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CD38 Is Required for the Peripheral Survival of Immunotolerogenic CD4+ Invariant NK T Cells in Nonobese Diabetic Mice

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T cell-mediated autoimmune type-1 diabetes (T1D) in NOD mice partly results from this strain’s numerical and functional defects in invariant NK T (iNKT) cells. T1D is inhibited in NOD mice treated with the iNKT cell superagonist α-galactosylceramide through a process involving enhanced accumulation of immunotolerogenic dendritic cells in pancreatic lymph nodes. Conversely, T1D is accelerated in NOD mice lacking CD38 molecules that play a role in dendritic cell migration to inflamed tissues. Unlike in standard NOD mice, α-galactosylceramide pretreatment did not protect the CD38-deficient stock from T1D induced by an adoptively transferred pancreatic β cell-autoreactive CD8 T cell clone (AI4). We found that in the absence of CD38, ADP-ribosyltransferase 2 preferentially activates apoptotic deletion of peripheral iNKT cells, especially the CD4+ subset. Therefore, this study documents a previously unrecognized role for CD38 in maintaining survival of an iNKT cell subset that preferentially contributes to the maintenance of immunological tolerance. The Journal of Immunology, 2006, 177: 2939–2947.

Most self-reactive T cells are eliminated by negative selection during their development in the thymus (1). However, this process is not complete. Several peripheral mechanisms exist to delete or functionally suppress autoreactive T cells escaping intrathymic negative selection (2). Defects in both central and peripheral tolerance induction mechanisms result in accumulation of the autoreactive T cells that destroy insulin-producing pancreatic β cells and cause type 1 diabetes (T1D) in NOD mice (3–7). Some of these defects are clearly T cell intrinsic (3, 5). However, abnormalities in other cell types also contribute to defective T cell tolerance induction in NOD mice (8).

NKT cells are a heterogeneous lymphoid population distinct from conventional T cells. The majority of murine NKT cells use an invariant Vα14Jα18 TCR chain preferentially paired with a Vβ8.2, Vβ2, or Vβ7 chain, and recognize lipid Ags presented by the MHC class I-like CD1d molecule (9, 10). CD1d-restricted NKT (invariant NK T; iNKT) cells are further divided into CD4+ and CD4−CD8− double-negative (DN) subpopulations that develop in the thymus (9, 10). NOD mice are characterized by numerical and functional defects in iNKT cells (11). Several lines of evidence indicate that increasing iNKT cell numbers or activity suppress T1D development in NOD mice. One is that transfer of iNKT cells protects NOD mice from T1D (12). Additionally, activation of iNKT cells by the superagonist α-galactosylceramide (α-GalCer) also inhibits T1D development (13–15).

Several mechanisms have been proposed for how α-GalCer activation of iNKT cells inhibits T1D development in NOD mice. One is that activated iNKT cells inhibit the pathogenicity of diabeticogenic CD4 T cells in NOD mice by skewing the pattern of cytokines they produce from a Th1 (IFN-γ) to a Th2 (IL-4 and IL-10) profile (13, 15). We do not favor this mechanism because α-GalCer treatment efficiently induced T1D resistance in a NOD stock made genetically deficient in the key Th2 cytokines IL-4 and IL-10 (our unpublished observation). Instead, our previous work supports a mechanism by which activated iNKT cells inhibit T1D development through downstream effects on dendritic cells (DCs) (14, 16). We have found that α-GalCer-activated iNKT cells in NOD mice secrete a novel protein that induces an enhanced preferential accumulation in pancreatic lymph nodes (PLNs) of tolerogenic myeloid DCs (14, 16). Subsequently, recruitment of β cell-autoreactive T cells to this site is also enhanced where they are deleted or rendered anergic (16).

The CD38 molecule contributes to multiple immunological processes that may be important to how activated iNKT cells inhibit T1D development in NOD mice. CD38 is an ectoenzyme that uses NAD+ to generate multiple calcium (Ca2+)–mobilizing metabolites, including ADP ribose and cyclic ADP ribose (17). In addition, CD38 functions as a cell surface receptor capable of initiating a signal transduction cascade in immune cells (18). CD38 regulates several important immunological processes, such as DC and neutrophil trafficking in response to inflammatory challenges (19, 20), B cell activation (21) and Ig isotype switching (22), as well as NK and T cell activation (23–25). Thus, most defects attributed to CD38 deficiency have heretofore been associated with attenuated immune responses rather than impaired tolerance induction (19,
20, 26). We were, therefore, surprised to find disruption of the CD38 gene markedly accelerated T1D development in NOD mice (27). Notable immunophenotypic alterations included an enrichment of islet-autoreactive CD8 T cells concomitant with a decrease in total numbers of CD8 T cells and in Foxp3-encoding RNA transcripts, a molecule essential to the function of regulatory T cells (Tregs) (27). However, we reasoned that perhaps by regulating the extent of DC migration to the PLNs, CD38 might also be important to the process by which iNKT cell activation inhibits T1D development in NOD mice. Thus, we analyzed the NKT-DC axis in NOD.CD38null mice. We show that CD38 is essential to the peripheral survival of iNKT cells, and the mechanism responsible for their loss. Our results also implicate the CD4+ subset of iNKT cells as preferentially providing essential contributions to the maintenance of immunological tolerance.

Materials and Methods
Mice and reagents
NOD/Lt and C57BL/6 (B6) mice are maintained by brother-sister mating at The Jackson Laboratory. A MHC class I and CD8 T cell-deficient NOD.B2mnull stock is maintained at the N11 backcross generation (28). NOD.B2mnull mice expressing human β2-microglobulin (β2m; design- ingated NOD.B2mnull/β2m) have been described previously (29). NOD mice transgenically expressing the TCR from the diabeticogenic CD8+ T cell clone AH (Voas/VB2) and a functionally inactivated Rag1 gene (design- ingated NOD.Rag1null/AH) have also been described (30). B6.CD38null (2m) mice (20) were originally provided by Dr. F. E. Lund (Trudeau Institute, Saranac Lake, NY). The CD38null mutation was backcrossed from this stock to the NOD background for 10 generations and then fixed to homozygosity. NOD.Art2null mice were created by 10 cycles of backcrossing of targeted Art2.1 and Art2.2 alleles from a previously described B6 stock (31). Linkage markers delineating all known Idd loci are fixed to homozygosity for NOD alleles in the NOD.CD38null and NOD.Art2null stocks. An intercross strategy was then used to generate a NOD stock genetically identical to the parental NOD.CD38null/CD38null strain, except that CD38null mice have one i.p. injection of α-GalCer (2 μg/recipient) or the same volume of vehicle on day 0. For DC migration experiments, single-cell suspensions were prepared from PLNs and mesenteric LNs (MLNs) on day 4 and analyzed by flow cytometry for surface marker expression and numbers of T and B lymphocytes and DCs.

Adaptive transfer of T1D
Beginning at 3–4 wk of age, female standard and CD38-deficient NOD mice received four once weekly i.p. injections of α-GalCer (2 μg) or the same volume of vehicle. Two days after the last treatment, mice were sublethally irradiated (600 rad from a 137Cs source) and injected i.v. with 5 × 10⁸ NOD.Rag1null/AH splenocytes (equivalent to 1 × 10⁶ A14 T cells). Recipient mice were then euthanized at 3 days posttransfer or monitored for T1D development over a period of 3 wk. For studying T1D development, recipient mice received one additional α-GalCer or vehicle treatment at 5 days after splenocyte transfer. T1D was assessed by daily monitoring of glycosuria development with Ames Diastrix (Bayer, Diagnostics Division), with disease onset defined by two consecutive values of ≥3. A14 T cells in the recipients were identified by flow cytometry analysis based on coexpression of CD8 and the TCR Vα8 chain.

Flow cytometric analyses
Single-cell suspensions were prepared from the spleen, thymus, liver, and LNs. RBC were removed. Liver mononuclear cells were further isolated using a Histopaque-1077 density gradient (Sigma-Aldrich). Cells were stained with the indicated Abs at 4°C for 30 min. The cells were then washed and resuspended in FACS buffer. For iNKT cell staining, cells were first treated with Fc block at room temperature for 10 min and then incubated with α-GalCer-loaded or empty CD1d dimers at 4°C for 30 min. Following CD1d dimer staining, the cells were washed once and then incubated with an Ab mixture containing anti-mouse IgG1, anti-CD4, and anti-TCRβ. Alternatively, cells were stained with an Ab mixture containing anti-CD4, anti-CD8, and anti-ART2 Ab and CD1d tetramer. Stained cells were washed and analyzed on a four-color FACScalibur or a Cytek-upgraded five-color FACSscan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences). Propidium iodide (PI) was used to gate out dead cells.

Generation of bone marrow (BM) chimeras
Lethally irradiated (1300 rad from a 137Cs source) female NOD.B2mnull/h2mnull mice were injected i.v. with a 1:1 mixture of BM cells (5 × 10⁶ cells) from sex-matched NOD.CD38null and NOD.B2mnull/h2mnull mice. Six weeks later, BM chimeras were treated with α-GalCer (2 μg/mouse) or same amount of vehicle. The numbers of DCs of each donor type in the PLNs and MLNs were determined at 4 days posttreatment.

Statistical analysis
All statistical comparisons between two groups were performed using the nonparametric Wilcoxon rank sum test.

Results
CD38 is required for iNKT cell-mediated T1D protection
Activation of iNKT cells by α-GalCer blocks T1D development in NOD mice by a process that includes an enhanced accumulation in PLNs of tolerogenic myeloid DCs followed by increased deletion or functional inactivation of β cell-autoreactive T cells (14, 16). CD38 is required for normal DC chemotaxis to inflammatory sites (20). These findings suggested accelerated T1D development in NOD.CD38null stock might, in part, entail the accumulation of even fewer tolerogenic DCs in PLNs than occurs in standard NOD mice. To initially test this possibility, we enumerated DCs in PLNs and MLNs from unmanipulated CD38 intact or deficient female NOD mice at 6 or 11 wk of age. No differences were found (data not shown). It should be noted that we chose to compare PLNs to MLNs because their cellular composition is similar (34). We next tested whether DCs in NOD.CD38null mice differed from those of NOD controls in ability to accumulate in PLNs following iNKT cell activation. As previously reported (16) and repeated in Fig.
IA, α-GalCer activation of iNKT cells in standard NOD mice resulted in dramatically increased numbers of DCs in PLNs but not MLNs. This differential response was previously found to be due to variations in the chemokine content of PLNs and MLNs following α-GalCer treatment (16). In sharp contrast, accumulation of DCs in PLNs was largely blocked in α-GalCer-treated NOD.CD38null mice (Fig. 1A). The increased numbers of DCs in PLNs of α-GalCer-treated WT NOD females was accompanied by significant increases in both T and B cells at this site (Fig. 1B). Conversely, as was the case for DCs, there was no increase in the numbers of T and B cells in PLNs of α-GalCer-treated NOD.CD38null mice (Fig. 1B).

T cells transgenically expressing the TCR from the β cell-autoreactive CD8+ AI4 clonotype rapidly induce T1D upon adoptive transfer into young sublethally irradiated NOD mice (16). However, this process is blocked if iNKT cells had been previously activated in the NOD recipients by α-GalCer treatment (16). If such protection is truly dependent upon an iNKT cell-induced accumulation of DCs in PLNs, we hypothesized pretreatment with α-GalCer would not protect NOD.CD38null mice from T1D induced by adoptively transferred AI4 T cells. As shown in Fig. 1C, this was indeed the case because α-GalCer pretreatment inhibited T1D development in standard NOD but not NOD.CD38null recipients of AI4 T cells.

Our previous study using WT NOD females showed α-GalCer pretreatment resulted in a significantly greater recruitment of adoptively transferred AI4 T cells to PLNs, where they were tolerized through both deletional and anergy induction mechanisms (16). Therefore, we determined whether this also occurred in NOD.CD38null recipients of AI4 T cells. As expected, α-GalCer pretreatment significantly increased the numbers of AI4 T cells at 3 days posttransfer in PLNs, but not MLNs, of standard NOD mice (Fig. 1D). Consistent with the inability of α-GalCer pretreatment to induce an accumulation of DCs in PLNs of NOD.CD38null mice, it also failed to enhance the recruitment of adoptively transferred AI4 T cells to this site. Therefore, augmented accumulation of DCs and AI4 T cells in PLNs only occurred in recipient mice protected from T1D by α-GalCer pretreatment.

DCs isolated from PLNs, but not other anatomical sites, in prediabetic NOD mice are capable of adoptively transferring T1D resistance (14, 35). In addition, some β cell-autoreactive NOD T cells are normally tolerized upon their entry into PLNs of NOD mice,

![Image](http://www.jimmunol.org/)

**FIGURE 1.** α-GalCer fails to induce accumulation of DCs, T lymphocytes, and B lymphocytes in the PLNs of NOD.CD38null, and as a result does not protect them from accelerated T1D. A and B. Female NOD and NOD.CD38null (6 wk old) mice were injected i.p. with α-GalCer (2 µg/mouse) or vehicle. Four days later, the numbers of DCs (A) and T and B lymphocytes (B) were determined in the PLNs and MLNs. Each value represents the number of DCs, T cells, or B cells in the PLNs or MLNs of individual mice. C. Incidence of T1D in AI4 T cell-recipient mice pretreated with α-GalCer or vehicle (n = 10/group). Recipient female NOD and NOD.CD38null (3–4 wk old) mice were pretreated with α-GalCer (2 µg/mouse) or vehicle once a week for 4 wk before being sublethally irradiated (600 rad) and injected i.v. with NOD.Rag1null AI4 spleen cells. D. Recipient female NOD and NOD.CD38null (3–4 wk old) mice were pretreated with α-GalCer (2 µg/mouse) or vehicle once a week for 4 wk before being sublethally irradiated (600 rad) and injected i.v. with NOD.Rag1null AI4 spleen cells. E. LN cells were analyzed for the presence of AI4 T cells at 3 days posttransfer. Each data point represents a pool of LN cells prepared from four mice each. E. DCs isolated from PLNs, but not spleens, of α-GalCer-treated NOD mice induced apoptosis of AI4 T cells. Dcs were purified from PLNs or spleens of NOD mice treated with α-GalCer (2 µg) 4 days earlier. AI4 T cells were then cocultured with each type of purified DCs for 3 days. Cells were harvested and stained with anti-CD8 and anti-TCR Vα8 Abs to delineate AI4 T cells as well as annexin V and PI. The FACS profiles depict annexin V and PI staining of AI4 T cells (gated on CD8+ and TCR Vα8+). The numbers represent the percentages of viable AI4 T cells.
but not at levels sufficient to inhibit progression to overt T1D (16). However, this tolerogenic process may be quantitatively increased to a level necessary to elicit T1D resistance as a consequence of the enhanced numbers of DCs that accumulate in PLNs following iNKT cell activation. To test whether DCs recruited to PLNs of 

\[ \text{H}9251 \text{GalCer-treated NOD mice} \]

promoted a higher level of AI4 T cell death than those of splenic origin (Fig. 1E).

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**FIGURE 2.** DCs do not require CD38 to migrate into PLNs in response to activated iNKT cells. Female NOD.\( \beta2m^{null} \cdot h\beta2m \) mice were lethally irradiated (1300 rad from a \( ^{137}\text{Cs} \) source) and reconstituted with a 1:1 mixture of BM cells (5 \( \times 10^6 \) cells of each type) from sex-matched NOD.\( CD38^{null} \) and NOD.\( \beta2m^{null} \cdot h\beta2m \) (CD38 intact) mice. Six weeks later, BM chimeras were treated with \( \alpha\text{-GalCer} \) (2 \( \mu\text{g/mouse} \)) or same volume of vehicle. The numbers (A) and origins (B) of DCs in the PLNs and MLNs were determined at 4 days posttreatment. The origin of DCs was determined by staining MHC class I molecules using an Ab (clone M1/42) that selectively recognizes MHC class I molecules associated with murine, but not human \( \beta2m \). The origins of DCs shown in B are presented as the portions of total CD11c\(^+\) cells in individual mice. Each symbol represents one animal.

**FIGURE 3.** Defective NKT-DC axis in the absence of CD38

A, DC maturation in \( \alpha\text{-GalCer}-\text{stimulated splenocyte culture} \) is compromised in the absence of CD38. NOD and NOD.\( CD38^{null} \) splenocytes were cultured with indicated concentrations of \( \alpha\text{-GalCer} \) for 48 h. Flow cytometry was used to assess CD86 expression levels on DCs (CD11c\(^+\)) as defined by mean fluorescence intensity (MFI) of Ab staining. Each data point represents the mean CD86 Ab staining ± SEM for DC from four mice in each group. Similar results were obtained in two other experiments.

B, Reduced ability of CM from \( \alpha\text{-GalCer} \)-stimulated NOD.\( CD38^{null} \) splenocyte cultures to induce DC maturation. CM samples were collected from four \( \alpha\text{-GalCer} \)-stimulated or control NOD or NOD.\( CD38^{null} \) splenocyte cultures and the corresponding vehicle control culture. Their ability to induce DC maturation was determined by incubating NOD.\( \beta2m^{null} \cdot h\beta2m \) splenocytes with CM at a concentration of 10% for 24 h. Expression of CD86 on DCs (CD11c\(^+\)) was analyzed by flow cytometry. Similar results were observed in two other experiments.

C, CD38-deficient DCs respond normally to the iNKT cell-derived maturation factor(s). Purified NOD or NOD.\( CD38^{null} \) splenic DCs (pooled from two mice of each strain) were incubated for 24 h with 10% CM generated from 48-h \( \alpha\text{-GalCer} \)-stimulated or control NOD splenocyte cultures. Expression of CD86 on DCs (CD11c\(^+\)) was analyzed by flow cytometry. The results are presented as mean ± SEM of triplicate evaluation of each DC pool. Similar results were obtained in two other experiments.

D, Reduced \( \alpha\text{-GalCer-induced IFN-}\gamma \) production in NOD.\( CD38^{null} \) mice. Splenocytes from four NOD or NOD.\( CD38^{null} \) mice were stimulated with \( \alpha\text{-GalCer} \) (250 ng/ml) for 48 h. Tissue culture media were collected and analyzed for IFN-\( \gamma \) by ELISA. Each value indicates the mean of each sample assayed in triplicate. Similar results were obtained in two other experiments.

E, DCs in the PLNs of NOD.\( CD38^{null} \) mice exhibit an impaired ability to differentiate in vivo following iNKT cell activation. NOD and NOD.\( CD38^{null} \) male mice were injected i.p. with \( \alpha\text{-GalCer} \) (2 \( \mu\text{g/mouse} \)) or vehicle, and 24 h later expression of CD86 on DCs (CD11c\(^+\)) was analyzed by flow cytometry. Data points represent MFI of CD86 Ab staining for DCs in PLNs of individual mice.
syngeneic and NOD. CD38null BM. We previously found the pan MHC class I Ab M1/42 recognizes MHC class I molecules associated with murine, but not human β2m (data not shown). Hence, similar to the use of Ly5.1 vs 5.2 allotypic markers to identify different populations of donor cells, positive or negative staining by the M1/42 Ab allowed for the respective delineation of CD38-deficient or intact DCs in the mixed BM chimeras. Furthermore, iNKT cell numbers and function (i.e., ability to induce up-regulation of CD86 expression by DC) in the NOD. β2mnull. hβ2m stock does not differ from that of standard NOD mice (Y.-G. Chen and D. V. Serreze, unpublished observation). As expected, α-GalCer treatment of the BM chimeras significantly increased the numbers of DCs in PLNs but not MLNs (Fig. 2A). Further analyses of DC origins showed comparable levels of CD38 intact and deficient DCs were recruited to the PLNs of α-GalCer-treated BM chimeras (Fig. 2B). CD38-deficient DCs are characterized by a defective chemotactic response to some chemokines (20). In contrast, these BM chimera analyses indicated CD38-dependent functions are not intrinsically required for DCs to migrate into PLNs in response to iNKT cell activation.

Next, we analyzed the function of iNKT cells from CD38 intact and deficient NOD mice by comparing their ability to induce DC differentiation upon α-GalCer stimulation in an in vitro splenocyte culture. As shown in Fig. 3A, α-GalCer stimulation of whole splenocyte preparations enhanced CD86 expression to a much lesser extent on resident DCs from NOD. CD38null mice than standard NOD controls. This could be due to an impaired ability of NOD. CD38null DCs to respond to the iNKT cell-derived maturation factors(s). Alternatively, lower levels of the iNKT cell-derived maturation factor(s) may have been produced in the α-GalCer-stimulated NOD. CD38null splenocyte culture. To distinguish between these possibilities, we collected CM from four separate sets of both control and α-GalCer-stimulated NOD and NOD. CD38null splenocytes and compared their ability in secondary cultures to induce the differentiation of DCs resident among NOD. β2mnull splenocytes. NOD. β2mnull mice lack iNKT cells and, hence, cannot respond to α-GalCer directly. For this reason, if splenic DCs from NOD. β2mnull mice were stimulated to mature by a particular source of CM, we could be assured the response was not due to an effect mediated by any residual α-GalCer in the test sample. It should also be noted that we previously found that CM derived from α-GalCer-stimulated splenocytes obtained from NOD stocks lacking conventional CD4 or CD8 T cells, or B cells, efficiently induced the maturation of NOD. β2mnull DCs (16). Thus, any differential ability of CM derived from α-GalCer-stimulated CD38 intact or deficient NOD splenocytes to induce DC maturation would likely reflect a direct variation(s) in iNKT cell function, rather than altered intermediary contributions from other lymphocyte populations. In addition, we incubated purified splenic DCs from either NOD or NOD. CD38null mice with CM generated from the α-GalCer-stimulated NOD splenocytes.

As shown in Fig. 3B, at a 10% concentration CM obtained from α-GalCer-stimulated NOD splenocytes induced differentiation of NOD. β2mnull DCs as assessed by up-regulation of CD86 expression. However, the same amount of CM generated from α-GalCer-stimulated CD38-deficient splenocytes failed to up-regulate CD86 expression on NOD. β2mnull DCs. In contrast, purified CD38-deficient and intact DCs responded equally well to the iNKT cell-derived maturation factor(s) of standard NOD origin (Fig. 3C). We further analyzed whether NOD and NOD. CD38null DCs were characterized by inherent functional differences by comparing their ability to produce the cytokines IL-12 p70 and IL-10 following LPS stimulation. No such differences were found (data not shown). The impaired iNKT cell activity observed in CD38-deficient splenocytes was not limited to the DC maturation factor(s), because IFN-γ production was also significantly decreased (Fig. 3D).

![Image](http://www.jimmunol.org/Downloadedfrom)
Another question was whether compared with those from standard NOD controls, did DCs in the PLNs of NOD.CD38null mice show an impaired ability to differentiate in vivo following iNKT cell activation. Based on up-regulation of CD86 expression, DCs in the PLNs of CD38 intact but not deficient NOD mice underwent significantly enhanced differentiation following iNKT cell activation in vivo (Fig. 3E). These collective results indicate that the diminished accumulation of mature DCs in PLNs of α-GalCer-treated NOD.CD38null mice cannot be fully explained by the previously reported defects in chemokine responsiveness. Rather, they suggest that CD38 is essential for maintenance of iNKT function that, in turn, regulates DC differentiation and recruitment into PLNs.

Preferential loss of peripheral CD4+ iNKT cells in CD38-deficient mice

It has been previously shown that CD38 is highly expressed on CD4/CD8 DN TCRαβ+ thymocytes (36), a population enriched for iNKT cells. Therefore, a decreased ability to produce iNKT cells could be one plausible explanation for why NOD.CD38null mice are unable to respond to α-GalCer treatment to the same extent as standard NOD controls. To test this possibility, numbers of iNKT cells in NOD and NOD.CD38null mice were compared. Thymocytes and splenocytes were initially stained with α-GalCer-loaded CD1d dimers. In the thymus, iNKT cell numbers and proportions were equivalent in WT and CD38-deficient NOD mice (Fig. 4A). In sharp contrast, numbers of splenic iNKT cells were significantly lower in NOD.CD38null mice (Fig. 4B). Most of the reduction is due to a preferential loss of CD4+ iNKT cells (Fig. 4B). In liver, the frequency of CD4+ iNKT cells is also significantly decreased in NOD.CD38null mice (data not shown). Therefore, the expansion and/or survival of iNKT cells in the periphery, especially the CD4+ subset, are CD38 dependent. These results agree with a previous report where no differences in DN TCRαβ+ thymocytes, presumably iNKT cells, were observed between WT and CD38 knockout (KO) mice (26). However, numbers of peripheral iNKT cells were not determined in the previous study. It

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**FIGURE 5.** The loss of peripheral iNKT cells in NOD.CD38null mice is mediated in an ART2-dependent, NAD substrate-induced manner. Single-cell suspensions were prepared from the thymus (A), spleen (B), and PLNs (C) of age-matched NOD (WT) and NOD.CD38null (CD38 KO) female mice (6–8 wk old), and stained with anti-CD3, CD4 and ART2 Abs and CD1d tetramers. The flow cytometric profiles of representative mice are shown. The summarized results of numbers of various subsets of iNKT cells in spleens from four mice of each strain are depicted in D, E. ART2-dependent NAD-induced cell death of iNKT cells. Splenocytes from four NOD, NOD.Art2null, and NOD.ART2null.CD38null female mice at 6–8 wk of age were incubated with 0, 2, or 8 μM of NAD at 37°C for 30 min. Subsets of iNKT cells were identified by anti-CD3 and CD4 Abs and CD1d tetramers. Apoptotic cells were determined by annexin V and PI staining. Annexin V binding was determined on PI-negative cells. Results are presented as mean percentages ± SEM of annexin V-positive cells among the DN and CD4+ iNKT populations in the spleens of the four mice from each strain. F, ART2-dependent loss of splenic iNKT cells in CD38 deficient NOD mice. Data depict numbers of total splenic iNKT cells as determined by α-GalCer-loaded CD1d tetramer staining in four to eight age-matched (6–8 wk old) females from each of the four indicated strains.
In mice, the duplicate the reduced peripheral numbers of iNKT cells in the absence of ART2 could explain the DN subset and are more sensitive to NAD-induced cell death (37). Interestingly, CD38 decreases ART2-mediated ADP-ribosylation of T cell surface proteins by reducing the available amount of the ecto-NAD substrate (31). Therefore, we asked whether iNKT cells express higher levels of ART2 than the DN subset that coexpresses ART2 (Fig. 5D).

We next determined whether iNKT cells are subject to NAD-induced death. For this purpose, we generated NOD mice deficient in ART2 only or also lacking CD38 expression. WT, ART2-deficient, and ART2/CD38 double KO NOD splenocytes were incubated with or without NAD for 30 min at 37°C. Exposure to NAD induced significantly higher levels of apoptosis (assessed by annexin V staining) among CD4+ than DN iNKT cells, and the process was completely dependent on intact ART2 (Fig. 5E). Furthermore, more CD4+ than DN iNKT cells from standard NOD mice also underwent spontaneous apoptosis (annexin V positive) in the absence of exogenous NAD. Such apoptosis is presumably due to the release of endogenous NAD from splenocytes that died while being prepared for culture, because this response not observed in the absence of ART2 (Fig. 5E).

We next genetically tested whether an absence of CD38 truly induces a loss of peripheral iNKT cells in an ART2-dependent manner. In contrast to the significantly reduced numbers in CD38-deficient mice, the stocks deficient only in ART2, or both CD38 and ART2, had comparable numbers of iNKT cells in the spleen (Fig. 5F) and thymus (data not shown) as standard NOD controls. These collective results indicate that the reduced level of peripheral iNKT cells in CD38-deficient mice is a result of ART2-dependent NAD substrate-induced apoptosis.

**ART2 is responsible for reduced α-GalCer-induced accumulation of CD4+ iNKT cells in CD38-deficient NOD mice**

The significantly reduced basal numbers of iNKT cells, particularly the CD4+ subset, in NOD.CD38null mice is clearly a consequence of ART2-driven apoptosis. However, another important question was to what extent does CD38 and ART2 expression affect α-GalCer-induced expansion of iNKT cells. To test this hypothesis, we injected standard NOD mice as well as those deficient in CD38 and/or ART2 with α-GalCer or vehicle, and then 4 days later compared the number of iNKT cells that accumulated in PLNs and spleens.

The balance of CD38 and ART2 expression had only a marginal effect on numbers of α-GalCer-induced DN iNKT cells that accumulated in PLNs and spleens (data not shown). In contrast, the balance of CD38 and ART2 expression had profound effects on the baseline and ART2/CD38-induced levels of CD4+ iNKT cells (Fig. 6). As expected, the levels of CD4+ iNKT cells in PLNs and spleens from vehicle-treated NOD.CD38null mice were significantly less than in NOD controls. In agreement with the previous experiment, this decrease was ART2 dependent because numbers of splenic CD4+ iNKT cells in vehicle-treated NOD mice were deficient in both CD38 and ART2 did not differ from standard NOD controls. Interestingly, baseline levels of CD4+ iNKT cells in PLNs from the stock deficient in both CD38 and ART2 were actually significantly higher than in NOD controls. It is tempting to speculate this could contribute to the previous observation that compared with standard NOD mice, the stock deficient in both CD38 and ART2 is significantly protected from spontaneous T1D (27).

Treatment with α-GalCer expanded CD4+ iNKT cell levels in both the PLNs and spleens of all four strains examined, but again the extent to which this expansion occurred was dependent on the balance of CD38 and ART2 expression (Fig. 6). Significantly fewer CD4+ iNKT cells accumulated in both PLNs and spleens of NOD.CD38null than standard NOD mice. Again, this decrease was ART2 dependent because the number of α-GalCer-induced CD4+ iNKT cells in PLNs and spleens of the NOD stock deficient in both CD38 and ART2 was, respectively, equivalent to or greater than the baseline level and α-GalCer-induced accumulation of CD4+ iNKT cells. Standard (WT), CD38-deficient only (CD38 KO), ART2-deficient only (Art2 KO), or ART2 and CD38-deficient (Art2/CD38 KO) NOD females (6 wk old) were injected i.p. with α-GalCer (2 μg/mouse) or vehicle control. Four days later, the numbers of CD4+ iNKT cells in PLNs (upper panel) and spleens (lower panel) were determined by flow cytometric analysis. Data depict numbers of the indicated iNKT cells in individual vehicle or α-GalCer-treated mice (4–7/group). In the upper panel, *p < 0.05, as compared with the corresponding vehicle or α-GalCer-treated NOD mice. In the lower panel, **p < 0.01, as compared with the corresponding vehicle or α-GalCer-treated NOD mice.

**Peripheral CD4+ iNKT cells express higher levels of ART2 than the DN subset and are more sensitive to NAD-induced cell death**

We reasoned that activity of the ectoenzyme ART2 could explain the reduced peripheral numbers of iNKT cells in the absence of CD38. In mice, the duplicate Art2 genes, Art2.1 and Art2.2, encode GPI-anchored cell surface enzymes on subsets of T cells (32). ART2 has been reported to regulate T cell function by transferring the ADP-ribose moiety of NAD onto other cell surface proteins, including LFA-1, CD8, CD27, CD43, CD44, CD45, and the P2X7 purinoceptor (37). ART2-expressing T cells function by transferring the ADP-ribosylation and activation of the P2X7 purinoceptor (37). Activated P2X7 induces calcium flux and formation of membrane pores, leading to cell death (37). Interestingly, CD38 decreases ART2-mediated ADP-ribosylation of T cell surface proteins by reducing the available amount of the ecto-NAD substrate (31). Therefore, we asked whether iNKT cells express ART2 and, as a result, are sensitive to NAD-induced cell death. CD1d tetramers were used to identify iNKT cells. Most thymic iNKT cells did not express ART2 in both WT and CD38-deficient NOD mice (Fig. 5A). In sharp contrast, a significant proportion of iNKT cells in the spleens and PLNs of standard NOD mice expressed high levels of ART2, and this was particularly pronounced in the CD4+ subset (Fig. 5, B and C).

![FIGURE 6. The balance of CD38 and ART2 expression regulates the baseline level and α-GalCer-induced accumulation of CD4+ iNKT cells. Standard (WT), CD38-deficient only (CD38 KO), ART2-deficient only (Art2 KO), or ART2 and CD38-deficient (Art2/CD38 KO) NOD females (6 wk old) were injected i.p. with α-GalCer (2 μg/mouse) or vehicle control. Four days later, the numbers of CD4+ iNKT cells in PLNs (upper panel) and spleens (lower panel) were determined by flow cytometric analysis. Data depict numbers of the indicated iNKT cells in individual vehicle or α-GalCer-treated mice (4–7/group). In the upper panel, *p < 0.05, as compared with the corresponding vehicle or α-GalCer-treated NOD mice. In the lower panel, **p < 0.01, as compared with the corresponding vehicle or α-GalCer-treated NOD mice.](http://www.jimmunol.org/)

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that of standard NOD controls. This latter finding suggests that CD4+ iNKT cells in the NOD stock deficient in both CD38 and ART2 may be even more responsive than those in standard NOD mice to antigenic ligand stimulation. If so, this result could also underlie the previous finding that the rate of spontaneous T1D in the stock deficient in both CD38 and ART2 is significantly less than in standard NOD mice (27). Regardless, the most important conclusion is that the impaired survival and α-GalCer-induced expansion of CD4+ iNKT cells lacking only CD38 expression is due to increased sensitivity of this immunoregulatory population to ART2-mediated NAD-dependent apoptosis.

**Discussion**

Pancreatic β cells from a CD38 KO stock with a mixed genetic background were originally reported to have a diminished insulin secretory capacity, resulting in impaired glucose tolerance compared with WT controls (38). If this premise is correct, accelerated T1D development in CD38-deficient NOD mice could entail dysfunctional β cells that renders them more susceptible to autoimmune attack. However, when fixed on either the B6 and NOD backgrounds, the CD38null mutation was found to not impair glucose tolerance (39). Although possible effects of CD38 deficiency on NOD β cell viability in the face of immunocytotoxic stresses cannot be completely excluded, we have uncovered an alternative mechanism underlying accelerated T1D development in NOD CD38null mice. Our data support a mechanism by which the absence of CD38 further diminishes the survival of peripheral iNKT cells that are already at insufficient levels to inhibit T1D development in standard NOD mice (11).

Decreased levels of iNKT cells have also been reported to be a contributory factor to T1D development in humans (40). However, this premise was questioned in another study reporting that NKT cell numbers did not differ in the peripheral blood of human T1D patients and controls (41). The basis for these discordant reports could include differences in the patient and control populations analyzed and/or methods used to detect iNKT cells (42). There is another issue to consider when assessing the possible contributions of iNKT cell deficiencies to TID susceptibility in humans and NOD mice. That is, in humans it is only possible to quantify iNKT cells in peripheral blood, whereas in NOD mice their numbers can also be assessed in lymphoid organs. In this regard, it should be noted that whereas iNKT cell numbers are reduced in NOD lymphoid organs, their numbers in peripheral blood are equivalent to or greater than that of T1D resistant strains (43).

Although the relative contribution of deficiencies in CD4+ or DN iNKT cells to T1D development in the NOD mouse model has not been studied in detail, there is evidence they are functionally distinct subsets (11). Specifically, the CD4+ subset has been proposed to preferentially mediate tolerance induction in several other experimental models (44–46). This paradigm is supported by our finding that accelerated T1D development in NOD CD38null mice is associated with a preferential loss of the CD4+ subset of iNKT cells in the periphery.

One function of CD38 is to serve as a NAD glycohydrolase for the generation of Ca2+-mobilizing metabolites that regulate chemokine-induced chemotaxis of DCs to LNs upon an inflammatory challenge (20). Collective results from this and previous studies (14, 16) indicate the inhibition of T1D in NOD mice by iNKT cell activation is likely to, at least in part, result from preferential accumulation in PLNs of tolerogenic DCs that subsequently delete or inactivate pathogenic T cells. In the current study, the failure of α-GalCer treatment to elicit T1D protective effects in NOD CD38null mice could be explained by an insufficient iNKT cell-induced influx of DCs into PLNs, and a subsequent failure to recruit and tolerate pathogenic T cells at that site. However, the inability of α-GalCer treatment to elicit T1D protective effects in NOD CD38null mice was not due to an inherent inability of their DCs to undergo chemotaxis in response to activated iNKT cells. Instead, the inability of α-GalCer treatment to inhibit T1D in NOD CD38null mice most likely results from their significantly reduced numbers of peripheral iNKT cells.

In addition to impairing the ability of DCs to directly delete or inactivate pathogenic T cells, there is another nonmutually exclusive mechanism by which a further loss in NKT cells induced by a deficiency in CD38 expression could accelerate T1D development in NOD mice. Such disease acceleration could also be at least partly due to a loss in Treg numbers or function previously shown to be elicited by DCs in a NKT cell-dependent fashion (45). Indeed, a loss of iNKT cells may also contribute to the decreased levels of Tregs we previously observed in NOD CD38null mice (27). However, a deficiency in CD4+ iNKT cells does not necessarily have to contribute to T1D development in NOD mice by inducing a downstream loss in numbers and functions of Tregs. Previous studies failed to demonstrate increased numbers or enhanced function of Tregs capable of suppressing T1D as the downstream mechanism of iNKT cell-mediated disease protection in standard NOD mice (13, 15). In addition, we used the quintessential FoxP3 marker to detect Tregs and found no increase in their frequency in the spleens or PLNs of standard NOD mice after α-GalCer administration (our unpublished observation).

Our studies indicate that the absence of CD38 does not block the original thymic development of iNKT cells, but rather limits their emigration to, or survival in, the periphery. We feel it is unlikely that the reduced numbers of peripheral iNKT cells is due to a thymic emigration defect, because no accumulation of these cells in the thymus was seen. Rather, our results support an ART2-dependent loss of peripheral iNKT cells in NOD CD38null mice. In the periphery, ART2 is expressed at much higher levels on CD4+ than DN iNKT cells. In contrast, neither iNKT cell subset expresses significant levels of ART2 in the thymus. Consistent with this expression pattern, iNKT cells, especially the CD4+ subset, are lost in the periphery but not in the thymus of CD38-deficient mice. In addition, it was found that peripheral CD4+ iNKT cells in NOD mice are more sensitive to ART2-dependent NAD substrate-induced apoptosis than the DN subset. Finally, the loss of splenic iNKT cells observed in the NOD CD38null stock is completely rescued if the mice are also made genetically deficient in ART2. Collectively, these results indicate the preferential loss of peripheral CD4+ iNKT cells in CD38-deficient mice is due to ART2-dependent NAD substrate-induced apoptosis.

In summary, CD38 is critical for the homeostasis of peripheral iNKT cells, especially the CD4+ subset that preferentially mediates tolerogenic processes. When this defect is superimposed on T1D-prone NOD mice, the further decrease in already subnormal levels of iNKT cells contributes to accelerated disease progression. Our results also provide additional evidence to highlight the role of CD4+ iNKT cells as being a subset that preferentially contributes to the maintenance of immunological tolerance.

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**Disclosures**

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**References**


