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A recombinant heavy chain antibody approach blocks ART2 mediated deletion of an iNKT cell population that upon activation inhibits autoimmune diabetes

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Abstract

The ectoenzyme ADP-ribosyltransferase 2.2 (ART2.2) can apoptotically delete various T-cell subsets. Depending on the involved apoptotic T-cell subset, enhanced ART2.2 activity could result in immunosuppression or autoimmunity. Diminished activity of the CD38 ectoenzyme that normally represents a counter-regulatory competitor for the NAD substrate represents one mechanism enhancing ART2.2 activity. Hence, it would be desirable to develop an agent that efficiently blocks ART2.2 activity *in vivo*. While the llama derived recombinant s+16 single domain antibody overcame the difficulty of specifically targeting the ART2.2 catalytic site potential therapeutic use of this reagent is limited due to short *in vivo* persistence. Thus, we tested if a modified version of s+16 incorporating the murine IgG1 Fc tail (s+16Fc) mediated long-term efficient *in vivo* suppression of ART2.2. We reasoned an ideal model to test the s+16Fc reagent were NOD mice in which genetic ablation of CD38 results in an ART2.2 mediated reduction in already subnormal numbers of immunoregulatory natural killer T-(NKT) cells to a level that no longer allows them when activated by the super-agonist alpha-galactosylceramide (α -GalCer) to elicit effects inhibiting autoimmune type 1 diabetes (T1D) development. Treatment with s+16Fc efficiently mediated long term *in vivo* inhibition of ART2.2 activity in NOD.*CD38^{null}* mice, restoring their iNKT cell numbers to levels that upon α -GalCer activation were capable of inhibiting T1D development.

Keywords

autoimmune diabetes; single chain antibodies; ectoenzymes; iNKT-cells

1. Introduction

ADP-ribosyltransferase 2 (ART2) belongs to a family of mono-ADP-ribosyltransferases (ART1-ART5) that catalyze the transfer of the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) to specific amino acids, that usually inactivates, but can sometimes activate the target proteins [1]. In mice, there are two duplicated *Art2* genes on Chromosome 7 (designated *Art2.1* and *Art2.2*). Naïve T-cells treated with the ART2-substrate NAD⁺ undergo apoptosis through ADP-ribosylation and activation of the P2X7 receptor [2,3]. Thus, under conditions where ART2 activity may become unfettered, a state of generalized

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immunosuppression could result. Alternatively, if ART2 activity were to selectively eliminate a particular population of regulatory T-cells, the development of various autoimmune diseases could be enhanced. Due to its ability to compete for the mutual NAD substrate, the CD38 glycohydrolase ectoenzyme normally provides one means for counter-regulating ART2 activity [4]. Thus, a diminution or loss of CD38 activity could represent one means by which potentially deleterious functions of ART2 could be enhanced. Indeed, our previous finding that the development of spontaneous T-cell mediated autoimmune type 1 diabetes (T1D) which normally occurs in standard NOD is further accelerated by the genetic ablation of CD38 [5] provides one illustration for why it would be valuable to develop pharmacological agents that can specifically and efficiently block ART2 activity *in vivo*. While humans lack a functional ART2 gene, they express other family members that may provide redundant functions [6]. Thus, the development of agents that efficiently block ART enzymatic activity may also ultimately be useful for the treatment of some human disease states.

An original hurdle in the development of an ART2 blocking reagent was the fact that available small molecule inhibitors affected diverse ART family members [7]. This difficulty was overcome by the previous development of the llama derived s+16 recombinant single domain antibody (sdAb) that could specifically block ART2.2 activity *in vitro* and *in vivo* [8,9]. However, the *in vivo* blockade of ART2.2 lasted only from 10 minutes to six hours after a single high dose (200 µg) i.v. injection of s+16, due to limited *in vivo* persistence resulting from both the small size of the sdAb (15 kD), and the lack of an Fc domain allowing for interactions with FcRn [10]. Thus, we generated a second generation reconstituted bivalent heavy chain only antibody (hcAb) version of S+16 (designated s+16Fc) that includes a fused mouse IgG1 Fc domain carrying the LSF mutation that prolongs its serum half life via interactions with FcRn [10]. We reasoned our previously described NOD.*CD38^{null}* mice would be a good model for testing whether the s+16Fc sdAb provided an means for the long term efficient *in vivo* blockade of deleterious ART2.2 functions that abrogates the ability of another pharmacological intervention to elicit T1D protective effects in this strain [5].

We found that the development of autoimmune type 1 diabetes (T1D) characterizing standard NOD mice [11,12] is further exacerbated by the genetic ablation of CD38 in part due to an ART2 mediated further depletion of the already subnormal levels of immunoregulatory invariant natural killer T (iNKT)-cells characterizing this strain, with the CD4 expressing subset being particularly affected [5]. This appears to result from the fact that iNKT-cells, particularly the CD4+ subset, express higher levels of ART2 than other T-cell sub-populations [5]. iNKT cells recognize through expression of relatively invariant T-cell receptors (TCR) glycolipid antigens presented by the major histocompatibility complex (MHC) class I related CD1d molecule [13,14]. At least in part, the relative paucity of iNKT cells in standard NOD mice appear to contribute to T1D development by limiting their ability upon activation to elicit the downstream differentiation of antigen presenting dendritic cells (DC) to state allowing them to induce several different T-cell tolerance induction mechanisms [15]. The CD4 expressing subset of iNKT-cells appears to be particularly important in the generation of tolerogenic DC [15]. T1D development can be inhibited in standard NOD mice by either increasing numbers of iNKT-cells by adoptive transfer, or through their activation with the superagonist alpha-galactosylceramide (α-GalCer) [16-19]. The maturation and entry into pancreatic lymph nodes (PLN) of tolerogenic DC is greatly enhanced following α-GalCer mediated activation of iNKT-cells in standard NOD mice [15]. However, most likely because of their further ART2 mediated reduction in iNKT cell numbers, α-GalCer treatment fails to elicit T1D protective effects in NOD.*CD38^{null}* mice [5]. Hence, in the current study we tested the possible ability of the s+16Fc sdAb to provide efficient long-term blockade of ART2.2 activity *in vivo*, by determining if it could enhance the survival of iNKT-cells in NOD.*CD38^{null}* mice and subsequently restore their ability to elicit T1D protective effects when activated by co-treatment with α-GalCer.

2. Materials and Methods

2.1 Mice and reagents

NOD/LtDvs mice are maintained by brother-sister matings. A previously described NOD stock transgenically expresses the TCR from the diabetogenic CD8 T-cell clone AI4 (V α 8/V β 2) and also carries a functionally inactivated *Rag1* gene (designated NOD.*Rag1*^{null}.AI4) [20]. NOD stocks genetically deficient in either CD38 or both ART2.1 and ART2.2 have also been previously described [5] and are maintained at the N10 backcross generation. Fluorochrome conjugated mAbs specific for CD11c (HL3), CD86 (GL-1), B220 (RA3-6B2), CD3 (145-2C11), CD8 (53-6.7) and CD4 (GK1.5) were used for flow cytometry. These mAbs and Annexin V, were purchased from BD Biosciences, San Diego, CA. Anti-ART2.2 mAb Nika102 [21] and polyclonal anti-P2X7 serum K1G have been described previously [3]. Mouse α -GalCer analog-loaded CD1d tetramers were provided by National Institutes of Health Tetramer Facility, Atlanta, GA. α -GalCer (KRN7000) was provided by Alexis, Farmingdale, NY, and was reconstituted to 1 mg/ml in DMSO. Etheno-NAD (eNAD) was purchased from Sigma, St. Louis, MO.

2.2 Recombinant antibody production and purification

The coding sequences of the llama derived VHH s+16a sdAb that blocks ART2.2 enzymatic activity [8] or the control l-15 sdAb were fused in frame with the coding sequence of mouse IgG1-Fc carrying the LSF mutation (mFc-LSF) allowing for increased interaction with FcRn (IgG1-Fc-LSF was kindly provided by Sally Ward, University of Texas [10]) into the mammalian expression vector pME18Sneo encoding an N-terminal CD8 Leader and Flag Tag [21]. l-15 is a control sdAb with unknown specificity picked from the primary library of the immunized llama [8]. COS7/HEK cells were transiently transfected with the expression vector by Jetpei (Polyplus-Transfection, New York, NY) according to the manufacturers protocol. Culture media were collected 6 days post-transfection and recombinant antibodies (henceforth designated s+16Fc or l-15Fc) were purified with a M2-mAb anti-FLAG Sepharose affinity column (Sigma, St. Louis, MO) and dialyzed into PBS.

2.3 Assays for etheno-ADP-ribosylation of cell surface proteins and NAD induced cell death (NICD)

Etheno-ADP-ribosylation of cell surface proteins was monitored as described previously [22]. Freshly prepared lymph node cells or splenic leukocytes were incubated for 15 min with 10 μ M etheno-NAD at 4°C and the Alexa 488-conjugated, etheno-adenosine specific mAb 1G4. Exposure of phosphatidyl-serine and uptake of propidium iodide induced by ADP-ribosylation of P2X7 was monitored as described previously [3] following incubation of leukocytes for 30min at 37°C or 4°C.

2.4 Short-term α -GalCer / Antibody treatment effects

Age-matched female NOD and NOD.*CD38*^{null} mice injected i.p. once weekly for 4 weeks with 5 μ g s+16Fc or l-15Fc, were given one i.p. injection of α -GalCer (2 μ g/recipient) or volume matched vehicle on day 0. Four days later, single-cell suspensions were prepared from individual Collagenase D digested PLNs (30 minutes at 37°C). Cells were counted and analyzed by flow cytometry for surface markers and numbers of DC, iNKT and T and B lymphocytes.

2.5 Long-term incidence studies

NOD females and NOD.*CD38*^{null} males were injected i.p. once weekly with 5 μ g s+16Fc or l-15Fc starting after weaning. T1D development was assessed by weekly monitoring of urinary glucose levels with Ames Diastrix (Bayer, Diagnostics Division), with disease onset defined

by two consecutive values of ≥ 3 . To assess the impact of iNKT cell activation on spontaneous T1D development in the NOD.*CD38^{null}* stock, mice were injected i.p. once weekly with 2 μ g α -GalCer or vehicle starting at 7 weeks of age. Additional NOD.*CD38^{null}* mice received both α -GalCer starting at 7 weeks of age and weekly 5 μ g injections of s+16Fc initiated at weaning. T1D onset was monitored as described above.

2.6 Adoptive transfer of T1D

Beginning at 3 weeks of age NOD.*CD38^{null}* mice were treated for 3 weeks with s+16Fc or 1–15Fc and then received four once weekly i.p. injections of α -GalCer (2 μ g) in combination with continued treatment with the antibodies. Age-matched NOD controls were treated with four α -GalCer injections only. Two days after the last treatment, mice were sublethally irradiated (600R from a ^{137}Cs source) and injected i.v. with $5\text{--}6 \times 10^6$ NOD.*Rag1^{null}.AI4* splenocytes (equivalent to 1×10^6 AI4 T-cells). Recipient mice were either euthanized at 3 days post-transfer or monitored for T1D development over a 2 wk follow up period. Recipient mice monitored for T1D development received one additional α -GalCer or vehicle treatment 5 days after AI4 T cell transfer. AI4 T-cells in the recipients were identified by flow cytometry analysis based on co-expression of CD8 and the TCR V α 8 and V β 2 elements.

2.7 Regulatory T-cell (Treg) Assay

Tregs were obtained by first purifying CD4⁺ T-cells from spleens of NOD and NOD.*CD38^{null}* mice by depleting B220⁺, CD8⁺ (clone 53-6.7, BD Bioscience), and CD11b⁺ cells with the previously described magnetic bead system [23]. The CD25⁺ fraction was isolated from the eluted CD4⁺ T-cells by staining with biotinylated anti-CD25 (clone 7D4; BD Bioscience) followed by streptavidin-conjugated microbeads (Miltenyi Biotec). The purity of CD4⁺CD25⁺ cells was >90%. NOD CD4⁺CD25[–] T-cells were purified as described above and labeled with carboxyfluorescein succinimidyl ester (CFSE) following a previously published protocol [15]. Labeled CD4⁺CD25[–] T-cells were co-cultured (5×10^4 cells/well) in triplicate with indicated ratios of Tregs in round-bottomed 96-well tissue culture plates with 5 μ g/ml anti-CD3 (clone 145-2C11, BD Bioscience) and 2×10^5 /well NOD-*scid* splenocytes. T-cell proliferation was assessed after incubation at 37°C for 3 days by CFSE dilution.

3. Results

3.1 ART2.2 and P2X7 are expressed at higher levels on peripheral iNKT than conventional T-cells

ART2.2 and P2X7 are the two key players in NAD induced cell death (NICD) [3]. Expression levels of these molecules on NOD thymocytes and splenocytes were assessed by flow cytometry. Consistent with developmental expression patterns in conventional T-cells [21], NOD iNKT-cells differentiating in the thymus were nearly all ART2.2 negative (Fig. 1a). Conventional T cells begin to express ART2.2 in the final stage of thymic differentiation (i.e. CD4 and CD8 single positive (SP) with low HSA) which is maintained following migration to the periphery [24]. This was also the case on peripheral iNKT-cells in NOD mice, however to a much greater extent than on conventional T cells (Fig. 1a). Similarly, P2X7 was expressed at very low levels on intrathymic iNKT cells in NOD mice, but increased dramatically to levels even higher than on conventional T cells in the periphery (Fig. 1a).

3.2 Long term Ab mediated in vivo blockade of ART2.2 activity

Our previous work indicated that due to a loss of substrate competition, an increase in ART2 enzymatic activity likely accounts for a further reduction in already subnormal numbers of immunoregulatory iNKT cells in NOD mice made genetically deficient in CD38 expression [25]. We reasoned ART2.2 rather than ART2.1 would contribute to any such loss of iNKT

cells, as the latter enzyme is only functional under reducing conditions [26]. Hence, we tested whether ART2.2 contributes to the further loss of iNKT cells in NOD.*CD38^{null}* mice by modification of a previous approach that utilized the llama derived s+16 sdAb to block activity of this ectoenzyme [8].

As previously observed [25], the loss of iNKT cells in NOD.*CD38^{null}* mice was most pronounced in the ART2.2+ subset (Fig. 1b, compare panels 3 and 4). ART2.2 enzymatic activity can be assessed by measuring cell surface ADP-ribosylation using the etheno-NAD analog as a substrate, and the 1G4 antibody that specifically recognizes etheno-ADP-ribosylated proteins [22]. There was no detectable etheno-ADP-ribosylation of cell surface proteins on iNKT cells from NOD.*ART2^{null}* mice (Fig. 1c, panel 1). The preferential loss of ART2.2-expressing iNKT cells in *CD38* deficient compared to standard NOD mice (Fig. 1b, panel 4) represented those most capable of mediating ADP-ribosylation activity (Fig. 1c panel 3). A 10 min pre-incubation with the s+16 sdAb completely abolished the ART2.2 activity of both standard NOD and NOD.*CD38^{null}* splenocytes (Fig. 1c, panels 5-6).

Due to both its small size (15 kD), and lack of an Fc domain allowing for interactions with FcRn [10], the sdAb s+16 has a short half-life *in vivo*, thus limiting its use as a potential therapeutic agent to short term blockade of ART2.2. Thus, we generated a bivalent heavy chain antibody (hcAb) version of S+16 (designated s+16Fc) that includes a fused mouse IgG1 Fc domain carrying the LSF mutation that prolongs its serum half life via interactions with FcRn [10]. A 5 µg i.p. injection of the s+16Fc hcAb was found to be sufficient to completely block the ART2.2 enzymatic activity of NOD.*CD38^{null}* leukocytes isolated at 24 h post-treatment (Fig. 2a). Another way of monitoring ART2.2 activity is measuring NICD by assessing apoptosis induction through Annexin V binding. NAD is released from a proportion of primary leukocytes that die or are stressed during their dispersion [27]. As a result, in the absence of *CD38*, a substantial proportion of dispersed T cells from untreated control mice underwent spontaneous NICD without the addition of external NAD (Fig. 2b, panel 1). While ADP-ribosylation proceeds efficiently at 4°C, downstream activation of P2X7 to induce NICD requires incubation at 37°C [27]. Thus, dispersed leukocytes from untreated NOD.*CD38^{null}* mice incubated at 4°C as a control, showed limited “spontaneous” NICD (Fig. 2b, panel 2). A single injection of s+16Fc completely abolished ART2.2-mediated NICD of NOD.*CD38^{null}* leukocytes up until 7 days post-treatment (Fig. 2b, panels 3,4). However, ART2.2 activity was regained after 14 days (Fig. 2b, panel 5).

3.3 Long-term treatment of NOD. *CD38^{null}* mice with s+16Fc increases peripheral iNKT-cell numbers and normalizes the CD4+/DN iNKT-cell ratio

To study the effects of long-term blockade of ART2.2 enzymatic activity *in vivo*, NOD.*CD38^{null}* mice were injected once weekly for up to four weeks with s+16Fc. Compared to controls, after 3 weeks of s+16Fc treatment both the percentage (Fig. 3a) and absolute numbers (Fig. 3b) of iNKT cells were significantly increased in PLN and spleen, corresponding well with the time it takes for a bone marrow derived T-cell progenitor to mature in the thymus and seed the periphery. Due to their differential rates of apoptotic loss, the ratio of CD4+ to double negative (DN) iNKT cells in the NOD.*CD38^{null}* stock is reduced to ~1:1 compared to the ~4:1 level in standard NOD mice (Fig. 3c). Treatment of the NOD.*CD38^{null}* stock with s+16Fc returned their CD4+/DN iNKT-cell ratio to that characterizing standard NOD mice (Fig. 3c, 3d). These data indicate s+16Fc treatment enhances the peripheral survival of the CD4+ subset of iNKT-cells in NOD.*CD38^{null}* mice, and furthermore, restores a standard NOD like ratio of CD4+ to DN iNKT-cells.

3.4 The ability of α -GalCer to activate iNKT — DC interactions blocking adoptively transferred T1D is restored in s+16Fc pre-treated NOD.CD38^{null} mice

We next tested if s+16Fc blockade of ART2.2 activity could restore the ability of α -GalCer treatment to elicit an expansion of DC and iNKT-cells in the PLNs of NOD.CD38^{null} mice and subsequent protect them from T1D induced by adoptively transferred AI4 TCR transgenic CD8 T cells. A set of NOD.CD38^{null} mice were injected once weekly for 4 weeks with s+16Fc. Untreated standard NOD and NOD.CD38^{null} mice served as controls. Mice in each group were then injected i.p. with α -GalCer or vehicle (DMSO). Numbers of DC and iNKT-cells within PLNs of mice in each treatment group were determined 4 days later. As expected [15], following α -GalCer injection, numbers of both DC and iNKT-cells were significantly increased in the PLNs of standard NOD mice (Fig. 4a, b). Also as expected [25], following α -GalCer treatment there was a much lower expansion of DC and iNKT-cells in the PLNs of CD38 deficient than standard NOD mice (Fig. 4a, b). However, long-term pretreatment of NOD.CD38^{null} mice with s+16Fc restored the ability of α -GalCer treatment to induce recruitment of DC to the PLN and the accompanying expansion of iNKT-cells to comparable levels as in standard NOD mice (Fig. 4a, b).

Another group of NOD.CD38^{null} mice were injected i.p. once weekly for 7 weeks with s+16Fc or control I-15Fc antibody. For the last 4 weeks the mice were additionally injected with α -GalCer. The mice were then sublethally irradiated and injected i.v. with 5×10^6 splenocytes from NOD.Rag1^{null}.AI4 TCR transgenic donors (equivalent to $\sim 1 \times 10^6$ AI4 T cells). All mice received an additional α -GalCer injection at day 5 after AI4 T cell transfer. T1D development was monitored daily for 2 weeks. As expected [25], α -GalCer treatment failed to protect NOD.CD38^{null} mice that received the control antibody from AI4 T cell induced T1D (Fig. 5a). Strikingly, the ability of α -GalCer to inhibit AI4 T cell induced T1D was restored in NOD.CD38^{null} mice pretreated with s+16Fc, but not the control Ab (Fig. 5a). It was possible that blocking of ART2.2 activity interferes with the diabetogenic capability of the AI4 T-cells. To test this possibility a group of NOD.CD38^{null} mice was pretreated with 4 once weekly injections of α -GalCer and then received s+16Fc just before irradiation and adoptive transfer of AI4 T cells. Clearly s+16Fc did not interfere with AI4 activity since all recipients were diabetic by day 6 post-transfer (Fig. 5a).

We previously found that compared to vehicle treated controls, α -GalCer treatment of standard, but not CD38 deficient NOD mice, leads to an increased recruitment of diabetogenic AI4 T-cells to the PLN where they are held and either deleted or inactivated [15,25]. Hence, at T1D onset or the end of the experiment, mice in each recipient group were sacrificed and assessed for numbers of AI4 T-cells and iNKT-cells within the PLNs. Indeed, in s+16Fc pre-treated NOD.CD38^{null} mice, the subsequent ability of α -GalCer to induce the accumulation of AI4 T-cells and the expansion of iNKT-cells in PLNs was restored (Fig. 5b).

3.5 Combined s+16Fc and α -GalCer treatment allows Treg expansion in the PLNs of NOD.CD38^{null} mice after AI4 T cell transfer

It has been reported α -GalCer mediated protection from T1D in NOD mice requires the presence of regulatory T cells (Tregs) [28]. Thus, we tested if s+16Fc inhibition of ART2.2 activity altered the numbers of Tregs that accumulated in the PLNs of NOD.CD38^{null} mice subsequently treated with α -GalCer and infused with AI4 T cells. The mice received the same schedules of s+16Fc antibody and/or α -GalCer injections prior to AI4 T cell infusion as described above. PLNs were analyzed for proportions and numbers of CD4+CD25+FoxP3+ Tregs four days after AI4 T cell transfer. Interestingly, previous blockade of ART2.2 activity allowed for a significant increase in both the proportion and numbers of Tregs in the PLNs of NOD.CD38^{null} mice subsequently treated with α -GalCer and infused with AI4 T cells (Fig. 6a). No expansion was seen in spleens (Fig. 6a). It should also be noted there was no alteration

in the numbers or proportions of Tregs in PLNs of NOD.*CD38^{null}* mice that received the s+16Fc antibody, but were not α -GalCer treated or infused with AI4 T cells (not shown). This indicated the accumulation of Tregs into the PLNs of NOD.*CD38^{null}* mice in which iNKT-cells were protected from ART2.2 mediated deletion by s+16Fc treatment, and subsequently activated by α -GalCer, also required the presence of events elicited by the presence of diabetogenic T-cells.

We next asked if in addition to the numerical alterations, can Treg activity in NOD.*CD38^{null}* mice be functionally improved as a consequence of ART2.2 blockade. Putative CD4+CD25+ Tregs were purified from both NOD and NOD.*CD38^{null}* mice. CD4+CD25- responder T cells were purified from NOD mice, stained with CFSE and co-incubated for 72 hours in the presence or absence of the indicated ratios of Tregs along with the TCR activating CD3 antibody and NOD-*scid* splenocytes as a source of co-stimulation. Tregs from control NOD.*CD38^{null}* mice demonstrated only very weak suppressive activity (Fig. 6b,c). We cannot exclude the possibility that putative Tregs from control NOD.*CD38^{null}* mice are eliminated during the *in vitro* culture period through an NICD mechanism. To test if the poor suppressive function of NOD.*CD38^{null}* Tregs is due to ART2.2 mediated effects (NICD or others), we assessed if their activity was enhanced in a group of mice injected with s+16Fc 24 hours previously. Indeed, blocking of ART2.2 activity increased the suppressive activity of NOD.*CD38^{null}* Tregs (Fig. 6b, c). Taken together these data support a contributory role for Tregs characterized by preferential sensitivity to ART2.2 mediated apoptosis in the absence of CD38 activity in how α -GalCer induced iNKT/DC interactions inhibit T1D development in NOD mice.

3.6 Long-term treatment with s+16Fc alone does not inhibit spontaneous T1D development in NOD.*CD38^{null}* mice, but restores their ability to protected from disease by α -GalCer treatment

We tested if increasing the numbers of iNKT-cells through ART2.2 mediated blockade would protect NOD.*CD38^{null}* mice from spontaneous T1D. NOD.*CD38^{null}* males were analyzed, since they show a 100% rate of T1D by 28 weeks of age, compared to an approximately 30% disease incidence in standard NOD males over the same time period [5]. Standard NOD females served as controls. Compared to those receiving the l-15Fc control antibody, weekly injections with the s+16Fc reagent, starting after weaning, did not change the rate or incidence of spontaneous T1D development in NOD.*CD38^{null}* males or NOD females (Fig. 7a). This was despite the fact that chronic ART2.2 blockade increased iNKT-cell numbers in NOD.*CD38^{null}* mice to levels comparable to that in standard NOD mice (Fig. 7b). These findings indicate that the accelerated T1D onset characterizing NOD.*CD38^{null}* mice is not solely due to ART2.2 mediated apoptotic deletion of peripheral iNKT-cells.

We further investigated whether the elevated numbers of iNKT-cells that are induced by blocking ART2.2 activity in NOD.*CD38^{null}* mice have a restored capacity to prevent spontaneous T1D onset when chronically activated by weekly α -GalCer injections. NOD.*CD38^{null}* females were injected once weekly from seven weeks of age with α -GalCer or vehicle. One group of α -GalCer treated mice were also injected once weekly with s+16Fc, starting at weaning. Clearly, the increased numbers of iNKT-cells that are elicited in NOD.*CD38^{null}* mice by treatment with the s+16Fc ART2.2 blocking antibody and then undergo α -GalCer mediated activation had a capacity to significantly inhibit the development of spontaneous T1D (Fig. 7c). Taken together these data indicate that in addition to being numerically increased by ART2.2 blockade, iNKT-cells must also be α -GalCer activated to protect NOD.*CD38^{null}* mice from spontaneous T1D.

4. Discussion

The llama derived s+16 sdAb was previously shown to effectively block ART2.2 enzymatic activity on the surface of T cells *in vivo* and protect these cells from NICD [8]. However, due to its small size and lack of an Fc component required for FcRn interactions, the original s+16 reagent has a short half-life *in vivo*, limiting its therapeutic potential to short term blockade of ART2.2. In order to develop a therapeutic agent capable of providing efficient long term suppression of ART2.2 activity, we describe here generation of a modified bivalent version of s+16, i.e. essentially a reconstituted heavy chain only antibody with a fused mouse IgG1 Fc domain (s+16Fc) that imparts a long *in vivo* half-life to the reagent. We investigated whether it was possible to utilize s+16Fc as a pharmacological agent for long-term blockade of ART2.2 mediated NICD *in vivo*. It was reasoned that an ideal model to test the pharmacologic capabilities of s+16Fc was the NOD.*CD38^{null}* mouse stock characterized by an accelerated onset of T1D due to enhanced ART2.2 mediated preferential deletion of immunoregulatory iNKT-cells [25]. While controversial, decreased levels of iNKT-cells have also been reported as a possible contributory factor to T1D development in humans [29,30]. Furthermore a recent genome-wide association study in T1D patients identified a new susceptibility locus that maps to the Chromosome 4p15 region where the polymorphic *CD38* gene is located [31]. Thus, a previously unconsidered possibility is that differences in CD38 expression levels or activity might contribute to T1D susceptibility or resistance in humans by determining the extent to which a counteracting ART family member mediates the apoptotic deletion of immunoregulatory iNKT cells. Such a finding would further support the value of developing reagents that can efficiently block the *in vivo* enzymatic activity of various ART family members. For this reason it was highly encouraging that by providing an efficient means to block ART2.2 enzymatic activity *in vivo*, treatment with the S+16Fc sdAb corrected the normal inability of NOD.*CD38^{null}* mice to maintain the survival of sufficient numbers of iNKT-cells which upon α -GalCer mediated activation exert T1D protective effects.

The CD4+ and DN subsets of iNKT-cells have been reported to be functionally distinct [32]. The CD4+ subset of iNKT-cells has been proposed to mediate tolerance induction mechanisms [33-35]. The fact that accelerated T1D development in NOD.*CD38^{null}* mice is associated with a preferential loss of the CD4+ subset of iNKT-cells in the periphery supports this paradigm. This appears to result from higher ART2.2 expression levels on CD4+ than DN iNKT-cells. Treatment with the ART2.2 blocking s+16Fc sdAb not only enhances the overall survival of iNKT-cells in NOD.*CD38^{null}* mice, but also normalizes the ratio of the CD4+ and DN subsets.

While the S+16Fc reagent allows for an increased overall survival of iNKT-cells, and normalizes the ratios of the CD4+ and DN subsets, this treatment by itself remains insufficient to protect NOD.*CD38^{null}* mice from spontaneous T1D development. This is likely because while s+16Fc treatment greatly enhances iNKT-cell survival in the CD38 deficient stock, they do not numerically exceed the level already known to be insufficient to protect standard NOD mice from T1D development. However, when activated by the super-agonist α -GalCer, the numbers of iNKT cells present in standard NOD mice are sufficient to elicit T1D protective effects. The ART2.2 mediated further reduction from already sub-normal levels of iNKT cells, likely accounts for our findings in a previous [25] and this study that α -GalCer treatment by itself fails to inhibit T1D development in NOD.*CD38^{null}* mice. However, a combination of s+16Fc and α -GalCer treatments were found to inhibit T1D development in NOD.*CD38^{null}* mice. Thus, a similar combination therapy approach might ultimately be considered for preventing progression to overt T1D in at least a subset of human subjects deemed to be at high future disease risk.

Tregs reportedly contribute to T1D protection mediated by α -GalCer activated iNKT-cells in NOD mice [28]. In addition to being characterized by a paucity of iNKT-cells, Tregs are also

reportedly decreased in NOD.*CD38^{null}* mice [5]. We now report that blocking of ART2.2 enzymatic activity allowed for a significant expansion of functional Tregs in NOD.*CD38^{null}* mice under conditions where both activated iNKT-cells and diabetogenic CD8 T-cells were also present. Thus, ART2.2 mediated NICD may play a greater than previously appreciated role in maintaining Treg homeostasis.

The use of a recombinant heavy chain antibody in this study is an important proof of principle demonstration that long-term inhibition of ecto-enzymes *in vivo* can enhance the peripheral survival of a particular cell type. Thus, it could be a valid future clinical approach to modulate cell populations in patients [9]. The VHH-Fc fusion protein unites the advantageous properties of a single domain VHH with extended CDR-3 loops that can protrude into active sites of enzymes [8,36,37] with advantageous low immunogenicity of same species Fc, to establish a high avidity antibody reagent with a long half-life. In this study we found no evidence for an immune response generating neutralizing antibodies against the VHH-Fc fusion protein. Our results are in line with those of a recent study using a similar strategy to reconstitute a bivalent heavy chain only antibody reagent for improved *in vivo* efficacy in the case of a scorpion toxin neutralizing dromedary sdAb fused to the Fc portion of human IgG1 [38].

Taken together this study demonstrates that blocking ART2.2 activity with a novel bivalent heavy only chain antibody corrects the strongly impaired peripheral survival of iNKT-cells in NOD.*CD38^{null}* mice and restores their ability upon α -GalCer mediated activation to inhibit T1D development through a mechanism likely including contributions from DC and Tregs. As such, this study is a proof of principle for the successful specific long-term blockade of a cell surface enzyme with a bivalent VHH-Fc fusion protein as well as the *in vivo* manipulation of the iNKT — DC axis. It could provide a possible future clinically useful means to inhibit progression to overt T1D in humans deemed to be at high future disease risk.

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Fig. 1. ART2 and P2X7 levels on iNKT cells at various developmental stages and comparison of iNKT cell numbers and ART2 activity in NOD and NOD.CD38^{null} mice

a) Total thymocytes and splenocytes from NOD mice were stained with antibodies specific for either ART2, P2X7, or CD3 and an iNKT TCR binding CD1d-tetramer and analyzed by flow cytometry. Gating was on conventional T-cells (tetramer negative) or iNKT cells. For P2X7 analyses staining with pre-immune serum and secondary antibodies is also shown for CD3+ cells. Data are representative profiles of multiple independent observations. **b)** Proportions and ART2 expression levels of splenic iNKT cells in the indicated strains. Data are representative profiles of multiple independent observations. **c)** ART2 enzymatic activity of splenic iNKT cells from the indicated strains. Splenocytes were incubated with or without 1 μg S+16 for 15

minutes followed by treatment with the etheno-NAD analog for 10 minutes at 4°C. Cells were washed and stained with a specific fluorochrome conjugated mAb (1G4) that detects eADP-ribosylated cell surface proteins and an iNKT TCR binding CD1d-tetramer and analyzed by flow cytometry. Data are representative profiles of multiple independent observations.

Fig. 2. Titration and serum persistence of the S+16Fc heavy chain ART2 blocking antibody
a) NOD.CD38^{null} mice were injected i.p. with indicated amounts of S+16Fc. After 24 h dispersed lymph node cells were incubated with or without etheno-NAD for 10 minutes at 4°C, washed, stained with 1G4-mAb that detects eADP-ribosylated cell surface proteins and analyzed by flow cytometry. White and gray histograms respectively indicate incubation with or without etheno-NAD. Data are representative of 3 independent experiments. **b)** NOD.CD38^{null} mice were injected i.p. with 5µg S+16Fc and sacrificed at the indicated timepoints. Spontaneous NICD was assessed by Annexin V staining after a 30 min incubation at 4°C (panel 2) or 37°C (panels 1,3-5). Data are representative of 3 independent experiments.

Fig. 3. Enhanced survival of peripheral iNKT cells in NOD.CD38^{null} mice after long term treatment with s+16Fc

NOD.CD38^{null} mice were injected i.p. once weekly with 5μg of S+16mFc-LSF. At the indicated time points PLN cells and splenocytes were assessed by flow cytometry for proportions (**a**) and numbers (**b**) of iNKT cells by tetramer analyses (n=4-6 per time point). Differences in proportions and numbers were compared by One-Way Analysis of Variance. **c**) Representative flow cytometric profiles of proportions of PLN origin iNKT cells expressing CD4 in NOD controls and NOD.CD38^{null} mice that did or did not receive four once weekly s+16Fc injections. Gating is on iNKT cells identified by positive tetramer staining. **d**) Mean ratio over time of CD4+/DN iNKT-cells in NOD.CD38^{null} mice receiving long term s+16Fc treatment (n=3 to 6 per group). Differences in ratios were compared by One-Way Analysis of Variance. Errorbars represent mean + SEM.

Fig. 4. Long term blocking of ART2 enzymatic activity restores iNKT — DC signaling axis in NOD.CD38^{null} mice

NOD.CD38^{null} mice were injected once weekly for 4 weeks with 5 μ g s+16Fc. Untreated standard NOD and NOD.CD38^{null} mice served as controls. Subsequently, mice in each group were then injected i.p. with 2 μ g α -GalCer or vehicle (DMSO). Numbers of dendritic cells (a) and iNKT cells (b) within PLNs of mice in each treatment group were assessed 4 days later by flow cytometry. In panel b, n=4 per group. Differences in cell numbers were compared by One-Way Analysis of Variance. Errorbars represent mean + SEM.

Fig. 5. Long term blocking of ART2 enzymatic activity restores ability of alpha-GalCer to protect CD38 deficient NOD from adoptively transferred T1D

NOD.*CD38^{null}* mice were injected i.p. once weekly for 7 weeks with 5 µg s+16Fc or control antibody (I-15Fc). For the last 4 weeks mice were additionally also injected i.p. with 2 µg α-GalCer. One group of mice received the α-GalCer injections only and then a single shot of s+16Fc just before irradiation. Mice were then sublethally irradiated (600R) and injected i.v. with 5×10^6 NOD.*Rag1^{null}.AI4* splenocytes. (a) Urinary glucose was monitored daily to determine T1D onset. Incidence curves were compared according to the Logrank Test. (b) At time of T1D onset or day 14 after transfer mice were sacrificed and numbers of AI4 CD8 T-cells and iNKT cells in PLNs were determined by flow cytometry (n=5 per group). Differences in numbers were compared using Wilcoxon rank sum test. Errorbars represent mean + SEM.

Fig. 6. Combination s+16Fc and α -GalCer treatment allows Treg expansion in the PLNs of NOD.CD38^{null} mice after AI4 T cell transfer

(a) NOD.CD38^{null} mice (n=5 per group) were treated once weekly for 7 weeks with 5 μ g s+16Fc. For the last 4 weeks mice were additionally injected with 2 μ g α -GalCer. Other groups were treated for 4 weeks with α -GalCer or DMSO only. Mice were then sublethally irradiated (600 R) and infused with 5×10^6 NOD.Rag1^{null}.AI4 splenocytes. At day 4 post-AI4 T cell transfer proportions and numbers of phenotypic CD4+CD25+FoxP3+ Tregs in PLNs and spleen were assessed by flow cytometry. Differences in percentage and numbers were analyzed using Wilcoxon rank sum test. Errorbars represent mean + SEM. (b, c) CD4+CD25- T-cells from NOD spleens were labeled with CFSE and incubated for 3 days with 5 μ g/ml anti-CD3 in the presence or absence of different ratios of putative CD4+CD25+ Tregs purified from spleens of NOD or NOD.CD38^{null} mice. One group of NOD.CD38^{null} mice were injected with 5 μ g s+16Fc 24 hours prior to purification of Tregs. CFSE dilution was measured by flow cytometry to assess proliferation of responder cells. Suppression assays were done in technical triplicates. (b) Treg activity shown as percent suppression of responder cell proliferation in the absence of Tregs. Responder cell to Treg ratios are indicated. Results are representative of 3 independent experiments. (c) Representative flow cytometric profiles of responder cell CFSE dilution in the presence of the indicated sources of Tregs at a 1:1 ratio.

Fig. 7. Long-term treatment with s+16Fc alone does not inhibit spontaneous T1D development in NOD.CD38^{null} mice, but restores their ability to respond to α-GalCer in a disease protective manner

(a) NOD.CD38^{null} males and standard NOD females were treated with weekly i.p. injections starting after weaning with 5μg of the s+16Fc ART2.2 blocking or the control antibody (I-15Fc). Urinary glucose levels were monitored weekly to determine spontaneous T1D onset. (b) Mice were sacrificed at T1D onset and numbers of viable iNKT cells in their PLNs were determined. (c) NOD.CD38^{null} females were injected with vehicle control or 2μg of α-GalCer once weekly from 6 weeks of age. A group of NOD.CD38^{null} mice were pretreated once weekly for 3 weeks with 5μg of the ART2.2 blocking s+16Fc with the treatment then continuing in combination with the α-GalCer injections. Urinary glucose levels were monitored weekly to determine spontaneous T1D onset. Incidence curves were compared according to the Logrank Test.