Human Immune System Development and Rejection of Human Islet Allografts in Spontaneously Diabetic NOD-Rag1\textsuperscript{null} IL2r\gamma\gamma\gamma\gamma\null Ins2\textsuperscript{Akita} Mice

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Running title: NRG-Akita model of hyperglycemia and human immunity

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**Objective**—To create an immunodeficient mouse model that spontaneously develops hyperglycemia to serve as a diabetic host for human islets and stem cell derived beta cells in the absence or presence of a functional human immune system.

**Research design and methods**—We backcrossed the Ins2<sup>Akita</sup> mutation onto the NOD-<sup>Rag1</sup><sup>null</sup> IL2<sup>γnull</sup> strain and determined 1) spontaneous development of hyperglycemia, 2) ability of human islets, mouse islets and dissociated mouse islet cells to restore euglycemia, 3) the generation of a human immune system following engraftment of human hematopoietic stem cells, and 4) the ability of the humanized mice to reject human islet allografts.

**Results**—We confirmed the defects in innate and adaptive immunity and the spontaneous development of hyperglycemia conferred by the IL2<sup>γnull</sup>, Rag1<sup>null</sup>, and Ins2<sup>Akita</sup> genes in NOD-Rag1<sup>null</sup> IL2<sup>γnull</sup> Ins2<sup>Akita</sup> (NRG-Akita) mice. Mouse and human islets restored NRG-Akita mice to normoglycemia. Insulin-positive cells in dissociated mouse islets required to restore euglycemia in chemically diabetic NOD-scid IL2<sup>γnull</sup> and spontaneously diabetic NRG-Akita mice were quantified following transplantation via the intra-pancreatic and subrenal routes. Engraftment of human hematopoietic stem cells in newborn NRG-Akita and NRG mice resulted in equivalent human immune system development in a normoglycemic or chronically hyperglycemic environment, with >50% of engrafted NRG-Akita mice capable of rejecting human islet allografts.

**Conclusions**—NRG-Akita mice provide a model system for validation of the function of human islets and human adult stem cell, embryonic stem cell, or induced pluripotent stem cell-derived beta cells in the absence or presence of an alloreactive human immune system.

Investigators are working to develop functional human beta cells from adult or embryonic stem cells (ESC), or from induced pluripotent stem (iPS) cells (1-4), and human ESC-derived beta cells can restore euglycemia in streptozotocin (STZ) diabetic immunodeficient mice (5). However, STZ can have detrimental effects on many organs in addition to beta cells (6,7), and there is sometimes recovery of the host beta cell function (8,9). In addition, it will be important to transplant human beta cells in the presence of an allogeneic human immune system.

We have developed an immunodeficient mouse model of spontaneous hyperglycemia based on NOD-Rag1<sup>null</sup> Prf1<sup>null</sup> mice bearing the Ins2<sup>Akita</sup> mutation (10). Mice heterozygous for Ins2<sup>Akita</sup> develop spontaneous hyperglycemia at 3-6 weeks of age (11,12), and transplantation of human islets restores euglycemia (10). However, human immune system engraftment is relatively inefficient in this model (13). Immunodeficient scid and Rag1<sup>null</sup> NOD mice bearing a mutation in the IL-2 receptor common gamma chain (IL2<sup>γnull</sup>) gene are now available (14,15) and can be engrafted with functional human immune systems (14-18).

We now describe the development of the NOD-Rag1<sup>null</sup> IL2<sup>γnull</sup> Ins2<sup>Akita</sup> (NRG-Akita) strain. These mice develop spontaneous hyperglycemia and euglycemia can be restored following transplantation with mouse or human islets or with dissociated
mouse islet cells. A human immune system develops following engraftment with human hematopoietic stem cells (HSC), and in some mice, this immune system is capable of rejecting human islet allografts.

**MATERIALS AND METHODS**

**Mice.** NOD-Rag1<sup>−/−</sup> IL2r<sup>−/−</sup> (NRG, 14), NOD-Rag1<sup>−/−</sup> Prf1<sup>−/−</sup> Ins2<sup>−/−</sup> Akita<sup>+</sup> (NRG-Akita), and NOD-scid IL2r<sup>−/−</sup> (NSG, 15) mice have been described. To generate NOD-Rag1<sup>−/−</sup> IL2r<sup>−/−</sup> Ins2<sup>−/−</sup> Akita<sup>+</sup> (NRG-Akita) mice, NOD.Cg-Rag1<sup>tm1Mom</sup> Prf1<sup>−/−</sup> Ins2<sup>−/−</sup> Akita<sup>+</sup> mice were crossed with NOD-Rag1<sup>−/−</sup> IL2r<sup>−/−</sup> strain. Additional crosses fixed the wild type Prf1 allele and the IL2r<sup>−/−</sup> mutation to homozygosity while maintaining the Ins2<sup>−/−</sup> Akita<sup>+</sup> mutation in the heterozygous state. Mice were housed in a specific pathogen free facility in microisolator cages (15). All animal use was in accordance with the guidelines of the IACUCs of the University of Massachusetts and The Jackson Laboratory.

**Antibodies and Flow Cytometry.** The phenotypes of murine cells were determined using four color analyses (15). Anti-human FoxP3 (eBioscience Inc., San Diego, CA), anti-human CD123 and BDCA2 (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-human CD11c (Biolegend, San Diego, CA) and anti-human CD235a (Coulter Immunotech, Miami, FL) were obtained as indicated. All other antibodies were purchased from BD Biosciences (San Jose, CA).

Bone marrow, spleen, and thymus cell suspensions and heparinized blood were incubated with anti-mouse FcR11b to block Fc binding (14,19). Cells were prepared for flow cytometry, and at least 50,000 events were acquired on BD Biosciences LSRII or FACScalibur instruments (BD Biosciences) (14,15,20). For rare populations we acquired at least 500 specific events. Data analysis was performed with FlowJo (Tree Star, Inc., Ashland, OR) software.

**Mouse and Human Islet Transplantation.** Handpicked BALB/c islets (21) were also dissociated into single cell suspensions (22). Dissociated islet cell suspensions were treated with Cytofix/Cytoperm (BD Biosciences) for intracellular staining using a biotinylated anti-insulin antibody and developed with SA-APC. Dissociated preparations contained 62±19% (mean±s.d, n=10) insulin-positive cells. Islets or dissociated cells were transplanted intrapancreatically or subrenally into STZ-diabetic NSG mice (150 mg/kg) or unmanipulated diabetic NRG-Akita recipients.

Human islets were obtained from JDRF Islet Isolation Centers or NIH Integrated Islet Distribution Program. Human IEQ (4000) were transplanted subrenally into diabetic NSG or NRG-Akita mice. Non-fasting blood glucose levels were determined twice weekly. Loss of graft function was determined by two consecutive blood glucose values >250 mg/dl.

**Engraftment of Mice with Human Hematopoietic Stem Cells (HSC).** NRG or progeny of (NRG x NRG-Akita) matings (~50% heterozygous for Ins2<sup>−/−</sup> Akita<sup>+</sup>) mice 1-3 days old were irradiated with 400cGy, and engrafted with umbilical cord blood (UCB) HSC provided by the University of Massachusetts Memorial Umbilical Cord Blood Donation Program under IRB approval (14,20). Mice were analyzed phenotypically 12-16 weeks later and/or transplanted with human islet allografts.

**Histology.** Pancreata and islet transplants were stained with H&E, and for insulin, glucagon, and huCD45 (16). Unilateral nephrectomy of the graft-bearing kidney was performed on selected cohorts to confirm recurrent hyperglycemia. In some cases, islet allograft survival was inferred from histological study.

**Statistical Analyses.** Most data is presented as the mean ± one s.d. while flow cytometry data is presented as mean ± s.e.m. Parametric
data were compared by one-way ANOVA with Bonferroni post-tests to compare individual pair-wise groupings. Nonparametric data were compared by a Kruskal-Wallis test with Dunns post-test to compare individual pair-wise groupings. Significant differences were assumed for p values <0.05. All statistical analyses were performed using GraphPad Prism software (version 4.0c, GraphPad, San Diego, CA).

RESULTS
Phenotype of the Hematopoietic System of NRG-Akita Mice. To develop an immunodeficient strain of mice bearing the \textit{Ins2} \textit{Akita} mutation that will support the development of a human immune system following engraftment with HSC, we generated NRG-Akita mice. As mice homozygous for \textit{Ins2} \textit{Akita} develop a rapid hyperglycemia and die shortly after weaning (10), all studies were performed using mice heterozygous for the \textit{Ins2} \textit{Akita} allele.

Flow cytometric analysis of NRG-Akita splenocytes confirmed the lack of mature T, B, and NK cells (Supplemental Table 1 in the online appendix which is available at http://diabetes.diabetesjournals.org). Although cells with an NK phenotype (DX5+/CD122+) were observed, these cells are not mature NK cells (15). Populations of granulocytes and macrophages in NRG and NRG-Akita mice were similar (Supplemental Table 1), documenting that NRG-Akita mice are deficient in adaptive immunity and NK cells.

Spontaneous Hyperglycemia in NRG-Akita Mice and Reversal by Islet Transplantation. All male and female NRG-Akita mice developed hyperglycemia between 3-5 weeks of age, and remained diabetic, viable, and healthy in the absence of exogenous insulin (Figure 1). In contrast, NRG (\textit{Ins2} \textit{Akita}+) littermates remained euglycemic throughout their lifespan.

Normal islet architecture and insulin and glucagon staining was observed in the pancreata of NRG mice at all ages (Figure 1). At ~3 weeks of age, the pancreata of NRG-Akita mice was only slightly disorganized, and strong insulin staining was observed. By ~32 weeks of age in hyperglycemic NRG-Akita mice, islet architecture was disorganized and condensed. Some insulin-positive cells were present but scattered throughout the islet. No inflammatory infiltrate was observed.

Subrenal transplantation of 20 mouse islets/gm body weight into hyperglycemic NRG-Akita mice restored normoglycemia (Figure 2). Islet graft recipients remained normoglycemic to the end of observation periods or in selected cases, reverted to hyperglycemia following removal of the graft-bearing kidney (data not shown).

Transplantation of 4000 IEQ human islets into the subrenal capsular space routinely restored euglycemia in NRG-Akita mice (Figure 2). These islets remained functional throughout the observation periods, in some cases over 300 days. Graft survival was confirmed by histology or by removal of the graft-bearing kidney and reversion to hyperglycemia (data not shown).

Restoration of Normoglycemia by Transplantation of Mouse Islets or Dissociated Islet Cells into Diabetic NSG or NRG-Akita Mice. Intra-pancreatic transplantation of 20 islets/gm body weight into STZ-diabetic NSG mice failed to restore normoglycemia whereas 40 islets/gm body weight restored normoglycemia in 2 of 7 recipients (Supplemental Figure 1). Using dissociated islet cells, $> \times 10^5$ insulin-positive beta cells were required for restoration of normoglycemia following subrenal transplantation in diabetic NSG mice (Supplemental Figure 1).

In diabetic NRG-Akita mice, intra-pancreatic transplantation of 40 mouse islets/gram body weight restored
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normoglycemia in 3 of 4 NRG-Akita mice whereas 20 islets/gram body weight restored normoglycemia in only 1 of 3 NRG-Akita recipients (Supplemental Figure 2). This is in contrast to restoration of normoglycemia in diabetic NRG-Akita recipients following subrenal transplantation of 20 mouse islets/gram body weight (Figure 2).

Intra-pancreatic transplantation of dissociated mouse islet cells containing 1-4x10^6 insulin-positive beta cells restored euglycemia in only 1 of 5 diabetic NRG-Akita recipients (Supplemental Figure 2). Only 2 of 6 hyperglycemic NRG-Akita mice became euglycemic after subrenal transplantation with 1-4x10^6 dissociated mouse islet cells (Supplemental Figure 2). These data document 1) dissociated mouse islets can restore normoglycemia in diabetic NSG and NRG-Akita mice, and 2) that fewer insulin-positive cells are required for restoration of normoglycemia following subrenal compared with intra-pancreatic transplantation.

Engraftment of Human HSC in NRG-Akita Mice. We have shown that NRG mice engraft with human HSC at levels similar to those observed in NSG mice (14), but the effect of chronic hyperglycemia on the development of a human immune system is unknown.

NRG-Akita and NRG littermates were injected with human HSC (14,20). Twelve weeks later, hyperglycemia was confirmed in NRG-Akita mice (data not shown). Similar percentages of splenic human CD45^+ cells and all human cell subsets were observed in hyperglycemic NRG-Akita as compared with euglycemic NRG mice (Table 1). Comparable levels of human CD45^+ and human cell subsets were also observed in the bone marrow, blood and thymus of hyperglycemic NRG-Akita and euglycemic NRG mice (Supplemental Tables 2-4). These data document that the human immune systems that develop in hyperglycemic NRG-Akita mice appear phenotypically comparable to those developing in normoglycemic NRG mice.

Transplantation of Human Islet Allografts in HSC-Engrafted NRG-Akita Mice

Eight of 9 human islet recipients of 4000 IEQ restored normoglycemia (<250 mg/dl, Figure 3) in non-HSC-engrafted diabetic NRG-Akita mice. In contrast, 8 of 13 human islet allografts were rejected in HSC-engrafted NRG-Akita mice (Figure 3). Insulin staining in the absence of mononuclear cell infiltration was detected in human islet allografts in non-HSC-engrafted NRG-Akita mice (Figure 3). In the 8 HSC-engrafted NRG-Akita human islet recipients that reverted to hyperglycemia, human CD45^+ cell infiltration into the graft site and loss of beta cells was observed. In the 5 HSC-engrafted NRG-Akita mice that did not revert to hyperglycemia, insulin-positive cells and human CD45^+ cell islet infiltration was observed (Figure 3)

DISCUSSION

We have previously described the development of spontaneously diabetic NOD-Rag1 null Prf1 null Ins2 Akita mice (10), but NOD-Rag1 null Prf1 null mice engraft poorly with human immune systems (23). To address this limitation, we generated NRG-Akita mice that spontaneously develop hyperglycemia. Euglycemia can be restored following transplantation with mouse or human islets or with dissociated mouse islet cells. Even in the presence of a state of chronic hyperglycemia, a human immune system develops following engraftment with human HSC that is capable of rejecting human islet allografts in some of the transplanted mice.

Unmanipulated NRG-Akita mice maintain a phenotype consistent with a severely immunodeficient mouse bearing IL2rgamma and Ins2 Akita mutated genes. Both male and female mice develop hyperglycemia between 3-5 weeks of age, and islets progressively lose insulin-positive cells with
age. However, small numbers of insulin-positive cells remain throughout life, perhaps explaining why administration of exogenous insulin is not required for long term survival.

We confirmed that transplantation of mouse or human islets restores euglycemia in diabetic NRG-Akita and NSG mice. Higher numbers of islets were required following intrapancreatic implantation than were needed following subrenal transplantation to restore euglycemia. Our data also suggest that single cell suspensions of insulin-producing cells may be less functional than comparable numbers contained within an islet structure.

We confirmed that a human immune system develops in diabetic NRG-Akita mice engrafted with human HSC and this immune system is phenotypically comparable to that generated in euglycemic NRG mice. However, only ~60% of human immune system engrafted NRG-Akita mice rejected human islet allografts. In separate studies, HSC-engrafted NSG mice readily reject human skin allografts (MAB, unpublished observations), but it is known that skin allografts prompt a more robust alloimmune response than do islet allografts (24,25). Alternatively, the human immune system may be impaired due to the hyperglycemic environment, or we may not have allowed sufficient time for graft rejection.

In summary, we have developed a new model of spontaneous hyperglycemia based on immunodeficient NOD mice bearing mutations in the $IL2r\gamma$ gene and the $Ins2^{Akita}$ gene. NRG-Akita mice can be engrafted with a human immune system, providing a recipient environment similar to that which will be encountered when transplanting allogeneic beta cells in the clinic. These mice provide a novel model system suitable for validation of the function of stem cell-derived human beta cells in the absence or presence of an alloreactive human immune system.

Author contributions: M.A.B researched data, wrote manuscript and reviewed/edited manuscript. R.B. reviewed/edited manuscript. P.D. researched data, contributed to discussion and reviewed/edited manuscript. J.L. researched data and contributed to discussion. J.L. reviewed/edited manuscript. A.C. researched data. C.Y. reviewed/edited manuscript. M.H. reviewed/edited manuscript. L.B. researched data and wrote manuscript. B.G. researched data and wrote manuscript. O.F. researched data. A.C.P. contributed to discussion and wrote manuscript. D.L.G. researched data, contributed to discussion, wrote manuscript and reviewed/edited manuscript. L.D.S. researched data, contributed to discussion, wrote manuscript and reviewed/edited manuscript.

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Figure Legends

Figure 1: Onset of Hyperglycemia and Islet Morphology in Male and Female NOD-Rag1null IL2rnull Ins2Akita Mice

Male (Panel A) or female (Panel C) NOD-Rag1null IL2rnull Ins2Akita and NOD-Rag1null IL2rnull littermate control mice were followed for >200 days monitoring blood glucose at the days of age indicated as described in Materials and Methods. Data points shown are of individual animals. Pancreata from male (Panel B) and female (Panel D) NOD-Rag1null IL2rnull Ins2Akita and NOD-Rag1null IL2rnull littermate control mice at the indicated ages were stained with H&E and immunohistochemically for insulin and glucagon. Young and older control NRG (+/+) mice and young NRG-Akita (+/Akita) mice display normal architecture and histochemical structure with insulin positive cells throughout the islets and a peripheral rim of glucagon positive cells. Older NRG-Akita mice display collapsed islet structure with fewer insulin positive beta cells and numerous glucagon positive cells scattered throughout the islets. Blood glucose levels of the mice at the time of recovery of the pancreas are shown at the bottom of each panel. Magnification X400

Figure 2. Transplantation of Mouse and Human Islets into Diabetic NRG-Akita Mice.

Diabetic NRG-Akita mice were transplanted in the renal subcapsular space with 4000 IEQ human islets or with 20 islets/gram body weight mouse islets as described in Materials and Methods. Blood glucose levels were determined, and the kidney bearing the islet transplant and the host pancreas were recovered at the end of the experiment for histological and immunohistochemical analyses. Panel A: H&E, insulin, and glucagon staining of transplanted mouse and human islets and host pancreas of the NRG-Akita transplant recipient at times indicated after islet transplantation. In the renal subcapsular space there is robust engraftment of mouse and human islets. Magnification X200. Panel B: Frequency of diabetes in mouse or human islet recipients. No significant differences were observed between recipients of mouse or human islets. Small vertical bars indicate censored data, i.e., mice that were found dead or were removed from the study for other analyses.

Figure 3. Transplantation of Human Islet Allografts into Diabetic NRG-Akita Mice Engrafted with Human HSC.

Diabetic NRG-Akita and HSC-engrafted NRG-Akita mice were transplanted subrenally with 4000 human IEQ as described in Methods and Materials. Panel A: Frequency of diabetes in islet allograft recipients. NRG-Akita vs. HSC-engrafted NRG-Akita, p=0.03. Panel B: Representative histology and immunohistochemical staining patterns are shown. Note the abundance of insulin-positive cells and the absence of human CD45-positive cells in non-HSC engrafted NRG-Akita mice (left column). Note presence of fewer insulin-positive cells and islet graft infiltration by human CD45+ cells in HSC engrafted NRG-Akita mice that were normoglycemic at the end of the experiment (middle column). Note the scarcity of insulin-positive cells and moderate numbers of human CD45+ cells in HSC engrafted NRG Akita mice that were hyperglycemic at the end of the experiment and had rejected their human islet allografts (right column). Top row, H&E, Middle row, human CD45 staining. Bottom row, insulin staining. Magnification X200.
Table 1. Human HSC Engraftment in the Spleen of NRG and NRG-Akita Mice.

Newborn NRG (n=16) and NRG-Akita (n=12) mice were irradiated with 400 cGy and injected with T cell-depleted UCB containing $3 \times 10^4$ human CD34$^+$ HSC by intracardiac injection as described in Materials and Methods. The percent of various human cell populations in the spleen was determined by flow cytometry 12 weeks later. No significant differences were observed.

| Splenocyte Number (x10^6) | NRG (n=16) | NRG-Akita (n=12) |
|----------------------------|------------|------------------|
| Total Splenocyte Number    | 17.4±4.3   | 23.1±3.5         |
| Total Human Leukocytes (%) |            |                  |
| CD45+                      | 58.8 ± 6.1 | 50.98 ± 4.9      |
| B Lineage (% of CD45)      |            |                  |
| CD20+                      | 69.4 ± 2.9 | 71.2 ± 2.8       |
| T Lineage (% of CD45)      |            |                  |
| CD3+                       | 5.2 ± 1.6  | 8.6 ± 3.9        |
| CD4+CD45RA+                | 5.7 ± 2.6  | 4.2 ± 1.2        |
| CD4+CD45RO+                | 3.3 ± 1.1  | 4.5 ± 1.4        |
| CD4+FoxP3+                 | 0.7 ± 0.2  | 0.8 ± 0.2        |
| CD8+CD45RA+                | 4.7 ± 1.7  | 6.3 ± 2.0        |
| CD8+CD45RO+                | 1.2 ± 0.4  | 1.6 ± 0.6        |
| Dendritic Subsets (from murine CD45 negative cells) |          |                  |
| (Monocytic) CD11c+BDCA2-   | 2.5 ± 0.3  | 3.6 ± 0.4        |
| (Plasmacytoid) CD123+BDCA2+ | 0.5 ± 0.1  | 0.4 ± 0.1        |
| Monocyte/Macrophage (% of CD45) |         |                  |
| CD14+                      | 1.3 ± 0.3  | 2.0 ± 0.4        |
Figure 1

A

B

C

D

NRG-Akita model of hyperglycemia and human immunity
Figure 2

A

| Mouse Islets | Human Islets |
|--------------|--------------|
| Kidney       | Kidney       |
| Pancreas     | Pancreas     |

- H&E
- Insulin
- Glucagon

17 weeks | 34 weeks

Days post-Transplant

B

Cumulative Percent Non-Diabetic

- Human Islets (N=18)
- Mouse Islets (N=7)

Days
Figure 3

Cumulative Percent Non-Diabetic

- HSC NRG-Akita (N=13)
- NRG-Akita (N=9)

Days

p=0.03

No HSC + Islets
HSC + Islets Normoglycemic
HSC + Islets Hyperglycemic

H&E
huCD45
Insulin