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Targeted Disruption of CD38 Accelerates Autoimmune Diabetes in NOD/Lt Mice by Enhancing Autoimmunity in an ADP-Ribosyltransferase 2-Dependent Fashion

Jing Chen,* Yi-Guang Chen,* Peter C. Reifsnyder,* William H. Schott,* Chul-Ho Lee,† Melissa Osborne,* Felix Scheuplein,‡ Friedrich Haag,‡ Friedrich Koch-Nolte,‡ David V. Serreze,* and Edward H. Leiter*‡

Ubiquitously expressed CD38 and T cell-expressed ADP-ribosyltransferase 2 (ART2) are ectoenzymes competing for NAD substrate. CD38 exerts pleiotropic actions in hemopoietic and nonhemopoietic compartments via effects on calcium mobilization. ART2 is an ADP-ribosyltransferase on naive CD4+ and CD8+ T cells. ART2-catalyzed ADP-ribosylation of the P2X7 purinoreceptor elicits apoptosis. Transfer of a genetically disrupted CD38 allele into the autoimmune diabetes-prone NOD/Lt background accelerated diabetes onset in both sexes, whereas transfer of a disrupted ART2 complex had no effect. However, the fact that the accelerated pathogenesis mediated by CD38 deficiency required ART2 activity was demonstrated by combining both ART2 and CD38 deficiencies. Reciprocal bone marrow reconstitution studies demonstrated accelerated diabetes only when CD38-deficient bone marrow was transferred into CD38-deficient recipients. Neither decreases in β cell function nor viability were indicated. Rather, the balance between T-effectors and T-regulatory cells was disturbed in CD38-deficient but ART2-intact NOD mice. In these mice, significant reductions in total viable CD8+ T cells were observed. This was accompanied by an age-dependent increase in a diabetogenic CD8 clone. This in turn correlated with impaired T-regulatory development (10-fold reduction in Foxp3 mRNA expression). These changes were corrected when CD38 deficiency was combined with ART2 deficiency. Both ART2-deficient and CD38/ART2 combined deficient T cells were resistant to NAD-induced killing in vitro, whereas CD38-deficient but ART2-intact T cells showed increased sensitivity, particularly the CD4+ CD25+ subset. Unexpectedly, diabetes development in the combined CD38/ART2 stock was strongly suppressed, possibly through epistatic interactions between genes linked to the targeted CD38 on Chromosome 5 and the ART2 complex on Chromosome 7.

Unlike the ubiquitously expressed CD38, ART2 expression is limited to murine T cell surfaces (18). It is the ortholog of RT6 in the rat (19, 20). Depletion of RT6-positive T cells in normally diabetes-resistant BB/Wor-DR rats enriches for autoreactive RT6-negative T-effectors capable of mediating diabetes when activated (21). In mice, levels of ART2 expression on CD4 and CD8 T cell subsets is developmentally regulated and strain dependent (18). In NOD and B6, ~80–90% of resting (naive) splenic CD8 T cells express ART2 compared with ~40% of splenic CD4 T cells (22). Following activation, ART2, like CD62L, is shed from the T cell surface by a TNFα-converting enzyme-like metalloproteinase rendering activated T cells resistant to NAD-induced cell death (NIDC) (16, 23). In B6 mice, it has recently been reported that the splenic CD4+CD25+ Foxp3-expressing subset of T-regulatory cells was particularly sensitive to NAD-mediated and ADP-ribosylation-catalyzed cell death (24).

NOD/Lt mice of both sexes spontaneously develop T cell-mediated autoimmune diabetes (25). T cell infiltration in the pancreatic islets is a key feature of this model, with disease penetrance higher in females than in males. Cyclophosphamide-accelerated diabetes in NOD/Lt males was associated with selective repopulation of spleen and islets with ART2-negative T cells (22). Furthermore, NAD-inhibited anti-CD3 induced NOD T cell activation, and retarded killing of β cell targets by islet-reactive CD8 effectors in vitro (22). CD38 controls the ART2-mediated ADP-ribosylation of T cell surface proteins by limiting the availability of NAD substrate (13). Hence, we hypothesized that congenic transfer to the NOD/Lt strain of a targeted CD38 null allele would enhance ART2-mediated ADP-ribosylation of T cell surface molecules regulating their function and survival. We had previously found that intragenoress into NOD/Lt mice of the genetically disrupted pair of tandem ART2-encoding genes (Art2a, Art2b) on Chromosome 7 alone had no protective or accelerative effect on diabetes (17). In common with other inbred strain backgrounds (C57BL/6, BALB/c) homozygous for CD38 deficiency (~90%), heterozygous for CD38 deficiency (~70%), and wild type (+/+), diabetes was diagnosed on two successive positive urinary testing results. Incidences observed under the same condition and time period were compared among genotypes by survival analysis. Mean insulin score (scale 0–4) was determined as described elsewhere (27).

Intraperitoneal glucose tolerance test

Overnight-fasted 5-wk-old NOD/Lt and NOD.CD38−/− mice of both sexes received injections i.p. with glucose at 2.5 g/kg body weight. The same two genotypes (both sexes) combined with the SCID mutation were challenged with 2.0 g/kg body weight glucose bolus at 7 wk of age. Blood was sampled at 0, 30, 60, and 120 min from retro-orbital sinus. Plasma glucose was determined using a Beckman Glucose Analyzer II (Beckman Instrument).

Detection of early β cell apoptosis

To test whether loss of CD38 function adversely affected β cell survival, we examined whether an early “wave of apoptosis” reported to initiate the onset of insulitis in NOD islets (28) was exacerbated. Pancreata were taken from 16-day-old to 4-wk-old mice, fixed with freshly prepared 4% paraformaldehyde, stained for cleaved caspase-3 using rabbit anti-cleaved caspase-3 (Asp175; Cell Signaling Technology) and a Vectastain ABC kit (Vector Laboratories) according to the instruction of the kit. Adjacent sections of each pancreas were also stained for granulated β cells by aldehyde fuchsin.

Serum and pancreatic insulin content measurement

Serum insulin was measured in blood taken by cardiac puncture immediately after euthanasia, and pancreatic tissue insulin content was measured as described previously (29) using a rat insulin RIA kit (Linco Research) and normalizing to pancreas wet weight.

Abs and fluorescence-activated cell sorter analysis

For PBL analysis, blood was taken from retro-orbital sinus at ages as specified in Results. For lymphocytes from spleen, pancreatic lymph nodes (PLN), and mesenteric lymph nodes (MLN), tissues were taken after euthanasia and pressed through Nitex membrane (100-μm mesh; Tetko) to 10 ml of HBSS (Sigma-Aldrich). Erythrocytes were removed by treatment with Gey’s solution. Fluorochrome-conjugated mAbs including anti-CD4 (L3T4), anti-CD8 (53–67.22), and anti-CD25 (PC61) were purchased from BD Pharmingen. Development of the ART2.2-specific mAb (clone Nika102) has been described previously (18). For viable cell gating, isotype-matched antibodies were used with Gey’s solution. Fluorochrome-conjugated mAbs including anti-CD4 (L3T4), anti-CD8 (53–67.22), and anti-CD25 (PC61) were purchased from BD Pharmingen. Development of the ART2.2-specific mAb (clone Nika102) has been described previously (18). For viable cell gating, isotype-matched antibodies were used with Gey’s solution. Flow cytometry analyses were performed using a FACScalibur (BD Biosciences), and data were analyzed by CellQuest software (BD Biosciences). Flow cytometric results were expressed as mean ± SE; statistical significance was analyzed by one-way ANOVA (JMP software; SAS Institute).

NAD-mediated T cell killing in vitro

T cell sensitivity to NAD-mediated apoptosis was tested in vitro using splenocytes from 6- to 8-wk-old male NOD, NOD.ART2−/−, NOD.CD38−/−, and NOD.CD38−/−ART2−/− mice, respectively (n = 3 each). Splenocytes were incubated with freshly prepared NAD (Sigma-Aldrich) at 0, 10, and 50 μM in DMEM supplemented with 10% FBS at 37°C for 30 min. Another aliquot of these splenocytes were kept at 4°C without NAD over this incubation period. After incubation, cells were washed and stained with Abs (anti-CD4, anti-CD8, anti-CD25), PI, and staining FITC-conjugated anti-ART2.2, clone Nika102; and PE-conjugated anti-CD38, clone 90).

CD38-deficient NOD.Prdksc−/− mice (henceforth abbreviated as NOD.SCID.CD38−/−) were created by crossing NOD.SCID with NOD.CD38−/− mice and backcrossing F1 progeny to NOD.SCID. At first backcross, SCID homozygotes were identified by flow cytometric confirmation of T and B cell deficiency. Those NOD.SCID homozygotes that were heterozygous for the targeted Cd38 allele were identified by PCR typing for D5Mit81. Such heterozygotes were intercrossed to fix the targeted Cd38 allele to homozygosity.

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annexin V, and analyzed by FACScan to detect viability in each population. Viable cells were defined as PI and annexin V-negative population.

Detection of autoreactive CD8+ T cells

β cell-autoimmune CD8+ NY8.3 clonotypic T cells were detected by specific tetramer staining as reported previously (30). A PE-conjugated Kd tetramer containing NRP-V7 mimotope (KYNKANVFL) of the islet specific glucose 6 phosphate catalytic subunit-related protein peptide recognized by NY8.3 T cells, and a control (TUM) PE-conjugated tetramer (KYQAVTTTL) were provided by Dr. P. Santamaria (University of Calgary, Alberta, Canada). Combinations of each PE-conjugated tetramer and FITC-conjugated anti-CD8 mAb were incubated with splenic leukocyte suspensions on ice for 120 min, and flow cytometric gating on 10^6 live cell events were performed. TUM was used to establish a background of non-specific tetramer binding.

Bone marrow reconstitution

NOD and CD38+/− recipients were lethally irradiated using two doses of 6 Gy and reciprocally reconstituted with 5 × 10^5 bone marrow cells isolated from nondiabetic NOD or NOD.CD38+/− femur as described previously (31). Bone marrow chimeras were screened for diabetes by weekly urinary glucose test.

CD4+ T cell purification and real-time PCR

CD4+ T cells were purified by negative selection from five pooled spleens per group. Splenocytes were incubated first with biotin-conjugated anti-mouse IgG (Sigma-Aldrich), anti-B220 (BD Pharmingen), anti-Mac-1 (BD Pharmingen), and anti-CD8 (BD Pharmingen), then incubated with streptavidin-microbeads for 15 min. Labeled cells were deleted by passing through the D column in SuperMACS separator (Miltenyi Biotec). Purity of CD4+ T cells was 85–91%. RNA was extracted from purified CD4+ T cells using RNAqueous-4PCR kit (Ambion). A RETRO script kit (Ambion) was used to synthesize cDNA, and real-time PCR was performed using CYBR Green PCR master mix (Applied Biosystems) and an ABI 7700 sequence detector. Flow cytometric detection of gated CD4+ T cells following permeabilization and intracellular staining for Foxp3 was performed using a commercially available kit (eBioscience).

Analysis of regulatory T cell (Treg) function

CD4+ T cells were purified as described above, except for the addition of anti-CD11c (HL3) to the negative selection mixture. CD4+CD25+ cells were further isolated by positive selection using biotin-conjugated anti-CD25 Abs and the MACS magnetic bead system (Miltenyi Biotec). The negative fraction was used as CD4+CD25− cells. Irradiated (2000 R) standard NOD splenocytes (5 × 10^5/well) were used as APC along with 5 μg/ml anti-CD3 to stimulate CD4+CD25− cells (5 × 10^5/well) in the presence or absence of different numbers of CD4+CD25+ cells in a 96-well plate for 3 days. [3H]Thymidine (1 μCi/well) was added during the last 18 h of culture.

Statistical methods

Significance of differences in diabetes incidences was analyzed by Kaplan-Meier survival analysis (JMP software; SAS Institute). Statistically significant differences in a given phenotype among the genotypes tested were assessed by one-way ANOVA with significance accepted at p ≤ 0.05.

Results

CD38 deficiency accelerated autoimmune diabetes in both sexes of NOD/Lt mice in an ART2-dependent fashion

Following 10 cycles of backcrossing to NOD/Lt, the length of the introgressed congenic segment around the targeted CD38 allele on Chromosome 5 was ~10 cm. Survival analysis showed no significant differences in the rate of diabetes development and the final frequencies between wild-type (+/+ ) and heterozygous (+/−) segregants of either sex (data not shown). In contrast, mice of both sexes homozygous for the congenic interval containing the targeted CD38 allele showed significant acceleration (p = 0.0012 for females; <0.0001 for males) compared with wild-type NOD/Lt (Fig. 1, A and B). The first diagnosis of diabetes in NOD.CD38−/− deficient mice came at 10 wk of age for both sexes vs 2 wk or more later in wild type. In addition, the final diabetes frequency in CD38-deficient males was increased, reaching 100% by 28 wk.

We expected that ART2-negative T cells would be resistant to NAD-mediated apoptosis (see below), and that this should exert some effect on the diabetogenic process. However, in contrast to the accelerated diabetes development elicited by introduction of a targeted CD38 allele, introgression into NOD/Lt of a disrupted ART2 complex had no significant modulatory effect on diabetogenesis in either sex (Fig. 1, A and B). As shown in Fig. 1, combining the CD38 deficiency with ART2 deficiency (combination CD38−/−/ART2−/− stock) not only reversed the accelerated diabetes produced by CD38 deficiency alone, but quite unexpectedly conferred strong protection in both sexes (p < 0.001 for both sexes vs wild type). Combination CD38/ART2-deficient females showed a diabetes frequency of only 25% by 28 wk compared with 100% in females lacking only CD38. Complete protection was observed in combination stock males vs development of 100% diabetes by 28 wk in males lacking only CD38.

CD38 deficiency does not overcome the requirement for ART2 in NAD-mediated T cell apoptosis

A requirement for intact ART2 has been demonstrated for NAD-mediated T cell apoptosis (16, 26, 32). NOD wild-type CD8+ (Fig. 2A) and CD4+ (Fig. 2B) T cells were sensitive to NAD-mediated killing in a dose-dependent fashion. In contrast, both T cell subsets from ART2-deficient NOD mice were completely resistant to
However, elimination of NAD-induced T cell apoptosis by disruption of the ART2 complex by itself cannot explain the enhanced diabetes resistance unexpectedly obtained in the combination CD38/ART2-deficient stock.

Consistent with another report that the CD4+CD25+ T cell subset from ART2-intact mice is particularly sensitive to NAD-mediated killing (24), data in Fig. 2C show that this subset from ART2-intact NOD/Lt wild-type mice was also highly sensitive to NAD. After 30 min in 10 μM NAD, the CD4+CD25+ T cell subset exhibited 16.0 ± 0.5% viability vs 27.7 ± 0.4% for total CD4+ cells and 23.8 ± 0.9% viability for CD8+ cells. The greatest loss of viability again was observed in the CD4+CD25+ T cells from CD38-deficient donors (11.6 ± 0.8% viable at 10 μM). Because this subset was resistant to NAD-mediated killing in both the ART2-deficient stock, which develops diabetes and the strongly protected CD38/ART2-deficient stock, the differential NAD sensitivity of this subset, presumably containing Tregs, alone cannot explain the diabetes protection observed in the CD38/ART2-deficient combination stock.

Bone marrow reconstitution shows diabetes acceleration requires both a CD38-deficient donor and CD38-deficient recipient

Diabetes was accelerated only when CD38-deficient bone marrow was transferred to CD38-deficient recipients. When bone marrow from NOD or from CD38-deficient mice was transferred to NOD recipients, the incidence of diabetes was comparable with NOD recipients receiving syngeneic NOD bone marrow (Fig. 3). This result suggests that the acceleration of diabetes by CD38 deficiency entails functional impairment in both hemopoietic and nonhemopoietic cells.

CD38 deficiency did not affect plasma insulin concentration or acute glucose-stimulated insulin secretion

Given the role of CD38 in calcium mobilization, the pancreatic β cell represented a logical nonhemopoietic component of increased diabetes susceptibility. We found that the plasma and pancreatic insulin content at 4 wk of age was comparable between NOD and NOD.CD38−/− (Table I), suggesting that accelerated diabetes was not related with an early loss of β cell function. We also found that CD38 deficiency had no effect on glucose tolerance in immune system intact 5-wk-old mice of either sex (data not shown), supporting a lack of effect previously seen on the B6 background (8).

![Figure 2](http://www.jimmunol.org/) Sensitivity of T cells to NAD-induced killing in vitro. Splenocytes were prepared from mice of the indicated strains at 8 wk of age and incubated for 30 min with or without exogenous NAD. Cells were then stained for cell surface markers and viability. Gating was performed on the indicated cell population, and viability was calculated as percentage of cells of the respective population not stained with annexin V or PI. ART2-deficient and CD38/ART2 combined deficient CD8+ (A), CD4+ (B), and CD4+CD25+ (C) T cells were resistant to NAD. In contrast, CD38-deficient T cells show an increased sensitivity compared with wild-type NOD/Lt controls. Some SE bars are not visible because of small values. n = 3 each group; *, p < 0.05.
To eliminate any potential effects of differences in insulitis development, we also analyzed glucose clearance rates in both genotypes combined with the SCID mutation after challenge at 7 wk of age with 2.0 g glucose/kg body weight. Again, no significant difference in glucose tolerance was detected (Fig. 4). Our results suggest that CD38 deficiency does not directly impair β cell secretory function before onset of autoimmune diabetes in NOD/Lt. As noted above, the discrepancy between our i.p. glucose tolerance test result and the original report likely reflects genetic background modification and possibly the use of different ES cell lines rather than a primary effect of the mutation (8).

**Insulitis onset and β cell destruction in prepubertal NOD.CD38−/− was not accelerated**

Diabetogenesis in NOD has been proposed to progress through several “checkpoints” (33). The earliest such checkpoint has been proposed to entail a prepuberty wave of β cell apoptosis that triggers early immune attention to the islets (28). A second checkpoint after puberty allows activation of T-effector cells in the insulitic infiltrate, perhaps associated with a decline in Treg function (34). To determine whether the accelerated diabetogenesis initiated at checkpoint 1, we studied islet cell apoptosis at 16–28 day and insulitis severity from 4 wk. No significant differences in number of apoptotic islet cells detected by Caspase-3 staining were noted between CD38−/− and wild-type NOD at 16 days to 4 wk of age (data not shown). Mean insulitis scores at multiple time points between 4 and 8 wk of age did not distinguish the two genotypes. However, insulitis progression was significantly more rapid in NOD.CD38−/− at 9 wk and later (Table II). Thus, CD38 deficiency exerted its accelerative effect not at the stage of early insulitis initiation (checkpoint 1), but at a later, postpubertal stage (checkpoint 2).

**NOD.CD38−/− peripheral lymphoid tissues show an ART2-dependent reduction in CD8+ T cell percentages**

Given the pivotal role of CD8+ T cells in both the initiation and progression of insulitis, we examined CD38-deficiency associated changes in this subset. Somewhat paradoxically in view of the accelerated diabetogenesis, we observed significant decreases in percentages of viable (PI negative) CD8+ T cells compared with wild type at 8 wk of age (Table III and representative flow cytometric profiles in Fig. 5). This decrease was seen in spleen, PLN, and MLN of both sexes at both 8 and 11 wk (data not shown). The decrease in CD8+ viability in CD38-deficient mice was ART2 dependent as shown by a restoration in viability when the disrupted ART2 complex was combined with the CD38-disrupted allele (Table III).

**Increased frequency of a β cell-autoreactive CD8+ clonotype is ART2 dependent**

Despite the overall decrease in percentages of CD8+ T cells in lymphoid organs of CD38-deficient mice, the frequency of the β cell-autoreactive clonotype detected by NRP-V7 tetramer staining was significantly higher than in wild-type NOD mice. This selective enrichment of a prevalent β cell-autoreactive CD8 clonotype was time dependent. No significant differences were detected at 8 wk of age (Fig. 6A), but a significant 6-fold increase was demonstrable by 11 wk (Fig. 6, B and C). In both CD38 intact and deficient mice, the majority (>75%) of splenic NRP-V7 tetramer-binding CD8+ T cells were CD62L negative, indicative of a memory/activated phenotype (data not shown). This temporal shift corresponds well with the rapid progression of insulitis after 9 wk at the so-called checkpoint 2. This age-dependent increase was also ART2 dependent, because combination of the disrupted ART2 complex genes with the disrupted CD38 allele prevented the increase in this highly diabetogenic clonotype (Fig. 6D). In fact, at 11 wk of age, the proportion of NRP-V7 tetramer-binding CD8+ T cells was less than half that in NOD wild-type mice (Fig. 6D).

### Table I. CD38 deficiency-accelerated diabetes does not entail earlier impairment of β cell function*

<table>
<thead>
<tr>
<th></th>
<th>Serum Insulin (ng/ml)</th>
<th>Pancreatic Insulin (ng/mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>NOD</td>
<td>0.90 ± 0.11</td>
<td>2.30 ± 0.50</td>
</tr>
<tr>
<td>NOD.CD38−/−</td>
<td>0.74 ± 0.10</td>
<td>1.92 ± 0.42</td>
</tr>
</tbody>
</table>

*Shown are serum and pancreatic insulin content at 4 wk of age (mean ± SE; n = 5 for each group).
NOD.CD38<sup>−/−</sup> mice exhibit an ART2-dependent reduction in peripheral CD4<sup>+</sup>CD25<sup>+</sup> T cells

CD4<sup>+</sup>CD25<sup>+</sup> T cells represent a phenotypic subset in mice with immunoregulatory potential (35, 36). In NOD mice, responsiveness of diabetogenic T-effectors to suppression mediated by CD4<sup>+</sup>CD25<sup>+</sup> Tregs declines with age (37, 38). As shown in Fig. 2, this subset in wild-type NOD mice, and to an even greater extent, in the CD38-deficient stock, exhibited heightened sensitivity to ART2-dependent NAD-mediated apoptosis. Consistent with our observation that severity of insulitis was comparable at 8 wk of age and at earlier time points, we found no difference in percentages of CD4<sup>+</sup>CD25<sup>+</sup> T cells in spleen and PLN at that age. Only in MLN (p < 0.01 for both sexes) was a significant decrease in the CD38-deficient donors noted at 8 wk (data not shown). However, by 11 wk of age, when insulitis was more advanced in CD38-deficient mice compared with NOD controls, modest, but significant reductions (p = 0.05) in CD4<sup>+</sup>CD25<sup>+</sup> T cell percentages were detected in PLN from CD38-deficient mice of both sexes and in the MLN of the males (Fig. 7A and B). The fact that this reduction in CD4<sup>+</sup>CD25<sup>+</sup> percentage was ART2 dependent was again demonstrated by comparison with the stock wherein the disrupted ART2 gene complex was combined with the disrupted CD38 allele (Fig. 7A).

The quintessential marker for the subset of CD4<sup>+</sup>CD25<sup>+</sup> T cells exerting Treg function is the transcription factor Foxp3 (39). As shown in Fig. 7C, at 10–11 wk of age the proportion of CD4<sup>+</sup>CD25<sup>+</sup> T cells in PLN that coexpressed Foxp3 was significantly less (p = 0.04) in CD38-deficient than intact females. Furthermore, Foxp3 mRNA transcript levels were 10-fold lower in 85–91% enriched splenic CD4<sup>+</sup> T cells isolated from CD38-deficient than intact 8-wk-old female donors (Fig. 7D). This is noteworthy because at this time point, percentages of splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells from both genotypes matched by sex were not statistically distinguishable (data not shown). To determine whether there was an inherent difference in the Treg activity of CD4<sup>+</sup>CD25<sup>+</sup> T cells from CD38 intact and deficient mice, we compared their ability to suppress the anti-CD3-driven proliferation of CD4<sup>+</sup>CD25<sup>+</sup> T cells. On a per cell basis, no significant differences were observed (data not shown).

### Table II. Acceleration of insulitis in CD38-deficient mice only demonstrable after 8 wk of age<sup>a</sup>

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>F&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>F&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>M&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>M&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.22 ± 0.27 (5)</td>
<td>0.46 ± 0.25 (6)</td>
<td>0.01 ± 0.22 (0)</td>
<td>0.17 ± 0.08 (7)</td>
</tr>
<tr>
<td>5</td>
<td>0.78 ± 0.39 (2)</td>
<td>0.74 ± 0.28 (4)</td>
<td>1.30 ± 0.43 (2)</td>
<td>0.42 ± 0.17 (13)</td>
</tr>
<tr>
<td>7</td>
<td>1.16 ± 0.24 (16)</td>
<td>0.78 ± 0.56 (3)</td>
<td>0.56 ± 0.28 (8)</td>
<td>0.86 ± 0.36 (5)</td>
</tr>
<tr>
<td>8</td>
<td>1.84 ± 0.36 (16)</td>
<td>1.48 ± 0.36 (5)</td>
<td>1.24 ± 0.30 (8)</td>
<td>1.40 ± 0.37 (5)</td>
</tr>
<tr>
<td>10</td>
<td>1.64 ± 0.31 (7)</td>
<td>2.88 ± 0.47* (3)</td>
<td>0.96 ± 0.22 (7)</td>
<td>2.18 ± 0.41* (2)</td>
</tr>
<tr>
<td>12</td>
<td>1.49 ± 0.27 (8)</td>
<td>3.31 ± 0.27* (8)</td>
<td>1.26 ± 0.54 (5)</td>
<td>2.55 ± 0.49* (6)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are mean insulitis score ± SEM for the number of mice indicated. F, Female; M, male. *p < 0.05 compared with wild-type NOD/Lt.

### Discussion

Given its multifaceted roles as a cell surface ectoenzyme, coactivator in Ig-, TCR-, and FcR complexes, as well as signal transducer and regulator of intracellular calcium, it is not surprising that CD38 exerts pleiotropic effects in both immune and nonimmune compartments (1, 10, 40–42). This pleiotropy is reflected in our demonstration using lethally irradiated bone marrow chimeras that CD38 deficiency at both hemopoietic and nonhemopoietic levels was required to reconstitute the accelerated diabetes observed in unmanipulated NOD.CD38<sup>−/−</sup> mice (Figs. 1 and 3). Impaired pancreatic β cell function would be a logical site for a nonhemopoietic requirement for earlier penetration of the clinical phenotype, particularly if, as previously reported by others, CD38 disruption impaired glucose-stimulated insulin secretion (6). Using insulin-free NOD-SCID mice of both sexes with either intact or disrupted CD38 alleles, we were unable to confirm that CD38 is essential for normal clearance of i.p.-injected glucose (Fig. 4). As noted elsewhere, the discrepancy likely reflects differences in additional modifier genes distinguishing inbred strain vs outbred strain backgrounds (8). Even though we could not demonstrate impaired responses to acute glucose challenge, CD38-deficient β cells might be more sensitive to induction of apoptosis (43). Checkpoint 1 of diabetogenesis has been hypothesized to entail a prepurvaly wave of apoptosis that, in NOD, provokes the early insulitis (28). We were unable to demonstrate either an earlier increase in Caspase 3 staining in islets of CD38-deficient mice between 16 and 28 days, or an earlier onset of β cell loss as reflected by either more advanced insulitis or decreased insulin content (Tables I and II). Thus, we have no evidence to support either impaired β cell function or survival as contributing to the accelerated rate of diabetogenesis in CD38-deficient mice.

CD38 deficiency has been associated with attenuated immune responses at multiple levels, including DC chemotaxis and migration (10). In separate studies we have found the absence of CD38 further enhances already present defects in the survival of CD4<sup>+</sup>NKT cells, and DC maturation/migration that contribute to type 1 diabetes in NOD mice (44). In this study, we find that accelerated pathogenesis correlated with a selective increase in the frequency of autoimmune effectors at a later postpuberty stage in insulitis.
progression (so-called checkpoint 2). Paradoxically, the accelerated insulitic destruction of β cells observed after 8 wk of age was marked by a systemic reduction in the percentages of viable CD8+ T cells (Table III), yet percentages of one of the most prevalent islet-specific autoreactive CD8+ clonotypes (NY8.3) was increased in the spleen (Fig. 5). This age- and ART2-dependent enrichment of a diabetogenic T-effector cell, in turn, correlated with an age-and ART2-dependent reduction in percentages of cells with a Treg phenotype (CD4+/CD25+ and CD4+/CD25-Foxp3+) (Fig. 7). Furthermore, the 10-fold decrease in Foxp3 expression observed at an age just before a loss of cells with a Treg phenotype in NOD.CD38−/− mice further supports a sudden shift in immunoregulatory capacity associated with accelerated β cell destruction.

CD4+/CD25+ T cells reportedly are hypersensitive to NAD-induced apoptosis (24), a finding supported by data in Fig. 2 of the current study. Flow cytometric gating on the CD4+/CD25+ T cell subset from NOD wild-type (and ART2-positive) donors showed a greater viability loss compared with total CD4 T cells following exposure to NAD. Some Treg phenotypic cells underwent spontaneous apoptosis in the absence of exogenously provided NAD. These spontaneously apoptotic Tregs most likely reflect an encounter with NAD released from cells that died while the splenocytes were being prepared for culture. Such NAD would be available to mediate ART2-catalyzed ADP-ribosylation of P2X7. Accordingly, cell preparations from CD38-deficient mice which do not efficiently hydrolyze NAD contain a higher proportion of spontaneously apoptotic cells, with much lower levels present in preparations from ART2-deficient mice that do not ADP-ribosylate cell surface proteins. Incubation of aliquots of the same cells in 10 μM NAD for 30 min at 37°C further affirmed the heightened sensitivity of CD4+/CD25+ T cells from ART-intact wild-type NOD mice, and at the same time showed the deleterious effect of CD38 disruption on viability that was blocked by ART2 disruption (Fig. 2). Conceivably, the reduction of cells with a Treg phenotype in CD38-deficient mice compared with wild-type NOD mice (Fig. 7) could reflect reduced survival in vivo elicited by sustained higher levels of extracellular NAD and, subsequently, more extensive ADP-ribosylation of P2X7.

Splenic NKT cells constitute an additional immunoregulatory subset whose numbers were shown to be significantly reduced in CD38-deficient NOD mice (44). NKT cells from standard NOD mice express ART2 at even higher mean fluorescence intensity than observed on naïve CD8+ T cells (our unpublished observation). Thus, the striking decrease in viable splenic NKT numbers previously found in CD38-deficient NOD mice very likely also requires an intact ART2 activity. In addition to maintaining CD4+NKT viability, CD38 is also required for DC mobilization from skin to inflammatory sites, including the PLN (10, 45). A CD4+NKT-secreted factor stimulates maturation of tolerogenic NOD DC in the PLN in response to α-galactosylceramide (45). Our finding that NOD mice lacking CD38 are unresponsive to the diabetes-protective effects of α-galactosylceramide (44) is consistent with a significant loss of CD4+NKT viability. Even though CD38 deficiency was impairing viability and/or function of both

FIGURE 5. Representative flow cytometric profiles depicting proportions of splenic CD4 and CD8 T cells in individual 8-wk-old NOD, NOD.CD38−/−, and NOD.CD38−/−/ART2−/− mice. Note reduced proportion of CD8 T cells in the CD38-deficient stock. Data are statistically summarized in Table III.

FIGURE 6. Effects of CD38 deficiency on an autoreactive CD8 clonotype. β cell-specific autoreactive CD8+ T cells, detected by NRP-V7 tetramer staining as described in Materials and Methods, are comparable at 8 wk of age (A) between CD38-deficient and intact NOD/Lt mice (n = 5 each), but significantly higher in CD38−/− when analyzed at 11 wk of age (B; n = 6 each). Representative staining of splenic CD8 T cells from 11-wk-old mice by the NRP-V7 and TUM-negative control tetramers (C). When CD38 deficiency and ART2 deficiency were combined, the NRP-V7+ autoreactive T cells were lower than in wild-type NOD/Lt controls (D). (▲, NOD; ■, NOD.CD38−/−; □, NOD.CD38−/−/ART2−/−).
NKT and CD4⁺CD25⁺ T cells regulatory subsets, early administration of CFA to 5-wk-old mice, known to up-regulate immunoregulatory networks in standard NOD mice (46), was effective in significantly retarding onset of hyperglycemia in NOD.CD38-deficient mice (data not shown). Hence, even under conditions in which quantitative deficits in CD4⁺CD25⁺ and CD4⁺NKT numbers may have persisted, the generalized immunostimulation provided by CFA treatment was capable of reversing the accelerated pathogenesis, possibly by promoting deletion of T-effectors.

Because CD38 is the major source of extracellular NAD hydrolysis, we had initially hypothesized that genetic ablation of CD38 may free NAD substrate, which can then be used by ART2 to mediate apoptosis of islet-infiltrating T cells and consequently delay diabetes in NOD mice. Rather, as the data clearly demonstrate, the reverse actually holds. When T cells are activated, ART2, like CD62L, is shed via a TNF-α converting enzyme-mediated proteolysis (23). Hence, naive or resting T cells are ART2 positive, whereas strongly activated cells are ART2-low or negative due to ectoenzyme shedding. Hence, our finding of a selective enrichment of autoreactive T-effectors in CD38-deficient NOD mice with an intact ART2 complex (Fig. 6) presumably reflected increased apoptosis of naive ART2-positive CD8 T cells and enhanced survival.
of ART2-negative CD8 effectors. Taken together, our results suggest that the balance of T-effector and T-regulatory cells, which is already disturbed in CD38 and ART2 intact wild-type NOD mice is even more disturbed in CD38-deficient but ART2 intact cells, most likely as a consequence of enhanced Nicd of Tregs in these mice. Inefficient removal of extracellular NAD in CD38-deficient mice presumably permits a stronger in vivo ADP-ribosylation of P2X7, resulting in a selective loss of NICD-sensitive Tregs.

Targeting the ART2 complex in NOD mice with an intact CD38 allele had no significant accelerative or retardative effect on diabetogenesis (Fig. 1). Accordingly, when we combined both targeted ART2 and CD38 alleles in the same stock, we anticipated that the CD38-mediated acceleration would be reversed if the mechanism was ART2 dependent as indeed it proved to be. However, we had not anticipated the astonishing degree of protection observed in the bicongenic stock; i.e., we anticipated that the bicongenic stock would show a diabetogenic progression not significantly different from that of standard NOD/Lt mice. One possible explanation for this enhanced protection is that elevated extra- and/or intracellular NAD concentrations in the absence of CD38 may exert protective effects, e.g., via other ecto-ARTs such as ART1 or via NAD-dependent intracellular enzymes such as the sirtuins or poly(ADP-ribos) polymerase (47, 48). In ART2 intact but CD38-deficient mice, such protective effects of elevated NAD would be masked by the potent ART2-dependent effects on Tregs. Another possible explanation for the almost complete protection from diabetes in the NOD/Lt mice presumably permits a stronger in vivo ADP-ribosylation of cell surface proteins by ART2 activates the cytolytic P2X7 receptor.

In conclusion, our data showed that CD38 deficiency accelerates diabetes in NOD/Lt mice by enhancing autoimmunity in an ART2-dependent fashion. The acceleration entailed both hemopoietic and nonhemopoietic events. The selective enrichment of CD8+ T-effectors accompanied by reductions in CD4+ T cells with immunoregulatory phenotypes represents one of the components underlying CD38 deficiency accelerated autoimmune diabetes in NOD/Lt mice. Our data failed to confirm the hypothesis proposed by others (6) that β cells represented a nonhemopoietic site of CD38 action required to prevent accelerated onset.

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Disclosures

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References


