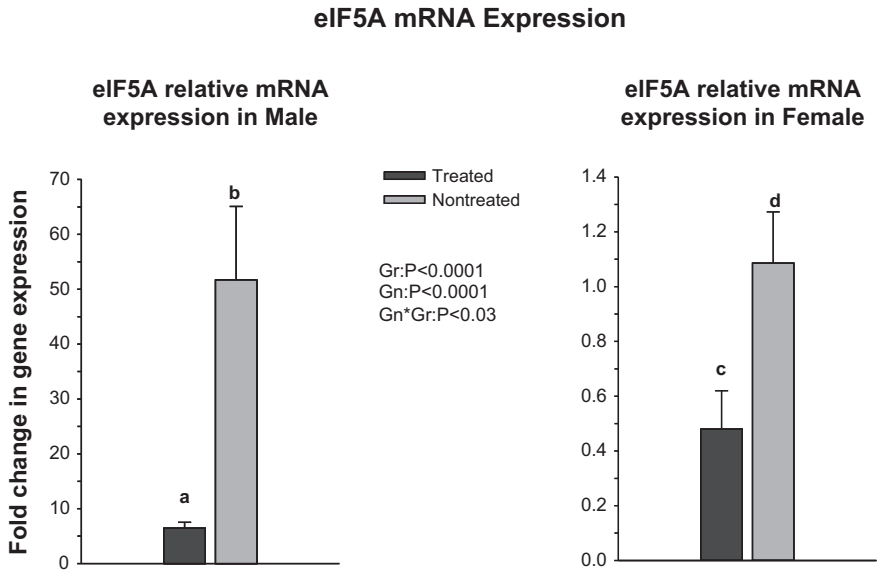


Fig. 5. Pancreatic eukaryotic translation initiation factor 5A (eIF5A) mRNA expression pattern was significantly higher in males (*left*) than in females (*right*) before GC7 treatment. The drop in expression after treatment (black bars) was significant in both males and females. The drop was higher in magnitude for males; however, the absolute level of expression was much lower for females. Statistical significance was determined at $P < 0.05$. Lowercase letters (a, b, c, and d) identify significant differences among the groups.

(21). Therefore, if the translation of CD25 were to be selectively impaired by DHS inhibition, the population of transient Tregs should be expected to be proportionally increased (as observed). Also, since the half-life of activated $CD4^+$ T cells is locally determined by Tregs, one would expect a proportional increase of the latter against the former (as observed).

Although the overall manifestation of delayed diabetes onset was common to both genders, we did observe subtle gender differences in the cellular outcomes described already. The difference in eIF5A mRNA expression (Fig. 5) is therefore worth noticing and may help explain these differences. Males had significant higher levels of pancreas eIF5A mRNA (which likely translated in higher protein levels) pretreatment, which might be more difficult to inhibit compared with females. Females lower levels might have been more sensitive to the GC7 inhibition. Consequently, CD4 decrease was more and Treg increase was less pronounced in females. Yet, since their actions physiologically counterbalance, the net effect over the diabetes outcome was the same as in males.

All together, our results show for the first time that down-regulation of eIF5A through inhibition of DHS alters the physiopathology and observed immune outcome in an animal model that closely resembles human T1D. Although the development of diabetes could not be abrogated by DHS inhibition in our model, the immunomodulatory capacity of this approach may supplement other interventions (4) directed at increasing regulation of autoreactive T cells in T1D.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.I. and J.C.J. performed experiments; S.I. and J.C.J. analyzed data; S.I. and J.C.J. prepared figures; S.I., R.G.M., and J.C.J. approved final version of manuscript; R.G.M. and J.C.J. conception and design of research; R.G.M. and J.C.J. interpreted results of experiments; J.C.J. drafted manuscript; J.C.J. edited and revised manuscript.

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Fig. 4. *A*: flow cytometry analysis of pancreatic and splenic lymphocytes of GC7-treated and nontreated (placebo-given) animals gated for CD4 positivity (dot plots) and further sorted for Foxp3 and CD25 (contour plots). The more than doubling of regulatory T cells (Tregs) in the GC7-treated sample (compare contour plots on *left*) is noteworthy. *B*: statistical differences (bar graphs) of pancreatic infiltrating and splenic lymphocytes of GC7-treated vs. nontreated animals carrying the depicted markers (CD25 and Foxp3) is also shown (*top middle*). Statistical significance was determined at $P < 0.05$. Lowercase letters (a, b, c, and d) identify significant differences among the groups. Means with different superscripts (* or #) have an approaching difference ($P = 0.06$ to $P < 0.1$).

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