Differential impact of chronic hyperglycemia on humoral versus cellular primary alloimmunity

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ABSTRACT

Diabetes is prevalent among solid organ transplant recipients and is universal among islet transplant recipients. While diabetes is often considered to result in an immune compromised state, the impact of chronic hyperglycemia on host alloimmunity is not clear. Potential immune modifying effects of obesity, autoimmunity, or diabetogenic agents like streptozotocin may confound understanding alloimmunity in experimental models of diabetes. Therefore, we sought to determine the role of chronic hyperglycemia due to insulinopenia on alloimmunity using the non-autoimmune, spontaneously diabetic H-2^b-expressing C57BL/6 Ins2^Akita^ mice (Akita). Akita mice harbor a mutated Ins2 allele that dominantly suppresses insulin secretion, resulting in lifelong diabetes. We used BALB/c donors (H-2^d^) in to assess alloimmunization and islet transplantation outcomes in Akita recipients. Surprisingly, chronic hyperglycemia had little effect on primary T cell reactivity following alloimmunization. Moreover, Akita mice readily rejected islet allografts and chronic hyperglycemia had no impact on the magnitude or quality of intragraft T cell responses. In contrast, allospecific IgM and IgG were significantly decreased in Akita mice following alloimmunization. Thus, while diabetes influences host immune defense, hyperglycemia itself does not cause generalized alloimmune impairment. Our data suggest that immune compromise in diabetes due to hyperglycemia may not apply to cellular rejection of transplants.
Diabetes is more prevalent among solid organ transplant recipients (based on public OPTN data) than in the general population (1) and is universal among islet and pancreas transplant recipients. Though diabetes impairs patients’ immunity to pathogens (2), it is not known whether alloimmunity is altered in chronic hyperglycemia. The heterogeneity of human diabetes and the use of potent immune suppressive drugs to prevent cellular alloresponses preclude addressing this question using clinical transplant data.

Numerous animal studies in both chemically induced (3-8) and obese (9-11) diabetic models report that diabetes decreases cellular (3-7; 9-11) and humoral immunity (3; 5; 8), including alloimmunity (4; 7; 9-11). However, confounding immune effects of streptozotocin (12; 13) and disrupted leptin signaling (14) complicate interpretation of these studies. Similarly, since autoimmunity can influence alloimmunity (15), the NOD model is also not suitable to address how chronic hyperglycemia itself affects alloimmunity. Therefore, we turned to the spontaneously diabetic, non-autoimmune C57BL/6 \( \text{Ins}^2\text{Akita} \) (Akita) model (16) to study alloimmunity. A single mutant \( \text{Ins}2 \) allele in Akita mice dominantly suppresses insulin secretion due to a protein-misfolding defect (17). Although Akita mice are reported to reject islet allografts (18; 19), a careful analysis of alloresponses is lacking in this newer animal model of diabetes. Using the Akita model, we evaluated the intrinsic impact of chronic hyperglycemia resulting from insulinopenia on primary cellular and humoral alloresponses.

**RESEARCH DESIGN AND METHODS**

**Animals.** C57BL/6 \( \text{Ins}^2\text{Akita} \) (Akita; H-2\(^b\)), C57BL/6J, and BALB/cByJ (H-2\(^d\)) mice were purchased from Jackson Laboratories. Male Akita mice treated with an islet isograft were crossed with C57BL/6 female mice and male offspring heterozygous for the Akita polymorphism (\( \text{Ins}^2\text{Akita}/+ \)) were used as diabetic recipients (blood glucose ≥20mM) at 8-12 weeks of age. Non-diabetic littermates (B6) were used as controls. Drs. Sang-Mo Kang (University of California San Francisco) and Anita Chong (University of Chicago) kindly provided C57BL/6 T cell receptor transgenic 4C (20) and TCR75 (21) mice, respectively. Mice were bred
and housed in AALAC-accredited facilities. The University of Colorado IACUC approved all practices and procedures.

**Assessment of polyclonal T cell responses to local alloimmunization.** Mice received $10^6$ BALB/cByJ splenocytes injected subcutaneously into each hind footpad. After five days, the draining popliteal lymph nodes were harvested for flow cytometry.

**Staining and flow cytometry.** Cells were stained with fixable live/dead discriminating dyes (BD or eBioscience) and antibodies to surface markers and intracellular proteins (using the FoxP3 staining Kit, eBioscience). CD4 (RM4-5), CD8 (53-6.72), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD90.2 (30-H12, Vb13 (MR12-3), Vb8.3 (1B3.3), FoxP3 (FJK-16s), IFNγ (XMG1.2), and Ki67 (B56) antibodies were obtained from BD or eBioscience. For intracellular cytokine staining, T cells were restimulated in Eagle’s minimum essential medium containing PMA (40 ng/mL) and ionomycin (500 ng/mL) in the presence of brefeldin A (1 µg/mL) for 4 hours. Samples were acquired on a BD LSR-II flow cytometer and analyzed using FlowJo software.

**Assessment of adoptively transferred allo-specific TCR-transgenic cells.** CD45.1 congenic 4C and TCR75 mice were depleted of CD8+ T cells using anti-CD8 (clone 2.43). $10^6$ 4C and $10^4$ TCR75 cells were transferred into CD45.2+ Akita mice and non-diabetic littermates. Alloimmunization and harvest were performed as above. To identify transferred cells, live singlet CD4+ cells were gated on CD45.1 and then Vb13+ (4C) by Vb8.3+ (TCR75).

**Streptozotocin treatment.** Where indicated, non-diabetic mice were given a single retro-orbital injection of streptozotocin (180 mg/kg; Sigma or Cayman). After three days, mice with two blood glucose readings ≥20 mM were considered diabetic. To control for streptozotocin, a group of diabetic Akita mice was also treated.

**Islet isolation and transplantation.** Islets from female BALB/cByJ retired breeders were isolated using Cizyme (Vitacyte) and Lympholyte 1.1 (Cedarlane). 400-450 islet equivalents were grafted beneath the left
kidney capsule. Blood glucose was measured to confirm engraftment (two consecutive readings ≤10mM) and rejection (two consecutive readings ≥20mM).

**Analysis of graft-infiltrating cells.** On day 7 post-transplant, a group of grafts was harvested, digested using dispase II (Roche) and further dissociated between frosted glass slides. Graft-infiltrating cell numbers were estimated using counting beads (eBioscience). Restimulation and staining were performed as above.

**Assessment of anti-donor antibody response.** Mice received $3 \times 10^7$ live BALB/cByJ splenocytes injected intraperitoneally or an islet allograft. Serum was harvested on days 0, 7, 14, and 21 or on the day of rejection of islet allografts (day 9-18). B6 mice were matched to a rejecting Akita. H-2d-expressing A20 target cells were exposed to sera (diluted 1:5) from immunized mice for 20 minutes at 37 degrees. Serum from a hyperimmune mouse was used as a positive control. Cells were then stained with secondary dye-conjugated anti-mouse IgM (Il/41) and anti-IgG antibody (11-4011-85, eBioscience).

**Statistics.** Data were graphed and statistically analyzed using Prism software. Statistical tests included multiple T tests with Bonferroni-Dunn corrections (alloimmunizations), Mann-Whitney U test (allograft survival) and two-way ANOVA tests with Bonferroni correction (graft-infiltrate analysis).

**RESULTS**

**No effect of chronic diabetes on polyclonal T cell responses to local allogeneic stimulation in vivo.** Naïve Akita mice demonstrate splenic lymphopenia (Supplemental Figure 1) but have otherwise normal B and T cell distribution (Supplemental Figure 2) and normal T cell memory phenotype (Supplemental Figure 3). To determine whether chronic hyperglycemia affected cellular alloreactivity, we immunized Akita mice and non-diabetic littermate controls with allogeneic splenocytes and analyzed the T cell response in the draining popliteal lymph node. Surprisingly, chronic diabetes did not significantly affect total cell numbers (Figure 1A) or CD4$^+$ and CD8$^+$ T cell numbers (Figure 1B) despite a tendency toward fewer cells.
We next determined whether diabetes affected markers of T cell antigen experience (CD44), proliferation (Ki67), or effector cytokine production (IFNγ). Again, chronic diabetes did not affect the frequency of responding \( \text{Ki67}^+ \text{CD44}^{\text{hi}} \) cells (Figure 1C) or their production of IFNγ (Figure 1D).

**No effect of chronic diabetes on adoptively transferred direct or indirect alloreactive T cell responses.** Although polyclonal alloresponses appeared normal, we next determined whether T cells recognizing alloantigens directly via *donor* antigen presenting cells (only recently exposed to hyperglycemia) would be differentially affected by hyperglycemia versus T cells recognizing alloantigen processed and presented indirectly on *host* antigen presenting cells (chronically exposed to hyperglycemia). We used 4C TCR transgenic mice (20) to model directly alloreactive T cells and TCR75 TCR transgenic mice (21) to model indirectly alloreactive T cells. Hyperglycemia did not affect the numbers (Figure 2A), proliferation (Figure 2B) or IFNγ production (Figure 2C) of adoptively transferred direct (4C) or indirect (TCR75) alloreactive TCR transgenic cells in draining popliteal lymph nodes of immunized mice. Thus, regardless of the pathway of allorecognition, T cell responses were unaffected by chronic diabetes.

**No effect of chronic diabetes on islet allograft rejection.** We next determined whether chronic diabetes affected acute islet allograft rejection. Since elevated blood glucose signifies graft rejection, Akita littermates were treated with streptozotocin to induce diabetes (SzB6). To control for streptozotocin (12; 13; 22; 23), some Akita mice were also treated with this agent (SzAkita). Akita recipients rejected allografts more rapidly than SzB6 recipients (Figure 3A). Since SzAkita mice rejected with the same tempo as SzB6 recipients, chronic hyperglycemia alone could not account for apparent accelerated rejection in Akita recipients.

**Streptozotocin, but not chronic hyperglycemia, alters the magnitude of the cellular response to islet allografts.** To further distinguish the effects of chronic hyperglycemia from those of streptozotocin, we examined intragraft T cells in B6, Akita, SzB6, and SzAkita recipients at day 7 post-transplant. Chronic hyperglycemia did not affect the total number of intragraft live cells or CD4\(^+\) and CD8\(^+\) cells, though all
mice exposed to streptozotocin had fewer graft-infiltrating cells (Figure 3B-C). Thus, chronic hyperglycemia did not affect the magnitude of the cellular alloresponse.

**Streptozotocin, but not chronic hyperglycemia, alters the phenotype of T cells responding to islet allografts.** We next determined whether chronic hyperglycemia affected intragraft T cell activation or regulation. Though prior chronic hyperglycemia did not affect the frequency of Ki67$^{+}$CD44$^{hi}$ responding T cells within islet allografts, streptozotocin decreased the frequency of CD8$^{+}$Ki67$^{+}$CD44$^{hi}$ cells (Figure 3D) by one third. Prior chronic hyperglycemia did not affect IFN$\gamma$ expression, but streptozotocin reduced the level of IFN$\gamma$ production in Ki67$^{+}$CD44$^{hi}$ T cells (Figure 3E) by nearly fifty percent (49%). While chronic hyperglycemia did not affect the frequency of FoxP3$^{+}$ regulatory T cells, streptozotocin doubled FoxP3$^{+}$ regulatory T cell frequency (Figure 3F). As a result, streptozotocin (but not chronic hyperglycemia) increased the ratio of regulatory T cells (CD4$^{+}$FoxP3$^{+}$) to effector T cells (FoxP3$^{+}$Ki67$^{+}$CD44$^{hi}$) in both CD4$^{+}$ (Figure 4H) and CD8$^{+}$ (Figure 4I) compartments. Thus, streptozotocin treatment - but not prior chronic hyperglycemia - shifted the quality of the intragraft T cell response.

**Severely impaired antibody response to alloantigen in vivo.** Finding no major differences in cellular alloresponses in chronic diabetes, we next assessed humoral alloresponses. Akita mice immunized with allogeneic splenocytes showed decreased allospecific IgM (Figure 4A) and IgG (Figure 4B) at 7, 14, and 21 days post-challenge. Only low levels of allospecific antibody (Figure 4C-D) were generated in response to islet allografts themselves in both diabetic and euglycemic animals, which is consistent with B cell dispensability for acute islet allograft rejection (24). Nevertheless, the marked reduction in alloantibody production after alloimmunization in Akita mice indicates that chronic hyperglycemia results in decreased humoral alloimmunity.

**DISCUSSION**
We have shown that chronic diabetes, without autoimmunity or chemical induction, specifically impairs humoral—but not cellular—alloimmunity \textit{in vivo}. Using four different experimental approaches (polyclonal alloimmunization, TCR transgenic alloimmunization, islet allograft survival, and islet allograft infiltrate analysis) we have demonstrated that primary cellular alloresponses are not affected by chronic hyperglycemia. Our finding of splenic lymphopenia in chronic hyperglycemia (Supplemental Figure 1) was consistent with other studies (7) but did not preclude normal cellular alloresponses. In contrast, chronic hyperglycemia resulted in decreased allospecific IgM and IgG responses. Our data do not determine whether the hyporeactive humoral immunity is T or B cell intrinsic, though others have reported normal T-independent but impaired T-dependent antibody responses in the streptozotocin model (3). Our finding of selective impairment of humoral versus cellular immunity in chronic hyperglycemia differs from previously published results in streptozotocin (3; 5) and obese models of diabetes (6; 10; 11), which indicated impairment of both cellular and humoral immunity in diabetic animals. Diabetes-independent immune modulating effects of streptozotocin and obesity could potentially explain why cellular immunity is decreased in those models but remains normal in Akita mice. While our interest was to study chronic hyperglycemia as an independent variable influencing alloimmunity, we should note that insulinopenia in Akita mice akin to Type 1 diabetes could also potentially affect results. As such, concomitant insulinopenia could potentially limit the general relevance of this study to all diabetic patients, many of whom are insulin-resistant and/or hyperinsulinemic. This latter point raises the additional prospect that the form of diabetes in the general transplant patient population could potentially influence host alloimmunity. While the present study suggests that alloimmunity is not impaired in the hyperglycemic, insulin-sensitive recipient (like may Type 1 diabetic patients), it is less clear whether this response is impacted by the insulin-resistant, hyperinsulinemic environment of the Type 2 diabetic patient. As such, this concept would encourage close interactions between dialectologists and transplant physicians. Nevertheless, our results qualify the tenet of immune compromise in diabetes by identifying a specific immune response (the primary cellular alloresponse) that is \textit{not} compromised in chronic hyperglycemia.
The nature of the underlying alloresponse of transplant recipients (including diabetic recipients) may become more relevant as the transplant immunology field seeks to replace lifelong, broad immunosuppression with short-term peri-transplant tolerance-promoting therapies. While we find here that chronic diabetes does little to prevent primary cellular alloimmunity, we have previously reported decreased anti-CD154-induced tolerance to islet allografts in Akita mice (18). The normal capacity for acute islet allograft rejection combined with decreased capacity for tolerance induction in chronic hyperglycemia unfortunately constitutes a formidable challenge for the future of tolerance induction in diabetic patients. This hitherto unappreciated challenge exists independent of autoimmunity, which further compounds the difficulty of tolerance induction in Type 1 diabetic patients.

Finally, our results highlight the importance of model selection in experimental islet transplantation studies. Despite early indications (13) and subsequent confirmation (12; 22; 23) of the immune modulating properties of streptozotocin, this drug has become the standard model for tolerance induction studies in islet transplantation (25). Our results confirm and extend those of previous groups by showing that streptozotocin itself dramatically alters the magnitude and quality of the cellular alloresponse within actual islet allografts. Due to these marked effects, our results reinforce the caveats related to the use streptozotocin in transplant studies and support the use of the Akita model as a potential alternative non-autoimmune model of diabetes.

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**FIGURE LEGENDS**

**Figure 1. No effect of chronic hyperglycemia on primary T cell responses to alloantigen.** A: Popliteal lymph node (PLN) cell counts in alloimmunized mice. B: CD4+ and CD8+ cell counts in PLN. C: Frequency of responding Ki67+CD44hi cells in PLN. E: Geometric mean fluorescence intensity (gMFI) of IFNγ in Ki67+CD44hi cells. Black circles=B6, white squares=Akita. n=6-8 per group from seven independent experiments. No significant differences after multiple T tests with Bonferroni-Dunn corrections.

**Figure 2. No effect of chronic hyperglycemia on responses of either direct or indirect alloreactive TCR transgenic adoptively transferred cells.** A: Number of direct (4C) and indirect (TCR75) alloreactive T cells in draining popliteal lymph nodes of Akita mice and euglycemic littermates 5 days after local alloimmunization. B: Frequency of proliferating (Ki67+) direct (4C) and indirect (TCR75) cells. C: gMFI of IFNγ in direct (4C) and indirect (TCR75) cells. Black circles=B6, white squares=Akita. Number of samples with no detectable cells in unchallenged mice indicated adjacent to “n.d.” n=4 per group from 4 independent experiments. No significant differences between immunized groups after multiple T tests with Bonferroni-Dunn corrections.
Figure 3. No impact of chronic hyperglycemia on cellular responses to islet allografts. A: Survival curves of islet allograft acute rejection. n=10-15. Akita versus SzB6 ***p<.001, Akita versus SzAkita *p<.05, SzB6 versus SzAkita not significant by non-parametric Mann-Whitney U-test. B: Total numbers of graft-infiltrating cells at day 7. C-D: Numbers of CD4$^+$ (C) and CD8$^+$ T cells (D) within CD90$^+$ graft infiltrating cells. E: Frequency of responding Ki67$^+$CD44$^{hi}$ cells within CD90$^+$CD8$^+$ graft-infiltrating cells. F: gMFI of IFN$\gamma$ in CD90$^+$CD8$^+$FoxP3$^+$Ki67$^+$CD44$^{hi}$ T cells. G: Frequency of FoxP3$^+$ cells within CD90$^+$CD4$^+$ graft-infiltrating cells. H-I: H: Ratio of CD90$^+$CD4$^+$FoxP3$^+$ regulatory T cells to CD90$^+$FoxP3$^+$Ki67$^+$CD44$^{hi}$CD4$^+$ (H) and CD8$^+$ (I) effector T cells. Black circles=B6, white squares=Akita, black triangles=SzB6, white diamonds=SzAkita. In B-I, n=4-6 per group from five independent experiments. Two-way ANOVA with Bonferroni corrections; *=p<.05, **=p<.01, ***=p<.001.

Figure 4. Impaired humoral response to alloantigen in chronic diabetes. H-2$^d$ expressing A20 target cells were exposed to serum from alloimmunized mice (A-B, n= 9-16 per group from four independent experiments) or islet allograft recipients (C-D, n=4-8 per group from two independent experiments). A: gMFI of anti-IgM. B: gMFI of anti-IgG. C: gMFI of anti-IgM. D: gMFI of anti-IgG. Multiple T tests with Bonferroni-Dunn corrections, *=p<.05, **=p<.01, ***=p<.001.
**Figure 1**

A. Total Cell Number

B. Cell Number

C. % Ki67+ CD44 hi

D. IFNγ gMFI
Figure 3

A

Percent survival

Days Post-Transplantation

B

# Lymphocytes

Ins2^{Akita} - + - +

Sz - - + +

C

# CD4^+

Ins2^{Akita} - + - +

Sz - - + +

D

# CD8^+

Ins2^{Akita} - + - +

Sz - - + +

E

% K67^+CD44^hi

Ins2^{Akita} - + - +

Sz - - + +

F

IFNγ oMFI

Ins2^{Akita} - + - +

Sz - - + +

G

% of CD4^+ FoxP3^+

Ins2^{Akita} - + - +

Sz - - + +

H

T_{reg} / CD4^+ T_{eff}

Ins2^{Akita} - + - +

Sz - - + +

I

T_{reg} / CD8^+ T_{eff}

Ins2^{Akita} - + - +

Sz - - + +

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Figure 4

A

B

Diabetes
Supplementary Figure 1. No effect of chronic hyperglycemia on cycling or effector cytokine production by either direct or indirect alloreactive adoptively transferred TCR transgenic cells. A-B: Frequency of Ki67+ direct (4C; A) and indirect (TCR75; B) alloreactive CD4 T cells. C-D: Number of Ki67+ direct (4C; C) and indirect (TCR75; D) alloreactive CD4 T cells. E-F: Geometric mean fluorescence intensity of IFNγ in Ki67+ direct (4C; E) and indirect (TCR75; F) alloreactive CD4 T cells. Black circles=B6, white squares=Akita. N=4 from 4 independent experiments. Number of samples with no detectable cells in unchallenged mice indicated adjacent to “n.d.” No significant differences by two-tailed Student T test.