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Testing the Role of P2X₇ Receptors in the Development of Type 1 Diabetes in Nonobese Diabetic Mice

Yi-Guang Chen,¹ Felix Scheuplein,² John P. Driver, Amanda A. Hewes, Peter C. Reifsnyder, Edward H. Leiter, and David V. Serreze

Although *P2rx7* has been proposed as a type 1 diabetes (T1D) susceptibility gene in NOD mice, its potential pathogenic role has not been directly determined. To test this possibility, we generated a new NOD stock deficient in P2X₇ receptors. T1D development was not altered by P2X₇ ablation. Previous studies found CD38 knockout (KO) NOD mice developed accelerated T1D partly because of a loss of CD4⁺ invariant NKT (iNKT) cells and Foxp3⁺ regulatory T cells (Tregs). These immunoregulatory T cell populations are highly sensitive to NAD-induced cell death activated by ADP ribosyltransferase-2 (ART2)-mediated ADP ribosylation of P2X₇ receptors. Therefore, we asked whether T1D acceleration was suppressed in a double-KO NOD stock lacking both P2X₇ and CD38 by rescuing CD4⁺ iNKT cells and Tregs from NAD-induced cell death. We demonstrated that P2X₇ was required for T1D acceleration induced by CD38 deficiency. The CD38 KO-induced defects in homeostasis of CD4⁺ iNKT cells and Tregs were corrected by coablation of P2X₇. T1D acceleration in CD38-deficient NOD mice also requires ART2 expression. If increased ADP ribosylation of P2X₇ in CD38-deficient NOD mice underlies disease acceleration, then a comparable T1D incidence should be induced by coablation of both CD38 and ART2, or CD38 and P2X₇. However, a previously established NOD stock deficient in both CD38 and ART2 expression is T1D resistant. This study demonstrated the presence of a T1D resistance gene closely linked to the ablated *Cd38* allele in the previously reported NOD stock also lacking ART2, but not in the newly generated CD38/P2X₇ double-KO line. *The Journal of Immunology*, 2011, 186: 4278–4284.

The P2X₇ receptor is an ATP-gated ion channel widely expressed in a variety of cell types including those in the immune system (1). Brief stimulation of the P2X₇ receptor with ATP reversibly opens a membrane channel and rapidly triggers influx of Na⁺ and Ca²⁺, and efflux of K⁺, whereas its prolonged activation leads to formation of a nonselective pore that allows the passage of molecules up to 900 Da (2, 3). In the immune system, the function of P2X₇ has been mostly studied in macrophages and T cells. ATP-mediated P2X₇ activation facilitates macrophage maturation and IL-1 β secretion (4). It has also been shown that effective T cell activation requires ATP release and the autocrine feedback through P2X₇ receptors (5, 6). Recent studies have identified an alternative mechanism that indirectly activates P2X₇ receptors. ADP ribosyltransferase-2 (ART2)-mediated and NAD-dependent ADP ribosylation of P2X₇ also activates this receptor (7). Two isoforms of ART2 (ART2.1 and ART2.2) encoded by tandem genes are expressed in mice (8).

Both ART2.1 and ART2.2 are expressed by T cells in most inbred mouse strains (9–12). Expression of ART2.1 is induced in macrophages in response to several inflammatory stimuli (13). ART2.1-mediated ADP ribosylation has been shown to lower the threshold required for P2X₇ activation in macrophages in response to extracellular ATP (14). In contrast, ART2-mediated ADP ribosylation of P2X₇ receptors on T cells results in NAD-induced cell death (NICD) (7).

Polymorphisms in the *P2rx7* gene resulting in a single change at amino acid residue 451 (Pro or Leu) of the encoded protein have been described in common inbred mouse strains (15). Autoimmune type 1 diabetes (T1D)-susceptible NOD mice harbor the “wild-type” allele encoding proline at position 451 that allows for high sensitivity to ATP (15). Conversely, the “mutant” allele expressed by C57BL/6 and C57BL/10 mice encodes a leucine at the same position that significantly increases the threshold required for ATP-induced P2X₇ activation (15). Considering the diverse roles of P2X₇ in the immune system, it has been proposed as a candidate T1D susceptibility (*Idd*) gene in NOD mice (16). However, this hypothesis has not been directly tested. We previously showed T cell-mediated autoimmune T1D was accelerated in CD38 knockout (KO) NOD mice (17). Disease acceleration in NOD.*CD38*^{-/-} mice required the expression of ART2 molecules (17). T1D acceleration in NOD.*CD38*^{-/-} mice was associated with a loss of CD4⁺ invariant NKT (iNKT) cells and Foxp3⁺ regulatory T cells (Tregs) (17, 18). CD38 is an ectoenzyme that uses NAD to generate multiple calcium (Ca²⁺)-mobilizing metabolites, including ADP-ribose and cyclic ADP-ribose (19). CD38 counterregulates ART2-dependent ADP ribosylation levels of T cell surface proteins including P2X₇ by competing for their common NAD substrate (20). Therefore, in the absence of the NAD hydrolyzing activity of CD38, increased concentrations of extracellular NAD allow for greater levels of ART2-mediated ADP ribosylation of P2X₇ receptors on T cells and their sub-

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Abbreviations used in this article: ART2, ADP ribosyltransferase-2; DKO, double knockout; DN, double negative; α -GalCer, α -galactosylceramide; iNKT, invariant NKT; KO, knockout; NICD, NAD-induced cell death; PLN, pancreatic lymph node; T1D, type 1 diabetes; Treg, regulatory T cell.

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sequent loss by NICD. CD4⁺ iNKT cells are extremely sensitive to NICD, most likely because of high expression levels of both ART2 and P2X₇ (18, 21). Similarly, Tregs have also been reported to be highly sensitive to NICD (22).

The collective results described earlier suggested, but did not directly assess, whether T1D acceleration in NOD.*CD38*^{-/-} mice also relies on the presence of P2X₇ molecules. It was also unknown whether concurrent deficiency of P2X₇ molecules could restore iNKT cells and Tregs in the CD38 KO stock to the levels observed in standard NOD mice. Furthermore, it remains to be determined whether independent of its role in mediating the loss of immunoregulatory populations through an NICD mechanism, P2X₇ might also contribute to T1D development through its capacity to serve as an ATP sensor in macrophages and T cells that respectively promotes IL-1 β secretion and effector function activation (23). To directly test these possibilities, we determined whether T1D development was altered in a newly generated P2X₇-deficient NOD stock. We also determined whether a functional interaction between CD38 and P2X₇ molecules modulates T1D development.

Materials and Methods

Mice

P2rx7 KO mice on the C57BL/6J background were originally obtained from the Induced Mutant Resource at The Jackson Laboratory (JAX stock no. 5576) (24) and the mutation backcrossed to the NOD/LtDvs strain. Linkage markers delineating all known *Idd* genetic loci have been fixed to homozygosity for NOD alleles in the strain now designated NOD.*P2X7*^{-/-}, currently maintained at the N9 backcross generation with a congenic interval of <49 Mb (the proximal boundary between *D5Mit259* and *P2rx7*; the distal boundary between *D5Mit138* and *D5Mit98*). The donor strain congenic interval of the previously reported NOD.*CD38*^{-/-} mouse stock has been further shortened (17) (Fig. 6A). The newly developed NOD.*CD38*^{-/-} stock was then used to generate NOD.*CD38*^{-/-}.*P2X7*^{-/-} mice. Because the *Cd38* and *P2rx7* genes are both on chromosome 5, this required identification and breeding of a double recombinant (17). NOD.*ART2*^{-/-} mice have been previously described (17). A stock of NOD mice deficient in both CD38 and ART2 molecules was also generated with the newly developed NOD.*CD38*^{-/-} strain, denoted as NOD.*CD38*^{-/-}<short>. *ART2*^{-/-} hereafter. To distinguish the new NOD.*CD38*^{-/-}<short>. *ART2*^{-/-} stock from the previously described NOD.*CD38*^{-/-}.*ART2*^{-/-} stock with the longer congenic interval around the *Cd38* locus, we denoted the latter as NOD.*CD38*^{-/-}<long>. *ART2*^{-/-} hereafter.

Flow cytometry

RBC-depleted single-cell suspensions were prepared from spleens and pancreatic lymph nodes (PLNs). P2X₇ expression by T cells was analyzed by staining leukocytes with the unconjugated HANO43 Ab and allophycocyanin-conjugated anti-CD3 ϵ (clone 145-2C11) followed by FITC-conjugated polyclonal goat anti-rat Ig. Tregs were identified by surface staining with anti-CD4 (clone RM4-5) and anti-CD25 (clone PC61), and intracellular staining with anti-Foxp3 (clone FJK-16s) according to instructions of the kit from eBioscience (San Diego, CA). For iNKT cell staining, cells were first treated with Fc block (anti-CD16/CD32, clone 2.4G2) at room temperature for 10 min and then incubated with a mixture containing anti-CD4, anti-TCR β , and the α -galactosylceramide (α -GalCer) analog PBS-57 loaded CD1d-tetramer (obtained from National Institutes of Health tetramer core facility) at 4°C for 30 min. Stained cells were washed and analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software. Anti-CD4, anti-CD3 ϵ , anti-CD25, and anti-TCR β were purchased from BD Biosciences (San Jose, CA). Anti-P2X₇ was obtained from Enzo Biosciences (Plymouth Meeting, PA). To analyze iNKT cell functional activity, we injected mice i.v. with 2 μ g α -GalCer (Enzo Life Sciences). Production of IFN- γ and IL-4 by iNKT cells was analyzed by intracellular staining at 2 h after α -GalCer administration. Stained cells were analyzed on an LSRII flow cytometer (Becton Dickinson). Both IFN- γ and IL-4 Abs were from eBioscience.

NICD analysis

To determine the effect of NAD on T cell viability, RBC-depleted splenocytes (5 \times 10⁶/ml) were incubated in previously described RPMI

1640 tissue culture medium (25) containing varying concentrations of NAD (Sigma, St. Louis, MO) at 37°C for 30 min. Cells were then washed and resuspended in Annexin V friendly FACS buffer, and stained with Annexin V (BD Biosciences) and anti-CD3 ϵ at 4°C for 30 min as described previously (18). Propidium iodine was added to all samples before analyses on a FACSCalibur flow cytometer (Becton Dickinson).

T1D incidence study

T1D was assessed by weekly monitoring of glycosuria development with Ames Diastrix (Diagnostics Division, Bayer, Elkhart, IN), and disease onset was defined as two consecutive values \geq 3.

In vitro Treg assay

Treg assays were performed as previously described (26). In brief, splenic CD4⁺ T cells were purified from 6–8-wk-old mice by depleting B220⁺, CD8⁺, and CD11b⁺ cells with the previously described magnetic bead system. The CD25⁺ fraction was further isolated by staining with biotinylated anti-CD25 (clone 7D4) followed by streptavidin-conjugated microbeads (Miltenyi Biotec). CD4⁺CD25⁺ cells were then enriched by positive selection using an LS column (Miltenyi Biotec). The CD4⁺CD25⁻ fraction was used as the effector population. Effector cells were labeled with CFSE as previously described. Labeled effector cells (5 \times 10⁴) were cocultured in triplicate with indicated numbers of CD4⁺CD25⁺ Tregs in the presence of 2 \times 10⁵ NOD.*scid* splenocytes and 5 μ g/ml anti-CD3 (clone 145-2C11, BD Bioscience) in round-bottom 96-well tissue culture plates in a final volume of 200 μ l of the previously described culture medium (25). Proliferation of effector cells was determined after 3 d of culture by CFSE dilution.

Results

Expression levels of P2X₇ receptors dictate the sensitivity of mature T cells to NICD

We first confirmed the absence of P2X₇ protein expression and function in NOD.*P2X7*^{-/-} mice. P2X₇ expression was assessed by flow cytometry and functional activity assayed by the sensitivity of mature T cells to NICD. As shown in Fig. 1A, expression of P2X₇ on the surface of splenic T cells was completely absent in

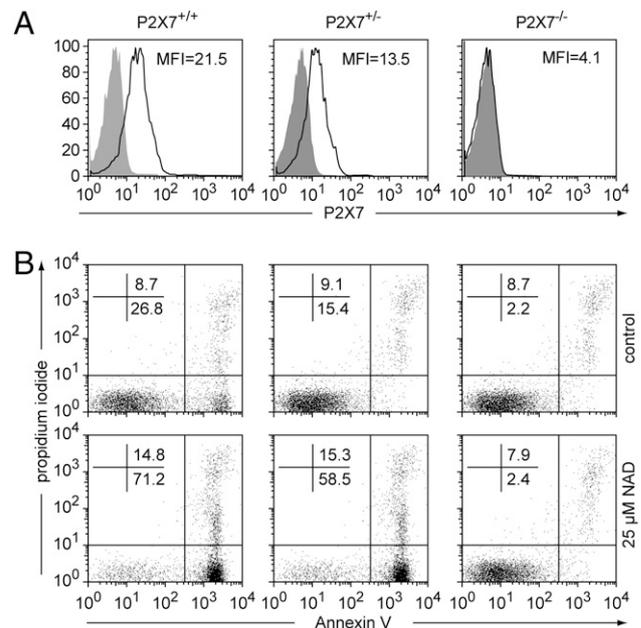


FIGURE 1. T cells from NOD.*P2X7*^{-/-} mice are resistant to NICD. *A*, Flow cytometric analysis of P2X₇ expression on total splenic T cells (gated on CD3⁺). A representative histogram from at least five mice per genotype is shown. Shaded areas represent unstained control; open areas represent with anti-P2X₇. *B*, Splenocytes were incubated with or without 25 μ M NAD at 37°C for 30 min. Apoptotic cells were determined by Annexin V and propidium iodine staining (gated on CD3⁺ cells). Similar results were obtained from another two experiments.

the NOD.*P2X₇^{-/-}* mice. P2X₇ was expressed at an intermediate level in NOD.*P2X₇^{+/-}* mice compared with wild-type and KO littermates (Fig. 1A). Splenic T cells from NOD.*P2X₇^{-/-}* mice were completely resistant to NICD (Fig. 1B). In contrast, splenic T cells from NOD.*P2X₇^{+/-}* and wild-type littermates were sensitive to NICD (Fig. 1B). Interestingly, P2X₇ expression levels directly correlated with the proportions of T cells undergoing NICD (Fig. 1B). A small but significant proportion of NOD.*P2X₇^{+/-}* and wild-type T cells also underwent apoptosis in the absence of exogenous NAD. This was most likely because of intracellular NAD released from lysed RBCs or other dead cells during the preparation process (27).

P2X₇ deficiency alone does not alter T1D development in NOD mice

To determine whether P2X₇ plays a role in the development of T1D in NOD mice, disease onset rates were compared for littermates carrying the wild-type (+/+), heterozygous (+/-), or homozygous (-/-) *P2rx7* KO alleles. In both male and female mice, no significant differences were found between any genotypes (Fig. 2).

CD38 deficiency-induced T1D acceleration in NOD mice is P2X₇ dependent

Our previous studies suggested, but did not definitively demonstrate, that *CD38* deficiency-induced T1D acceleration in NOD mice needs an intact NICD pathway, that is, ART2-mediated ADP ribosylation and activation of P2X₇ molecules (17). To directly test this hypothesis, we intercrossed *CD38* and *P2X₇*-deficient NOD stocks and generated NOD.*CD38^{-/-}.P2X₇^{-/-}* mice. We directly compared the T1D development rates of wild-type, *CD38* KO, *P2X₇* KO, and *CD38/P2X₇* double-KO (DKO) NOD mice side by side. Consistent with the previously published results (17), the NOD.*CD38^{-/-}* stock exhibited greatly accelerated T1D development compared with standard NOD mice (Fig. 3). Although

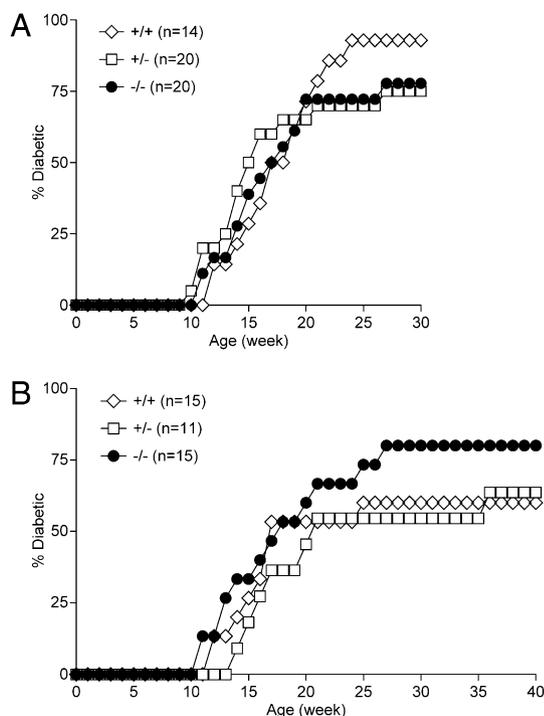


FIGURE 2. T1D incidence of NOD.*P2X₇^{-/-}* female (A) and male (B) mice and their wild-type and heterozygous littermate controls. No statistically significant differences were found between genotypes in both sexes.

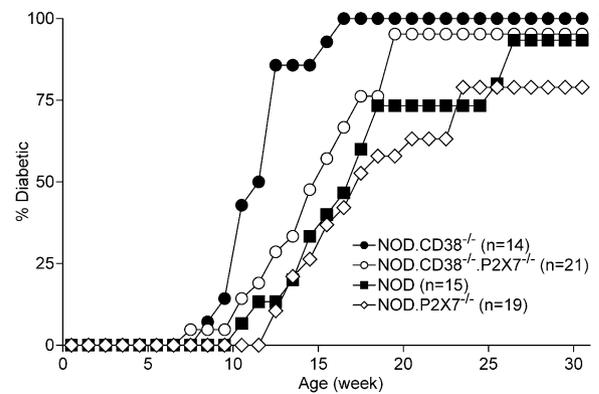


FIGURE 3. T1D incidence of NOD, NOD.*CD38^{-/-}*, NOD.*P2X₇^{-/-}*, and NOD.*CD38^{-/-}.P2X₇^{-/-}* female mice. **p* < 0.005 compared with other three strains (Kaplan–Meier log-rank analysis). There is no statistical difference between NOD and NOD.*P2X₇^{-/-}* or NOD and NOD.*CD38^{-/-}*. *P2X₇^{-/-}* mice. The T1D development rate between NOD.*P2X₇^{-/-}* and NOD.*CD38^{-/-}.P2X₇^{-/-}* mice is significantly different. #*p* = 0.0304, Kaplan–Meier log-rank analysis.

P2X₇ deficiency alone did not significantly affect disease onset (Fig. 2), its absence completely abrogated accelerated T1D development in *CD38*-deficient DKO mice (*p* = 0.2712, NOD versus NOD.*CD38^{-/-}.P2X₇^{-/-}*; Fig. 3). Therefore, we conclude that ADP ribosylation of P2X₇ receptors and consequent triggering of NICD play an important role to promote accelerated T1D development in NOD.*CD38^{-/-}* mice. We noted that the rate of T1D development is slightly greater in NOD.*CD38^{-/-}.P2X₇^{-/-}* than in NOD.*P2X₇^{-/-}* mice. This suggests that in the absence of *CD38* molecules, ART2 may promote T1D development independent of P2X₇ by ADP ribosylation of other proteins. This possibility will be the subject of future studies.

P2X₇ deficiency rescues the depleted iNKT and Treg compartments in NOD.CD38^{-/-} mice

T1D acceleration in NOD.*CD38^{-/-}* mice is associated with a loss of peripheral CD4⁺ iNKT cells (18). Therefore, we asked whether

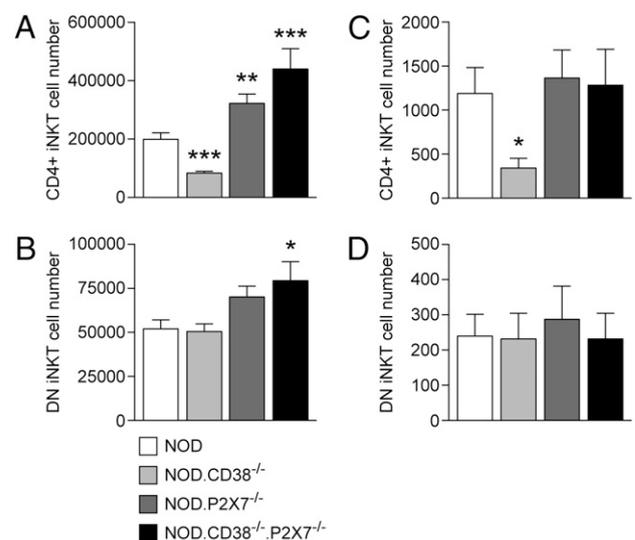
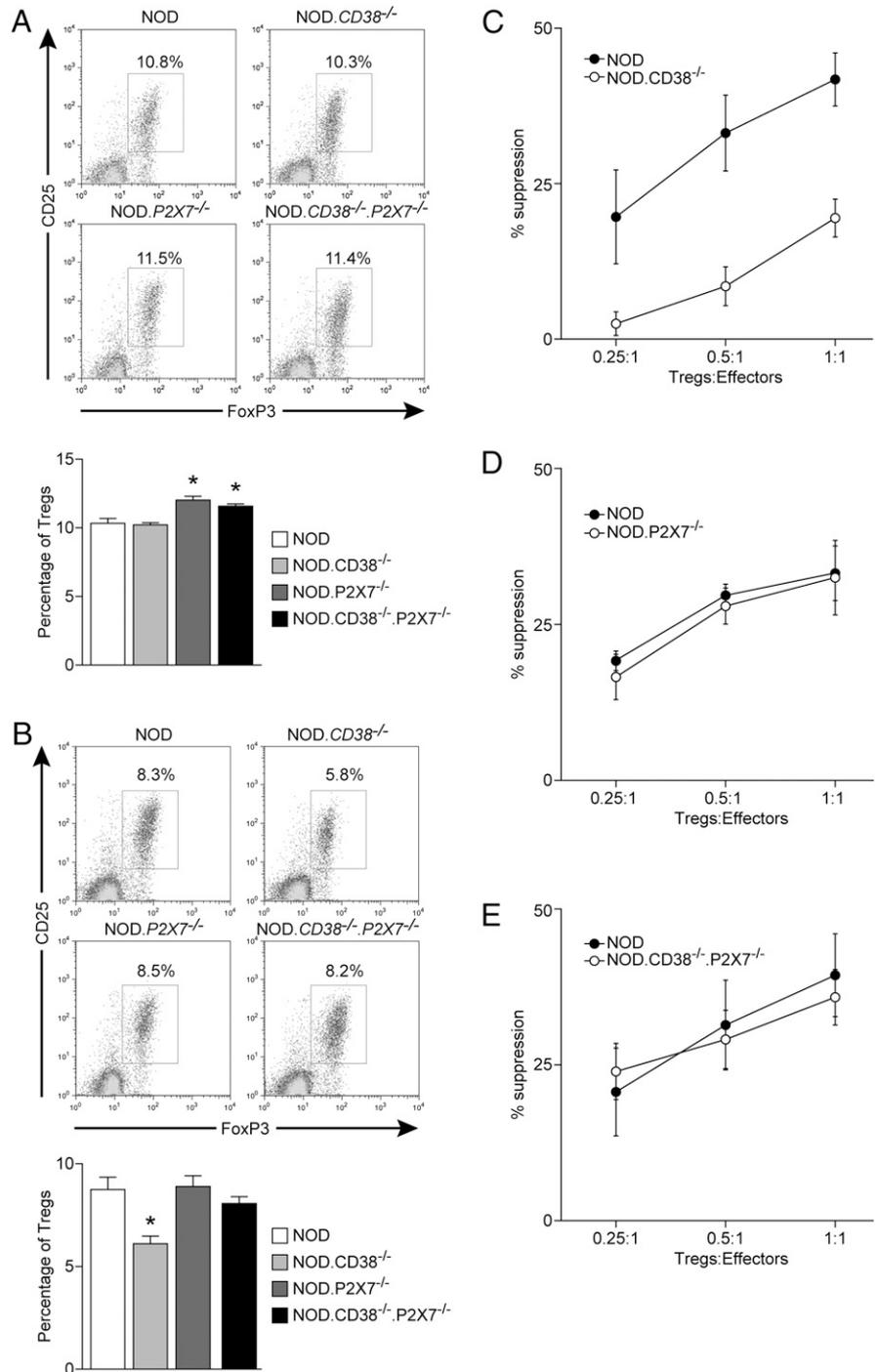


FIGURE 4. Coablation of *P2X₇* improves the survival of CD4⁺ iNKT cells in *CD38*-deficient NOD mice. The numbers of iNKT cell subsets were determined in 6–8-wk-old female mice. Splenic (A, B) and PLN (C, D) iNKT cells were identified by CD1d tetramer staining and further separated into CD4⁺ (A, C) and DN (B, D) subsets. **p* < 0.05, ****p* < 0.01, *****p* < 0.005, compared with NOD mice (Mann–Whitney *U* test, *n* = 6–9 per genotype).

the frequencies and numbers of CD4⁺ iNKT cells in the NOD. *CD38*^{-/-}.*P2X7*^{-/-} stock are restored to the levels in standard NOD mice. We determined the numbers of CD4⁺ and double negative (DN) iNKT cells in the spleens and PLNs of standard NOD, NOD.*CD38*^{-/-}, NOD.*P2X7*^{-/-}, and NOD.*CD38*^{-/-}.*P2X7*^{-/-} mice. As expected, the NOD.*CD38*^{-/-} stock had significantly reduced numbers of splenic CD4⁺ iNKT cells compared with standard NOD mice (Fig. 4A). Interestingly, numbers of splenic CD4⁺ iNKT cells were not only restored in the NOD.*CD38*^{-/-}.*P2X7*^{-/-} stock but actually greater than in standard NOD mice (Fig. 4A). This was also true for NOD.*P2X7*^{-/-} mice (Fig. 4A). Therefore, the loss of immunoregulatory CD4⁺ iNKT cells in NOD.*CD38*^{-/-} mice is a *P2X7*-dependent process. However, *P2X7* ablation also allows for an expansion of CD4⁺ iNKT

cells in CD38 intact NOD mice. Consistent with our previous results (18), standard NOD and NOD.*CD38*^{-/-} mice had similar levels of splenic DN iNKT cells (Fig. 4B). Compared with standard NOD mice, the NOD.*CD38*^{-/-}.*P2X7*^{-/-} stock, but not NOD.*P2X7*^{-/-} mice, also had increased numbers of splenic DN iNKT cells (Fig. 4B). Similarly, the loss of CD4⁺ iNKT cells in the PLNs of NOD.*CD38*^{-/-} mice was also rescued by the concomitant deficiency of *P2X7* molecules (Fig. 4C). No differences in the numbers of PLN DN iNKT cells was observed in standard NOD mice and those deficient in CD38 and/or *P2X7* molecules (Fig. 4D). Although CD38-deficient mice have fewer iNKT cells, we previously showed that the remaining ones responded normally to α -GalCer stimulation (18). In this study, we also tested the function of CD38 and *P2X7* double-deficient iNKT cells. On α -GalCer

FIGURE 5. Coablation of *P2X7* improves the survival and function of Tregs in CD38-deficient NOD mice. The frequency of CD4 Tregs (CD25⁺Foxp3⁺) was determined in the spleens (A) and PLNs (B) of 10–11-wk-old male NOD mice and those deficient in *CD38* and/or *P2X7*. A, The upper panel shows the representative CD25 and Foxp3 staining (gated on CD4 T cells). Results are summarized in the lower panel. **p* < 0.05, compared with NOD mice (Mann–Whitney *U* test, *n* = 5–6 per genotype). B, The upper panel shows the representative CD25 and Foxp3 staining (gated on CD4 T cells). Results are summarized in the lower panel. **p* < 0.05, compared with NOD mice (Mann–Whitney *U* test, *n* = 5–6 per genotype). C–E, In vitro Treg assay comparing the suppressive activity between indicated strains (age-matched female mice at 6–8 wk). NOD effector T cells (CD4⁺CD25⁻) were labeled with CFSE and cocultured at indicated ratios with Tregs (CD4⁺CD25⁺) in triplicate in a 96-well plate in the presence of NOD.*scid* splenocytes (2×10^5) and 5 μ g/ml anti-CD3 for 3 d. Proliferation of effector T cells was determined by CFSE dilution. The percentage of suppression is defined by the reduction in the proportion of divided effector T cells relative to that of the control without Tregs. Results indicate the mean \pm SEM of three independent experiments.



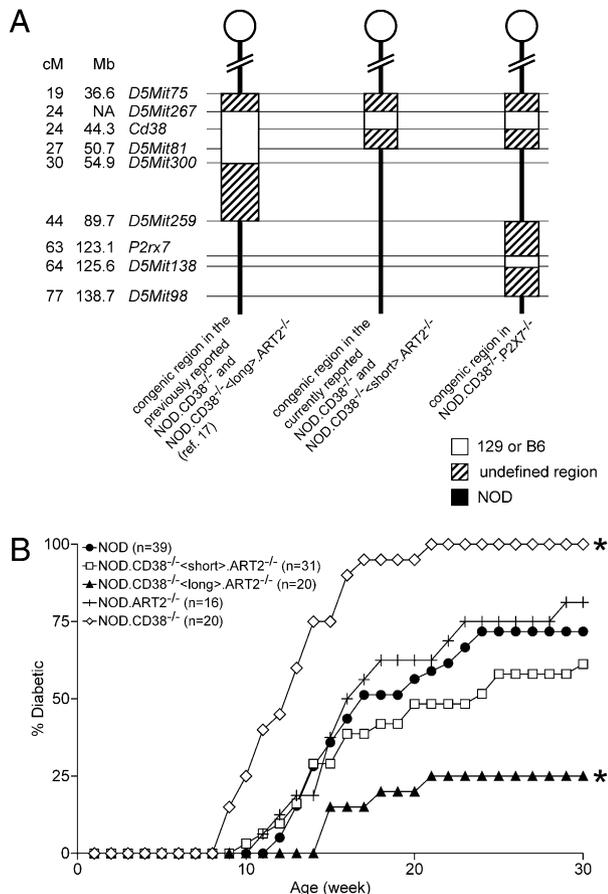


FIGURE 6. Identification of a NOD T1D susceptibility genetic locus closely linked to *Cd38* on chromosome 5. **A**, Genetic map showing the *Cd38* and *P2rx7* congenic regions in the indicated strains. The physical locations of the markers/genes are based on NCBI Build 37. NA, not available. **B**, T1D incidence of female NOD mice and stocks deficient in CD38 and/or ART2 molecules. **p* < 0.005, compared with NOD mice (Kaplan–Meier log-rank analysis).

stimulation, iNKT cells in standard NOD mice and the stock deficient in both CD38 and P2X₇ responded similarly as determined by intracellular IFN- γ and IL-4 staining (data not shown).

NOD.CD38^{-/-} mice also have reduced frequencies of Tregs (CD4⁺CD25⁺Foxp3⁺) in PLNs (17). Therefore, we determined whether Tregs in the NOD.CD38^{-/-}.P2X₇^{-/-} stock also return to the level characterizing standard NOD mice. Although the frequencies of Tregs were not significantly different in the spleens of NOD and NOD.CD38^{-/-} mice, we observed a trend of reduced Foxp3 protein expression (based on mean fluorescence intensity of specific Ab staining) in the latter stock, consistent with our previous analysis at the RNA transcript level (17) (Fig. 5A). In contrast, as shown in Fig. 5A, the ablation of P2X₇ resulted in a marginal, but statistically significant increase in the percentages of splenic Tregs in both CD38-deficient and intact NOD mice. Also in agreement with previous studies (17), the NOD.CD38^{-/-} stock had reduced percentages of Tregs in PLNs compared with standard NOD mice (Fig. 5B). Similarly, Foxp3 expression in CD38-deficient Tregs was also reduced compared with those in standard NOD mice (Fig. 5B). These defects were completely reversed in the concurrent absence of P2X₇ molecules (Fig. 5B). To compare the function of Tregs in standard NOD mice and those deficient in CD38 and/or P2X₇ molecules, we determined their suppressive activity in vitro. As previously described (21), Tregs from the NOD.CD38^{-/-} stock had reduced suppressive activity

compared with those in standard NOD mice (Fig. 5C). Although P2X₇ deficiency alone did not alter the suppressive activity of Tregs (Fig. 5D), it completely reversed the defect of those from CD38-deficient NOD mice (Fig. 5E versus 5C). These results indicate that the impaired function of Tregs in CD38-deficient NOD mice depends on the presence of intact P2X₇ molecules.

A locus closely linked to CD38 regulates T1D development

We previously showed that further depletion of the iNKT and Treg compartments apparently contributing to accelerated T1D development in NOD.CD38^{-/-} mice depended on the expression of ART2 molecules (17, 18). Together with the results reported in this study, we concluded that the further loss of iNKT cells, particularly the CD4⁺ subset, and Tregs in NOD.CD38^{-/-} mice are due to P2X₇-dependent NICD. However, in contrast with the observation in this study that codepletion of CD38 and P2X₇ did not alter the rate of T1D onset in NOD background mice, disease development was strongly suppressed in a previously reported stock lacking both CD38 and ART2 (denoted as NOD.CD38^{-/-}<long>.ART2^{-/-} in this study) (17). This suggested that, in the absence of both CD38 and ART2-mediated NAD-degrading activities, increased levels of extracellular and/or intracellular NAD may exert T1D suppressive effects. It was also possible that a 129/Sv or C57BL/6 (B6)-derived T1D resistance gene(s) closely linked to the targeted *Cd38* and/or *Art2* alleles was inadvertently introduced into the previously reported NOD DKO stock during the backcrossing process. The latter possibility is supported by a recent study identifying a novel 129/Sv-derived T1D resistance locus on chromosome 5 in close linkage to the *Cd38* gene (28). To test these possibilities, we used the currently reported NOD.CD38^{-/-} mice with a shortened congenic interval (Fig. 6A) to generate a new stock lacking both CD38 and ART2 (denoted as NOD.CD38^{-/-}<short>.ART2^{-/-}) and monitored them for T1D development. It should be noted that the chromosome 5 congenic interval carrying the *P2rx7* targeted allele does not overlap the 129/Sv and/or B6-derived region linked to the ablated *Cd38* gene present in the original NOD.CD38^{-/-}<long>.ART2^{-/-} stock (Fig. 6A).

Consistent with previous results, NOD.CD38^{-/-} and NOD.CD38^{-/-}<long>.ART2^{-/-} stocks were respectively more susceptible or resistant to T1D compared with standard NOD mice (17) (Fig. 6B). Also as shown previously, ART2 deficiency alone did not alter the course of T1D development in NOD mice (17) (Fig. 6B). Interestingly, the newly generated NOD.CD38^{-/-}<short>.ART2^{-/-} stock displayed a similar T1D incidence as standard NOD mice (Fig. 6B). Collectively, these results indicate that T1D resistance observed in NOD.CD38^{-/-}<long>.ART2^{-/-} mice is largely due to the presence of a 129/Sv- and/or B6-derived disease protective gene(s) closely linked to the inactivated *Cd38* allele.

Discussion

A P2X₇ receptor variant has been previously suggested as a candidate T1D susceptibility gene in NOD mice (16). In this study, we found that P2X₇ deficiency alone does not alter T1D development in NOD background mice. However, P2X₇ is required to promote the accelerated rate of T1D onset characterizing NOD.CD38^{-/-} mice. This was demonstrated by the finding that a newly generated NOD.CD38^{-/-}.P2X₇^{-/-} stock developed T1D at the same rate as standard NOD mice. In the T cell compartment, CD4⁺ iNKT cells and Tregs are highly sensitive to P2X₇-dependent ATP or NICD (18, 22). Although tight control mechanisms normally do not allow plasma ATP and NAD to reach levels sufficient to activate P2X₇-mediated cell death, local concen-

trations of these small molecules could be significantly increased as a result of inflammation or tissue destruction (29). Indeed, it has recently been shown that high concentrations of extracellular NAD depleted Tregs in vivo and promoted immunogenic responses (30). Therefore, it is tempting to speculate that the suboptimal survival of Tregs in inflamed pancreatic islets in NOD mice is, in part, due to P2X₇-dependent NICD in addition to the previously reported reduction in IL-2/IL-2R signaling (31). As a result of in-traitlet Treg loss, the pathogenic functions of β cell-specific effector T cells are further unleashed, possibly through enhanced accumulation of islet infiltrated and fully activated dendritic cells (32). Activated T cells shed ART2 molecules and become resistant to NICD (33). Therefore, P2X₇-dependent NICD can also promote effector T cell activity by eliminating bystander naive T cells that express high levels of ART2 (29). P2X₇ receptors can also regulate T cell activation directly. It has recently been demonstrated that stimulated T cells release ATP, which, in turn, stimulates P2X₇ molecules in an autocrine fashion to further promote their activation (5, 6).

The previously reported findings described earlier would seemingly have predicted suppressed T1D development in NOD.P2X₇^{-/-} mice, a result that was not observed. In contrast, the P2X₇-dependent cell death induction mechanism may also limit the initial activation of effector T cells. It has been reported that exacerbation of experimental autoimmune encephalomyelitis in P2X₇-deficient mice is associated with reduced lymphocyte apoptosis in the inflamed tissues (34). Therefore, it is possible that pathogenic T cells remain susceptible to P2X₇-dependent T cell death induction during the initial phase of their activation after encountering self-Ags and before shedding of ART2 molecules. Lack of P2X₇ may allow β cell autoreactive T cells to escape from this tolerance induction mechanism that normally limits the extent of self-reactivity. In this case, one would predict accelerated T1D onset in NOD.P2X₇^{-/-} mice. However, our results demonstrate that T1D development in NOD.P2X₇^{-/-} mice is not altered. It is possible that a combination of multiple T1D-promoting and -suppressing functions of the P2X₇ receptor on various cell populations in NOD mice masks its regulatory effects in this disease model.

In the absence of CD38 molecules, the balance is tipped to favor the proinflammatory functions of P2X₇ receptors as a result of increased extracellular levels of NAD. At the cellular level, the most apparent outcome of CD38 deficiency on the NOD background is the loss of CD4⁺ iNKT cells and Tregs, which, in turn, contributes to accelerated T1D development in these mice. Both NOD.CD38^{-/-}.P2X₇^{-/-} and NOD.CD38^{-/-}<short>.ART2^{-/-} exhibited similar T1D levels as standard NOD mice, indicating an important role of NICD in the regulation of disease development. In addition, increased levels of extracellular NAD may also increase ADP ribosylation of P2X₇ receptors on macrophages, which, in turn, promotes their production of the proinflammatory cytokine IL-1 β (4).

In this study, we also demonstrated a NOD-derived T1D susceptibility locus closely linked to the *Cd38* gene. The most apparent candidate region to harbor this T1D susceptibility locus is defined by the *Cd38* gene (44.3 Mb) and the microsatellite marker *D5Mit259* (89.7 Mb) (Fig. 6A). However, we could not rule out the possibility that the region proximal to the *Cd38* gene was also truncated but not revealed by the markers used to define the congenic region. This possibility is supported by the discovery of a 129/Sv-derived T1D resistance locus with the logarithm of odds score peaking at 31 Mb, proximal to the *Cd38* gene, in a previous quantitative trait locus analysis (28). Therefore, the location of this T1D susceptibility gene(s) reported in this study can be defined only by the markers *D5Mit75* (36.6 Mb) and *D5Mit259*

(89.7 Mb). It is also possible that the congenic region carrying the *Art2* mutant allele on chromosome 7 was also truncated without being revealed by the microsatellite markers used to type the interval in the newly generated NOD.CD38^{-/-}<short>.ART2^{-/-} mice. However, this is not likely to be the reason the newly generated NOD.CD38^{-/-}<short>.ART2^{-/-} stock became T1D susceptible in contrast with the marked T1D resistance in the previously reported DKO line. This is because NOD.ART2^{-/-} and standard NOD mice developed T1D at the same rate, indicating the 129/Sv-derived congenic region carrying the *Art2* mutant allele alone does not suppress disease development. In addition, no 129/Sv T1D resistance loci were found on chromosome 7 in the previously reported QTL analysis (28).

In conclusion, we demonstrated that P2X₇ deficiency alone does not alter T1D incidence in NOD mice. However, T1D acceleration in NOD.CD38^{-/-} mice requires intact P2X₇ molecules. Similarly, expression of ART2 is also essential for accelerated T1D development in CD38-deficient NOD mice. Collectively, these results indicate an important role of NICD in the regulation of T1D in NOD mice. Our study also provides an example of how molecules in the same pathway can interactively modulate T1D development.

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Disclosures

The authors have no financial conflicts of interest.

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