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Cutting Edge: Nonobese Diabetic Mice Deficient in Chromogranin A Are Protected from Autoimmune Diabetes

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T cells reactive to β cell Ags are critical players in the development of autoimmune type 1 diabetes. Using a panel of diabetogenic CD4 T cell clones derived from the NOD mouse, we recently identified the β cell secretory granule protein, chromogranin A (ChgA), as a new autoantigen in type 1 diabetes. CD4 T cells reactive to ChgA are pathogenic and rapidly transfer diabetes into young NOD recipients. We report in this article that NOD.ChgA−/− mice do not develop diabetes and show little evidence of autoimmunity in the pancreatic islets. Using tetramer analysis, we demonstrate that ChgA-reactive T cells are present in these mice but remain naive. In contrast, in NOD.ChgA+/+ mice, a majority of the ChgA-reactive T cells are Ag experienced. Our results suggest that the presence of ChgA and subsequent activation of ChgA-reactive T cells are essential for the initiation and development of autoimmune diabetes in NOD mice. The Journal of Immunology, 2016, 196: 39–43.

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of pancreatic islet β cells and orchestrated by T cells responding to β cell Ags. Therefore, identifying the autoantigens that trigger the autoimmune process is key to preventing or halting disease development. We reported recently on the discovery of a new autoantigen in T1D, chromogranin A (ChgA), in both NOD mice (1) and in human T1D patients (2). ChgA, like insulin, is a protein present in the secretory granules of β cells. We demonstrated that a peptide ligand from ChgA is antigenic for the diabetogenic CD4 T cell clones BDC-2.5, BDC-10.1, and BDC-9.46 (1, 3) and for CD4 T cells from BDC-2.5 TCR-transgenic (Tg) NOD mice (3).

One method for assessing the relative importance of β cell autoantigens in the progression of T1D is through elimination of the Ag in NOD mice. Several Ag-deficient NOD models have been described and include mice deficient in islet amyloid polypeptide (IAPP) (4), glutamic acid decarboxylase (5), and islet Ag-2 (6). With the exception of insulin 1 and insulin 2 (7), knockout (KO) of these Ags had little or no effect on the development of the disease. We report in this article that, in the absence of ChgA, NOD mice do not develop diabetes, a result that indicates a critical role for this protein as an autoantigen in T1D.

Materials and Methods

Mice

NOD and NOD.scid breeding mice were acquired from The Jackson Laboratory and were bred and housed in specific pathogen–free conditions at National Jewish Health. NOD.ChgA−/− mice were bred in our colony by backcrossing C57BL/6.129.ChgA−/− mice (8) onto the NOD background for 10 generations and then intercrossed to generate homozygous KO mice, verifying genotype by PCR. For the microsatellite analysis of NOD.ChgA−/− mice, a low-density panel of 73 microsatellite markers, equally spaced throughout the genome (~30-cM intervals), were used to differentiate between the genetic background of the originating/donor (B6/129) and target/recipient (NOD/ShiLtJ) mouse strains. NOD.BDC-2.5 TCR-Tg (9) and NOD.BDC-6.9 TCR-Tg (10) mice were generated as previously described. Breeding mice and experimental animals were monitored for development of disease by urine glucose testing (Diastix; Bayer), and hyperglycemia was confirmed by blood glucose testing using a OneTouch Ultra glucometer (LifeScan). Mice were considered diabetic when blood glucose levels were >15 mmol/l (270 mg/dl) for two consecutive readings. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Culture of T cell clones

T cell clones (BDC-2.5, BDC-10.1, BDC-5.2.9, and BDC-4.38) were restimulated every 2 wk, as previously described (11). For expansion of cell numbers before transfer, T cells were subcultured for 4 d after restimulation with additional IL-2.

Ex vivo analysis of pancreas

Single-cell suspensions from pancreata were prepared, as previously described (4), before being stained with an appropriate master mix of Abs and tetramers.
Histology
Pancreas was fixed in 10% formalin and embedded in paraffin. Sections were stained with H&E. Insulitis was determined by scoring the mononuclear infiltrate in NOD, NOD.ChgA<sup>−/−</sup>, and BALB/c islets. Islets were scored as follows: 0: no infiltrate, 1: 10–50% infiltrated, 2: 50–75% infiltrated, 3: islet is completely infiltrated.

Adoptive transfer
T cell clones were expanded in secondary cultures, and 1 × 10<sup>7</sup> cells were injected i.p. into young (<14-d-old) NOD, NOD.ChgA<sup>−/−</sup>, or NOD.scid pups. CD4<sup>+</sup> cells from BDC-2.5 and BDC-6.9 TCR-Tg mice were isolated by negative selection using a kit from STEMCELL Technologies (Vancouver, BC, Canada). Purified CD4 populations were then stained with a vital dye, CFSE (Invitrogen) or Violet Proliferation Dye 450 (BD Biosciences), and 5 × 10<sup>5</sup> cells from each population were cotransferred i.v. into adult (>8 wk old) wild-type (WT) NOD or NOD.ChgA<sup>−/−</sup> mouse. Dyes were switched in repeat experiments.

Ag assay
T cell responses were assessed as described previously (4). Forms of Ags used included islet cell suspensions (1 × 10<sup>6</sup> cells) from BALB/c, NOD, NOD, ChgA<sup>−/−</sup>, or NOD.IAPP<sup>−/−</sup> mice or synthetic peptides, including ChgA peptide WE14 (WSRMDQLAKELTAE; 200, 400 μg/ml), Insulin B9-23 (SHLVEALLYVCGERG; 25, 100 μg/ml), or the IAPP peptide KS20 (KCNATCATQRLANFLVRSS; 25, 100 μg/ml). After 18 h, IFN-γ concentrations were determined in culture supernatants by ELISA.

Tissue harvest
After sacrifice of experimental transfer mice, islets were isolated and dissociated as previously described (12). In some experiments, blood was collected by cardiocentesis. Inguinal lymph nodes (LNs), pancreatic LNs, and spleen were also harvested for analysis of T cells.

Salivary gland digestion
The submaximal salivary gland was isolated from the mouse following careful removal of nearby LNs. The salivary glands were digested using collagenase D (Roche), with some of the collagenase solution injected directly into the salivary gland for 30 min at 37°C and pressed through a 40-μm filter to yield a single-cell suspension.

Flow cytometry
Abs were conjugated with the following fluorophores: FITC, PE, PerCP-Cy5.5, allophycocyanin, allophycocyanin-Cy7, Pacific Blue, Brilliant Violet 421, Brilliant Violet 510, and Brilliant Violet 711. Gating strategies are indicated in each figure; the lymphocyte gate was based on forward scatter/side scatter properties, and the singlets gate was based on the pulse-width parameter. Samples were run on a CyAn (Beckman Coulter) or BD Fortessa flow cytometer, and data were analyzed using FlowJo software (Tree Star, Ashland, OR). Tetramer staining was performed as previously described (4).

Statistical analysis
Statistical analysis included the Wilcoxon rank-sum test and the Student t test. Statistical significance was defined as p < 0.05.

Results and Discussion

The Ag for ChgA-reactive CD4 T cells is not present in NOD.ChgA<sup>−/−</sup> mice

CD4<sup>+</sup> T cell clones from the BDC panel were cultured with pancreatic islet cells from different mouse strains (BALB/c, NOD, NOD, ChgA<sup>−/−</sup>, and NOD.IAPP<sup>−/−</sup>), and IFN-γ secretion was used as a measure of T cell activation. The BDC T cell clones BDC-2.5 and BDC-10.1, which express different TCRs, are reactive with ChgA (1). Islets from NOD.ChgA<sup>−/−</sup> mice did not stimulate BDC-2.5 or the second ChgA-reactive T cell clone BDC-10.1, indicating that the ligand for these clones was absent from NOD.ChgA<sup>−/−</sup> islets (Fig. 1). BDC-2.5 and BDC-10.1 responded to positive-control Ags, such as islets from NOD and NOD.IAPP<sup>−/−</sup> mice and the ChgA peptide WE14. NOD.ChgA<sup>−/−</sup> islets elicited responses from BDC clones reactive to other Ags, including an IAPP-reactive T cell clone BDC-5.2.9 and an insulin-reactive T cell clone BDC-4.38.

In a second set of experiments, T cell transfer was used to test for the presence of Ag in vivo in different NOD mouse variants. T cell clones from the BDC panel rapidly transfer diabetes into young (<2-wk-old) WT NOD recipient mice. The BDC-2.5 T cell clone was adoptively transferred into NOD or NOD.ChgA<sup>−/−</sup> mice, and the recipients were subsequently monitored for diabetes (Supplemental Fig. 1A). NOD mice became diabetic within a few weeks after transfer, but BDC-2.5 failed to transfer diabetes to the NOD.ChgA<sup>−/−</sup> mouse, confirming the absence of the Ag for BDC-2.5 in this mouse. As a control, BDC-4.38, an insulin-reactive T cell clone, was transferred into NOD or NOD.ChgA<sup>−/−</sup> mice, and all recipients became diabetic within 3 wk following transfer (Supplemental Fig. 1A).

In a third set of experiments, CD4<sup>+</sup> T cells isolated from BDC-2.5 and BDC-6.9 TCR-Tg NOD mice (9, 10) were cotransferred into WT NOD or NOD.ChgA<sup>−/−</sup> mice. The BDC-6.9 TCR-Tg mouse was derived from a diabetogenic BDC T cell clone that is not reactive with ChgA. Each population was labeled with a vital dye (CFSE or Violet Proliferation Dye 450), which allowed us to distinguish among host cells, BDC-2.5 TCR-Tg cells, and BDC-6.9 TCR-Tg cells and to monitor T cell proliferation as an indicator of T cell activation (see gating strategy in Supplemental Fig. 1B). T cells from BDC-2.5 TCR-Tg mice proliferated and accumulated in the islets and pancreatic LNs of WT NOD mice (Supplemental Fig. 1C, 1D). In NOD.ChgA<sup>−/−</sup> mice, BDC-2.5 TCR-Tg T cells were present, but fewer in number, and they did not proliferate in the islets or pancreatic LNs. In contrast, we observed that BDC-6.9 TCR-Tg cells could readily proliferate and accumulate in the islets and pancreatic LNs of both WT NOD and NOD.ChgA<sup>−/−</sup> mice (Supplemental Fig. 1C, 1D). Results obtained with Tg CD4 T cells provide further confirmation that ChgA-deficient NOD mice lack the Ag for BDC-2.5.

NOD mice deficient in ChgA do not develop diabetes

To determine the effect of ChgA deficiency in the islets on the development of autoimmunity in the islets, we followed co-housing of male and female WT NOD and NOD.ChgA<sup>−/−</sup> mice in parallel for up to 12 mo. Mice were monitored for disease by checking weekly urine glucose levels as a sign of hyperglycemia and were considered diabetic when blood glucose was >15 mM for two consecutive days. Cumulative incidence in our WT NOD colony indicates that ~90% of female mice become diabetic by 52 wk. In contrast, in NOD.ChgA<sup>−/−</sup> mice during the same time period, males were completely protected...
from disease and only three females (of 118 total) became diabetic, demonstrating that, in the absence of ChgA, the incidence of autoimmune diabetes is greatly reduced (Fig. 2).

**NOD.ChgA−/− mice are protected from insulitis but not sialitis**

Histological analysis was performed on sections from the pancreas of BALB/c, NOD, or NOD.ChgA−/− mice. Tissue from BALB/c mice was used as a negative control because these mice do not develop autoimmunity, and the pancreas does not contain infiltrate (Fig. 3A). In pancreatic sections from NOD.ChgA−/− mice, we observed that >80% of the islets were completely protected from insulinitis (Fig. 3A), with no difference between younger and older mice. In contrast, in WT NOD mice, we observed a clear progression of insulinitis as the mice aged; up to 50% of the islets in older mice had a score of 3, indicating extensive infiltration. The histological differences between the pancreatic islets of WT NOD and NOD.ChgA−/− mice indicate that the autoimmune process is markedly reduced in the pancreas of mice lacking ChgA.

NOD mice often exhibit signs of autoimmunity in sites other than the pancreas and, for example, provide a model of Sjögren’s syndrome (13). We examined the salivary glands to determine whether ChgA−/− mice develop inflammatory infiltrates outside the pancreas. Sialitis is the characteristic lymphocytic infiltrate of the salivary glands previously described in NOD mice (13). We observed a focal infiltrate in the salivary glands of both WT NOD and NOD.ChgA−/− mice (data not shown). Flow cytometric analysis of the salivary glands showed that, in the CD45+ lymphoid cell population, CD4+ cells were significantly increased in WT NOD and NOD.ChgA−/− mice compared with BALB/c mice (Fig. 3B), indicating that, in NOD mice, the autoimmune process in the salivary gland was intact, either in the presence or absence of ChgA. Although the Ags driving Sjögren’s syndrome are not clearly defined, ChgA is not expressed in the salivary glands (14). These data indicate that NOD.ChgA−/− mice are still prone to autoimmunity. Additionally, our findings suggest that ChgA is necessary for development of the infiltrate present in the pancreas of NOD mice but apparently is not a target for the autoreactive CD4 T cells responsible for sialitis.

**CD4 T cells do not accumulate in the pancreas of NOD.ChgA−/− mice**

To gain further insights into the cells present in the pancreas of NOD.ChgA−/− mice, we performed flow cytometric analysis of the pancreatic infiltrate. Compared with WT NOD mice, the total number of CD4+ cells present in the pancreas of NOD.ChgA−/− mice was significantly reduced (Fig. 2C). The numbers of CD4+, CD8+, and CD19+ cells were also lower (Fig. 3C). We evaluated the overall number of CD4 cells/islet by flow cytometry; our data indicate that there was a significant increase in the number of CD4 T cells/islet in WT NOD mice compared with NOD.ChgA−/− mice (Fig. 3D). These data confirm and expand our observations shown in Fig. 3A that, overall, there are decreased leukocyte numbers present in the pancreas of NOD.ChgA−/− mice.

We used tetramer analysis to further investigate Ag specificity of T cells present in the spleen and the pancreas of NOD.ChgA−/− mice. BDC-2.5-like cells were monitored using the 2.5mi tetramer (15), which contains a BDC-2.5 mimotope peptide (AHHPIWARMDA). As a negative control, we used a tetramer loaded with a peptide from hen egg lysosome (HEL12–25). In the pancreas of NOD.ChgA−/− mice, 2.5mi tetramer-positive (tet+) cells were virtually absent (Fig. 4A, Supplemental Fig. 2C), indicating that, in the absence of ChgA, 2.5mi tet+ cells do not migrate and accumulate in the pancreas. In contrast, the pancreas of WT NOD mice contained large populations of 2.5mi tet+ cells. We observed 2.5mi tet+ cells in the spleen of both WT NOD and NOD.ChgA−/− mice and examined the activation status of these cells by examining markers of T cell memory (CD44hiCD62Llo) (Supplemental Fig. 2A). In the spleen of NOD.ChgA−/− mice, 2.5mi tet+ cells were present but remained mostly CD44loCD62Lhi. Both memory and naive populations of 2.5mi tet+ cells were present in the spleen of WT NOD mice (Fig. 4B, Supplemental Fig. 2B). These data indicate that 2.5mi tet+ cells are present in NOD.ChgA−/− mice but remain naive.

We also analyzed WT NOD and NOD.ChgA−/− mice for the presence of insulin-reactive T cells using a recently described insulin tetramer (16) that stains one of the first insulin-reactive CD4 T cell clones described, PD12.4.4 (17). Insulin tet+ cells were significantly increased in the pancreas of
polyclonal CD4 population for memory and naive populations. Memory CD4 T cells were defined as CD44hiCD62Llo, and naive cells were defined as CD44loCD62Lhi.

14.6 wk (n = 6, 66 islets scored) for younger NOD mice and 13.25 wk for younger NOD ChgA−/− (n = 4, 61 islets scored); for older mice, the average age was 42 wk for NOD mice (n = 13, 179 islets scored), and 52 wk for NOD ChgA−/− (n = 14, 275 islets scored). (B) Submaximal salivary glands from 6–10-mo-old WT NOD, NOD ChgA−/−, or BALB/c mice were analyzed by flow cytometry. The percentages of CD4 cells present in submaximal salivary glands are indicated. Gates were set on the lymphocyte gate, singlets, and on CD45+ and dump (PerCP-Cy5.5) and analyzed by flow cytometry. Data are a summary of four independent experiments with two mice/group/experiment. Averages are represented by black horizontal lines. (C) Data are representative of two independent experiments, with two mice/group/experiment. Averages are represented by black horizontal lines. **p < 0.01.

WT NOD mice compared with NOD ChgA−/− mice (Fig. 4A). We observed that insulin tet+ cells were also present in the spleen of both WT NOD and NOD ChgA−/− mice, but there were no significant differences between the two mouse strains.

The ultimate goal of this study was to investigate whether the lack of ChgA had any impact on disease incidence in NOD mice. Because KO of other Ags, such as glutamic acid decarboxylase 65 and IAPP, does not affect development of diabetes in NOD mice (4), the lack of disease development in NOD ChgA−/− mice was unexpected and may signify a different, more critical role for this secretory granule protein. In other attempts to pinpoint the importance of specific T cell epitopes, investigators mutated peptide sequences from islet-specific glucose 6 phosphatase or insulin and then determined the effect on disease development. Although mutating islet-specific glucose 6 phosphatase had no effect (18), a single amino acid substitution at position 16 in the B:9-23 peptide of the insulin B chain protected mice from the development of diabetes (7). We observed some infiltrate in the pancreatic islets and in the salivary gland of NOD ChgA−/− mice, suggesting that there is not a complete absence of autoimmunity, but with the rare exception, these animals do not develop diabetes. Thus, our data provide evidence that ChgA is required for spontaneous disease development in NOD mice and underscore the importance of ChgA in the initiation and overall pathogenesis of the disease.

Our results suggesting that the presence of ChgA is necessary for the development of autoimmune diabetes in NOD mice indicate new future directions for the study of this autoantigen. Although the peptide WE14 from ChgA elicited responses in T cells from human T1D patients (2), it is not clear that this is the only, or optimal, ChgA ligand for autoreactive T cells in humans. Therefore, it is of great importance to determine the natural peptide ligands for ChgA-reactive T cells, both from the standpoint of determining the role of ChgA in pathogenesis and because better reagents are needed to identify autoreactive T cells as biomarkers. For example, can peptide sequences from ChgA be used to design better tetramers for monitoring the ongoing autoimmune process directed toward ChgA and identify subjects at risk for developing T1D? Another important area of interest is whether peptides from ChgA could be used in strategies to...

FIGURE 3. ChgA-deficient mice are protected from insulitis but not sialitis. (A) Pancreatic sections from BALB/c, NOD (WT), or NOD ChgA−/− (KO) mice were stained with H&E and analyzed for the presence of mononuclear infiltrate in the islets. Thirty-six islets were scored for BALB/c mice. The average age was 14.6 wk (n = 6, 66 islets scored) for younger NOD mice and 13.25 wk for younger NOD ChgA−/− mice (n = 4, 61 islets scored); for older mice, the average age was 42 wk for NOD mice (n = 13, 179 islets scored) and 52 wk for NOD ChgA−/− mice (n = 14, 275 islets scored). (B) Submaximal salivary glands from 6–10-mo-old WT NOD, NOD ChgA−/−, or BALB/c mice were analyzed by flow cytometry. The percentages of CD4 cells present in submaximal salivary glands are indicated. Gates were set on the lymphocyte gate, singlets, and on CD45+ and dump (PerCP-Cy5.5) and analyzed by flow cytometry. Data are a summary of four independent experiments with two mice/group/experiment. Averages are represented by black horizontal lines. **p < 0.01.

FIGURE 4. ChgA-reactive T cells develop in the absence of ChgA but remain naive. Single-cell suspensions from pancreas (A) and spleen (B) were stained with tetramers specific for T cells reactive to hen egg lysosome, ChgA (2.5mi), or insulin (Insp8G), and a master mix of Abs. Gates were set on the lymphocyte gate, singlets, and on CD45+, dump (CD8+CD11b+CD11c+CD19-). Each symbol represents an individual mouse. Data are expressed as ratios of tet+ cells relative to the polyclonal CD4 population for memory and naive populations. Memory CD4 T cells were defined as CD44hiCD62Llo, and naive cells were defined as CD44loCD62Lhi; each symbol represents an individual mouse. The dotted line indicates a ratio of 1. Data are from four (A) or three (B) independent experiments, with two mice/group/experiment. **p < 0.01. n.s., no significance (p > 0.05).
induce Ag-specific tolerance. Trials are in progress to test the ability of myelin peptides to suppress multiple sclerosis in humans (19). It may be that peptides from ChgA could contribute to controlling autoimmunity in T1D.

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Disclosures

The authors have no financial conflicts of interest.

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