Nardilysin-dependent proteolysis of cell-associated VTCN1 (B7-H4) marks type 1 diabetes development.

Ilian A. Radichev¹, Lilia V. Maneva-Radicheva¹, Christina Amatya¹, Camille Parker¹, Jacob Ellefson¹, Clive Wasserfall², Mark Atkinson², Paul Burn¹, Alexei Y. Savinov¹

¹Sanford Project/Children’s Health Research Center at Sanford Research; Department of Pediatrics, University of South Dakota School of Medicine, Sioux Falls, South Dakota, USA
²Department of Pathology, University of Florida, College of Medicine, Gainesville, Florida, USA

Corresponding author: Alexei Savinov, Sanford Project/Children’s Health Research Center, Sanford Research, 2310 East 60th Street North, Sioux Falls, SD, 57104, USA. Phone: (605) 312-6019; Fax: (605) 312-6071; E-mail: Alexei.Savinov@sanfordhealth.org

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ABSTRACT

T cell responses directed against insulin-secreting pancreatic β cells are the key events highlighting type 1 diabetes (T1D). Therefore, a defective control of T cell activation is thought to underlie T1D development. Recent studies implicated a B7-like negative co-stimulatory protein, VTCN1 (V-set domain-containing T cell activation inhibitor-1), as a molecule capable of inhibiting T cell activation and, potentially, an important constituent in experimental models of T1D. Here, we unravel a general deficiency within the VTCN1 pathway that is shared between diabetes-prone mice and a subset of T1D patients. Gradual loss of membrane-tethered VTCN1 from antigen-presenting cells combined with an increased release of soluble VTCN1 (sVTCN1) occurs in parallel to natural T1D development, potentiating hyper-proliferation of diabetogenic T cells. Mechanistically, we demonstrate that the loss of membrane-tethered VTCN1 is linked to proteolytic cleavage mediated by the metalloproteinase nardilysin (NRD1). The cleaved sVTCN1 fragment was detected at high levels in the peripheral blood of 53% T1D patients compared to only 9% of the healthy subjects. Elevated blood sVTCN1 levels appeared early in the disease progression and correlated with the aggressive pace of disease, highlighting the potential use of sVTCN1 as a new T1D biomarker, and identifying NRD1 as a potential therapeutic target.
INTRODUCTION

Type 1 diabetes (T1D) is a complex autoimmune disease. Despite extensive research, a detailed understanding of the mechanisms driving diabetogenic autoimmunity is lacking, and a need for reliable biomarkers of this disorder remains pressing. The continuous destruction of insulin-producing β-cells within the pancreatic islets, a hallmark of T1D, is mediated by autoreactive islet-specific T lymphocytes (1; 2). In healthy individuals, a small subset of autoreactive T cells escapes thymic negative selection circulating in the periphery in an unprimed naïve state (3; 4). Failure to silence self-reactive T cells would, therefore, create an autoimmune condition. Accordingly, activation of islet-specific T cells is the key feature of T1D-associated autoimmunity (5).

T cell activation entails integration of two independent signals delivered by antigen-presenting cells (APCs): antigen-specific and co-stimulatory. Different co-stimulatory ligands expressed on APCs bind to T cells providing for activation or anergy, depending on the nature of co-stimulatory signal (6). The “classical” B7-1 and B7-2 co-stimulatory molecules transduce an activation signal. Lately, several B7-homologous negative co-stimulatory ligands have been discovered and characterized (7-9).

V-set domain-containing T cell activation inhibitor-1, VTCN1, also known as B7-H4, B7S1, and B7X, is a negative co-stimulatory molecule (8; 10) that binds to an unidentified receptor on T cells, delivering downstream signaling through ERK, JNK, and Akt kinases (11). VTCN1 suppresses T cell responses to antigenic stimulation, decreasing cytokine production and reducing proliferation of both CD4+ and CD8+ T cells (8; 10; 12). Accumulating evidence indicates that VTCN1-mediated negative co-stimulation provides a crucial balance between abnormal T cell activation and anergy. Accordingly, experimental interference with VTCN1
signaling exacerbates multiple autoimmune conditions, as was reported for rheumatoid arthritis (RA) (13) and multiple sclerosis (MS) models (10; 14).

Persistence of autoreactive T cell responses during T1D implies that impaired VTCN1 co-inhibition may contribute to diabetogenic autoimmunity. Accordingly, matrix-surface-bound VTCN1-Ig fusion protein suppressed proliferation of islet-specific T1D patient-derived T cell clones, while VTCN1-Ig transfection protected human islets from these clones (15). Furthermore, treatment of diabetes-susceptible NOD mice with VTCN1-Ig protein significantly attenuated T1D (16). Ex-vivo VTCN1 over-expression in mouse islets shielded them from T cell cytotoxicity in transplantation experiments (17). In vivo β-cell-specific VTCN1 over-expression protected against diabetes induced by both CD4+ and CD8+ islet-specific clonal T cells (14; 18).

All recent studies addressing effects of VTCN1-mediated negative co-stimulation on the development of diabetogenic autoimmunity, however, relied on experimental models and utilized artificial interference and/or enhancement of VTCN1 signaling. The state of endogenous VTCN1 in T1D-susceptible animals and, most importantly, human patients, therefore, remained overlooked. Here we show that T1D pathogenesis incorporates a previously unidentified endogenous functional defect of VTCN1-mediated inhibitory co-stimulation, which augments activation of diabetogenic T cells. We also demonstrate that a proteolytic cleavage by the metalloproteinase nardilsyn (NRD1) is being involved in VTCN1 inactivation during T1D development. Finally, we identify NRD1 as a presumptive novel therapeutic target, and point out soluble VTCN1 as a potential biomarker of human T1D diagnosis.
RESEARCH DESIGN AND METHODS

Mice

Female NOD/ShiLtJ (NOD), NOD.CB17-Prkdcsid/J (NOD-scid), B6.NOD-(D17Mit21-D17Mit10)/LtJ (B6g7) and DBA/2J (DBA) mice were from The Jackson Laboratory. B6.G9C8 mice, transgenic for TCR derived from InsB15-23-specific CD8+ T cell clone G9C8, and H-2Kd MHC allele (19) were provided by Dr. A. Chervonsky (University of Chicago).

Human samples

Sera from T1D cohorts, collected under IRB guidelines with informed consent, were from the University of Florida. Blood samples for PBMC isolation were obtained according to Sanford Research IRB guidelines. Sera from T2D patients and controls were from BioChemed Services.

Antibodies

All antibodies/dilutions used are listed in Table S1.

Immune cells isolation and activation

Thioglycollate-elicited peritoneal macrophages were prepared as described (20). Bone marrow-derived macrophages (BMDMs) were generated by culturing bone marrow cells in L929-conditioned medium (21). Dendritic cells (DCs) were isolated using CD11c MicroBeads (Miltenyi Biotec). To obtain human PBMC-derived macrophages, leukocytes isolated from blood by Ficoll-Paque Premium (GE Healthcare) density centrifugation were adhesion-enhanced and cultured for 10 days with 50ng/ml of M-CSF.

T cells were purified from NOD or B6.G9C8 spleens using Pan T-Cell Isolation KitII (Miltenyi Biotec), and labeled with CFSE (22). G9C8 T cells were co-cultured for 5 days with macrophages pulsed with InsB15-23 peptide (10µg/ml). IL-2 (5U/mL) was added on day 3. NOD T cells were cultured with NIH3T3 cells and activated by anti-CD3/anti-CD28-coated beads.
Recombinant mouse VTCN1 or IgG was added 1 hr before beads. Medium aliquots for ELISA analysis were collected 48 hrs after activation. T cell proliferation was evaluated by FACS on day 5.

**Immunofluorescence**

Fixed in 4% PFA macrophages were stained for VTCN1, F4/80 or CD14, and NRD1. Cells were mounted in DAPI-Vectashield medium (Vector Laboratories) and examined under a Nikon-AI microscope. Imaging conditions were constant between all samples within an experiment. Results expressed in relative fluorescent units (RFU) were calculated for ≥100 individual cells by subtracting mean fluorescent intensity (MFI) of the control antibody from MFI of the test antibody.

**FACS analysis**

Single-cell suspensions were blocked with Fc-receptor antibodies (eBioscience) and stained for VTCN1 and either F4/80 or CD14. FACS was performed on the Accuri-C6 (BD). Data were analyzed using FlowJo (Tree Star) software.

**Immunobloting**

Serum-free medium conditioned by macrophages (1-3 days) was concentrated in Amicon Ultra-10K concentrators (Millipore). Macrophages' lysates were prepared in RIPA buffer, as described (23). Samples (7 µg protein) were subjected to immunoblot analysis using a chemiluminiscent detection system. The membranes were scanned on a UVP Biospectrum 500 imaging system.

**ELISA**

Mouse IL-2 and IFNγ were analyzed using ELISA Construction Kits (Antigenix). sVTCN1 was measured by sandwich ELISA. Briefly, Nunc-Immuno MaxiSorp 96-well plates were coated with capturing antibodies, blocked, and incubated with 1:10-diluted sera. Recombinant VTCN1
(R&D) was used for standard curves. Consequent incubations with detection antibodies, HRP-conjugated secondary antibodies, and BioFX TMB/M substrate (SurModics) followed. Reactions were stopped with 50µL of 1M HCl, and measured at 450nm on SpectraMax-M5 microplate reader (Molecular Devices).

**RNA and cDNA**

Total RNA was isolated using Direct-zol RNA MiniPrep kit (Zymo Research). RNA integrity was confirmed on 2100 Bioanalyzer (Agilent). cDNA was prepared using GoScript Reverse Transcription System (Promega).

**RT-qPCR**

cDNAs were amplified with gene-specific primers (Table S2) on Stratagene MX3005P using RT² SYBR-Green ROX qPCR Mastermix (QIAGEN). Assays were normalized to multiple housekeeping genes including β-actin and GAPDH. The qPCR results were analyzed by the 2^-ΔΔCT method (24).

**Cloning and stable expression of VTCN1**

Full-length mouse *VTCN1* was cloned into a modified pOZ retroviral vector after PCR amplification of cDNA from NOD spleen using 5’-GGATCCATGGCTTCCTTGGGGCAGAT-3’ and 5’-CTCGAGTCATCTTAGCATCAGGCAACAGGAG-3’ as forward and reverse primers. Cloned *VTCN1* corresponded to sequences with accession numbers NM_178594 and BC032925. For truncated VTCN1 (a.a.1-256) generation, the reverse primer 5’-CTCGAGTCAGTCATCTTACGTCAGGCAACAGGAG-3’ with STOP-codon before Gly257, was used. *VTCN1* sequence was integrated upstream of puromycin-N-acetyl-transferase gene containing IRES. 3T3 cells stably expressing recombinant VTCN1 proteins were established after viral transduction and puromycin selection (25).
**NRD1 knockdown**

Silencer Select siRNAs were from Life Technologies. 2×10⁵ cells were transfected with 20nM Silencer Negative Control #1 siRNA or mouse NRD1 siRNA (ID s106505) using Lipofectamine RNAiMAX (Life Technologies). 24hrs after transfection cells were washed and incubated for additional 24hrs in serum-free medium. The conditioned medium and cell lysates were then collected and analyzed by immunoblot.

**Statistics**

Group statistics was computed in GraphPad Prism (GraphPad Software). Differences between two groups were analyzed by Student's *t*-test. One-way ANOVA with Tukey or Dunnet’s post-hoc tests was used to analyze multiple groups. Two-way ANOVA with Bonferroni post-test was calculated for multiple effects. sVTCN1 levels within human cohorts were compared by Mann-Whitney (nonparametric) unpaired U-test. Frequencies of sVTCN1⁺ individuals were compared with Fisher’s exact test. The correlation analyses (Figs. 1I, 5G) were based on a linear regression model with *t*-statistics for parameter estimate computed in SPSS for Windows v.17 (SPSS). Nominal logistic regression analyses and standard least squares linear models were computed using JMP software v.8 (SAS Institute).
RESULTS

Defective surface presentation and elevated shedding of VTCN1 in APCs from NOD mice and T1D patients

To examine the state of endogenous VTCN1 in T1D, we compared VTCN1 immunostaining of peritoneal macrophages from pre-diabetic and diabetic NOD mice to that of diabetes-resistant strains: DBA and B6\textsuperscript{g7}. Surface-localized VTCN1 was significantly decreased in pre-diabetic NOD macrophages, and nearly undetectable after diabetes development (Fig. 1A). FACS analysis of peritoneal F4/80\textsuperscript{+} cells confirmed the reduction of membrane VTCN1 in diabetes-prone animals (Fig. 1B). A similar decline (NOD \textit{vs}. B6\textsuperscript{g7}) was observed in BMDMs and APCs (DCs and macrophages) from pancreatic lymph nodes (Fig. 1C, D and Fig. S1), pointing towards generalized defect of surface VTCN1 presentation in diabetes-susceptible mice.

Gene expression analysis, however, showed a marked increase of \textit{VTCN1} mRNA in NOD compared to B6\textsuperscript{g7} macrophages (Fig. 1E). Dissecting the discrepancy between elevated mRNA and low surface-associated VTCN1 protein levels, we found an extensive release of soluble VTCN1 (sVTCN1) fragment from NOD, but not DBA or B6\textsuperscript{g7} macrophages (Fig. 1F). ELISA analysis also showed increased sVTCN1 in NOD compared to B6\textsuperscript{g7} blood sera (Fig. 1G), confirming that shed VTCN1 is readily detectable \textit{in vivo}.

Extending our murine observations, we analyzed a small cohort of T1D patients, their first-degree relatives, and healthy age-matched controls for whom both sera and PBMCs were available. Obtained PBMCs, were differentiated into macrophages (PBMC-MΦs) and used for FACS and immunofluorescence analyses (Fig. 1H). Measured sVTCN1 levels in sera were then correlated to the respective surface VTCN1 intensity on PBMC-MΦs (Fig. 1I). Similarly to murine data, membrane-associated VTCN1 on PBMC-MΦs was significantly lower in T1D
patients compared to healthy controls (Fig. 1J). Moreover, all subjects displayed a significant inverse correlation ($R^2 = 0.62$, $p = 0.0001$) between VTCN1 on PBMC-MΦs and sVTCN1 in sera, with T1D patients largely showing decreased surface and elevated soluble VTCN1 levels, while healthy subjects displaying an opposite trend (Fig. 1I). Hence, VTCN1 shedding is driving its defective presentation in APCs from diabetes-susceptible mice and T1D patients.

Next, we investigated whether surface-associated VTCN1 deficiency can be restored by the physiological stimuli known to facilitate $VTCN1$ expression (26; 27). Accordingly, macrophages treated with a combination of IL-10 and TGF-β increased both membrane-tethered VTCN1 and its mRNA levels (Fig. 2A, B). However, cytokine treatment had no effect on VTCN1 shedding in NOD macrophages, and was insufficient in restoring their surface VTCN1 levels (Fig. 2A, C). Interestingly, cytokine treatment produced sVTCN1 degradation product (Fig. 2C), suggesting that shedding compromises sVTCN1 integrity.

**Loss of surface-associated VTCN1 promotes hyper-proliferation of diabetogenic T cells**

To assess VTCN1 loss during T1D development, macrophages were isolated from 5-, 10- and 15-week old female mice of NOD, NOD-$scid$, and B6$^{g7}$ strains. Immunostaining revealed an age-dependent reduction of cell-associated VTCN1 in macrophages from all strains, most prominently seen in NOD and NOD-$scid$ animals (Fig. 3A). A moderate reduction of surface VTCN1 in B6$^{g7}$ macrophages was not dependent on sVTCN1 release (Fig. 3A, B), and likely relied on down-regulation of mRNA expression, which declined at a similar rate (Fig. 3C). Conversely, a massive sVTCN1 release, accompanied by near-complete diminishment of surface-associated VTCN1, despite significant $VTCN1$ mRNA up-regulation, was associated with aging in NOD macrophages. Interestingly, NOD and NOD-$scid$ macrophages showed
similar patterns of VTCN1 loss, indicating that sVTCN1 release arises independently from lymphocyte-mediated responses.

Next, we compared the ability of B6$^{g7}$ and NOD macrophages, loaded with exogenous insulin-derived InsB$^{15-23}$ peptide, to induce proliferation of diabetogenic G9C8 CD8$^+$ T cells, bearing clonal InsB$^{15-23}$-recognizing TCR (19). NOD macrophages stimulated proliferation of G9C8 cells significantly better than B6$^{g7}$ macrophages (Fig. 3D). Addition of VTCN1-neutralizing antibody (28) to the B6$^{g7}$ macrophages improved propagation of co-cultured G9C8 cells to levels achieved by NOD macrophages, confirming that VTCN1 membrane presentation is crucial for its function.

**Nardilysin mediates sVTCN1 shedding**

To examine the mechanism of sVTCN1 release, we generated NIH-3T3 cells (3T3) stably expressing full-length (flVTCN1) or soluble, truncated (trVTCN1; aa1-256) VTCN1 (Fig. 4A). *In silico* analysis via Eukaryotic Linear Motif database (http://elm.eu.org/) revealed a potential cleavage site (K$^{246}$RRS$^{249}$), located before the transmembrane domain, predicted to be targeted by the metalloproteinase NRD1 (nardilysin), and two subtilisin-like proprotein convertases: PCSK1, and PCSK2.

Expression of these proteinases in NIH-3T3-based cell lines and macrophages from mice of different ages was evaluated as substantial levels of *NRD1* and *PCSK2* mRNAs were measured in the macrophages, while only *NRD1* was expressed in 3T3-cells (Fig. 4B, C). Interestingly, both *NRD1* and *PCSK2* mRNAs gradually increased with aging in NOD and NOD-scid macrophages, although *PCSK2* mRNA levels decreased with age in B6$^{g7}$ macrophages. *PCSK1* mRNA was barely detected in macrophages of all strains at any age. Since 3T3-flVTCN1 cells shed sVTCN1 (Fig. 4D), but lack *PCSK1* and *PCSK2* expression, these
endopeptidases are likely dispensable for VTCN1 processing. Overall, these results, together with the partial co-localization of VTCN1 with NRD1 detected in permeabilized NOD macrophages (Fig. S2), identify NRD1 as a likely VTCN1-shedding enzyme.

This prospect was further addressed using the NRD1-specific inhibitor 1,10-Phenanthroline (phenanthroline) (29). Phenanthroline treatment suppressed sVTCN1 release by macrophages and 3T3-flVTCN1 cells (Fig. 4D). To exclude involvement of PSCK1, PCSK2, as well as other metalloproteinases, we used a panel of inhibitors, which included: cathepsin/subtilisin inhibitor, known to inhibit cysteine and serine proteases; furin inhibitor-II, which alongside furin inhibition increases PCSK2 activity; and the aminopeptidase inhibitors amastatin and bestatin, which were shown to inhibit NRD1, but not other metalloproteases (30). All three NRD1-specific inhibitors (phenanthroline, amastatin, and bestatin) constrained sVTCN1 secretion, while simultaneously stabilizing membrane VTCN1 (Fig. 4E and F). Conversely, NRD1-irrelevant cathepsin/subtilisin inhibitor and furin inhibitor-II had no effect on either of these phenotypes.

In functional assays, treatment with NRD1 inhibitors reduced macrophages’ ability to induce InsB$_{15-23}$ peptide-dependent proliferation of G9C8 cells (Fig. 4G). Additionally, immunoblot analysis of media conditioned by macrophages, collected from NOD mice at different ages, showed a strong correlation between released sVTCN1 and NRD1 levels (Fig. 4H). Finally, NRD1 siRNA knockdown in 3T3-flVTCN1 cells provided a considerable inhibitory effect on VTCN1 shedding (Fig. 4I).

**Elevated serum sVTCN1 marks disease in T1D patients**

To assess sVTCN1 as a potential T1D biomarker, we analyzed sera from 223 individuals: healthy controls (n=74), T1D patients (n=67), and non-diabetic first-degree relatives of T1D
patients (n=82) (Table 1). A significant 6.5-fold increase of mean sVTCN1 levels was observed in the T1D group, compared to the control group (57.7±14.2ng/ml and 8.9±2.8ng/ml, respectively; p<0.0001; Fig. 5A). The mean blood sVTCN1 concentration in relatives of T1D patients (17.53±5.7ng/ml) was not significantly different from the control group (p=0.5). Since T1D is largely a childhood-age onset disease, the sub-cohort of pediatric subjects from each group was analyzed separately. Similarly to the all-ages cohort, a significant 6.9-fold increase in mean sVTCN1 levels was evident in pediatric T1D patients vs. controls (45.3±13.7ng/ml and 6.6±2.1ng/ml, respectively; p=0.001; Fig. 5B), while no statistical significance (p=0.33) was observed between pediatric relatives and respective controls (Fig. 5B, Table 1). Thus, elevated blood sVTCN1 is characteristic for T1D patients of any age.

To exclude the possibility that sVTCN1 increase arises from diabetes-associated metabolic alterations unrelated to autoimmunity, we evaluated a separate cohort of T2D patients (n=29) and respective age-, sex-, race-, and BMI-matched control subjects (n=17) (Table 1). No significant differences in mean sVTCN1 concentrations were observed between T2D and control groups (7.8±2.5ng/ml and 13.6±7.8ng/ml, respectively; p=0.82; Fig. 5C), indicating that sVTCN1 marks specifically autoimmune process.

sVTCN1/T1D association was strengthened by nominal logistic regression analysis, which determined that log-transformed, sex- and age-adjusted sVTCN1 levels can stratify subjects from both all-ages and pediatric cohorts for T1D diagnosis (adjusted odds ratios: OR=1.32 for all-ages cohort, p<0.0001; and OR=1.31 for pediatric sub-cohort, p=0.0002), and therefore can serve as a marker of clinically active T1D. Analysis of patients from T2D cohort found no association of sVTCN1 levels with T2D diagnosis (OR=0.99, p=0.59).
Next, we performed a secondary analysis, evaluating sVTCN1 levels during disease progression. Accordingly, T1D patients from all-ages cohort were classified, based on the length of clinical disease, into either new-onset group (N.O.; <6mo. from diagnosis), 1-5 years from diagnosis group (established disease, Est.1), or >5 years from diagnosis group (Est.5) (Table 1). Control subjects were used as a reference. Strikingly, mean sVTCN1 levels did not decline alongside disease progression and remained steadily elevated in all T1D groups (46.3±23.3ng/ml, p=0.0001 for new-onset; 32.4±16.1ng/ml, p=0.01 for Est.1; and 81.3±26.7ng/ml, p=0.003 for Est.5 groups, vs. control’s levels of 8.9±2.8ng/ml; Fig. 5D). Multivariate regression analysis (sex- and age-adjusted) also did not reveal association between log-transformed sVTCN1 concentrations and T1D duration (p=0.4). Hence, sVTCN1 likely manifests itself rather early in the disease and persists throughout T1D.

In context of these findings, we explored whether sVTCN1 levels rise in concert with the established autoimmune markers (anti-islet autoantibodies) or follow a separate pattern. Consequently, autoantibodies to GAD65, IA2, and ZnT8 were measured (31), and pediatric T1D patients and relatives were assigned into one of the two subgroups: autoantibody-negative subjects (AAB⁻), or subjects with elevated titer of two or more islet-specific autoantibodies (AAB⁺). Subjects positive for one autoantibody were excluded from this secondary analysis of means. Control pediatric subjects were used as a reference. Both AAB⁻ and AAB⁺ pediatric T1D patients exhibited prominently elevated sVTCN1 levels (54.9±24.2ng/ml, p=0.002; and 64.5±32.9ng/ml, p=0.066) vs. control group (6.6±2.1ng/ml; Fig. 5E). These levels were not significantly different from each other (p=0.87). Similarly, mean sVTCN1 levels in AAB⁺ and AAB⁻ subgroups of pediatric relatives were slightly up vs. control and not significantly different from each other (Fig. 5E, Table 1). Nominal logistic regression analysis revealed that log-
transformed, age- and sex-adjusted sVTCN1 levels were able to successfully stratify pediatric T1D patients and relatives for T1D diagnosis (OR=1.53, p<0.0003), outperforming both GAD65 and IA2 autoantibodies (log-transformed) as single continuous variables (OR=1.00, p=0.35 for GAD65; OR=1.09, p=0.74 for IA2), and even GAD65/IA2 interaction term (p=0.01) in our population. Therefore, augmentation of blood sVTCN1 likely transpires autonomously from the islet-specific antibodies, and can serve as an independent indicator of T1D.

Next, we examined the frequencies of high blood sVTCN1 in human subjects in relation to T1D diagnosis. As the median sVTCN1 concentration in the all-ages control group was 1.1ng/ml, we defined subjects with ~10-fold increase of sVTCN1 concentrations (≥10ng/ml) as “sVTCN1-positive” (sVTCN1⁺). 55.2% of all-ages T1D patients and 52.8% of pediatric T1D-diagnosed subjects were sVTCN1⁺, which was significantly higher than 14.9% (p<0.0001) and 8.7% (p<0.0001) for the respective control groups (Fig. 5F), suggesting that VTCN1 processing is a relevant endogenous pathway influencing a large cluster of T1D patients.

Additionally, the analysis of T1D patients grouped by the length of disease (N.O., Est.1, or Est.5), revealed that all groups displayed significantly higher frequency of sVTCN1⁺ subjects (61.9%, p=0.0004; 53.3%, p=0.003; and 58.6%, p<0.0001, respectively) vs. control group (Table 1), confirming that sVTCN1 can be considered as an early-arising long-lasting marker of clinically active T1D.

Noticeably, autoantibody status did not affect frequencies of sVTCN1⁺ subjects neither in pediatric T1D patients, nor in pediatric relatives (Fig. 5F), supporting our suggestion that VTCN1 shedding occurs independently from anti-islet autoantibody rise, and likely marks a separate, previously uncharacterized cluster of T1D-affected individuals.
Finally, analysis of sVTCN1+ pediatric T1D patients revealed that blood sVTCN1 levels displayed a statistically significant, moderate correlation with the disease aggressiveness, defined as the patients’ age at diagnosis ($R^2=0.3$, $p=0.008$; Fig. 5G). In contrast, sVTCN1 levels did not show similar correlation with age in the controls and relatives groups (Fig. S3).

**Reduced functionality of sVTCN1 fragment**

Previous *in vitro* studies suggested that VTCN1 exerts inhibitory activity only in a solid surface-bound state (13). Since soluble VTCN1-Ig was diabetes-protective in NOD mice (16), we assessed sVTCN1 activity in comparison to conventionally membrane-tethered VTCN1. sVTCN1 inhibited T cell proliferation and cytokine production only at concentrations significantly higher than those detected in peripheral blood of NOD mice and T1D patients (Fig. 6A, B). A VTCN1 shedding within T cells priming sites might result in a higher local sVTCN1 concentrations we mimicked intra-tissue VTCN1 discharge by co-culturing activated T cells with 3T3-flVTCN1 and 3T3-trVTCN1 cell lines. In conditions of massive sVTCN1 release (occurring in both cell lines, Fig. 4D), membrane-bound VTCN1 on 3T3-flVTCN1 cells was notably more potent in reducing the mean division cycle of T lymphocytes than sVTCN1 produced by 3T3-trVTCN1 cells (Fig. 6C). Addition of recombinant sVTCN1 or VTCN1-Ig in these conditions decreased T cell proliferation only in high concentrations roughly corresponding to those reported to be therapeutic *in vivo* in NOD mice (Fig. 6D).
DISCUSSION

Recent studies implicate the negative co-stimulatory molecules B7-H1, VISTA, and CR1g as important players in experimental models of T1D, RA, and MS (9; 32-36). Negative co-stimulation is generally portrayed as a mechanism limiting antigen-specific activation of autoreactive T cells. These observations triggered our study addressing the role of VTCN1 in the disease-underlying mechanisms of T1D. We found that surface VTCN1 levels were remarkably reduced in NOD APCs and in PBMC-derived macrophages from human T1D patients (Fig. 1). In mouse macrophages, VTCN1 reduction: progressed alongside disease development; occurred despite a likely compensatory increase in VTCN1 mRNA; strongly depended on the shedding of an extracellular sVTCN1 fragment; and developed on diabetes-susceptible NOD genetic background independently of T and B cell signaling (Figs. 1, 2). In humans, defective surface VTCN1 presentation on PBMC-MΦs was evident in T1D patients (Fig. 1H, J), and correlated with the elevated blood sVTCN1 levels (Fig. 1I), which were significantly higher in T1D patients compared to controls (Fig 5A, B).

In functional aspect, defective VTCN1 presentation on NOD-originated macrophages conferred hyper-proliferation of diabetogenic G9C8 cells ex-vivo (Fig. 3D). Since monocyte/macrophage recruitment initiates formation and drives progression of islet infiltrates (37), VTCN1 reduction on islet-resident macrophages is likely to translate into over-aggressive insulitis and accelerated diabetes. A recent report linking diabetes protection in NOD mice to the enrichment of islet infiltrates with macrophages highly expressing another co-inhibitory molecule, CR1g (32), supports this suggestion.

Combining proteinase inhibition and gene silencing approaches, we identified a novel pathway of VTCN1 metabolism, namely proteolytic cleavage by NRD1 (Fig. 4), a
metalloproteinase recently implicated in governing immune response via processing antigenic self-peptides (38). Reports showing elevated serum sVTCN1 levels (13) and increased PBMCs NRD1 expression in RA patients (39), coupled with our observations of augmented sVTCN1 in NOD mice and T1D patients (Figs. 1, 5) together with the correlation between released sVTCN1 and NRD1 (Fig. 4H), suggest a fundamental proteolysis-driven impairment of VTCN1-mediated negative co-stimulation being associated with autoimmunity.

Concentrations of sVTCN1 detected in peripheral blood of human subjects (Fig. 5A-C) were similar to those reported in (13), therefore they can be considered physiological. However, in co-culture experiments sVTCN1 at such physiological levels was functionally inactive (Fig. 6 A-B). Moreover, sVTCN1 was significantly less potent than membrane-tethered VTCN1 in attenuation of T cells proliferation (Fig. 3D and 6C). While treatment with soluble VTCN1-Ig protein mildly attenuated diabetes in NOD mice with a reduction from ~80% diabetes in controls to 38% diabetes in treated mice (16), concentrations of naturally shed sVTCN1 we detected in NOD blood were at least 150-times lower, than ones used in the above-referenced study. Thus, sVTCN1 levels, generated during T1D development, are likely below its functional threshold. Moreover, sVTCN1 appeared to be targeted for rapid degradation (Fig. 2C). Additionaly, a recent report (13) suggested that sVTCN1 acts as a decoy for the putative VTCN1 receptor, blocking the regulatory function of membrane VTCN1. Linking sVTCN1 to the Fc-portion of IgG, though, significantly improved its inhibitory activity most probably fixing the molecule to the Fc-receptors widely expressed on surfaces macrophages and other APCs.

The significant increase in mean blood sVTCN1 levels in T1D patients (Fig. 5A, B), coupled with the lack of significant differences in low sVTCN1 levels between T2D patients and matched controls (Fig. 5C), indicates that sVTCN1 may serve as a biomarker for autoimmune
diabetes. The observed significant proportion of diabetic patients (~50%) displaying sVTCN1-positivity (Fig. 5F), corroborates this suggestion, and advocates that sVTCN1 can and should be further evaluated and validated as a marker of human T1D. We should not, though, exclude the possibility that the elevated sVTCN1 levels could be a general phenomenon relevant to multiple autoimmune conditions, as sVTCN1 was also reported to be elevated in RA patients (13). Further studies analyzing sVTCN1 in other autoimmune disorders should be conducted to address the question if changes of sVTCN1 levels are characteristic only for T1D or for autoimmunity in general.

An almost identical prominent increase of mean blood sVTCN1 levels in AAB+ and AAB− pediatric T1D patients, combined with the similarly identical modest sVTCN1 elevation in AAB+ and AAB− pediatric relatives (Fig 5E), together with observations of roughly equal frequencies of sVTCN1+ subjects in AAB+ and AAB− subgroups of pediatric T1D patients, and, similarly, their first-degree relatives (Fig. 5F) indicated that augmentation of blood sVTCN1 is uncoupled from islet-specific antibody responses. Moreover, sVTCN1 clearly stratifies pediatric population for T1D diagnosis independently of autoantibodies, therefore confirming that sVTCN1 levels can serve as an autonomous and powerful marker of T1D. Human VTCN1 processing, consequently, appears to be neither associated, nor dependent on the development of anti-islet B cell responses and, in certain similarity to NOD-scid mice, who lack B cells but shed VTCN1 extensively (Fig. 3A, B), is expected to mark patients likely harboring predisposition for diabetogenic autoimmunity. The positioning of the VTCN1 gene within the genomic regions associated with T1D susceptibility (Idd10 in mouse and Iddm26 in rat) and in immediate proximity to the human 1p13.2 T1D-susceptibility locus (www.t1Dbase.org), corroborates this hypothesis.
Our observation that sVTCN1 levels were elevated in the new onset T1D patients (Fig. 5D), suggests that sVTCN1 shedding is likely initiated at the earliest disease stages, and, therefore, might contribute to the conditions favoring progression of aggressive diabetogenic autoimmunity. The direct correlation between high sVTCN1 concentrations and the disease aggressiveness (Fig. 5G) further upholds this suggestion and indicates that sVTCN1 may be developed as a parameter for clinical T1D monitoring and for evaluation of the effectiveness of future T1D treatments.
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Author contributions: I.A.R., P.B. and A.Y.S. designed the experiments; I.A.R. and L.V.M.-R. performed the inhibitors studies, in vitro T-cell activation, immunofluorescence and ELISA; I.A.R. and C.A. executed the RNA analysis; C.P. completed FACS analysis; C.W. contributed to human serum studies; I.A.R., C.P. and A.Y.S. analyzed the data; I.A.R., J.E., C.P., M.A. and A.Y.S. wrote the paper.

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Guarantor: A.Y.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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FIGURE LEGENDS

Figure 1

Surface VTCN1 presentation is altered in APCs from NOD mice and T1D patients.

(A) Quantitative analysis (left) and representative images (right) of peritoneal macrophages (P.MΦ) stained for VTCN1 (red) and F4/80 (green). Data in mean relative fluorescence units (RFU) ±SEM (n=3-5 mice/group). Bars, 10 µm. ND, non-diabetic; D, diabetic. (B) FACS of F4/80⁺ peritoneal cells from mice. In grey – isotope control. (C) Representative images of BMDMs stained as in (A). (D) FACS of CD11c⁺ DCs from pancreatic lymph nodes (PLN). (E) RT-qPCR of VTCN1 mRNA in B6 and ND-NOD P.MΦ. Data in mean arbitrary units (AU) ±SEM (n=3 mice/group). (F) VTCN1 immunoblot of medium conditioned by P.MΦ. Black line separates noncontiguous lanes from the same gel. (G) ELISA of sVTCN1 in mouse sera. Data are mean ±SEM (n=12 mice/group). (H) FACS (left) and immunohistochemistry (right) of human CD14⁺ PBMC-MΦs. cont.(P), non-diabetic parent of T1D-1 patient; in grey – isotype control; Bars, 20 µm. (I) Linear regression analysis of serum sVTCN1 and VTCN1 on PBMC-MΦs from humans. The overall correlation (solid black line) and individual correlations for each group (dashed lines of the corresponding color) are shown. (J) Quantitative immunofluorescent analysis of VTCN1 on PBMC-MΦs from six T1D patients and five matching control subjects. Data in mean RFU ±SEM (n>100 cells/subject). Black lines show mean for each group. Statistics were calculated by one-way ANOVA with Tukey post-hoc test (A and G) and unpaired Student’s t-test (E).

Figure 2

IL-10 and TGF-β increase VTCN1 expression and its surface presentation in P.MΦ.
(A) VTCN1 immunostaining of (left) and quantitative analysis of the fluorescent signal (right) F4/80⁺ P.MΦ from B6<sup>g7</sup> and pre-diabetic NOD mice after incubation for 72 h in the presence or in the absence of 10 ng/ml IL-10 and 10 ng/ml TGF-β. Bars, 10 µm. (B) RT-qPCR analysis of VTCN1 mRNA in P.MΦ treated as in (A). Data are mean ±SEM from two experiments (n=3 mice/group). The effect of cytokine treatment in B6<sup>g7</sup> and NOD mice was evaluated by two-way ANOVA followed by Bonferroni post-hoc test. (C) Immunoblot (7 µg per lane) of conditioned medium (left) and cell lysates (right) of P.MΦ. sVTCN1 – soluble VTCN1; mVTCN1 – membrane-tethered VTCN1; Asterisks indicate non-specific bands. Blot was split between 50 kD and 30 kD to use a darker exposure for the lower molecular weight degraded products.

**Figure 3**

Shedding-dependent loss of surface VTCN1 impairs its functionality.

(A) VTCN1 immunostaining of F4/80⁺ P.MΦ isolated from B6<sup>g7</sup>, NOD and NOD-scid mice of indicated ages. Representative images (left) and quantification (right). Data are mean RFU ±SEM (n=3-5 mice/group). Bars, 10 µm. (B) Detection and quantification of sVTCN1 in medium conditioned by P.MΦ from B6<sup>g7</sup>, NOD and NOD-scid mice of different ages. Relative intensities of shed VTCN1 bands were expressed as percentages of sVTCN1 detected in media from 5 week-old B6<sup>g7</sup> P.MΦ. Data are mean ±SEM (n=4-6 group/mice). Bottom panel – representative immunoblots. (C) RT-qPCR analysis of VTCN1 mRNA levels in P.MΦ from B6<sup>g7</sup>, NOD or NOD-scid mice at 5 and 15 weeks of age. Data are mean ±SEM (n=3-5). (D) FACS analysis of proliferating CFSE-labeled insulin-reactive G9C8 TCR-transgenic CD3⁺ cells co-cultured with NOD or B6<sup>g7</sup> macrophages in the presence of InsB<sup>15-23</sup> peptide (Ins), inhibitory anti-VTCN1 antibody (αV1), or an isotype control (IgG). The percentages of proliferating cells
within the gated region are shown. Statistics was calculated by one-way ANOVA with Dunnet’s post-hoc test for each mouse strain vs. 5 week group (A, B) or two-way ANOVA (C).

**Figure 4**

Nardilysin (NRD1) cleaves cell surface VTCN1.

(A) Schematics of VTCN1. SP-signal peptide; DS-disulfide bond; TM-transmembrane domain; Ig1/2-immunoglobulin-like domains; arrow - predicted cleavage site (K246R↓RS249). (B) RT-qPCR of P.MΦ and 3T3-cells. Data are mean AU ±SEM (n=3). (C) Age-related changes in NRD1 and PCSK2 expression. Data are mean AU ±SEM (n=3-5 mice/group); *p<0.05; **p<0.001. (D) Detection and quantification of sVTCN1 in conditioned medium after phenanthroline (Phen.) treatment. Data are mean ±SEM (n=2). Representative immunoblots (right). (E) Detection and quantification of sVTCN1 in conditioned medium after treatment with indicated inhibitor. Ama.–Amastatin; Bes.–Bestatin; FurinII–Furin inhibitorII; Cat./Sub.–Cathepsin/Subtilisin inhibitor. Representative immunoblot (right). The arrow indicates sVTCN1. The asterisk indicates a nonspecific band. Data are mean ±SEM (n=2). (F) Quantification (top) and representative images (bottom) of VTCN1 on P.MΦ treated with indicated inhibitor. Data are mean ±SEM (n>100 cells/treatment). Bars, 10 µm. (G) Proliferation of CFSE-labeled G9C8 cells co-cultured with P.MΦ and treated with indicated inhibitor. Representative FACS (top). Mean division cycle (bottom) ±SEM (n=3). (H) Regression analysis fit of relative immunoblot intensities of NRD1 and sVTCN1 in conditioned media from P.MΦ (top). Representative immunoblots (bottom). Black line separates noncontiguous lanes from the same gel. (I) Immunoblots of cell lysates (left) and conditioned media (right) after siRNA silencing of NRD1. C, control; N, NRD1 siRNA; S, scrambled siRNA. Statistics was calculated by two-way
ANOVA (C), unpaired Student’s t-test (D) and one-way ANOVA with Dunnet’s post-hoc test (E, F and G).

**Figure 5**

Elevated serum sVTCN1 levels are characteristic for T1D.

(A) Serum sVTCN1 measured by ELISA in three groups of patients: healthy controls (Control), type 1 diabetics (T1D) and non-diabetic first-degree relatives of T1D patients (Rel.). (B) sVTCN1 concentrations in sera from the sub-cohort of pediatric subjects. (C) Serum sVTCN1 in type 2 diabetic (T2D) patients and their matching healthy controls. (A-C) The horizontal red lines indicate the mean of the results. The y axis is set up at 1 ng/ml; individual as values <1 ng/ml even though not shown on graph, were included into the analysis of means. (D) sVTCN1 levels in controls and T1D patients with new onset of disease (<6mo, N.O.,) or with established T1D lasting >5 years (Est.5). Data are mean ±SEM. (E) sVTCN1 levels in pediatric sub-cohort of control subjects and T1D patients and relatives identified as negative (AAB−) or positive (AAB+) for at least two islet-specific autoantibodies. Data are mean ±SEM. (F) Frequencies (%) of sVTCN1-negative (<10ng/ml) and +positive (≥10ng/ml) subjects within studied groups. Data are mean ±95%CI for sVTCN1+. Statistics was performed by Mann-Whitney U-test (A-E) and Fisher’s exact test (F); N.S., non-significant. (G) Linear regression analysis of ln-transformed sVTCN1 levels and the age at diagnosis in sVTCN1+ pediatric T1D patients. The p value was calculated by t-statistics for parameter estimate.

**Figure 6**

Functional impairment of sVTCN1 fragment.
(A) FACS analysis of proliferating CFSE-labeled NOD T cells incubated for 5 days with anti-CD3/anti-CD28-coated beads in the presence of indicated concentrations of sVTCN1 or IgG, as a non-specific control. Percentages of proliferating T cells within the marked region are indicated. (B) ELISA of IL-2 (left), and IFNγ (right) secreted by activated NOD T cells 48h after activation as in panel A. Mean ±SEM (n=3). (C) FACS analysis of proliferating CFSE-labeled NOD T cells co-cultured with 3T3-cells expressing full length (3T3-flVTCNI) or soluble VTCN1 (3T3-trVTCNI) and activated by anti-CD3/anti-CD28-coated beads for 5 days. 3T3-parental cell line was used as a control. Percentages of proliferating T cells within the marked region are indicated. The mean division cycle of the activated T cells is shown on the right histogram. Mean ±SEM (n=3). (D) FACS analysis of proliferating NOD T cells after activation with anti-CD3/anti-CD28-coated beads performed in co-culture with 3T3-parental cells with or without addition of indicated concentrations of sVTCN-Ig or sVTCN1. Percentages of proliferating T cells within the marked region are indicated. One-way ANOVA with Tukey post-hoc test (B and C).
### TABLES

#### Table 1. Sample size, age and sex distribution of the subjects in the study.

| Cohort          | Sample size | Age (years) | Sex (M/F) | BMI* (ng/ml)* | C-peptide (ng/ml)* | sVTCN1 | p value vs. control | sVTCN1+ | p value vs. control |
|-----------------|-------------|-------------|-----------|---------------|--------------------|--------|---------------------|--------|---------------------|
| **All-ages cohort†** |             |             |           |               |                    |        |                     |        |                     |
| Controls        | 74          | 21.1±1.3    | 55/45     | -             | -                  | 8.9±2.8 | N/A                 | 14.9   | N/A                 |
| T1D§            | 67          | 17.3±1.2    | 51/49     | -             | -                  | 57.7±14.2| <0.0001             | 55.2   | <0.0001             |
| *N.O.-T1D*      | 22          | 13.3±1.5    | 55/45     | -             | 0.79±0.11          | 46.3±23.3| **0.0001**          | 61.9   | **0.0004**          |
| *Est.T1D-1*     | 15          | 13.1±1.0    | 40/60     | -             | BD‡                | 32.4±16.1| 0.01                | 53.3   | 0.003               |
| *Est.T1D-5*     | 29          | 23.7±2.4    | 55/45     | -             | BD‡                | 81.3±26.7| **0.003**          | 58.6   | <0.0001             |
| Relatives       | 82          | 28.9±2.0    | 54/46     | -             | -                  | 17.53±5.7| 0.5                 | 18.3   | 0.67                |
| *AAB+||| 24          | 17.4±2.8    | 62/38     | -             | -                  | 13.8±8.4 | 0.83               | 20.8   | 0.53                |
| *AAB−| 39          | 33.2±2.5    | 51/49     | -             | -                  | 12.6±5.4 | 0.25               | 12.8   | 1.0                 |
| **Pediatric sub-cohort** |             |             |           |               |                    |        |                     |        |                     |
| Controls        | 46          | 14.1±0.5    | 59/41     | -             | 1.2±0.09           | 6.6±2.1 | N/A                 | 8.7    | N/A                 |
| T1D             | 53          | 13.7±0.5    | 55/45     | -             | -                  | 45.3±13.7| **0.001**          | 52.8   | <0.0001             |
| *AAB+||| 22          | 12.5±0.8    | 55/45     | -             | -                  | 54.9±24.2| **0.002**          | 59.1   | <0.0001             |
| *AAB−| 15          | 15.0±1.0    | 60/40     | -             | -                  | 64.5±32.9| 0.066              | 53.3   | **0.0006**          |
| Relatives       | 38          | 11.7±0.7    | 61/39     | -             | -                  | 14.9±6.4 | 0.33               | 23.7   | 0.073               |
|        | AAB⁺ || | 19 | 10.9±0.9 | 63/37 | - | - | 17.1±10.5 | 0.49 | 26.3 | 0.1 |
|--------|--------|--------|-----|-----|--------|------|----|----|--------|------|------|----|
| AAB⁻   | 12     | 13.0±1.5 | 69/31 | -  | -      | 18.9±11.5 | 0.52 | 25.0 | 0.1 |

**T2D cohort**

|          |        |        |        |        |        |        |        |        |        |        |        |
|----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|          | Controls | 17 | 61.1±4.2 | 53/47 | 25.6±0.8 | 2.5±0.4 | 13.6±7.8 | N/A | 17.6 | N/A |
|          | T2D     | 29 | 58.0±3.3 | 55/45 | 25.2±0.6 | 2.1±0.2 | 7.8±2.5 | 0.82 | 17.2 | 1.0 |

* Mean ±SEM  
† “All-ages cohort” does not include subjects with T2D and their respective matching controls  
‡ BD; below detection  
§ Total number of T1D patients is higher than the sum of the respective subgroups because one patient was removed due to lack of information about the date of blood withdrawal.  
‖ AAB⁺ subpopulation includes only subjects positive for two or more autoantibodies
Surface VTCN1 presentation is altered in APCs from NOD mice and T1D patients.

(A) Quantitative analysis (left) and representative images (right) of peritoneal macrophages (P.MΦ) stained for VTCN1 (red) and F4/80 (green). Data in mean relative fluorescence units (RFU) ±SEM (n=3-5 mice/group). Bars, 10 µm. ND, non-diabetic; D, diabetic. (B) FACS of F4/80+ peritoneal cells from mice. In grey – isotype control. (C) Representative images of BMDMs stained as in (A). (D) FACS of CD11c+ DCs from pancreatic lymph nodes (PLN). (E) RT-qPCR of VTCN1 mRNA in B6g7 and ND-NOD P.MΦ. Data in mean arbitrary units (AU) ±SEM (n=3 mice/group). (F) VTCN1 immunoblot of medium conditioned by P.MΦ. Black line separates noncontiguous lanes from the same gel. (G) ELISA of sVTCN1 in mouse sera. Data are mean ±SEM (n=12 mice/group). (H) FACS (left) and immunohistochemistry (right) of human CD14+ PBMCEMΦs. cont.(P), non-diabetic parent of T1D-1 patient; in grey – isotype control; Bars, 20 µm. (I) Linear regression analysis of serum sVTCN1 and VTCN1 on PBMCEMΦs from humans. The overall correlation (solid black line) and individual correlations for each group (dashed lines of the corresponding color) are shown. (J) Quantitative immunofluorescent analysis of VTCN1 on PBMCEMΦs from six T1D patients and five matching control subjects. Data in mean RFU ±SEM (n>100 cells/subject). Black lines show mean for each group. Statistics were calculated by one-way ANOVA with Tukey post-hoc test (A and G) and unpaired Student’s t-test (E).
IL-10 and TGF-β increase VTCN1 expression and its surface presentation in P.MΦ.

(A) VTCN1 immunostaining of (left) and quantitative analysis of the fluorescent signal (right) F4/80+ P.MΦ from B6g7 and pre-diabetic NOD mice after incubation for 72 h in the presence or in the absence of 10 ng/ml IL-10 and 10 ng/ml TGF-β. Bars, 10 µm. (B) RT-qPCR analysis of VTCN1 mRNA in P.MΦ treated as in (A). Data are mean ±SEM from two experiments (n=3 mice/group). The effect of cytokine treatment in B6g7 and NOD mice was evaluated by two-way ANOVA followed by Bonferroni post-hoc test. (C) Immunoblot (7 µg per lane) of conditioned medium (left) and cell lysates (right) of P.MΦ. sVTCN1 – soluble VTCN1; mVTCN1 – membrane-tethered VTCN1; Asterisks indicate non-specific bands. Blot was split between 50 kD and 30 kD to use a darker exposure for the lower molecular weight degraded products.

114x79mm (300 x 300 DPI)
Shedding-dependent loss of surface VTCN1 impairs its functionality.

(A) VTCN1 immunostaining of F4/80+ P.MΦ isolated from B6g7, NOD and NOD-scid mice of indicated ages. Representative images (left) and quantification (right). Data are mean RFU ±SEM (n=3-5 mice/group). Bars, 10 µm. (B) Detection and quantification of sVTCN1 in medium conditioned by P.MΦ from B6g7, NOD and NOD-scid mice of different ages. Relative intensities of shed VTCN1 bands were expressed as percentages of sVTCN1 detected in media from 5 week-old B6g7 P.MΦ. Data are mean ±SEM (n=4-6 group/mice). Bottom panel –representative immunoblots. (C) RT-qPCR analysis of VTCN1 mRNA levels in P.MΦ from B6g7, NOD or NOD-scid mice at 5 and 15 weeks of age. Data are mean ±SEM (n=3-5). (D) FACS analysis of proliferating CFSE-labeled insulin-reactive G9C8 TCR-transgenic CD3+ cells co-cultured with NOD or B6g7 macrophages in the presence of InsB15–23 peptide (Ins), inhibitory anti-VTCN1 antibody (αV1), or an isotype control (IgG). The percentages of proliferating cells within the gated region are shown. Statistics was calculated by one-way ANOVA with Dunnet’s post-hoc test for each mouse strain vs. 5 week group (A, B) or two-way ANOVA (C).
Nardilsin (NRD1) cleaves cell surface VTCN1.

(A) Schematics of VTCN1. SP—signal peptide; DS—disulfide bond; TM—transmembrane domain; Ig1/2—immunoglobulin-like domains; arrow—predicted cleavage site (K246R RS249).

(B) RT-qPCR of P.MΦ and 3T3-cells. Data are mean AU ±SEM (n=3). (C) Age-related changes in NRD1 and PCSK2 expression. Data are mean AU ±SEM (n=36 mice/group); *p<0.05; **p<0.001. (D) Detection and quantification of sVTCN1 in conditioned medium after phenanthroline (Phen.) treatment. Data are mean ±SEM (n=2). Representative immunoblots (right). The arrow indicates sVTCN1. The asterisk indicates a nonspecific band. Data are mean ±SEM (n=2). (F) Quantification (top) and representative images (bottom) of VTCN1 on P.MΦ treated with indicated inhibitor. Bars, 10 µm. (G) Proliferation of CFSE-labeled G9C8 cells co-cultured with P.MΦ and treated with indicated inhibitor. Representative FACS (top). Mean division cycle (bottom) ±SEM (n=3). (H) Regression analysis fit of relative

Diabetes
immunoblot intensities of NRD1 and sVTCN1 in conditioned media from P.MΦ (top). Representative immunoblots (bottom). Black line separates noncontiguous lanes from the same gel. (I) Immunoblots of cell lysates (left) and conditioned media (right) after siRNA silencing of NRD1. C, control; N, NRD1 siRNA; S, scrambled siRNA. Statistics was calculated by two-way ANOVA (C), unpaired Student’s t-test (D) and one-way ANOVA with Dunnet’s post-hoc test (E, F and G).
Elevated serum sVTCN1 levels are characteristic for T1D.

(A) Serum sVTCN1 measured by ELISA in three groups of patients: healthy controls (Control), type 1 diabetics (T1D) and non-diabetic first-degree relatives of T1D patients (Rel.). (B) sVTCN1 concentrations in sera from the sub-cohort of pediatric subjects. (C) Serum sVTCN1 in type 2 diabetic (T2D) patients and their matching healthy controls. (A-C) The horizontal red lines indicate the mean of the results. The y axis is set up at 1 ng/ml; individual as values <1 ng/ml even though not shown on graph, were included into the analysis of means. (D) sVTCN1 levels in controls and T1D patients with new onset of disease (<6mo, N.O.,), established disease lasting for 1-5 years (Est.1) or with established T1D lasting >5 years (Est.5). Data are mean ±SEM. (E) sVTCN1 levels in pediatric sub-cohort of control subjects and T1D patients and relatives identified as negative (AAB-) or positive (AAB+) for at least two islet-specific autoantibodies. Data are mean ±SEM. (F) Frequencies (%) of sVTCN1-negative (<10ng/ml) and -positive (≥10ng/ml) subjects within studied groups. Data are mean ±95%CI for sVTCN1+. Statistics was performed by Mann-Whitney U-test (A-E) and Fisher's exact test (F); N.S., non-significant. (G) Linear regression analysis of ln-transformed sVTCN1 levels in T1D patients.
levels and the age at diagnosis in sVTCN1+ pediatric T1D patients. The p value was calculated by t-statistics for parameter estimate.

170x209mm (600 x 600 DPI)
Functional impairment of sVTCN1 fragment.

(A) FACS analysis of proliferating CFSE-labeled NOD T cells incubated for 5 days with anti-CD3/anti-CD28-coated beads in the presence of indicated concentrations of sVTCN1 or IgG, as a non-specific control. Percentages of proliferating T cells within the marked region are indicated. (B) ELISA of IL-2 (left), and IFNγ (right) secreted by activated NOD T cells 48h after activation as in panel A. Mean ±SEM (n=3). (C) FACS analysis of proliferating CFSE-labeled NOD T cells co-cultured with 3T3-cells expressing full length (3T3-flVTCN1) or soluble VTCN1 (3T3-trVTCN1) and activated by anti-CD3/anti-CD28-coated beads for 5 days. 3T3-parental cell line was used as a control. Percentages of proliferating T cells within the marked region are indicated. The mean division cycle of the activated T cells is shown on the right histogram. Mean ±SEM (n=3). (D) FACS analysis of proliferating NOD T cells after activation with anti-CD3/anti-CD28-coated beads performed in co-culture with 3T3-parental cells with or without addition of indicated concentrations of sVTCN-Ig or sVTCN1. Percentages of proliferating T cells within the marked region are indicated. One-way ANOVA with Tukey post-hoc test (B and C).

104x66mm (600 x 600 DPI)
Surface VTCN1 levels are decreased in residential PLN macrophages from NOD mice in comparison to those from B6g7 mice.

Analysis of VTCN1 intensity on F4/80+ cells in cells isolated from pancreatic lymph nodes (PLN) by FACS (top). The mean VTCN1 intensity in each F4/80+ fraction is shown on the lower table.

|                | Mean VTCN1 intensity |
|----------------|----------------------|
| Ig control     | 474                  |
| NOD            | 635                  |
| B6g7           | 1050                 |

Surface VTCN1 levels are decreased in residential PLN macrophages from NOD mice in comparison to those from B6g7 mice.

Analysis of VTCN1 intensity on F4/80+ cells in cells isolated from pancreatic lymph nodes (PLN) by FACS (top). The mean VTCN1 intensity in each F4/80+ fraction is shown on the lower table.
VTCN1 partially co-localizes with NRD1. Immunofluorescent image of permeabilized mouse P.MΦ stained for VTCN1 (red) and NRD1 (green). Arrow indicates VTCN1/NRD1 co-localization.
36x19mm (300 x 300 DPI)
sVTCN1 levels in peripheral blood do not correlate with age in healthy subjects. Linear regression analysis of ln-transformed sVTCN1 levels and the age of the patients in sVTCN1+ controls and T1D-relative populations. The p value was calculated by t-statistics for parameter estimate.
ONLINE SUPPLEMENTAL MATERIAL

Figure S1. Surface VTCN1 levels are decreased in residential PLN macrophages from NOD mice in comparison to those from B6g7 mice.

Analysis of VTCN1 intensity on F4/80+ cells in cells isolated from pancreatic lymph nodes (PLN) by FACS (top). The mean VTCN1 intensity in each F4/80+ fraction is shown on the lower table.

Figure S2. VTCN1 partially co-localizes with NRD1.

Immunofluorescent image of permeabilized mouse P.MΦ stained for VTCN1 (red) and NRD1 (green). Arrow indicates VTCN1/NRD1 co-localization.

Figure S3. sVTCN1 levels in peripheral blood do not correlate with age in healthy subjects.

Linear regression analysis of ln-transformed sVTCN1 levels and the age of the patients in sVTCN1+ controls and T1D-relative populations. The p value was calculated by t-statistics for parameter estimate.

Table S1

List of the primers used in current study.

| gene                | direction | primer sequence/source           | amplicon size (bp) |
|---------------------|-----------|---------------------------------|--------------------|
| mouse β-actin       | F/R       | QIAGEN; cat#PPM02945B           | 154                |
| mouse GAPDH         | F/R       | QIAGEN; cat#PPM02946E           | 140                |
| human β-actin       | F/R       | QIAGEN; cat#PPH00073G           | 174                |
| Method | Primary or capturing antibody (conc. and commercial source); conjugate | Secondary or detection antibody (conc. and commercial source); conjugate |
|--------|-------------------------------------------------|-------------------------------------------------|
| IF     | Rat anti-mouse B7-H4 (5µg/ml; R&D Systems) | Donkey anti-rat (1µg/ml; Jackson Immunoresearch); AlexaFluor-594 |
|        | Mouse anti-human B7-H4 (5µg/ml; eBioscience) | Donkey anti-mouse (1µg/ml; Jackson Immunoresearch); AlexaFluor-594 |
|        | Rabbit anti-mouse NRD1 (2.5µg/ml; Proteintech) | Donkey anti-mouse (1µg/ml; Jackson Immunoresearch); AlexaFluor-488 |
|        | Rat anti-mouse F4/80 (10µg/ml; BioLegend); AlexaFluor-488 | N/A |

**Table S2**

List of the antibodies used in current study.
| Method  | Antibody Description                                                                 | Detection Antibody                        |
|---------|--------------------------------------------------------------------------------------|-------------------------------------------|
| FACS    | Mouse anti-human CD14 (20 µl/test; BioLegend); FITC                                  | N/A                                       |
|         | Rat anti-mouse B7-H4 (4µg/ml; eBioscience); PE                                       | N/A                                       |
|         | Rat anti-mouse F4/80 (10µg/ml; BioLegend); AlexaFluor-488                            | N/A                                       |
|         | Mouse anti-human B7-H4 (5µg/ml; eBioscience)                                         | Donkey anti-mouse (5µg/ml; Jackson Immunoresearch); PE |
|         | Mouse anti-human CD14 (BioLegend); FITC                                             | N/A                                       |
| WB      | Goat anti-B7-H4 (1µg/ml; LifeSpan Biosciences)                                       | Donkey anti-goat (0.2µg/ml; Jackson Immunoresearch); HRP |
|         | Rabbit anti-mouse NRD1 (1µg/ml; Abcam, respectively)                                 | Donkey anti-rabbit (0.1µg/ml; Jackson Immunoresearch); HRP |
|         | Rabbit anti-GAPDH (Proteintech)                                                      | Donkey anti-rabbit (0.1µg/ml; Jackson Immunoresearch); HRP |
| ELISA   | Mouse anti-human B7-H4 (1µg/ml; eBioscience); **capturing** antibody                 | N/A                                       |
|         | Rat anti-mouse B7-H4 (1µg/ml; R&D Systems); **capturing** antibody                  | N/A                                       |
|         | Goat anti-B7-H4 (0.5µg/ml; LifeSpan Biosciences); **detection** antibody             | Donkey anti-goat (0.2µg/ml; Jackson Immunoresearch); HRP |