

Slc11a1 Enhances the Autoimmune Diabetogenic T-Cell Response by Altering Processing and Presentation of Pancreatic Islet Antigens

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OBJECTIVE—Efforts to map non-major histocompatibility complex (MHC) genes causing type 1 diabetes in NOD mice identified *Slc11a1*, formerly *Nramp1*, as the leading candidate gene in the *Idd5.2* region. *Slc11a1* is a membrane transporter of bivalent cations that is expressed in late endosomes and lysosomes of macrophages and dendritic cells (DCs). Because DCs are antigen-presenting cells (APCs) known to be critically involved in the immunopathogenic events leading to type 1 diabetes, we hypothesized that *Slc11a1* alters the processing or presentation of islet-derived antigens to T-cells.

RESEARCH DESIGN AND METHODS—NOD mice having wild-type (WT) or mutant *Slc11a1* molecules and 129 mice having WT or null *Slc11a1* alleles were examined for parameters associated with antigen presentation.

RESULTS—We found that *Slc11a1* enhanced the presentation of a diabetes-related T-cell determinant of GAD65, and its function contributed to the activation of a pathogenic T-cell clone, BDC2.5. An enhanced generation of interferon (IFN)- γ -producing T-cells was also associated with functional *Slc11a1*. The alteration of immune responsiveness by *Slc11a1* genotype did not correlate with altered MHC class II expression in DCs; however, functional *Slc11a1* was associated with accelerated phagocytosis and phagosomal acidification in DCs.

CONCLUSIONS—The association of variants encoding *Slc11a1* with type 1 diabetes may reflect its function in processing and presentation of islet self-antigens in DCs. Thus, non-MHC genes could affect the MHC-restricted T-cell response through altered antigen processing and presentation. *Diabetes* 58:156–164, 2009

Evidence has been presented supporting *Slc11a1* as a non-major histocompatibility complex (MHC) gene contributing to the development of spontaneous type 1 diabetes in NOD mice (1–4). *Slc11a1* has been characterized primarily for its ability to mediate resistance to intracellular pathogens such as

Salmonella and *Leishmania* via its expression in macrophages (5,6). Moreover, recently, *Slc11a1* was shown to be expressed in dendritic cells (7). The protein structural characteristics of *Slc11a1*, i.e., 12-transmembrane domains and a transport motif, indicate a function as an ion channel and a transporter (5,8). Intracellular staining of *Slc11a1* in macrophages and dendritic cells demonstrated that the protein is restricted to intravacuolar compartments, especially late endosomes and lysosomes (7,9,10). A glycine to aspartic acid substitution at position 169 (G169D) within the fourth transmembrane domain of *Slc11a1* is present in many mouse strains and reduces *Slc11a1* expression on the phagosomal membrane (11,12). Mouse inbred strains, such as 129/sv and A/J, resistant to particular intracellular bacteria/parasite infections, have the wild-type *Slc11a1* protein (G169), whereas infection-susceptible strains, C57BL/6/J and BALB/c, express the mutant *Slc11a1* protein (D169) (13). In contrast, for autoimmune disease such as type 1 diabetes, the resistant allele encodes the mutant *Slc11a1* protein. In humans, polymorphisms correlated with expression differences have been identified in the promoter of *Slc11a1* mRNA, which contains several lipopolysaccharide (LPS)-related response elements (14,15). Among several polymorphic alleles of the *Slc11a1* gene that vary in transcription efficiency, allele 3, the allele having the greatest expression, has the highest association with several autoimmune diseases, including rheumatoid arthritis, type 1 diabetes, multiple sclerosis, and Crohn's disease, whereas the allele having the lowest expression, allele 2, is commonly associated with susceptibility to many infectious agents (16).

For a given MHC haplotype, the antigen processing machineries for class I and II MHC presentation pathways play a crucial role in selecting which peptides will be presented efficiently, poorly, or not at all on APC. T-cells recognizing well-presented self-peptides should have been eliminated in the thymus or tolerized/exhausted in the periphery, and thus the poorly presented (cryptic) peptides are considered as major players in autoimmunity (17). In this study, we have tested the hypothesis that *Slc11a1* contributes to type 1 diabetes susceptibility by altering antigen processing and presentation of certain poorly presented, diabetes-associated T-cell determinants in dendritic cells. The expression of *Slc11a1* in DCs and its function on T-cell response were examined in 129/sv mice with a disrupted *Slc11a1* gene (*Slc11a1* KO) and two NOD.B10 congenic mouse strains differing at *Slc11a1*, NOD.B10 *Idd5.1/5.3* (R193), and NOD.B10 *Idd5.1/5.3/5.2* (R444). The R193 strain has the NOD-derived functional *Slc11a1* gene and R444 mice have the B10-derived mutant *Slc11a1* gene. We found that R193 DCs could present a diabetes-associated GAD65 determinant more efficiently

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than the R444 APC, as well as an islet ligand recognized by the well-known diabetogenic T-cell clone, BDC2.5. In addition, Slc11a1 WT mice generated a much stronger Th1 response after immunization with a model antigen, hen eggwhite lysozyme (HEL), and this Th1 proinflammatory activity of Slc11a1 was similarly observed in 129/Sv mice when compared with Slc11a1 KO mice. Slc11a1-dependent MHC class II expression differences on DCs were not observed; however, evidence supported the ability of Slc11a1 to accelerate the acidification process in DCs. Thus, altered and/or enhanced antigen processing and presentation may attribute to the Slc11a1-mediated Th1 response.

RESEARCH DESIGN AND METHODS

Mice. Inbred mouse strains [129S1/SvImJ (129/Sv), NOD/LtJ (NOD), NOR/LtJ (NOR), NOD.CB17-Prkdc^{scid}/J (NOD.scid)] were purchased from The Jackson Laboratory (Bar Harbor, ME). The Slc11a1-null mutant (Slc11a1 KO) was generated using 129/Sv embryonic stem cells (18), and the KO mice were maintained at the animal facility of Torrey Pines Institute for Molecular Studies (TPIMS). The NOD.B10 congenic strains NOD.B10 *Idd5.1/5.3* (R193) and NOD.B10 *Idd5.1/5.3/5.2* (R444) were developed as described in Hunter et al. (4) and were obtained from Taconic (Hudson, NY) and maintained as inbred strains at TPIMS. BDC2.5 TcR transgenic NOD mice were maintained at TPIMS by backcrossing with NOD mice. Experimental protocols were conducted with approvals from the TPIMS Ethical Review Committee.

APC preparation. To prepare bone marrow (BM)-derived DCs, bone marrow was collected from femurs and tibias, and red blood cells in the marrow were lysed with red blood cell lysing buffer (Sigma). Fresh granulocyte-macrophage colony-stimulating factor (GM-CSF) (~100 units/ml) was added every other day to stimulate the bone marrow cells (10^6 cells/well) adherent to a 24-well plate. The proliferating immature DCs were harvested by gentle dislodging on day 6 and plated in a 60-mm² culture Petri dish (10^7 cells/ml) in complete medium with GM-CSF. The mature DCs present after 24-h incubation become nonadherent, after which they are collected for further studies. Splenic APCs were enriched after plastic adherence. Briefly, red blood cell-lysed splenocytes (10^7 /ml) are allowed to adhere to plastic in serum-free RPMI media at 37°C for 60–90 min, followed by repeated gentle washes with medium to remove nonadherent splenocytes until the plate bottom becomes transparent. Then, complete 10% FCS medium is added to continue the incubation overnight to release the adherent DCs. Splenic CD11c⁺ DCs were isolated using anti-CD11c-coated magnetic microbeads (Miltenyi Biotec, Auburn, CA) according to the product instructions.

Antigens, peptide synthesis, and islet isolation. GAD65 peptides p206–220, p217–236, p246–266, p286–300, p524–538, p530–543, and p524–543 were synthesized at the peptide synthesis laboratory of the University of California, Los Angeles, on an Advanced Chemtech 395 Synthesizer using F-moc chemistry and purified by high-performance liquid chromatography to >90% purity. The Ig-GAD1 and Ig-HEL chimeric molecules were constructed by Dr. Zaghouni by inserting the GAD65 p524–543 or HEL p11–25 nucleotide sequences into the variable region of the H-chain of Ig molecules (19). Baculovirus-expressing recombinant GAD65 (rGAD65) was obtained from Dr. Nora Sarvetnick at the Scripps Research Institute (La Jolla, CA). Isolation of islets was performed as follows: 3 ml 0.46 mg/ml collagenase dissolved in Hanks' balanced salt solution buffer was injected into pancreatic glands through the bile duct, followed by digestion in a 37°C water bath for 18–20 min and disruption by vigorous shaking at the end of incubation. The digestion was terminated by washing once with ice-cold Hanks' balanced salt solution buffer, and the debris was resuspended in 10 ml of 10% fetal calf serum (FCS) RPMI medium. Single islets were picked under a dissection microscope. An average of 100–250 islets per adult NOD.scid pancreas can be expected.

T-cell proliferation and activation assay. T-cell lines specific for GAD65 peptides, p530–543 or p524–543, were generated by immunizing NOD mice with 100 µg of the peptides emulsified in complete Freund's adjuvant (Difco). Draining lymph nodes and spleens were collected 9–12 days later and expanded in vitro with 10 µg/ml of the respective peptide for 3 days followed by resting in 10 units/ml of rIL-2 in complete RPMI media, containing 10% FCS, penicillin (100 units/ml) and streptomycin (100 µg/ml), 2 mmol/l L-glutamine, 10 mmol/l HEPES buffer, and 0.1 mmol/l of minimal essential medium with nonessential amino acids (Gibco, Invitrogen, San Diego, CA). The lines were further expanded once with p524–543, using irradiated NOD splenocytes as APCs, and rested for at least 7 days in 10 units/ml of rIL-2 before testing for an antigen-specific T-cell response. For T-cell proliferation or activation assays, $1-2 \times 10^5$ peptide-specific T-cells or purified CD4⁺ BDC2.5 T-cells (isolated with anti-CD4-coated magnetic microbeads; Miltenyi) were mixed

with various antigens or the pancreatic islets and APCs or purified DCs. After 3 days of culture, ³H-thymidine (International Chemical and Nuclear, Irvine, CA) was added for another 18 h of culture. Cells were harvested with a Micro Cell Harvester (Skatron Instruments, Sterling, VA), and incorporation of ³H-thymidine was measured on a Wallac MicroBeta Trilux counter (Perkin Elmer, Boston, MA). In some experiments, culture supernatants were collected at 72 h for measuring interferon (IFN)-γ concentrations using a commercial enzyme-linked immunosorbent assay (ELISA) kit (BD Bioscience, San Diego, CA).

ELISPOT. The number of HEL-specific, IFN-γ-secreting T-cells was evaluated in R193 and R444 mice immunized with HEL, using an enzyme-linked immunosorbent spot (ELISPOT) assay (BD Biosciences). Briefly, ELISPOT plates (MAHA S45 10, Millipore) were first coated overnight at 4°C with anti-IFN-γ mAb (5 µg/ml; BD Biosciences) and blocked with 1% BSA (Fraction V; Sigma Aldrich) in PBS. HEL-primed splenocytes (10 days after immunization) were added at 5×10^5 /well for 48 h at 37°C in the presence of 100 or 500 µg/ml of HEL, or 5 µg/ml of Concanavalin A as a positive control, or media only. IFN-γ secretion was detected with a second biotinylated anti-IFN-γ mAb (2 µg/ml; BD Biosciences). Spots were developed using streptavidin-horseradish peroxidase and 3-amino-9-ethyl-carbazole (AEC) substrate.

Western blotting for Slc11a1 in macrophages and DCs. Macrophages were collected from the peritoneal cavity 3 days after injection of 3 ml of 3% thioglycollate media. BM-derived DCs were stimulated with 2 µg/ml LPS for 24 h for further maturation. A total of $1-2 \times 10^7$ cells were lysed in 100–200 µl cell-lysing buffer (containing 20 mmol/l Tris-HCl, 5 mmol/l EDTA, 1% NP-40, 1 mmol/l NaVO₄, 1% SDS, and 10 µg/ml aprotinin/leupeptin) for 15 min on ice, followed by centrifugation at 15,000 rpm for 20 min at 4°C. Cell lysates were collected for SDS-PAGE. Samples were denatured at 37°C for 30 min using a 5× sample buffer that contains 10% SDS, 10 mmol/l β-mercaptoethanol, 20% glycerol, 0.2 mol/l Tris-HCl (pH 6.8), and 0.05% bromophenol blue. SDS-PAGE-separated protein samples were electro-transferred to a polyvinyl derivative membrane (Amersham Biosciences, Piscataway, NJ) using a semi-dry trans-blot (Bio-Rad Laboratories, Hercules, CA). The membrane was immunoblotted with the anti-Slc11a1 antibody (1:1,000 dilution) or a control anti-ERK antibody (1:500) (Cell Signaling Technology, Danvers, MA), followed by horseradish peroxidase-conjugated secondary antibodies and visualized with an enhanced chemiluminescence (ECL) detection system (Amersham).

FACS and intracellular flow cytometry. Antibodies specific for MHC class II I-A^{g7} (AMS-32.1), I-A^b (AF6–120.1), CD80 (16–10A1), CD11c (HL3), and fluorescence-labeled anti-Ig antibodies were purchased from BD BioScience (San Diego, CA). Slc11a1-specific polyclonal antibody was produced in rabbits after immunization with a NH₂-terminal peptide from the mouse Slc11a1 molecule as described (12). For intracellular staining of Slc11a1 protein, splenic plastic adherent APCs were fixed with 3.7% paraformaldehyde for 10 min, followed by membrane perforation with 0.5% Saponin dissolved in 1% BSA/PBS for 10 min on ice. The perforated cells were then blocked with 1 µg/ml anti-CD16 (FcγR) before staining with the Slc11a1-specific antibody (1:500 dilution in 0.1% Saponin). After three washes with 0.1% Saponin, cells were further incubated with a second anti-rabbit Ig labeled with fluorescein (BD BioScience) or the anti-CD11c antibody, and the staining was detected using FACSCalibur (BD BioScience, San Jose, CA).

Phagocytosis and phagosomal acidification. A modified protocol to measure the rates of phagocytosis and phagosomal acidification using flow cytometry was established after a described protocol by Savina et al. (20), in which DC phagocytosis was induced using iron-coated microbeads with a diameter of 40–50 nm (Miltenyi) that were pre-conjugated with pH-sensitive dyes (FITC) and pH-insensitive dyes (PE). The conjugation was performed by incubation of 250 µl protein-G microbeads (Miltenyi) with 20 µg of each FITC- and PE-labeled rat Igs for 30 min on ice, followed by purification with a magnetic cell separation (MACS) MS column (Miltenyi) to remove excess antibodies. The FITC/PE-labeled microbeads were eluted in 250 µl MACS buffer and used to pulse DCs. At different time points, the fluorescence intensity of FITC and PE of the pulsed DCs was monitored by FACS. Because PE is relatively stable to pH change or phagosomal degradation, the accumulation of PE on DCs reflects the rate of phagocytosis. The pH-sensitive FITC dye is an indicator of phagosomal degradation, and a change of the FITC/PE ratio will reflect the relative pH values or the rate of acidification process within the DC phagosomes.

RESULTS

Slc11a1 enhances processing and presentation of a diabetogenic T-cell determinant, p524–543, of GAD65. Spontaneous autoreactive T-cell responses to several GAD65 peptides correlate with the development of diabe-

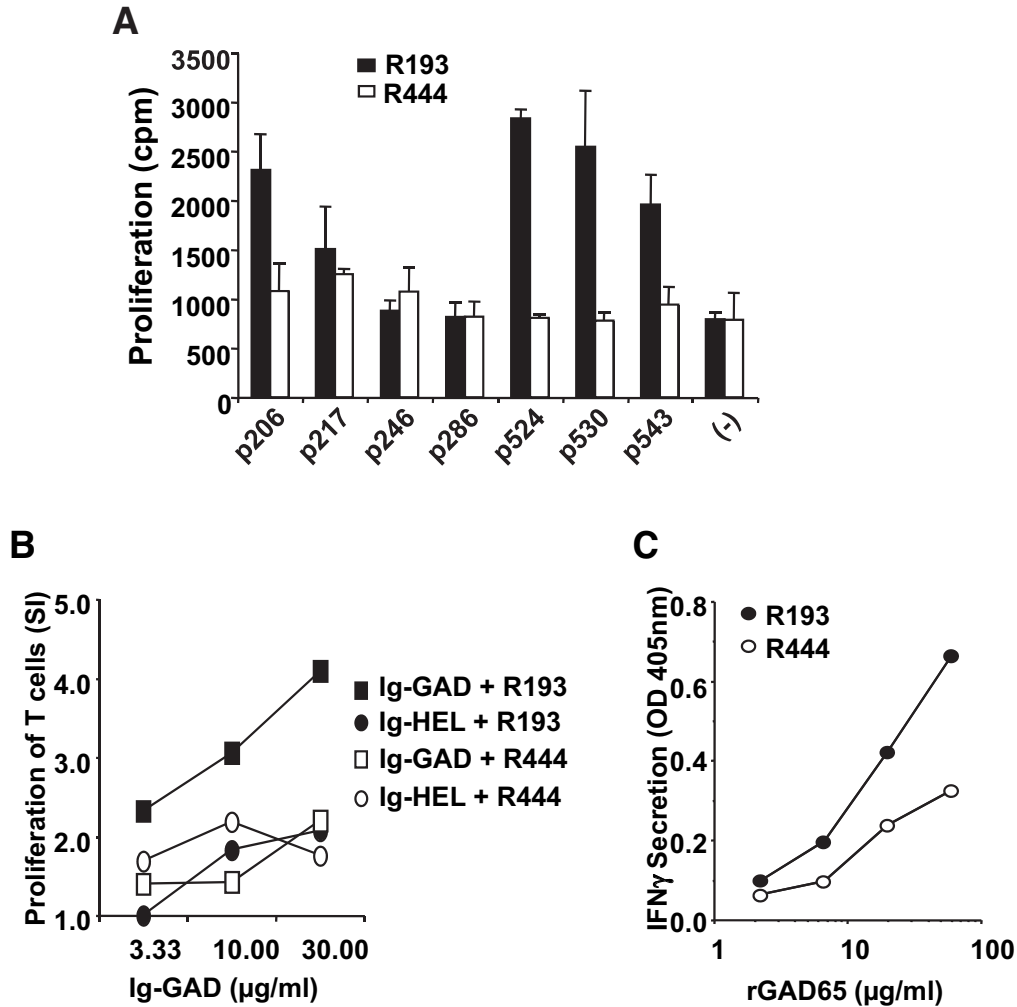


FIG. 1. Slc11a1 enhanced processing and presentation of GAD65 T-cell determinants. **A:** Spontaneous T-cell response to GAD65 in R193 (Slc11a1 WT) and R444 (Slc11a1 mutant) *Idd5* congenic mice. Spleen cells were prepared from 16-week-old female R193 or R444 mice and cultured with synthetic peptides derived from the mouse GAD65 molecule: p206 = 206–220, p217 = 217–236, p246 = 246–266, p286 = 286–300, p524 = 524–538, p530 = p530–543, and p543 = 524–543. **B:** An Ig-GAD chimeric molecule was used as the recall antigen for T-cells specific for GAD65 p530–543 peptide. **C:** Recombinant GAD65 (rGAD65) was used to stimulate a p524–543-specific T-cell line. Positive controls (with 5 µg/ml of p524–543 peptide): R193.APC = 2.80 ± 0.06 , R444.APC = 2.95 ± 0.12 . Background odds density (OD) values were subtracted from the data shown: R193.APC = 0.19 ± 0.004 , R444.APC = 0.20 ± 0.003 . Each experiment is representative of two experiments performed.

tes in NOD mice (21,22). We thus compared GAD65-specific T-cell responses in two NOD.B10/*Idd5* congenic strains, R193 and R444, which carry functional versus nonfunctional mutant Slc11a1 alleles, respectively. Only the R193 strain showed a proliferative response to several known GAD65 T-cell determinants: p206–220, p524–538, p530–543, and p524–543 (Fig. 1A). In contrast, splenocytes collected from the Slc11a1-nonfunctional R444 mice showed no reactivity to any of the tested peptides, indicating a lack of peripheral activation or expansion of GAD65-reactive T-cells in the R444 strain. To examine whether Slc11a1 could affect the processing and presentation of GAD65, we used an Ig-chimeric antigen that includes one of the dominant determinants of GAD65, p524–543, in the variable region of the Ig H-chain (19); In Fig. 1B, presentation of the p524–543 determinant after processing of the Ig-chimeric antigen by splenic adherent APC was detected using a p530–543-specific T-cell line; it was found that only the R193 APCs were efficient in processing the chimeric molecule; a control chimeric molecule, Ig-HEL, as well as the R444 APC proved to be inactive for stimulating the T-cells. Similar results were obtained when recombinant GAD65 protein was used as antigen, in

that R193 APCs were more efficient in activating the p524–543-specific T-cells to secrete IFN- γ (Fig. 1C). These data are consistent with a conclusion that Slc11a1-expressing APCs are more efficient in processing and presenting this diabetes-associated GAD65 determinant.

Activation of a highly pathogenic diabetes-associated T-cell clone, BDC2.5, requires Slc11a1 expression in dendritic cells. The BDC2.5 T-cell clone is diabetogenic in NOD mice (23,24) and strongly reactive to a membrane protein of 50–85 kDa expressed in normal islet β -cells (25). To examine whether Slc11a1 could affect the processing and presentation of the ligand to stimulate this islet-infiltrating T-cell clone, we first performed an in vitro T-cell proliferation assay, in which CD4⁺ BDC2.5 T-cells were cultured with freshly isolated splenic APCs in the presence of different numbers of the pancreatic islets or media. We were able to stimulate BDC2.5 T-cells using only two islets per well (Fig. 2A), whereas BDC2.5 cells were unresponsive in the absence of the islets despite the addition of LPS-activated splenic APCs (data not shown), suggesting that a specific high-affinity antigen presented in the islets was necessary to activate naive BDC2.5 T-cells. At 20 islets per well, a stimulation index of 20 could be

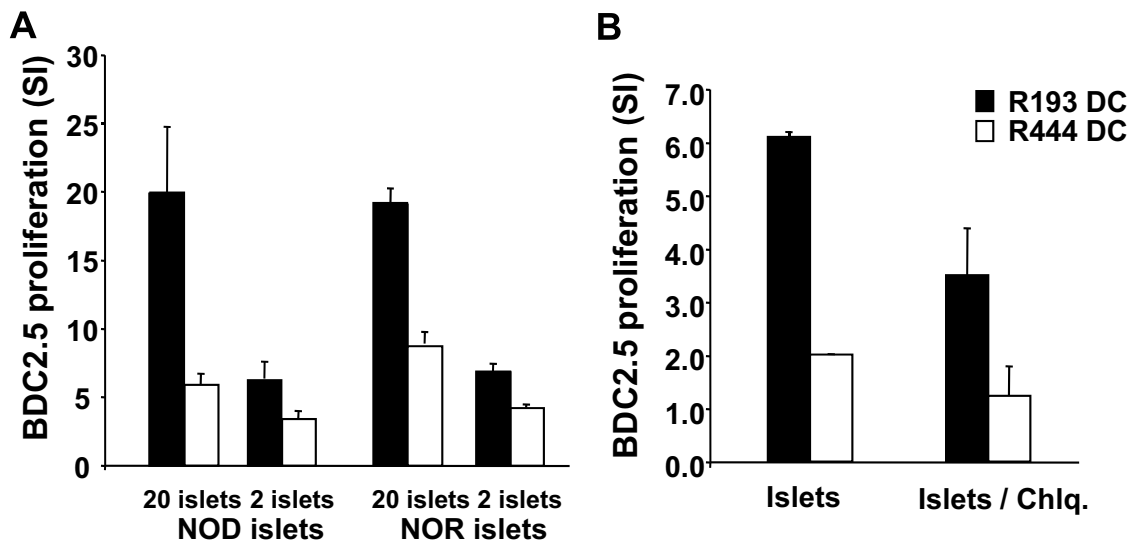


FIG. 2. Pancreatic islet antigens are processed more efficiently by the R193 than by the R444 DCs. **A:** BDC2.5 T-cells were isolated from transgenic mice with anti-CD4 MACS beads; DCs were enriched from R193 or R444 splenocytes by plastic adherence; islets were handpicked from digested NOD, NOD.*scid*, or NOR pancreas. For proliferation assays, BDC2.5 (100,000/well) were cultured with DCs (20,000/well) and 20 or 2 islets for 72 h followed by an additional 18 h with ^3H -thymidine. Negative controls had no islets: R193 = 1,460 cpm, R444 = 4,675 cpm. Each number represents the mean of triplicate wells. **B:** The ratio of BDC2.5 T-cells: DCs remained at 5:1. The difference in this experiment was that DCs were purified using anti-CD11c microbeads, and 100 $\mu\text{mol/l}$ chloroquine was added for inhibition of processing. Background: R193 = 2,325 cpm, R444 = 3,390 cpm; with addition of chloroquine: R193 = 120 cpm, R444 = 130 cpm. Each experiment is representative of two experiments performed that yielded similar results.

reached for BDC2.5 cells by R193 APCs, whereas under the same conditions, the R444 APCs were not as efficient in processing the islets, resulting in a lesser proliferation of BDC2.5 T-cells with a stimulation index of ~ 5 (Fig. 2A). Additionally, we observed that islets isolated from a diabetes-resistant, NOD-congenic mouse strain, NOR, were essentially equivalent to the islets from NOD mice in stimulating BDC2.5 T-cells (Fig. 2A), indicating that the ligand of BDC2.5 cells is also presented in the NOR islets. Finally, this Slc11a1-dependent processing was sensitive to chloroquine treatment, since the activation of BDC2.5 T-cells by islets was reduced by addition of 100 $\mu\text{mol/l}$ chloroquine in the culture (Fig. 2B). Therefore, insufficient processing and presentation of some T-cell determinants such as the BDC2.5 ligand correlated with the diabetes resistance observed in the R444 and the NOR strains.

Slc11a1 enhanced Th1-mediated response to HEL immunization. Slc11a1 is proinflammatory, but it is not clear whether Slc11a1 directly affects the production of inflammatory cytokines by DCs and macrophages or through indirect activation of T-cells. Nevertheless, enhanced Th1 bias would accelerate autoimmune destruction in the pancreas. We therefore examined the immune response and the cytokine profiles in the congenic NOD strains, R193 and R444, after HEL immunization. The Slc11a1-nonfunctional R444 mice mounted a similar proliferative response to HEL compared with R193 mice, as assessed using their HEL-primed splenocytes (Fig. 3A); however, there was a dramatic increase in IFN- γ production by the R193 lymphocytes, over two to threefold more than that produced by the R444 cells, as detected by ELISA (Fig. 3B) as well as by ELISPOT assays (Fig. 3C). Similar ELISPOT results were observed when Slc11a1 WT vs. KO mice were immunized with HEL (Fig. 3D), confirming a strong bias toward Th1 response in the presence of Slc11a1.

Slc11a1 does not affect the expression levels of MHC class II and costimulatory molecules. It has been reported that Slc11a1 function correlates with IFN- γ -induced MHC class II expression on BM-derived macro-

phage cell lines (26). We therefore examined MHC class II expression on freshly isolated, as well as LPS-stimulated splenic, DCs. CD11c was used to identify or isolate the myeloid DC population. Splenic DCs were isolated from R193 versus R444, or WT versus KO splenocytes by anti-CD11c-coated magnetic microbeads, followed by staining using fluorescent anti-I-A^b, anti-I-A^{g7}, anti-CD80, or anti-CD11c antibodies. We observed that in three independent experiments, DCs from both the NOD congenic pair (Fig. 4A) and the WT versus KO mice (Fig. 4B) expressed comparable levels of MHC class II (I-A^{g7} or I-A^b, respectively) and CD80 (B7.1) molecules; and this observation remained unchanged when LPS-activated CD11c⁺ DCs (Fig. 4A, lower panel) were examined. In addition, knockout of Slc11a1 did not change the percentage of the CD11c⁺CD80⁺ DC subset within the isolated splenic CD11c⁺ DC population, as shown in Fig. 4C. Therefore, the pro-Th1 function of Slc11a1 should not be attributable to enhanced expression of the MHC class II or B7.1 costimulatory molecules on APCs. An alternate hypothesis is that the observed pro-Th1 function of Slc11a1 could be an indirect result of differential activation of T-cells with higher affinity due to altered antigen processing in Slc11a1-expressing APCs.

Slc11a1 enhances phagocytosis and acidification. To compare the rates of phagocytosis and phagosomal acidification between Slc11a1 functional DCs (R193 and Slc11a1 WT) and nonfunctional DCs (R444 and Slc11a1 KO), mature DCs generated separately from the bone marrows of the R193, R444, WT, and KO mice were incubated with the preformed FITC/PE-microbeads (see RESEARCH DESIGN AND METHODS) at 37°C for various periods, followed by FACS analysis to monitor the DC-bound FITC and PE dyes. Within certain periods (5–7 h in our experiments), the fluorescence intensity is positively correlated with incubation times. In four separate experiments, we repeatedly observed a faster accumulation of the fluorescent dyes in the R193 or WT DCs, in comparison with DCs from the R444 or KO mice (Fig. 5A), suggesting that Slc11a1 can enhance the phagocytosis of the FITC/PE-

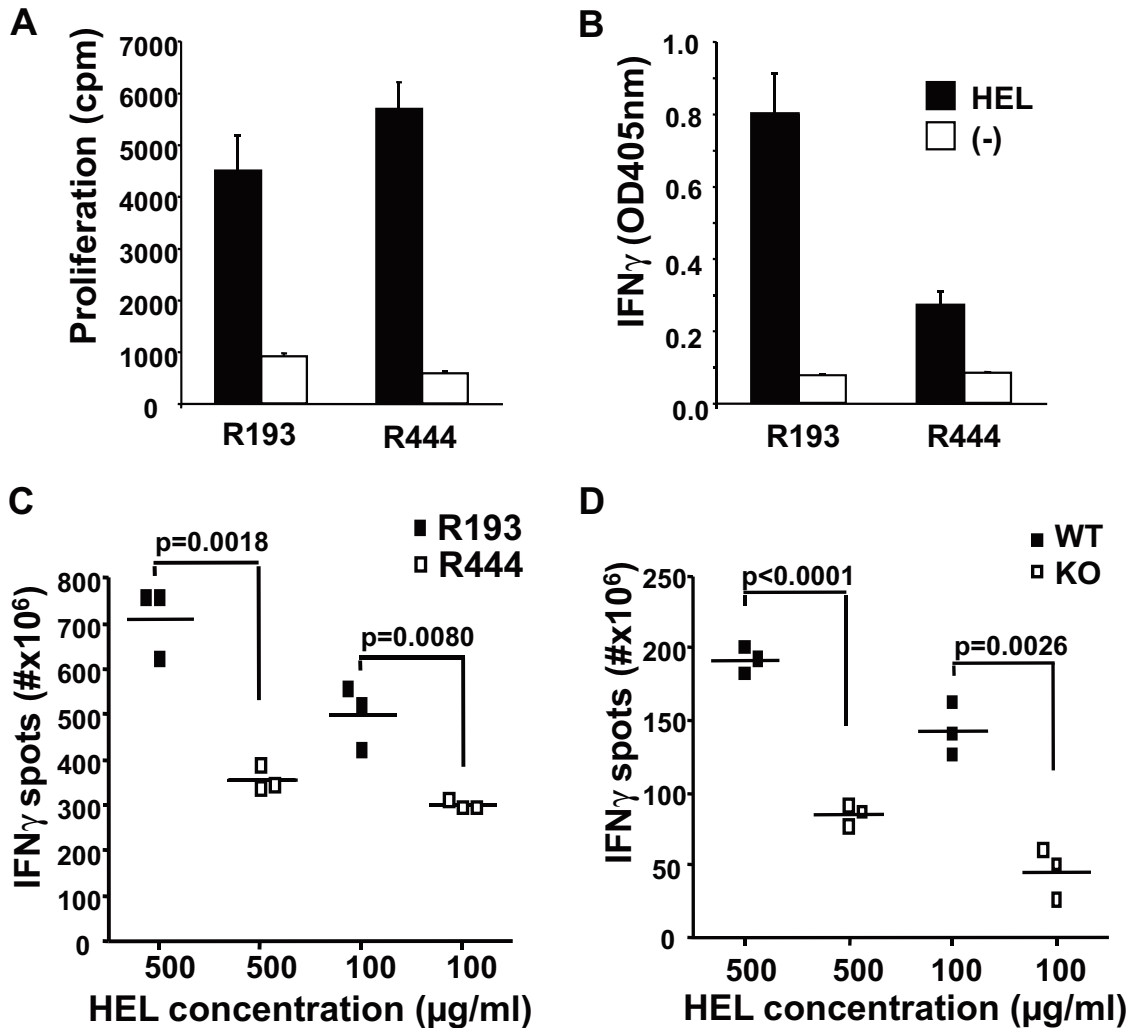


FIG. 3. Increased Th1 response to HEL in Slc11a1-sufficient mice. Mice were immunized with HEL emulsified in complete Freund's adjuvant. After 9–12 days, primed splenocytes were collected to measure proliferation in the presence or absence of 200 $\mu\text{g/ml}$ HEL (A), IFN- γ production in 48-h culture supernatants (B), and IFN- γ -producing cells by ELISPOT in R193 versus R444 mice (C) or Slc11a1 WT 129/Sv versus Slc11a1 KO mice (D). Background spots with no HEL antigen in C: R193 = 4.7, R444 = 20.1, and in D: WT = 29, KO = 9 spots per 10^6 splenocytes. Each result is representative of two experiments performed that yielded similar results.

microbeads. Because only FITC, but not PE, is a pH-sensitive dye that degrades in an acidic environment, a drop in the FITC/PE ratio would indicate a more acidic environment. Thus, this ratio can be used to monitor pH change or the acidification process in phagosomes. Figure 5B demonstrates that this ratio decreased more rapidly in the R193 or WT DCs, suggesting that Slc11a1 mediates an enhanced acidification process in DCs after taking up the FITC/PE-microbeads. In four separate experiments using both the R193 and R444 congenic pair and the Slc11a1 WT and KO mice in parallel, Slc11a1 functional DCs persistently showed a lower FITC/PE ratio and a faster drop of this ratio, although the differences were relatively small except for one of the experiments. Similar results were also observed when splenic DCs, collected by plastic adherence, were used. In addition, the NOD background of the R193 and R444 mice clearly contributes to a much more vigorous phagocytic activity in comparison with the 129/sv background of the WT and KO mice (Fig. 5A).

Slc11a1 is expressed in subsets of activated DCs. Western blotting of Slc11a1 is problematic owing to its highly hydrophobic features (12 transmembrane domains). However, using an antibody specific for the NH₂-terminal region of the Slc11a1 molecule, we were able to

detect a band in WT but absent from KO peritoneal macrophages (data not shown), which confirmed a previous report by Vidal et al. (12). Importantly, we demonstrate, for the first time, a similar band (~100 kDa) in WT BM-derived DC samples, and the density of this band was enhanced after LPS stimulation (Fig. 6A). The fuzziness of the band can be ascribed to the heavy glycosylation and/or phosphorylation of the Slc11a1 protein (12). To examine Slc11a1 protein expression in splenic DCs, we established a flow cytometry protocol to stain intracellular Slc11a1 molecules. Figure 6B shows that despite a high background of the anti-Slc11a1 antibody, a majority of the LPS-activated, CD11c⁺ WT, but not KO, splenic DCs express Slc11a1. Therefore, Slc11a1 can be upregulated in certain subsets of splenic DCs after stimulation with LPS.

DISCUSSION

Slc11a1 expressed in DCs. In this study, we demonstrated that both BM-derived DCs and splenic CD11c⁺ DCs express Slc11a1, confirming a recent report that Slc11a1 is expressed in dendritic cells (7). However, by comparing Slc11a1 WT and KO DCs on the 129 genetic background as well as DCs from congenic NOD strains expressing WT

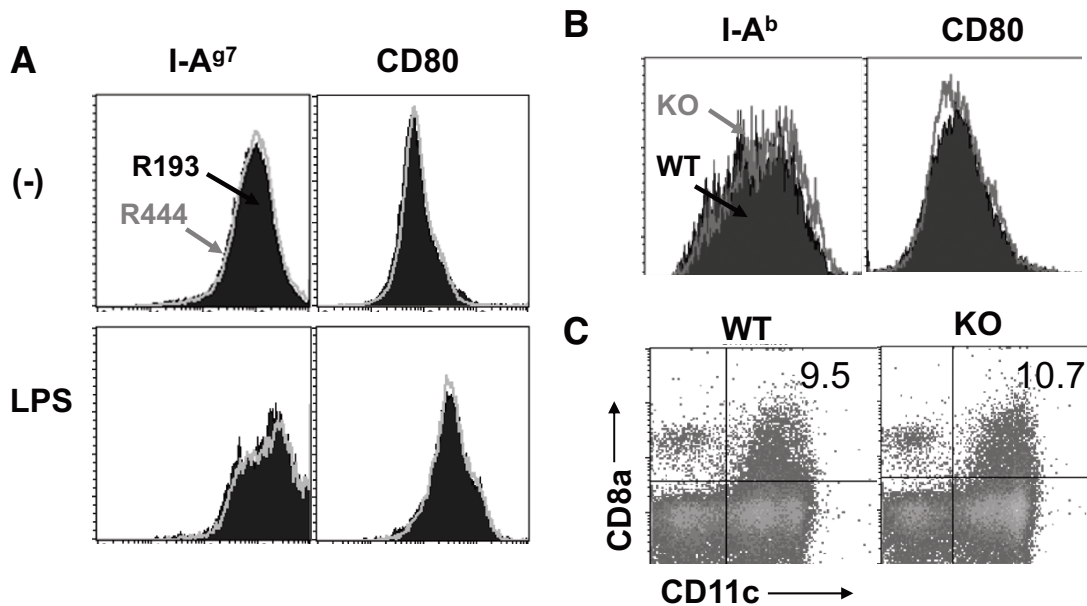


FIG. 4. Expression of MHC class II and CD80 molecules on splenic DCs. **A:** DCs were isolated from collagenase-digested splenocytes from R193 (black) or R444 (gray) mice using anti-CD11c-coated microbeads, followed by culture for 24 h in the presence or absence of 2 μ g/ml LPS. I-A^{g7} MHC class II and CD80 expression was examined using specific antibodies. **B:** Splenic CD11c⁺ DCs were similarly isolated but from WT (black) or KO (gray) mice and then stained with antibodies specific for I-A^b or CD80. **C:** WT or KO CD11c⁺ splenic DCs were examined for CD8 expression. Numbers shown are percentages of CD11c⁺CD8⁺ splenic DCs.

Slc11a1 or a nonfunctional mutant of Slc11a1, we did not confirm the difference in MHC class II expression previously reported using BM-derived DCs from Slc11a1 congenic mice on the B10 genetic background (7). Instead we saw equivalent class II expression on freshly isolated as well as LPS-stimulated splenic DCs (Fig. 4). We also examined unstimulated CD8⁺ or CD8⁻ CD11c⁺ DC subsets, and similarly, we found no difference between the Slc11a1 WT and KO in their MHC class II expression level. In addition to the genetic background differences present

in the two studies, it is possible that the cell types examined (BM-derived DCs in the case of Stober et al. and splenic DCs in our study) could account for the discrepant observations. Because different DC subsets or maturation stages vary in their MHC class II expression level, the

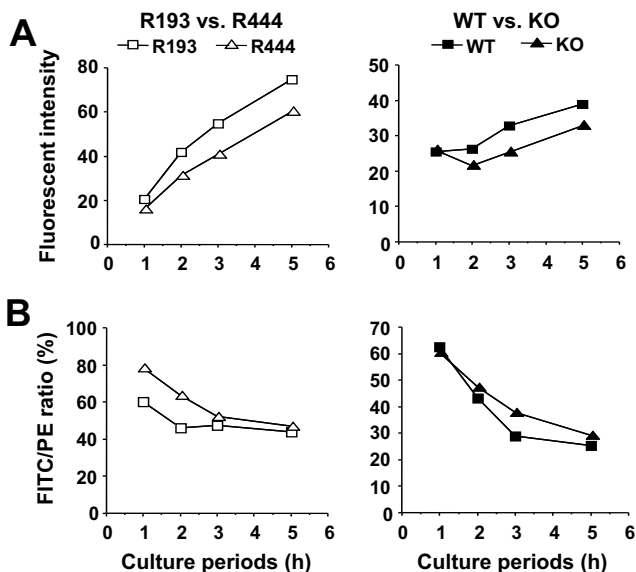


FIG. 5. Effects of Slc11a1 on DC phagocytosis and acidification. BM-DCs were incubated with preformed FITC/PE microbeads (see RESEARCH DESIGN AND METHODS) for various times at 37°C, followed by FACS analysis of DC-bound FITC and PE fluorescent dyes. **A:** Rate of phagocytosis: the accumulation of the PE dye in the microbeads-pulsed DCs generated from the bone marrow of R193, R444, Slc11a1 WT, and KO mice. **B:** Rate of phagosomal acidification: FITC/PE ratio = (average FITC intensity/average PE intensity in DCs) \times 100%. Similar results were observed in four independent experiments.

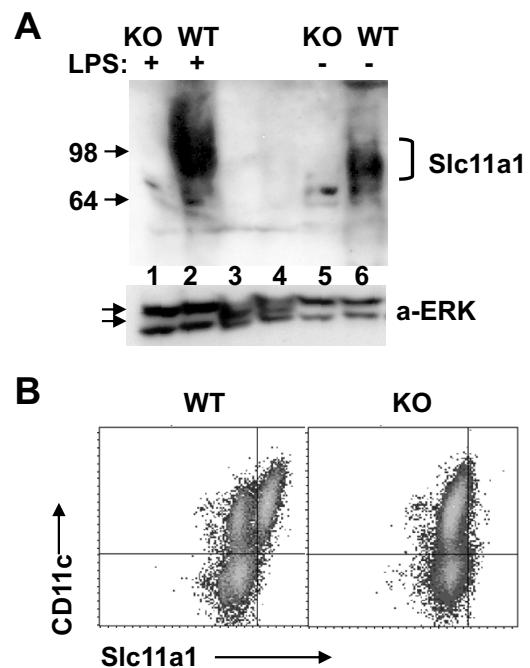


FIG. 6. Slc11a1 is expressed in activated dendritic cells. **A:** BM-derived DCs were analyzed for Slc11a1 expression by Western blot using a polyclonal Slc11a1-specific antibody (12). Lanes 1 and 2 are cell lysates from LPS-stimulated BM-derived DCs generated from 129.Slc11a1 KO and 129 WT mice, respectively. Lanes 3 and 4 are total splenocytes ($\sim 5 \times 10^6$ /lane) from KO and WT mice, respectively. Lanes 5 and 6 are immature BM-derived DCs with no LPS stimulation. **B:** Splenic-derived CD11c⁺ DCs were enriched by plastic adherence, followed by overnight LPS stimulation. Intracellular FACS staining was performed using the Slc11a1-specific antibody, and DCs were identified by co-staining with labeled anti-CD11c antibody.

functional status of Slc11a1 could influence the maturation rate of BM-derived DCs *in vitro*, whereas splenic DC subsets have differentiated entirely *in vivo*.

Selectively enhanced antigen presentation by Slc11a1. Because we lacked evidence that the level of MHC class II molecules on the surface of DCs was altered by the status of Slc11a1, we hypothesized that Slc11a1 alters antigen processing. We presented evidence that Slc11a1 enhances the rates of phagocytosis and phagosomal acidification in DCs (Fig. 5) and enhances the processing and presentation of certain T-cell determinants (Figs. 1 and 2); therefore, we propose that Slc11a1 alters antigen presentation by altering the processing machinery, rather than increasing the expression of MHC class II molecules on DCs. In other ongoing studies of the processing and presentation of HEL protein, we have observed that for particular HEL-specific T-cell clones, APCs lacking Slc11a1 are twofold more efficient than the APCs from WT mice in processing HEL and activating such clones (Y.D.D., I.G.M., E.E.S., and C.R. Gabaglia, unpublished data). Thus, the enhanced antigen presentation by Slc11a1 in the current study is likely selective, rather than owing to nonspecific enhancement of any available T-cell determinants.

Proinflammatory activity of Slc11a1. We observed a strong induction of IFN- γ -secreting Th1 cells after HEL immunization (Fig. 3). This pro-Th1 activity of Slc11a1 does not necessarily require a strong adjuvant such as complete Freund's adjuvant, since a similar increase of IFN- γ -producing cells was also observed in R193 mice after immunization with HEL emulsified in incomplete Freund's adjuvant (data not shown). Stober et al. (7) reported that there was a significant increase of the ratio of IL-10/IL-12 mRNA in mutant DCs, but the difference was mainly obvious in unstimulated DCs. Again, these DCs were produced *in vitro* from bone marrow after stimulation with GM-CSF and IL-4 for over 10–15 days. It remains to be addressed whether Slc11a1 could exhibit a similar effect on the ratio of IL-10/IL-12 production in freshly isolated peripheral DCs. Alternatively, as proposed in this study, Slc11a1 may promote a proinflammatory state and a response with a Th1 bias by altering antigen processing and presentation, e.g., by inducing T-cell clones with higher affinity, or by reducing the specific regulatory T-cell population. Future experiments will address whether such proinflammatory activity of Slc11a1 is seen for antigens other than GAD65 and HEL. Such altered processing may favor enhanced generation of usually cryptic determinants from the target antigens, or eliminate certain determinants related to regulatory function, or create modified determinants of the antigens with higher affinity.

Enhanced intracellular vesicle fusion and/or acidification by Slc11a1. The intracellular interaction of Slc11a1 with antigens or pathogens remains unclear. By fusing with intracellular vesicles and/or recruiting newly synthesized molecules, primary endocytic vesicles or phagosomes enter a gradual maturation process, which includes early endosomes, later endosomes, and lysosomes. During this maturation process, the hydrolytic activity of the endocytic vesicles increases gradually as the luminal pH value decreases from pH 6.3–6.8 in early endosomes to pH 4.5–5.5 in lysosomes; this pH drop might vary in different cell types and/or depend on their activation status. It has been suggested that Slc11a1 functions as a membrane transporter of divalent cations (27), but its mechanism of mediating natural resistance to bacterial

and parasitic infections remains unclear. Previous studies on the innate response of macrophages during intracellular bacterial infection suggested that Slc11a1-mediated deprivation of divalent cations might change the phagosomal microenvironment that impairs the pathogenesis of intracellular pathogens (27). Alternatively, the transporter activity of Slc11a1 might accelerate the acidification process by either enhancing the fusion of phagosomes with late endosomes/lysosomes, or by increasing V-ATPase activity and proton accumulation in the phagosomes (28,29). Our data from measuring intracellular pH values in DCs (Fig. 5) are consistent with previous findings that Slc11a1 contributes to enhanced phagosomal acidification in macrophages (28), although the differences we observed between DCs from the wild-type versus the mutant strains were less robust, which may be due to a less sensitive method (flow cytometry) that we used. If we could monitor the FITC/PE ratio within the intracellular later endosomes or lysosomes where the Slc11a1 molecules are expressed, we might see a stronger difference. We propose that in the presence of Slc11a1 protein, altered antigen transport or an increased intravascular acidic environment might expose the antigens to certain hydrolytic enzymes, such as aspartic and cysteine peptidases and amino- or carboxy-peptidases, or to an overall stronger hydrolytic activity, leading to differential presentation of T-cell epitopes on MHC molecules. It can be expected that such Slc11a1-mediated changes in antigen processing within the vesicular system could have general effects in autoimmune as well as in infectious systems.

Altered antigen processing and presentation in type 1 diabetes. APCs have long been considered as contributing to diabetes susceptibility (30–32) owing to their exclusive expression of MHC class II, the major genetic factor underlying type 1 diabetes. Some efforts have also been made to identify diabetes-associated genes that are involved in antigen-processing pathways (33–37), but most of these results have been either contradictory or inconclusive. This is indicative of the difficulty in using genetic tools to link individual genes to a particular phenotype in such a complicated multigene-involved autoimmune disease, although it has been suggested that diabetogenic genes affecting pathogenesis influenced by APCs are independent from those genes regulating T-cell activity (38). A few genes involved in class II processing pathways have been studied for their effects on diabetes in NOD mice using gene knockout NOD mice, instead of natural mutants. Cathepsin (Cat) L-deficient NOD mice failed to develop insulinitis as well as diabetes (39). The authors found decreases in the percentage of total CD4 T-cells as well as of the diabetogenic BDC2.5 T-cell, but a relative increase of CD4⁺CD25⁺ regulatory T-cells. Loss of the invariant (Ii) chain, the chaperone for the class II processing pathway, also protected NOD mice from diabetes (40). Similarly, decreased CD4 numbers were found in both the thymus and the periphery, but the percentage of CD4⁺CD25⁺ regulatory T-cells remained constant, indicating that these regulatory cells may mature in the thymus and/or differentiate in the periphery through an Ii-independent pathway. The decreased ratio of pathogenic versus regulatory T-cells could be an explanation in the above two studies, but more likely, the altered antigen processing of self-antigens in the thymus versus the periphery may interfere with the specific association of I-A^{g7} with diabetes in NOD mice—an essential pathway in which non-MHC genes contribute to MHC-associated autoimmune disease.

It is likely that a shift of peptide and T-cell repertoire would be expected after such drastic alterations of the antigen-processing machinery.

An interesting question for autoimmunity in general is why defective processing machinery should affect a T-cell-dominant autoimmune response. A short answer is that some pathogenic T-cell determinants require a particular enzymatic activity within the APCs processing machinery, which is also able to prevent or reduce the generation of regulatory determinants for presentation to MHC class II-restricted T-cells. Our early idea of MHC-guided processing as a major pathway in antigen presentation (42) was strongly supported by data showing that very long lysozyme peptides of approximately 70 amino acids could be detected in peptide elution experiments (41). Subsequently, the MHC-bound peptide is trimmed at both ends (43) to yield the typical short 13–25 residue peptides still bound to class II MHC (44,45). Recently, we demonstrated that trimming of the flanking residues of a diabetes-associated GAD65 peptide could affect the selection of individual T-cell clones for activation (46), and importantly, such trimming events varied between diabetes-susceptible and -resistant strains, with the susceptible strain exhibiting an enhanced selection of diabetogenic T-cells. How this selection process, catalyzed by MHC-guided trimming, is associated with disease development, and whether Slc11a1 is involved in this process remains to be learned. Further studies of this heterogeneous epitope-specific repertoire and its diverse functional phenotypes are indicated.

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