Anti-Islet Autoantibodies Trigger Autoimmune Diabetes in the Presence of an Increased Frequency of Islet-Reactive CD4 T Cells

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OBJECTIVE—To define cellular mechanisms by which B cells promote type 1 diabetes.

RESEARCH DESIGN AND METHODS—The study measured islet-specific CD4 T cell regulation in T-cell receptor transgenic mice with elevated frequencies of CD4 T cells recognizing hen egg lysozyme (HEL) autoantigen expressed in islet β-cells and thymic epithelium under control of the insulin-gene promoter. The effects of a mutation in *Roquin* that dysregulates T follicular helper (Tfh) cells to promote B-cell activation and anti-islet autoantibodies were studied, as were the effects of HEL antigen–presenting B cells and passively transferred or maternally transmitted anti-islet HEL antibodies.

RESULTS—Mouse anti-islet IgG antibodies—either formed as a consequence of excessive Tfh activity, maternally transmitted, or passively transferred—caused a breakdown of tolerance in islet-reactive $CD4^+$ cells and fast progression to diabetes. Progression to diabetes was ameliorated in the absence of B cells or when the B cells could not secrete islet-specific IgG. Anti-islet antibodies increased the survival of proliferating islet-reactive $CD4^+$ T cells. FcγR blockade delayed and reduced the incidence of autoimmune diabetes.

CONCLUSIONS—B cells can promote type 1 diabetes by secreting anti-islet autoantibodies that act in an FcγR-mediated manner to enhance the expansion of islet-reactive CD4 T cells and cooperate with inherited defects in thymic and peripheral CD4 T–cell tolerance. Cooperation between inherited variants affecting CD4 T–cell tolerance and anti-islet autoantibodies should be examined in epidemiological studies and in studies examining the efficacy of B-cell depletion. ***Diabetes*** **60:2102–2111, 2011**

Type 1 diabetes is an autoimmune disease caused by islet-reactive T cells that destroy insulin-producing β-cells. T-cell tolerance mechanisms normally prevent autoimmune diabetes in mice and humans (1,2), and T cell–directed treatment with anti-CD3 monoclonal antibodies slows the loss of insulin production in the 1st year after diabetes diagnosis (3). With the focus on T cells, the role of antibodies and B cells in type 1 diabetes etiopathogenesis is often neglected. A single clinical case of type 1 diabetes in a person with X-linked agammaglobulinemia indicates that type 1 diabetes can develop in the absence of antibodies and B cells (4). However, antibodies to insulin and other islet antigens are predictive of subsequent type 1 diabetes (5), and B cells are required for diabetes development in the nonobese diabetic (NOD) mouse model of type 1 diabetes (6–8). A recent clinical trial of B-cell depletion with anti-CD20 in humans with newly diagnosed type 1 diabetes demonstrated a significant delay in further loss of insulin synthesis and established a role for B cells (9), but how B cells contribute to type 1 diabetes pathology remains unclear. Better understanding of mechanisms by which B cells can promote type 1 diabetes may improve the efficacy of interventions that target B cells.

B cells may contribute to type 1 diabetes pathology by capturing and presenting autoantigens to islet-reactive CD4+ T cells (10–12). The effect on CD4 T cells is particularly relevant because B cells present captured antigens to CD4 cells via major histocompatibility complex (MHC) II molecules, and type 1 diabetes is strongly associated with particular HLA-DR haplotypes. However, an alternative possibility is that B cells contribute to type 1 diabetes pathology by secreting autoantibodies. In humans, the high predictive value of anti-islet autoantibodies for progression to type 1 diabetes is well documented (13–17). However, it remains unresolved whether or not autoantibodies are simply a biomarker of breakdown in T-cell tolerance (5). Indirect evidence against a pathogenic role for autoantibodies comes from the decreased incidence of type 1 diabetes in offspring of diabetic mothers compared with diabetic fathers, despite transmission of maternal anti-islet autoantibodies (18–20). Paradoxical protection from diabetes in children with maternally derived islet-autoantibodies has been reported (21), although this protection was not observed in children with the high-risk HLA-DR3/DR4-DQ8 genotype. This observation could indicate that the effects of anti-islet antibodies are influenced by underlying heterogeneity in the efficiency of CD4 T–cell tolerance mechanisms, which are affected by variability in MHC II antigen presentation.

Only a few experimental studies have investigated whether or not secreted autoantibodies influence type 1 diabetes progression, and none have examined their influence on cellular mechanisms of tolerance in islet-reactive CD4 T cells. In NOD mice, diabetes was reduced when maternal transmission of antibodies from NOD mothers was prevented (22,23). It is not yet known whether these maternal effects reflect transmission of anti-islet autoantibodies, antibodies against microbial flora, or changes in

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maternal transmission of microbial commensals themselves. In one study, type 1 diabetes incidence in litters from NOD mothers clustered according to the titer of anti-insulin antibodies in the mother's serum (24); however, in another study there was no correlation (25). Supporting a pathogenic role for autoantibodies, NOD mice lacking different activating Fcγ receptors were shown to be protected from diabetes (26). Also, passive transfer of rabbit or mouse antibodies against islet-expressed ovalbumin (OVA) has been shown to enhance activation of islet-reactive CD8+ T cells and break tolerance (27). Hence, experimental studies are needed to directly investigate the effects of anti-islet autoantibodies on islet-specific CD4 T cells and how these may interact with inherited defects in islet-specific CD4 T–cell tolerance. Here we find that anti-islet autoantibodies are potent cofactors in type 1 diabetes progression and not simply markers of breakdown in CD4+ T–cell tolerance.

RESEARCH DESIGN AND METHODS

Mice. 3A9TCR-transgenic (T-cell receptor [TCR]+) (28), insulin promoter-driven hen egg lysozyme (HEL)-transgenic (HEL+, originally called ILK3) (29), MD4 ($IgMD^{HEL}$) (30), γγ4 (HyHEL10-IgG1) (31), and $Ptprc^{SJL}$ (CD45.1) mice were backcrossed seven or more generations to B10.BR. $Roquin^{san}$ C57BL/6 mice were backcrossed to TCR+HEL+ mice with a mixed (CBA × B10.BR) background, fixing $H2^{k}$. Bim-deficient mice have been described (32), and $Cd79a^{ken}$ is an N-ethyl-nitrosourea–induced null allele resulting from a premature stop codon in exon 2 that abolishes *CD79a* mRNA and results in a completely nonleaky block in B-cell development and antibody formation (33). Mice in Fig. 4 were third, fourth, and fifth generation backcross of the TCR and HEL transgenes from B10.BR to CBA. The Animal Ethics and Experimentation Committee of the Australian National University approved all procedures.

Analysis of anti-HEL IgG. HEL-binding IgG was measured by ELISA on Nunc MaxiSorp plates coated with 5 μg/mL HEL (Fluka). Serial serum dilutions were applied followed by alkaline phosphatase-conjugated goat anti-mouse IgG or IgG2a (Southern Biotechnology) and then Phosphatase Substrate (Sigma). Plates were read at 405 nm with a Thermomax Microplate Reader (Molecular Devices). Anti-HEL IgG titers were interpolated from a standard curve made using serum from HyHEL10-IgG1 mice (31) analyzed on the same plate, whereas pooled serum from $Roquin^{san/san}$ TCR+HEL+ mice was used to generate a standard curve for anti-HEL IgG2a.

Production of anti-HEL and anti-OVA immune serum or IgG. HEL-immune serum was collected from mice immunized intraperitoneally 4 weeks earlier with 100 μg HEL protein in 4.5% alum, or 4.5% alum alone for control serum. Alternatively, a 50 μL emulsion containing 50 μg HEL or OVA mixed 1:1 with complete Freund's adjuvant (CFA; Sigma) was injected subcutaneously in each flank. To purify IgG, serum was clarified by centrifugation, diluted 20-fold with binding buffer (300 mM NaCl, 100 mM Tris/HCl, pH 8.0) and filtered through a 0.45 mm Millipore (Billerica) membrane. Diluted serum aliquots of 20 mL were applied to HiTrap Protein G Sepharose column (GE Healthcare). IgG eluted with 0.1 M glycine/HCl (pH3) was collected in 1.0 mL fractions buffered with 30 μL of 3.0 M Tris/HCl (pH8).

Injections of serum, purified IgG, or monoclonal antibodies. Neonates were injected intraperitoneally with 17 μg purified anti-HEL or anti-OVA IgG per gram of body weight or 50 μL serum on days 1, 3, and 5 after birth. Mice were injected intraperitoneally with 20 μg of monoclonal Fcγ*R*-blocking antibody (2.4G2; BioXCell) or rat IgG2b isotype control (LTF2; BioXCell) on days 1, 3, 5, 8, 11, 14, and 17 after birth. These neonates were also injected intraperitoneally with 50 μL anti-HEL immune serum on days 2, 4, and 6 after birth. Adult mice were injected with 200 μL serum per dose.

Diabetes incidence and pancreas histology. Urine glucose was tested using Uristix (Bayer) twice per week. In mice with two successive positive urine glucose tests, blood glucose was measured, and mice with blood glucose >10 mmol/L were called diabetic. In the Fig. 4 cohort, urine glucose was tested from individual mice when their cage was observed to be wet, and mice with glucosuria >111 mmol/L (++++ on Uristix) were called diabetic. For histology, the pancreas was fixed in 10% formalin, paraffin embedded, and stained with hematoxylin and eosin.

Islet transplantation. To isolate islets, the pancreas of CBA mice was infused via the common bile duct with 3 mL of Hanks' buffered saline solution (HBSS) containing 3 mg/mL collagenase P (Roche, 11 213873 001), 10 μg/mL DNAse I (Roche, 0104159), 0.15% w/v bovine serum albumin plus penicillin, and streptomycin. Infused pancreata were dissected free and incubated for 15 min at 37°C before replacement of the medium with HBSS containing 10% bovine fetal serum. Islets were separated by two rounds of "hand-picking" using a drawn glass pipette under a dissecting microscope. For transplantation, two depots of 100–125 islets each were incorporated into clots of recipient blood and placed under the kidney capsule, and blood glucose was measured weekly. Eighty-five percent of transplanted diabetic TCR+HEL+ animals became euglycemic within 1–2 weeks.

Flow cytometry. Lymphocytes were stained as described previously (34) using the following antibodies: 1G12 anticlonotype (mouse IgG1, detecting the 3A9 αβ TCR [TCR^{HEL}]) (35) culture supernatant followed by rat anti-mouse IgG1-APC; anti–CD8a-PerCP; anti–CD4-FITC-PerCP or -PE, anti–CD3-PE, anti-CXCR5-biotin, anti–CD69-PE, and anti-B220-APCCy7 (BD Biosciences); anti–CD5-FITC and anti–CD25-PE (Caltag); anti-CD45.2-PacificBlue and anti-CD45.1-Alexa700 (BioLegend); and anti–PD-1-PE (eBioscience). An LSR II flow cytometer (BD Biosciences) was used for data acquisition, and FlowJo software (Treestar) was used for analysis.

Cell culture. Spleen and lymph node single-cell suspensions containing 1×10^5 cells in tissue culture medium (RPMI containing 5% fetal calf serum, 2 mM L-glutamine, penicillin, streptomycin, and nonessential amino acids [Gibco], 1 mM sodium pyruvate plus 10 mM Hepes buffer [Sigma]) were placed into flat-bottomed 96-well plates with or without 50 μg/mL HEL protein for 5 days; cell supernatants were then collected and stored at −20°C. Cytokines in supernatants were measured using the Cytometric Bead Array kit (BD Biosciences).

Cell transfer. Whole splenocytes from TCR+ mice were labeled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE) and injected intravenously into recipients at a TCR^{HEL}+ CD4+ cell dose of $\sim 3 \times 10^5$ per mouse.

Statistical analysis. Diabetes incidences were compared with the log-rank test using GraphPad Prism. Otherwise, Mann-Whitney *U* tests were used except for the "ratio *t* test" used in Figs. 6*B* and 6*C*, the rationale of which is described in GraphPad Prism Statistics Guide (http://www.graphpad.com).

RESULTS

Accelerated diabetes in $Roquin^{san/san}$ TCR+HEL+ mice. A point mutation in the *Roquin* gene (*Rc3h1*, $Roquin^{san}$) exaggerates inducible T-cell costimulator expression and formation of T follicular helper (Tfh) cells, and causes systemic autoimmunity (36,37). To investigate its effect on islet-specific CD4 T–cell tolerance mechanisms, $Roquin^{san}$ mice were crossed with TCR+HEL+ double-transgenic mice that have an increased frequency of islet-reactive CD4+ T cells. The HEL transgene encodes HEL under the insulin gene promoter, and mirrors the pattern of insulin expression with high expression in islet β-cells, nanomolar concentrations in serum, and *Aire*-dependent expression in thymic medullary epithelial cells (29,38). The TCR transgene encodes the T-cell receptor from clone 3A9, which recognizes a dominant HEL peptide (46–61) presented by $I\text{-}A^{k}$ (28).

The resulting TCR+HEL+ mice make it possible to measure cellular mechanisms of CD4 T–cell tolerance. They are, on the one hand, an experimentally contrived model because of their increased thymic production of islet-reactive CD4+ T cells recognizing a single, experimentally expressed autoantigen that mirrors but may not exactly replicate insulin itself. On the other hand, only ~20% of TCR+HEL+ animals progress to β-cell destruction and diabetes on the diabetes-resistant B10BR strain background due to the combined action of thymic deletion, Treg function, and peripheral anergy. Perturbations found to precipitate diabetes in TCR+HEL+ mice—such as inherited defects in AIRE, Bim-induction, interleukin (IL)-2 signaling, and Cbl-b—have revealed cellular mechanisms that are highly relevant to human type 1 diabetes and to antigenically complex models such as the NOD mouse strain or the KDP rat strain, in which mechanisms regulating islet-reactive CD4 cells are difficult or impossible to measure (29,34,39–41). Hence we used this model to investigate how dysregulated formation of Tfh cells affects the regulation of islet-reactive CD4 T cells.

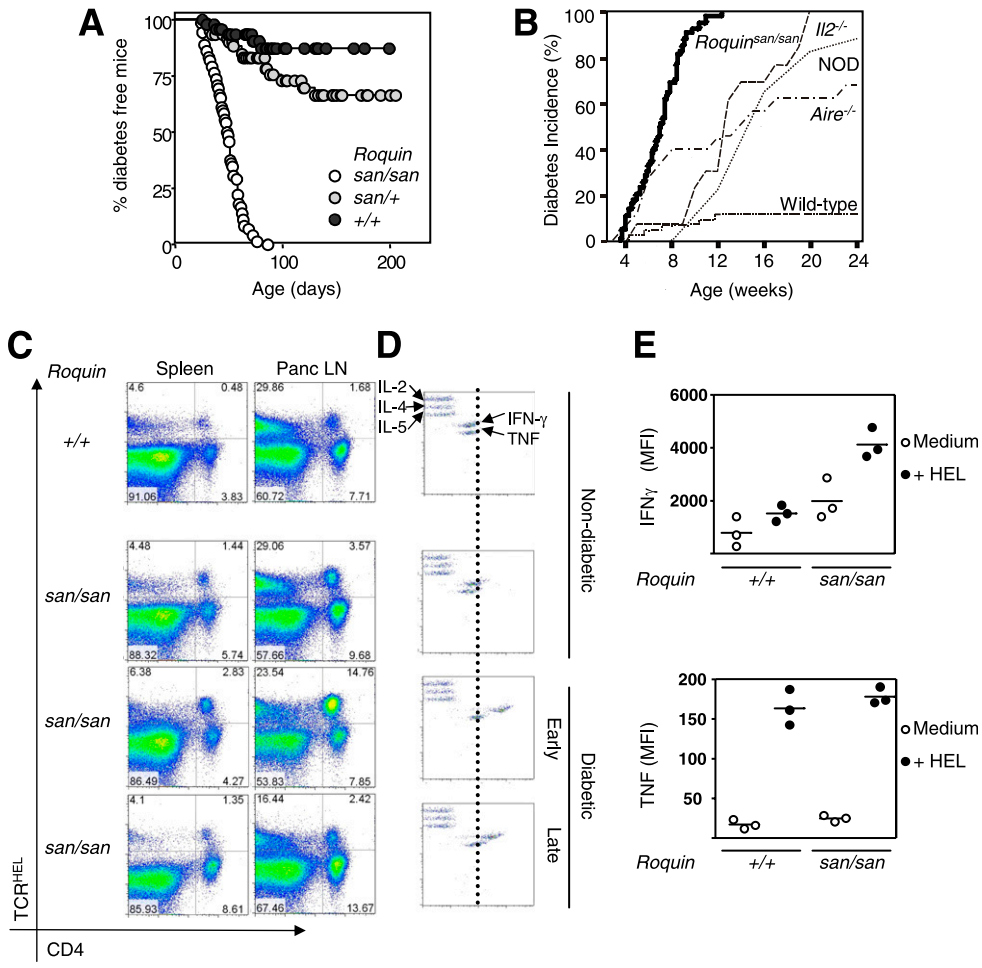

FIG. 1. Cooperation between *Roquin*^{san} mutation and increased frequency of islet-specific CD4 cells for progression to diabetes. *A*: Diabetes incidence curves in TCR+HEL+ mice that were either *Roquin*$^{san/san}$ (white), *Roquin*$^{san/+}$ (gray), or *Roquin*$^{+/+}$ (black). *B*: Comparison of diabetes incidence in *Roquin*$^{san/san}$ TCR+HEL+ mice with previously described TCR+HEL+ mice bearing other diabetes susceptibility mutations. *C*: Representative flow cytometry showing the frequency of TCR^{HEL}+ (detected with the anticlonotype [1G12] antibody) CD4+ cells (*right upper quadrant*) in spleen (*left column*) and draining PLNs (right column) in: (from top to bottom) nondiabetic *Roquin*$^{+/+}$, nondiabetic *Roquin*$^{san/san}$, early diabetic (<1 week after onset) *Roquin*$^{san/san}$, or late diabetic (>1 week after onset) *Roquin*$^{san/san}$ TCR+HEL+ mice. A summary of data from multiple mice is shown in Supplementary Fig. 2. *D*: Splenocytes were cultured with 50 μg/mL HEL protein for 5 days; cell supernatants were then collected and analyzed for cytokines using the Cytometric Bead Array system. Representative flow cytometry plots displaying from top to bottom the beads that detect IL-2, IL-4, IL-5, γ-interferon, and tumor necrosis factor for the same mice as in (*C*). *E*: Quantification of cytokines in the culture supernatants of splenocytes from multiple nondiabetic *Roquin*$^{+/+}$ or early diabetic *Roquin*$^{san/san}$ TCR+HEL+ mice stimulated with HEL protein (50 μg/mL) for 5 days. The experiments in *C–E* were performed twice with at least three mice per group. (A high-quality color representation of this figure is available in the online issue.)

The *Roquin*^{san} mutation dramatically increased progression to type 1 diabetes in TCR+HEL+ mice such that 100% of *Roquin*$^{san/san}$ homozygotes developed diabetes by 8 weeks of age (Fig. 1*A*). The median onset of type 1 diabetes in these mice was earlier than TCR+HEL+ mice with mutations in *Aire*, *Il2* or NOD non-MHC diabetes-susceptibility genes (Fig. 1*B*) (34,38,40). The *Roquin*^{san} mutation had no discernable effect on thymic deletion of islet-reactive CD4 T cells bearing the 3A9 TCR (TCR^{HEL}) (Supplementary Fig. 1), but the frequency of these cells was increased in the pancreatic lymph node (PLN) of *Roquin*$^{san/san}$ mice (Fig. 1*C* and Supplementary Fig. 2). TCR^{HEL}+CD4+ cells from *Roquin*$^{san/san}$ mice divided extensively ex vivo in response to HEL (not shown) and produced elevated levels of γ-interferon (Fig. 1*D* and *E*), indicating that they were not anergic, unlike their counterparts from wild-type TCR+HEL+ mice (29). There was no detectable increase in secreted IL-4 or IL-5, suggesting that the islet-reactive cells in *Roquin*$^{san/san}$ TCR+HEL+ mice are predominantly Th1 cells. Thus, *Roquin*$^{san/san}$ TCR+HEL+ animals provide an experimental model of spontaneous, rapidly developing diabetes that stems from increased frequency of islet-reactive CD4 cells (due to TCR and HEL transgenes) and breakdown in peripheral tolerance (due to *Roquin* mutation).

***Roquin*^{san}-accelerated diabetes requires B cells.** B cells were of particular interest in the accelerated diabetes caused by the *Roquin*^{san} mutation because accumulation of Tfh cells causes spontaneous germinal centers and IgG autoantibodies that characterize nontransgenic *Roquin*$^{san/san}$ mice (36,42). *Roquin*$^{san/san}$ TCR+HEL+ mice had elevated Tfh cell frequencies in PLN compared with those in *Roquin*$^{san/+}$ TCR+HEL+ mice, which reached 10-fold when the heightened CD4+ T–cell frequency was taken into account (Fig. 2*A*). Germinal center B cells were also detected at increased frequencies in PLN of *Roquin*$^{san/san}$ TCR+HEL+ mice (Fig. 2*B*). *Roquin*$^{san/san}$ TCR+HEL+ mice examined 11 days after birth exhibited lymphocytic

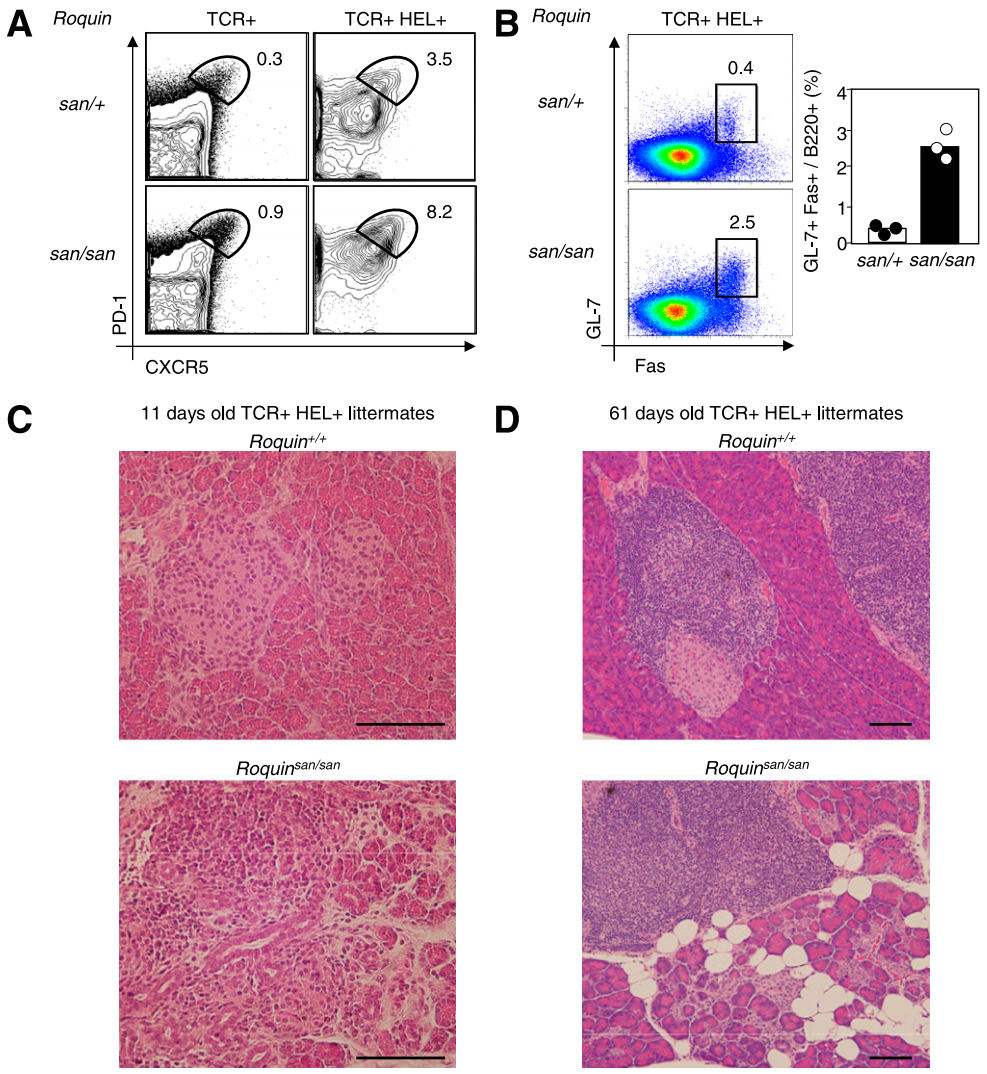

FIG. 2. Increased Tfh cells and germinal centers in PLNs of *Roquin*$^{san/san}$ TCR+HEL+ mice. *A* and *B*: Flow cytometric analysis and percentage of PD-1^{high}CXCR5^{high} (Tfh) cells among CD4+ T cells (*A*) and GL-7+ Fas+ germinal center B cells among B220+ cells (*B*) in PLNs of 4-week-old *Roquin*$^{san/san}$ or *Roquin*$^{san/+}$ TCR+ mice with or without the insHEL transgene; mice in (*B*) are HEL+TCR+. *C* and *D*: Pancreas sections from 11-day-old (*C*) and 61-day-old (*D*) *Roquin*$^{+/+}$ TCR+HEL+ or *Roquin*$^{san/san}$ TCR+HEL+ littermates stained with hematoxylin and eosin. All scale bars represent 100 μm. Images are representative of three infant and at least six adult pancreata per group. (A high-quality digital representation of this figure is available in the online issue.)

insulitis, whereas *Roquin*$^{+/+}$ TCR+HEL+ littermates did not (Fig. 2*C*). Regardless of *Roquin* genotype, nearly all islets in adult TCR+HEL+ mice exhibited peri-insulitis or insulitis (Fig. 2*D*), but focal to generalized exocrine pancreatitis was only observed in *Roquin*$^{san/san}$ mice (Fig. 2*D*).

Roquin$^{san/san}$ TCR+HEL+ mice also developed high titers of anti-HEL IgG autoantibodies in serum (Fig. 3*A*), including antibodies of the IgG2a isotype (Fig. 3*B*) characteristic of Th1-driven immune responses. Autoantibody titers correlated with progression to diabetes in heterozygous *Roquin*$^{san/+}$ TCR+HEL+ mice (Fig. 3*A*), and were characteristic of type 1 diabetes progression in *Roquin*$^{san/san}$ TCR+HEL+ mice, as high titers were not observed in diabetic TCR+HEL+ mice with mutations in *Aire* or *Il-2* (Fig. 3*C*). Remarkably, the diabetes incidence in *Roquin*$^{san/san}$ TCR+HEL+ mice rendered B cell deficient by a null mutation in *Cd79a* (*Cd79a*^{ken}) was no greater than in B cell–deficient *Roquin*$^{+/+}$ TCR+HEL+ mice (Fig. 3*D*).

Roquin$^{san/san}$ serum is sufficient to drive _Roquin_^{san}-accelerated diabetes, whereas intact B-cell antigen presentation is not. The requirement for B cells in *Roquin*^{san}-accelerated autoimmune diabetes could reflect the function of B cells acting as antigen-presenting cells or the function of secreted IgG autoantibodies. To investigate this, we generated *Roquin*$^{san/san}$ TCR+HEL+ mice in which high frequencies of B cells expressed HEL-specific IgM and IgD transgenes (MD4 [30]). Surprisingly, inheritance of IgMD^{HEL} decreased the diabetes incidence in *Roquin*$^{san/san}$ TCR+HEL+ mice so that diabetes incidence was no greater than in *Roquin*$^{+/+}$ TCR+HEL+ mice (Fig. 3*E*). This result demonstrates that intact antigen presentation by B cells is not sufficient to drive *Roquin*^{san}-accelerated diabetes when the B cells cannot secrete islet-specific IgG.

Next, to test whether *Roquin*$^{san/san}$ serum was sufficient to trigger *Roquin*^{san}-accelerated diabetes, we transferred serum from diabetic *Roquin*$^{san/san}$ TCR+HEL+ mice or control serum pooled from diabetic *Roquin*$^{san/+}$ and *Roquin*$^{+/+}$

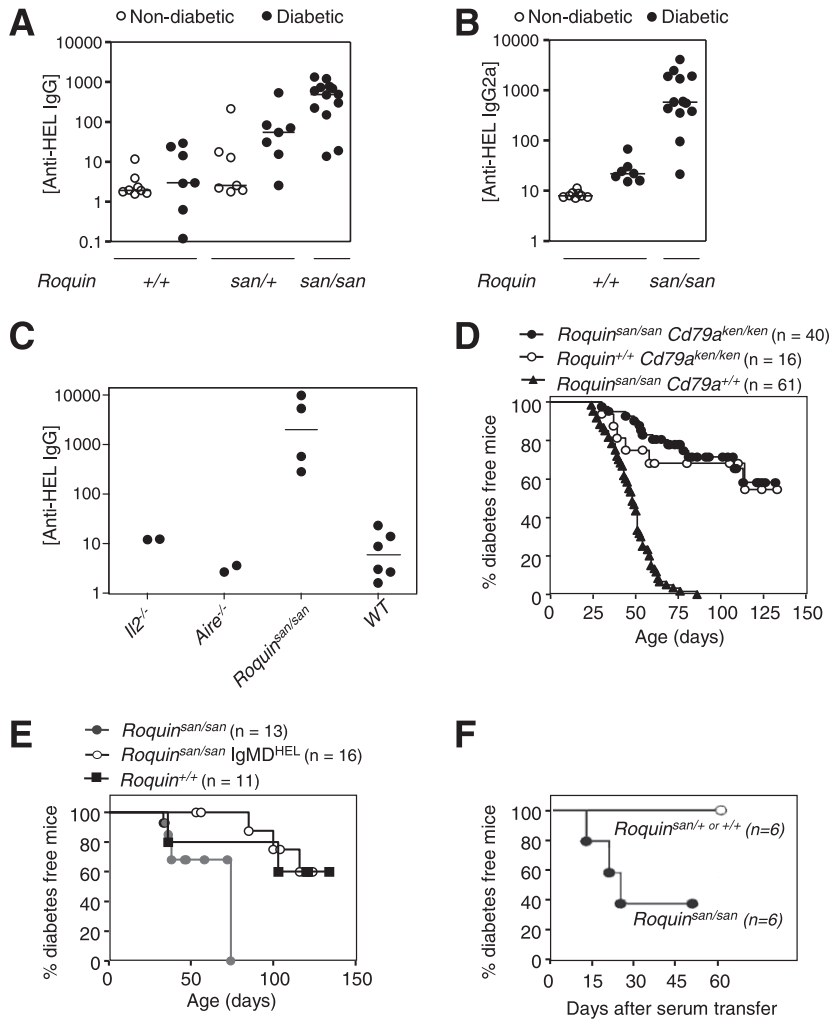

FIG. 3. High autoantibodies and requirement for B cells for accelerated progression to diabetes in *Roquin*$^{san/san}$ TCR+HEL+ mice. *A* and *B*: Titers of anti-HEL IgG (*A*) and IgG2a (*B*) in TCR+HEL+ mice of the indicated *Roquin* genotypes and diabetes status. *C*: Anti-HEL IgG titers in diabetic TCR+HEL+ mice of the indicated genotypes. *D*: Diabetes incidence in *Roquin*$^{san/san}$ TCR+HEL+ mice that were either B cell deficient (*Cd79a*$^{ken/ken}$; filled circles) or B cell sufficient (*Cd79a*$^{+/+}$; triangles) and in *Roquin*$^{+/+}$ *Cd79a*$^{ken/ken}$ TCR+HEL+ mice (open circles). *E*: Diabetes incidence in *Roquin*$^{san/san}$ TCR+HEL+ mice with or without an IgMD^{HEL} transgene that prevents isotype switching of HEL-specific B cells, and control TCR+HEL+ mice with normal *Roquin* and B-cell repertoire. *F*: Diabetes incidence in *Roquin*$^{san/+}$ TCR+HEL+ mice after transfer of a single 200 μL dose of serum from diabetic *Roquin*$^{san/san}$ TCR+HEL+ mice (closed symbols) or from diabetic *Roquin*$^{san/+}$ or *Roquin*$^{+/+}$ TCR+HEL+ mice (open symbols).

TCR+HEL+ mice into nondiabetic *Roquin*$^{san/+}$ TCR+HEL+ mice. Four of six recipients of *Roquin*$^{san/san}$ serum developed diabetes within 25 days, whereas six recipients of the control serum remained nondiabetic (Fig. 3*F*).

Maternal transmission of anti-islet antibodies promotes diabetes in predisposed offspring. We also investigated the role of anti-islet IgG in contexts other than the *Roquin*^{san} mutation. A unique feature of the breeding strategy used to produce the TCR+HEL+ animals represented in Fig. 4 was that one parent was TCR+HEL+ and had previously developed diabetes that was cured by transplanting nontransgenic islets lacking the HEL autoantigen. Once euglycemia was restored, the mouse was bred with a nontransgenic partner. Fifty-five percent of TCR+HEL+ offspring of diabetes-cured TCR+HEL+ mothers were diabetic by 150 days old, compared with 20% of otherwise matched offspring of diabetes-cured fathers (Fig. 4*A*). The increase in diabetes incidence occurred in both male and female offspring but was more severe in males (Fig. 4*B*). This demonstrates that there is a naturally occurring diabetogenic factor that is transmitted from mother to offspring throughout the breeding life of a formerly diabetic mother. Furthermore, this maternal factor discloses an unexpected sex dimorphism in susceptibility to diabetes in the TCR+HEL+ offspring. Anti-HEL IgG was present in the serum of diabetes-cured mothers around the time of transplantation and also >200 days after transplantation (Fig. 4*C*), indicating that their offspring were open to vertical transmission of anti-HEL IgG (43).

To test whether a maternally transmitted cofactor could sensitize the offspring to diabetes in a setting where mothers were themselves diabetes free, wild-type nontransgenic females were immunized with HEL antigen in CFA to elicit HEL-specific IgG antibodies and then mated with TCR+HEL+ males (Fig. 5*A*). The neonates had high titers of anti-HEL IgG, which declined by 70 days after birth, confirming their maternal origin (Fig. 5*B*). All of the TCR+HEL+ offspring developed diabetes 3–6 weeks after birth, whereas littermates lacking either or both transgenes

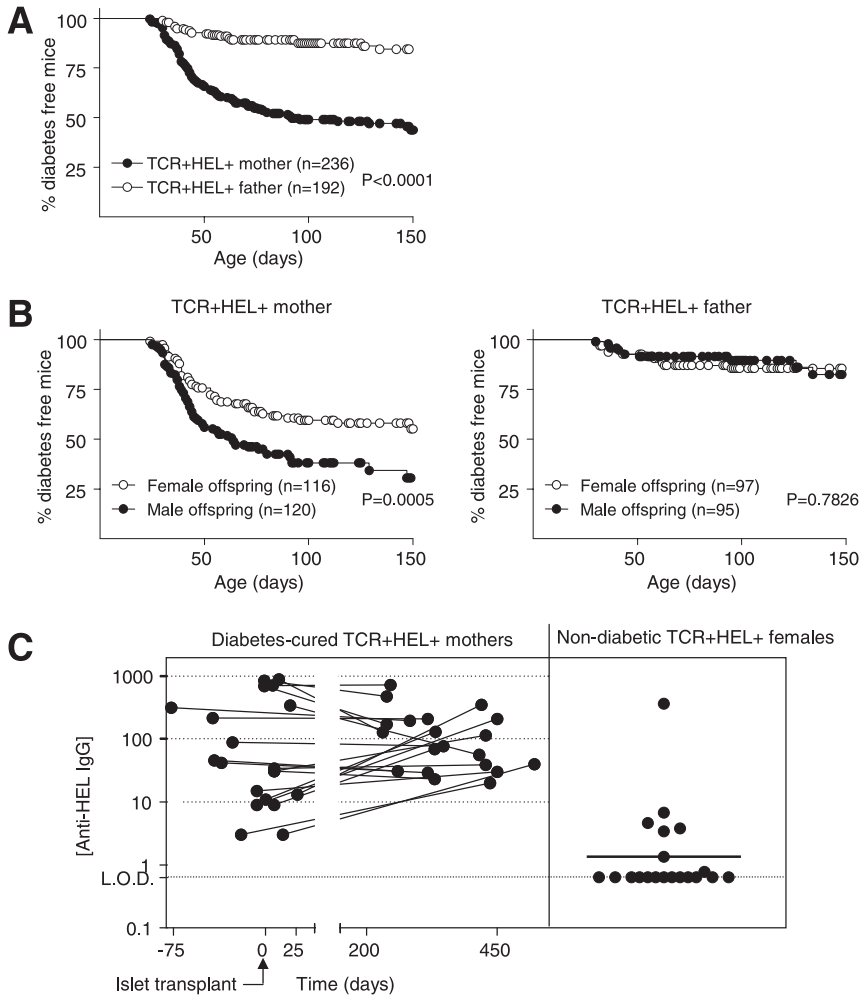

FIG. 4. A maternally transmitted epigenetic factor increases diabetes incidence in the TCR+HEL+ offspring of formerly diabetic mothers. *A*: Diabetes incidence in TCR+HEL+ mice bred from TCR+HEL+ mothers or fathers that had spontaneously developed diabetes and had euglycemia stably restored by transplanting nontransgenic islets. The diabetes-cured TCR+HEL+ parents were bred with nontransgenic partners. Statistical analysis used the log-rank method. *B*: Diabetes incidence for the cohort described in (*A*) stratified by sex of the offspring. *C*: For each of 20 diabetes-cured TCR+HEL+ female breeders used to generate the cohort described in (*A*), two serial serum anti-HEL IgG titers (connected by a line) are plotted against the time of blood sampling relative to islet transplantation on day 0 (*left*). Serum anti-HEL IgG titers for 20 nondiabetic TCR+HEL+ females from the same colony as the diabetes-cured females (*right*).

did not become diabetic (Fig. 5*C*). Diabetes was not increased when the mothers were immunized with a control antigen OVA (Fig. 5*D*).

To determine whether anti-HEL IgG was sufficient to trigger diabetes, neonatal TCR+HEL+ mice from unmanipulated breeders were given serum, or in a separate experiment purified IgG, from mice immunized with HEL/CFA. Diabetes developed at 3–4 weeks of age in all neonates receiving HEL-immune serum and by 15 days of age in neonates receiving purified anti-HEL IgG but not in controls receiving anti-OVA serum or IgG (Fig. 5*E* and *F*).

Anti-islet antibody acts through Fcγ*R*-bearing cells. That diabetes did not occur in HEL+ single-transgenic offspring suggests that the antibodies do not cause direct lysis of islets but rather act indirectly by enhancing the activation of islet-reactive CD4+ T cells. To test antibody-mediated effects on CD4+ cells, splenocytes from CD45.1 TCR+ transgenic donors were labeled with CFSE and injected into CD45.2 HEL+ or HEL– recipients. HEL+ recipients were given a course of injections of HEL-immune or control serum, and the fate of the HEL-specific T cells was tracked by flow cytometry of recipient spleen (Fig. 6*A*) using the ratio of donor CD4+ cells to donor B cells to normalize any mouse-to-mouse differences in the number of cells injected. Treatment of recipients with HEL-immune serum increased the numbers of HEL-reactive CD4+ cells present at days 3, 5, and 7 (Fig. 6*B*) and their CFSE dilution at each time-point analyzed (Fig. 6*C*).

To determine whether the HEL-immune serum enhances CD4+ cell proliferation and/or survival, the experiment was repeated using donor splenocytes that were Bim-deficient and therefore resistant to cell death (32). Interestingly, although the Bim-deficient T cells were triggered to proliferate specifically in the HEL+ recipients as expected, CFSE dilution within Bim-deficient T cells was equivalent regardless of whether mice were treated with HEL-immune or control serum (Fig. 6*D*). This suggests that HEL-immune serum does not enhance cell proliferation, but rather it inhibits the death of cells that have proliferated.

Antibody-mediated activation of T cells would appear to occur through antigen-presenting cells expressing receptors for the Fc portion of IgG (FcγR), given that $CD4^+$ T cells themselves do not express FcγR. To test this, we

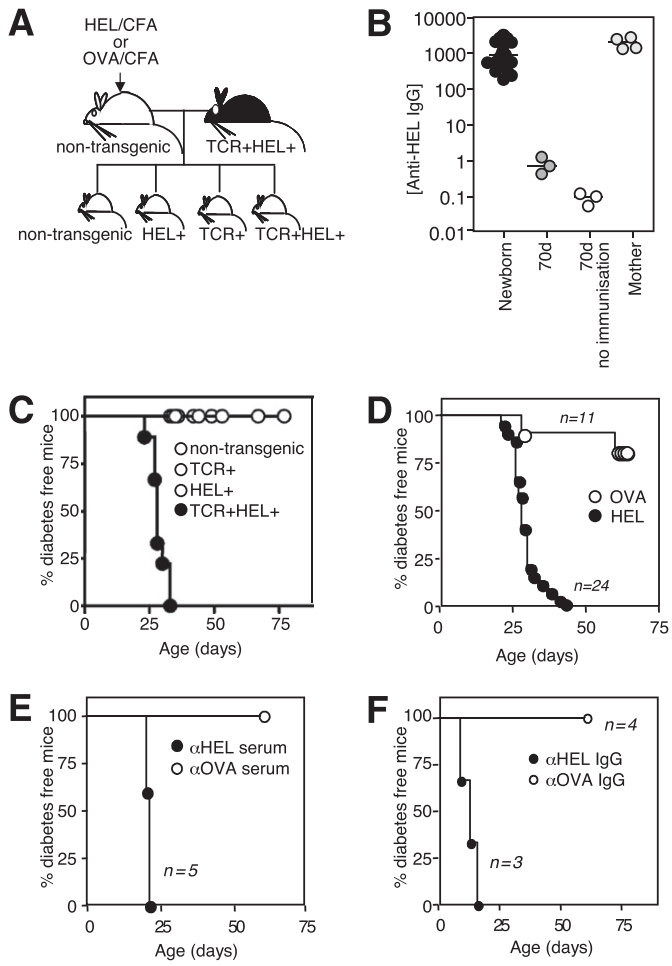

FIG. 5. Effect of passive transfer of antibody on diabetes in TCR+ HEL+ mice. *A*: Breeding strategy for maternal antibody transfer experiments. Nontransgenic B10.BR females were immunized with HEL or OVA protein emulsified in CFA to induce specific antibodies and then mated with TCR+HEL+ males to yield 25% TCR+HEL+ offspring and equal numbers of singly transgenic or nontransgenic littermates. *B*: Anti-HEL IgG titers in newborns from mothers immunized as illustrated in (*A*); 70-day-old pups from immunized or unimmunized mothers and mothers 5 weeks after immunization. *C*: Diabetes incidence in offspring with the indicated genotypes bred from HEL-immunized mothers. *D*: Diabetes incidence in TCR+HEL+ offspring of mothers immunized with either HEL (closed symbols) or OVA (open symbols). *E* and *F*: Diabetes incidence in TCR+HEL+ neonates injected on days 1, 3, and 5 after birth with serum (*E*) or purified IgG (*F*) from HEL- or OVA-immunized mice.

injected neonates with three doses of anti-HEL serum on days 2, 4, and 6 after birth and either a monoclonal Fcγ*R*-blocking antibody or rat IgG2b isotype control on days 1, 3, and 5 and twice a week thereafter until day 17. FcγR blockade delayed the onset and reduced the incidence of diabetes (Fig. 6*E*). Thus, in the presence of islet-reactive CD4 T cells, anti-islet autoantibodies trigger diabetes via a mechanism that requires antibody recognition by FcγR+ cells.

DISCUSSION

Here we study the role of B cells in type 1 diabetes and show that anti-islet IgG autoantibodies accelerate CD4 T cell–driven diabetes. The anti-islet antibodies were insufficient to precipitate diabetes on their own, arguing against a direct antibody-mediated destruction of β-cells. Instead, they acted in an Fcγ*R*-mediated manner to enhance accumulation of islet-reactive CD4+ T cells and promote diabetes in animals that were genetically predisposed because of increased frequency of islet-reactive CD4+ T cells. The accelerated progression to type 1 diabetes in *Roquin*^{san} TCR+HEL+ animals with high-titer anti-islet antibodies demonstrates a principle of positive feedback between dysregulation of autoreactive helper T cells and the resulting autoantibodies.

Although the experimental evidence that B cells contribute to diabetes induction in NOD mice is strong, whether autoantibodies play a prominent role is unclear. Evidence for a contribution by autoantibodies came from reports of diabetes suppression when maternal antibody transfer was abrogated (22,23). New data have raised the question of whether the epigenetic factor is islet-specific autoantibody transmitted from mother to offspring or altered colonization of the offspring by microbial commensals whose interaction with the innate immune system influences diabetes incidence in NOD mice (44). Furthermore, maternal hyperglycemia can perturb fetal islet development (45), which could affect tolerance to the islets. Evidence against a role for antibodies came from the observation that fortnightly injections of 70 μg purified Ig from diabetic NOD mice could not induce diabetes in B cell–deficient NOD.*Ighm*^{null} mice (10), although it is possible that the concentration of active antibody achieved was suboptimal. Furthermore, anti-insulin VH IgM transgene (VH125Tg) restored diabetes in NOD.*Ighm*^{null} mice in the absence of measurable anti-insulin antibodies (12). Given the lack of diabetes enhancement observed here with an anti-HEL IgM transgene and dramatic enhancement by anti-HEL IgG antibody, these collective experimental findings imply that antigen presentation by islet-specific B cells and secretion of anti-islet IgG may both serve as positive feedbacks compounding the dysregulation of islet-reactive T cells but with varying significance in different individuals.

Cross-presentation of islet autoantigen by dendritic cells (DCs) to islet-specific CD8+ cells was enhanced by coinfusion of rabbit anti-islet antibodies, and this promoted diabetes (27). Our results reinforce and complement these findings by demonstrating that spontaneously produced anti-islet autoantibodies, either made by the animal itself or passively transferred as maternal antibody, can overcome thymic and peripheral tolerance mechanisms to trigger CD4+ cell–mediated autoimmune diabetes in an Fcγ*R*-dependent manner. Although the inhibition of HEL-immune serum-induced diabetes by anti-FcγR (2.4G2) reveals a crucial role for FcγRs, the specific FcγR+ cell types that are pivotal in unleashing the autoimmune cascade remain unclear. 2.4G2 binds to both the inhibitory FcγRIIb (CD32) and the activatory FcγRIII (CD16) molecules (46), but its immunosuppressive effect is presumably due to its binding to FcγRIII. Blockade of FcγRIII expressed by antigen-presenting cells would inhibit the uptake of antigen-antibody complexes and would prevent antigen-presenting cell maturation, but the blockade may also inhibit the activation of immune effector cells such as macrophages, natural killer cells, and granulocytes (47). Indeed, reinstating FcγR+ cells in either the afferent (purified DCs) or the efferent (purified natural killer cells) arm of the immune response was sufficient to increase diabetes in NOD mice lacking the FcR common γ chain (26). Although we cannot exclude a direct action on effector cells, increased autoantigen uptake by and maturation of DCs is likely to underlie the enhancement of islet-specific CD4+ T–cell survival by HEL-immune serum.

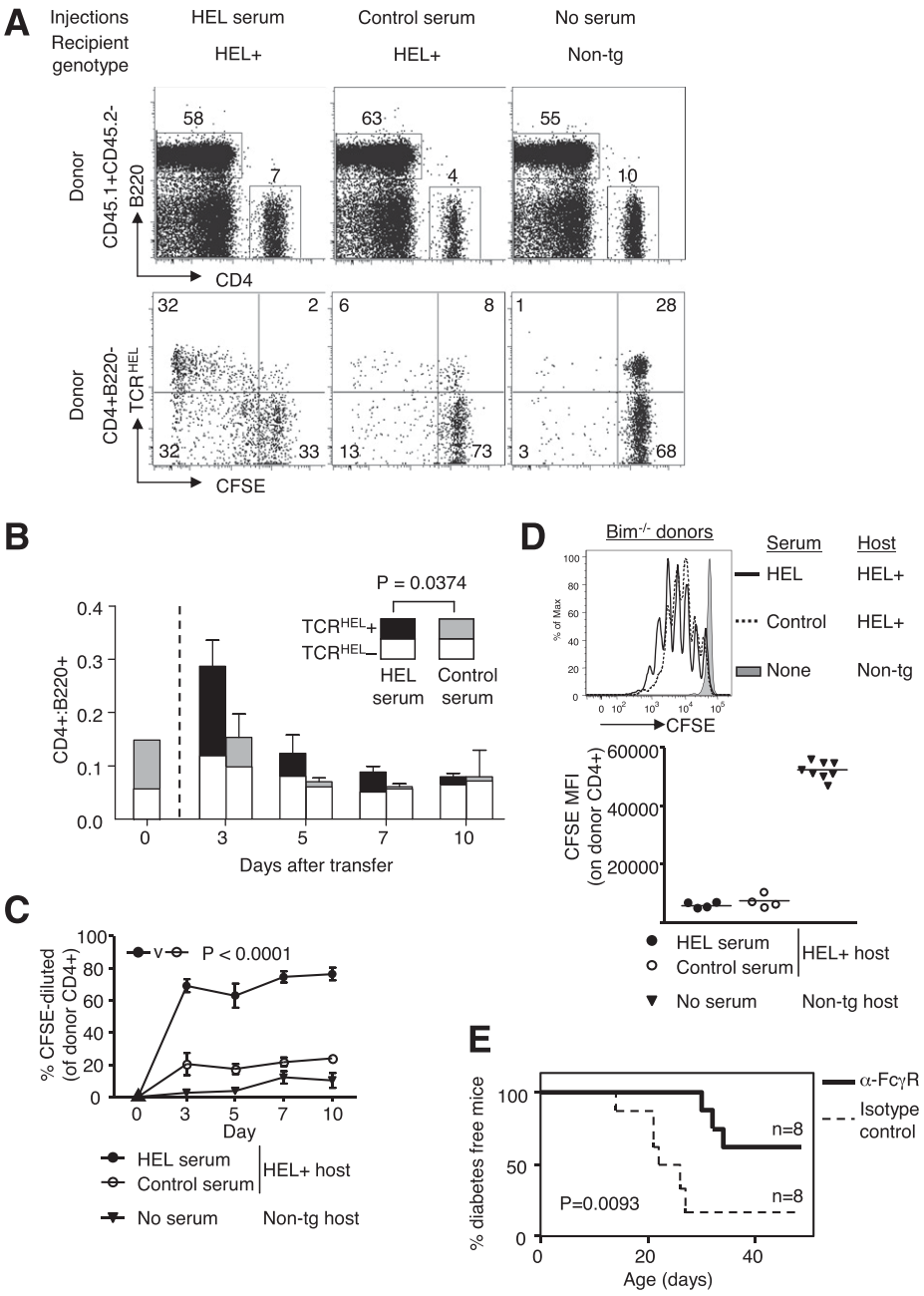

FIG. 6. Anti-HEL autoantibodies enhance accumulation of HEL-specific CD4+ T cells. Splenocytes from TCR+ mice (bearing the CD45.1 allelic marker) were labeled with the cell division dye CFSE and injected intravenously into 24 HEL+ recipients (CD45.2) on day 0. Twelve recipients were given 200 μL HEL-immune serum and twelve were given 200 μL control serum i.p. starting on day −1 and on alternate days thereafter in surviving mice. Flow cytometry on recipient spleens was performed on days 3, 5, 7, and 10, each time using three mice from each treatment group plus two control nontransgenic mice that had received cells but no serum injections. *A*: Representative flow cytometric plots (from day 5 after transfer) showing gates used to track donor-derived CD4$^+$ cells and B220$^+$ "tracer" B cells (*upper panel*), plus TCR^{HEL} expression and CFSE dilution on the donor-derived CD4$^+$ cells (*lower panel*). *B*: Graphs show the ratio of donor CD4$^+$ cells to donor B220$^+$ cells, and the shaded areas represent the proportion of TCR^{HEL}+ cells among donor-derived CD4$^+$ cells. *C*: Percentage of CFSE-diluted cells among donor-derived CD4$^+$ cells. In (*B*) and (*C*), symbols indicate group means; error bars show the SD. Statistical differences between HEL-immune and control serum groups were analyzed using a ratio *t* test (see RESEARCH DESIGN AND METHODS). The data shown are from one of three experiments that yielded comparable results. *D*: The experiment described in *A–C* was repeated, except that the TCR+ donors were CD45.2+ Bim-deficient mice and the recipients were CD45.1+. Histogram (*top*) displays CFSE on donor (CD45.2+) CD4+ cells in HEL+ hosts that received HEL-immune or control serum, or in nontransgenic hosts as indicated, with a summary of the data (*bottom*) for multiple recipients. *E*: Diabetes incidence in TCR+HEL+ neonates injected on days 2, 4, and 6 after birth with serum from HEL-immunized mice and on days 1, 3, 5, 8, 11, 14, and 17 after birth with Fcγ*R*-blocking antibody or rat IgG2b isotype control. Statistical analysis used the log-rank method.

The role of B cells and autoantibodies in human type 1 diabetes is complex and poorly understood because although anti-islet autoantibodies are the best current predictors of future type 1 diabetes, the observational studies that followed multiple cohorts of subjects with either mothers or fathers with type 1 diabetes have revealed paradoxical protection associated with transmission of anti-islet autoantibodies—albeit dependent on HLA-DR status. Genome-wide association studies and studies of HLA polymorphisms indicate that the pathogenesis of human type 1

diabetes is likely to be heterogeneous, and hence it is conceivable that the diabetes-promoting role of B cells and anti-islet antibodies may not be uniform. Islet autoantibodies may be active cofactors in human type 1 diabetes pathogenesis once islet-reactive CD4+ T cells have escaped thymic or peripheral tolerance. In our transgenic mouse studies, this occurs congenitally because of the high frequency of islet-specific CD4 cells formed in the thymus even before birth, whereas it may only occur years after birth in humans, for example because of inherited gene variants of *HLA-DR*, *CTLA4*, or *AIRE*. The findings here, by defining mechanisms by which anti-islet IgG autoantibodies cooperate with incomplete thymic deletion to promote clonal expansion of islet-reactive CD4 T cells in vivo and progression to diabetes, provide an experimental foundation for future studies in humans and more complex rodent models where it is more difficult to trace the regulation of islet-specific CD4 cells.

The Fcγ*R*-blocking study also shows that inhibiting immune complex recognition by FcγR+ cells is attractive as a therapy. The first B cell–depleting trials with rituximab (rituxan, monoclonal CD20-specific antibody) in type 1 diabetes have reported responsiveness to therapy broadly comparable with anti-CD3 monoclonal antibody, highlighting an important role for B cells or antibody in type 1 diabetes in humans (9). Rituximab is highly effective in depleting recirculating and memory B cells and causes a rapid decrease in autoantibodies in a number of autoimmune diseases (48), although its effect on anti-islet antibodies in the recent phase 2 clinical trial was not described. Our study provides experimental evidence that it will be valuable both to measure anti-islet antibodies as an internal surrogate end point and pathogenic cofactor in future B cell–depleting trials, and to investigate clinical interventions that more specifically target autoantibody secretion or its effects on T-cell activation.

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D.G.S. researched data. S.R.D. researched data and helped write the manuscript. J.H., S.K.L., C.E.T., and D.Y.H. conducted experiments. K.-P.L. helped plan the study. C.C.G. and C.G.V. planned the study and wrote the manuscript.

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