

# Killer cell Ig-like receptor (KIR) 3DL1 down-regulation enhances inhibition of type 1 diabetes by autoantigen-specific regulatory T cells

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**Both Foxp3<sup>+</sup> regulatory T cells (Tregs) and antigen-expanded Foxp3<sup>-</sup> Tregs play an important role in regulating immune responses as well as in preventing autoimmune diseases and graft rejection. Molecular mechanisms modulating Treg function remain largely unclear, however. We report here on the expression and function of an inhibitory killer cell Ig-like receptor, KIR3DL1, in a nonobese diabetic (NOD) mouse-derived autoantigen-specific Treg (2D2), which protects from type 1 diabetes (T1D) in adoptive transfer experiments. This gene is not expressed in T1D pathogenic T cells (Tpaths) or non-Tpath T cells. KIR genes are known to play an important role in regulating natural killer (NK) cell function, but their role in Tregs and T1D is unknown. To examine whether KIR3DL1 expression may modulate Treg function, we used shRNA to down-regulate KIR3DL1 expression (2D2-shKIR). We find that KIR3DL1 down-regulation enhances *in vitro* function, as measured by improved suppression of target cell proliferation. Antibody blockade of IL-10 but not IL-4 partially abrogated suppressive function. *In vivo* function is also improved. Adoptive transfer of 2D2-shKIR into 10-wk-old NOD mice prevented spontaneous insulinitis and T1D, and the inhibitory effect was further improved if the cells were transferred earlier into 6-wk-old NOD mice. These studies indicate that KIR3DL1 expression may negatively regulate Treg function and suggest a previously undescribed target for improving immune tolerance for potential treatment of autoimmune diseases like T1D.**

autoimmunity | KIR gene family

**R**egulatory T cells (Tregs) help maintain homeostatic balance in the immune system (1–4). The loss of Tregs, either as a result of failed development or by experimental depletion, leads to multiorgan autoimmune diseases (1–4). Conversely, augmenting Treg populations by adoptive transfer can prevent autoimmune diseases, including type 1 diabetes (T1D) (1–6). Both Foxp3<sup>+</sup>CD4<sup>+</sup> Tregs and antigen-expanded Foxp3<sup>-</sup>CD4<sup>+</sup> Tregs play an important role in regulating immune responses as well as in preventing autoimmune diseases and graft rejection. Antigen-specific Tregs are often more potent than polyclonal Tregs at abrogating disease (5–8), but the molecular mechanisms modulating antigen-specific Treg function remain to be determined.

We previously isolated several CD4<sup>+</sup>Foxp3<sup>-</sup> Tregs that are specific for glutamic acid decarboxylase (GAD), a major autoantigen in T1D (9–12). These autoantigen-specific and cytokine-dependent Tregs are able to inhibit proliferation of pathogenic T cells (Tpaths) effectively (9–12). Some of these Tregs required cell contact, but others did not. For example, Treg N206, which is a GAD peptide p206-specific CD4<sup>+</sup> Treg line isolated from nonobese diabetic (NOD) mice, does not require cell contact, but it inhibits Tpath proliferation and prevents T1D in adoptive transfer experiments (9). The regulatory function of NR206, another p206-specific Treg line isolated from nonobese diabetes-resistant (NOR) mice, is dependent on both IFN- $\gamma$  production and cell contact with target cells (11). It is not known how these Tregs acquire or perform their regulatory function.

To identify factors that may be involved in modulating Treg function, we performed genome-wide comparative expression analyses of CD4<sup>+</sup> Tregs, Tpaths, and non-Tpaths and noted unique expression of killer cell Ig-like receptor (KIR) 3DL1 in Tregs. KIRs are transmembrane glycoproteins expressed by natural killer (NK) cells. KIR genes have been studied mainly in humans, where they are known to be involved in self-nonself recognition by binding to ligands on target cells (13–19). For some KIRs, the ligand is an HLA class I membrane protein. By recognition of HLA ligands on prospective target cells, KIRs play a critical role in regulating NK cell function (17, 18, 20, 21). One outcome is that NK cells are inactivated as a result of KIRs recognizing self-HLA ligands; thus, they will not attack target cells bearing self-HLA molecules. NK cells express a wide range of both activating and inhibitory KIRs, and the varied expression profile and balance of these receptors can dictate the NK cell function and activities (14, 17, 18). Inhibitory KIRs may play an important role in immune regulation by actively promoting peripheral tolerance, enhancing effector cell survival, or dampening immune responses (16, 20–22). On the other hand, activating KIRs are implicated in conditions including active host defense against infectious organisms (23–25). Normal T cells express very few or no KIRs, but KIR expression can be detected on a small subset of T cells in patients with autoimmune diseases, such as lupus and rheumatoid arthritis (22, 26–31). Based on these studies, it has been proposed that KIR expression may play a role in aberrant T-cell responses (13, 15, 22, 29, 30). Beyond this, little is known, and expression of KIRs in Tregs has not been studied.

Herein, we report results obtained from comparative gene array analyses and further functional studies of mouse KIR3DL1. We find that this KIR is expressed at relatively high levels in Tregs but not in Tpaths or non-Tpaths. Because KIR3DL1 is homologous to the human inhibitory KIR3DL1, we hypothesized that expression of KIR3DL1 in Tregs will inhibit their regulatory functions. To address this hypothesis, we used gene-specific shRNA to down-regulate KIR3DL1 expression in 2D2 cells, a Treg clone derived from N206 cells. Our studies demonstrate that KIR3DL1 expression indeed can negatively regulate their Treg function and suggest that KIR3DL1 is a previously undescribed important target for modulation of Treg function for treatment of autoimmune diseases like T1D.

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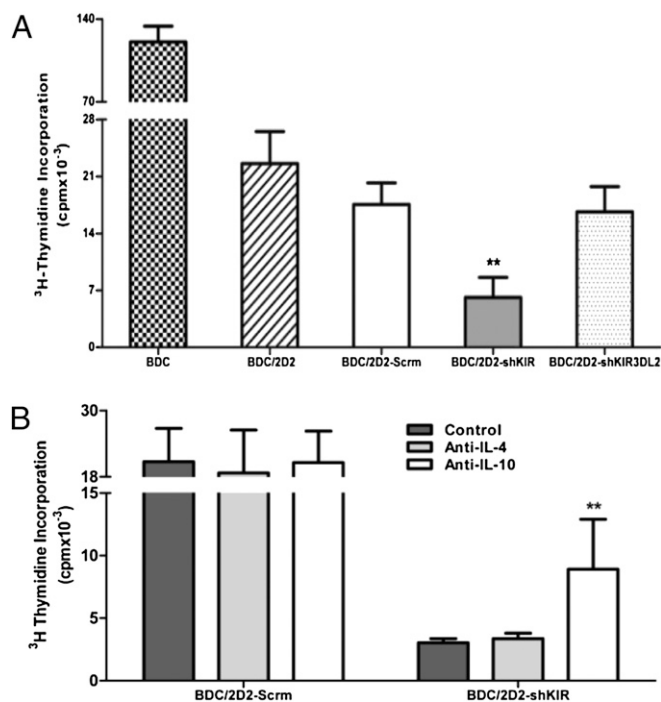
Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE26456).

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**Fig. 3.** Down-regulation of KIR3DL1 significantly improves the regulatory function of 2D2 Tregs in suppressing proliferation of Tpaths. (A) Suppression of BDC cell proliferation by Tregs in a mixed cell inhibition assay (*Materials and Methods*). BDC cells were cultured either alone or with various Tregs, including 2D2, 2D2-Scrm, 2D2-shKIR (harboring shRNA against KIR3DL1), and 2D2-shKIR3DL2 (harboring shRNA against KIR3DL2) cells. At least four measurements were made, and the SD is shown. (B) Regulatory function of 2D2-shKIR is partially dependent on IL-10 production. Inhibition assays were performed in which the cells were cultured either alone or with the presence of a saturating amount (24  $\mu$ g/mL) of either anti-IL-4 or anti-IL-10 antibodies. At least five measurements were made, and the SD is shown. \*\* $P < 0.01$ .

2D2-shKIR Tregs exert their enhanced regulatory function, at least in part, by increased secretion of IL-10.

**Down-Regulation of KIR3DL1 Significantly Increases the Ability of 2D2-shKIR Cells to Inhibit Spontaneous T1D in Both Early-Preonset and Late-Preonset Models of NOD Mice.** Does down-regulation of KIR3DL1 in 2D2-shKIR cells increase their ability to inhibit T1D in NOD mice? To address this question, we transferred 2D2-Scrm or 2D2-shKIR cells into 10-wk-old late prediabetic NOD mice. At this age, 2 wk before the onset of overt diabetes, it is known that destructive invasive insulinitis has developed for several weeks (usually starting from 3–4 wk of age). As shown in Fig. 5A, 2D2-shKIR Treg treatment delayed the onset of spontaneous T1D in the treated NOD mice by 10–11 wk compared with that in either untreated NOD mice or mice treated with 2D2-Scrm cells. Moreover, only 30% of 2D2-shKIR cell-treated NOD mice eventually developed T1D, compared with 75–80% of untreated or 2D2-Scrm cell-treated NOD mice. Although invasive insulinitis has already developed in 10-wk-old NOD mice, 2D2-shKIR cell treatment was still able to inhibit the progression of T1D significantly in these mice. We then performed studies to determine whether transfer of 2D2-shKIR Tregs to NOD mice at a younger age (6 wk) could prevent the initiation of T1D. Fig. 5B shows that none of the 6-wk-old NOD mice transferred with 2D2-shKIR cells developed diabetes at 30 wk of age (24 wk after cell transfer). In comparison, mice transferred with 2D2-Scrm cells developed diabetes at a rate comparable to that found in untreated NOD mice. Therefore, in young mice, 2D2-shKIR Tregs are very effective in preventing the development of T1D.

Because invasive insulinitis develops before T1D development, we histologically examined the insulinitis status of islets using pancreas sections obtained from 2D2-shKIR-treated or 2D2-Scrm-treated animals. Fig. 5C and D shows that islets in 2D2-shKIR-treated mice remained intact or not invasively infiltrated with leukocytes, whereas the islets of 2D2-Scrm-treated NOD mice were heavily infiltrated with leukocytes. Altogether, our data support the model that 2D2-shKIR Tregs are able to inhibit spontaneous T1D, at least partly, through the suppression of invasive insulinitis development.

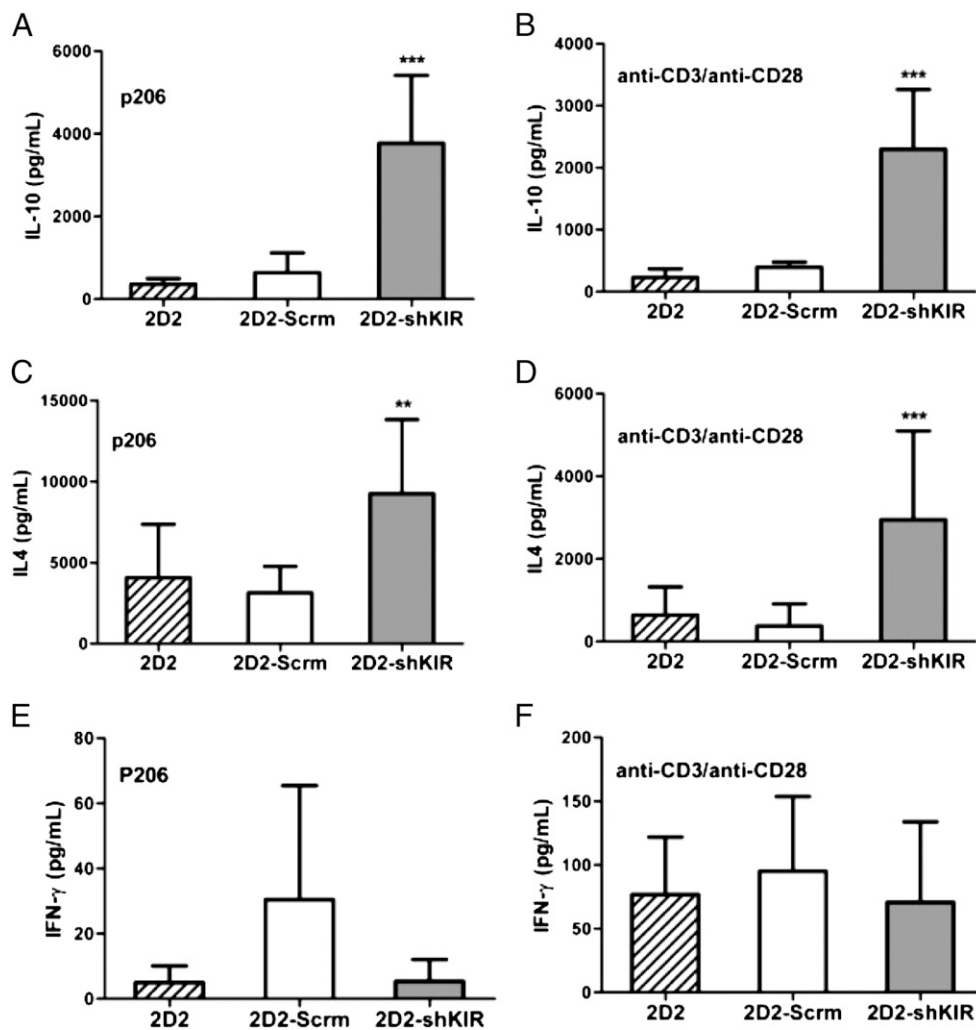
## Discussion

It is known that the regulatory function of autoantigen-specific CD4<sup>+</sup> Tregs can be dependent on cytokine production and, depending on the Treg line, may or may not require contact with target cells (1–4, 9–12, 32, 34). Beyond this, the molecular mechanisms that control their regulatory function are still largely elusive. We report here the identification of a gene that may play a key role in modulating the regulatory function of autoantigen-specific Tregs. We found that KIR3DL1 is expressed at a high level in two GAD peptide-specific Tregs but not in either pathogenic BDC cells or neutral nonpathogenic N79 cells. We then found that down-regulation of KIR3DL1 in the 2D2 Tregs, which did not affect their proliferation and survival in response to stimulation, dramatically enhanced their suppressive effect on the proliferation of target T cells in vitro and their ability to prevent spontaneous T1D in NOD mice. The enhanced Treg function is partly attributable to increased IL-10 production, because blockade of IL-10 partially abrogated the suppressive function of Tregs. Altogether, our results support the hypothesis that KIR3DL1 is a negative regulator of Treg function both in vitro and in vivo.

KIRs play a critical role in regulating NK cell function (13–19). T cells normally express very few or no KIRs, although KIR expression can be detected on a small subset of T cells in patients with autoimmune diseases, such as lupus and rheumatoid arthritis (22, 26–31). It is suspected that KIR expression on the subset of T cells in these conditions may play a role in regulating aberrant T-cell responses that are associated with the initiation and/or progression of the diseases (13, 15, 22, 29, 30). Beyond this, little is known about the role of KIRs in T cells, and expression of KIRs in Tregs has not been studied. Previous studies, mostly in humans, have suggested that expression of different sets of inhibitory or activating KIRs could contribute to the function of NK cells by modulating immune responses to target cells, including tumor cells and autoreactive pathogenic cells (20, 21, 35–38). Inhibitory KIRs have a long cytoplasmic domain containing immune receptor tyrosine-based inhibitory motifs (ITIMs) (13–19). Both mouse and human KIR3DL1 contains ITIMs and may function as inhibitory KIRs (13–19, 39). Therefore, a hypothesis consistent with our results is that expression of KIR3DL1 in Tregs serves as a functional brake to fine-tune their suppressive effect on immune responses. Without such a molecular brake, Tregs may overly inhibit immune responses. According to this model, expression of KIRs, such as KIR3DL1, in Tregs may play a key regulatory role in orchestrating immunity and autoimmunity.

Down-regulation of KIR3DL1 in 2D2 Tregs significantly increased both IL-10 and IL-4 production. Moreover, blockade of IL-10 but not IL-4 partially reversed the effect of KIR3DL1 down-regulation, suggesting that KIR3DL1 exerts its negative effect, in part, by controlling production of suppressive cytokines, such as IL-10. Previous studies on human KIRs have demonstrated that several KIRs are capable of mediating their function by recognizing HLA class I molecules as their ligands. For example, human KIR3DL1 recognizes HLA-B Bw4 (13–18). Based on the amino acid sequence homology and functional similarity between mouse and human KIR, it is likely but not yet experimentally shown that mouse KIR3DL1 also recognizes





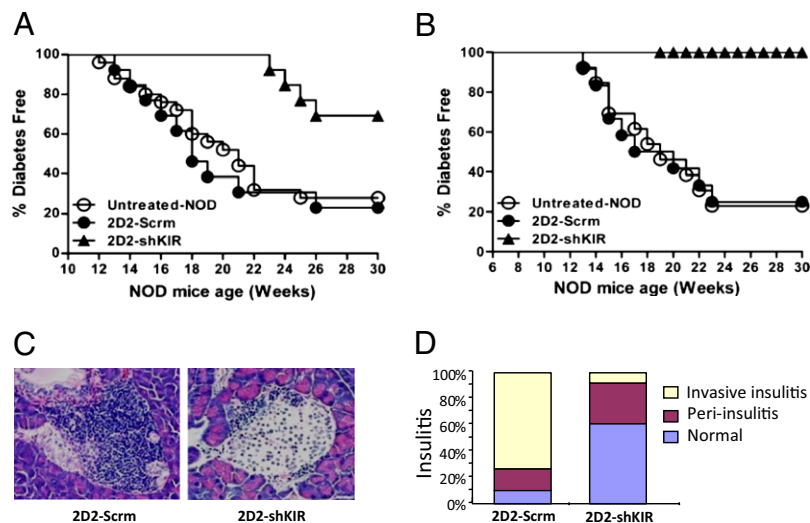
**Fig. 4.** Down-regulation of KIR3DL1 significantly increases IL-10 production. Tregs were activated with either p206 plus irradiated APCs (A, C, and E) or with anti-CD3/anti-CD28 antibodies (B, D, and F) for 24 h. Cell culture supernatant was harvested for ELISA to detect the presence of IL-10 (A and B), IL-4 (C and D), or IFN- $\gamma$  (E and F). At least three measurements were made, and the SD is shown. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

MHC class I molecules. One likely outcome following recognition of self-MHC molecules by KIRs is inactivation of NK cells so that they will not attack self but will only attack foreign cells not presenting the correct MHC or malignant host cells that have lost self-MHC expression. If the mouse KIR3DL1 also recognizes self-MHC class I molecules, it could inactivate or down-regulate Treg function. One possible biological consequence is self-control of the functional strength of Treg during the induction of immune tolerance to prevent autoimmune disease outcomes while ensuring effective immunity against infection or malignant cells. Considering the important role of KIR3DL1 in modulating Treg function and the potential of using it as a target to enhance Treg function in inducing more effective immune tolerance, it would be important to identify the ligands for mouse KIR3DL1 and whether MHC class I molecules can bind to KIR3DL1. Further studies would be necessary to examine how ligand-activated KIR3DL1 in 2D2 cells may modulate their function at both biochemical and molecular levels.

We found that adoptive transfer of a Treg with down-regulated KIR3DL1 into NOD mice dramatically decreases the incidence of T1D. Even after invasive insulinitis had already developed in 10-wk-old NOD mice, 2D2-shKIR cell treatment was still able to prevent T1D. Importantly, the protective effect against the disease was further improved when the mice were

treated at an early-preonset stage at 6 wk of age. In addition, our results from histological analyses of insulinitis development in NOD mice showed that the 2D2-shKIR cells were able to inhibit T1D, perhaps, at least partly, through the suppression of spontaneous invasive insulinitis development in treated NOD mice. Based on these results, it is likely that down-regulation of KIR3DL1 improves the ability of 2D2 Tregs to inhibit Tpath trafficking and infiltration into the islets.

In summary, we report that KIR3DL1 expression in Tregs can have a negative effect on their *in vitro* and *in vivo* function. Down-regulation of KIR3DL1 was able to enhance the regulatory function of Tregs and their ability to inhibit insulinitis and T1D. Our studies provide evidence that expression of a KIR, previously thought to function in NK cells, may play an important role in CD4<sup>+</sup> Tregs by regulating their function in modulating immune responses and inducing immune tolerance. This suggests that KIRs may be good molecular targets for improvement of Treg functional potency. A requirement, and challenge, for successful Treg-based cellular immunotherapy is to produce a sufficient number of Tregs for clinical use. By down-regulating KIR3DL1 expression in Tregs, significantly fewer cells may be needed for treatment of T1D or other autoimmune diseases and in preventing graft rejection and graft-vs.-host diseases.



**Fig. 5.** Down-regulation of KIR3DL1 significantly increases the ability of 2D2-shKIR cells to inhibit spontaneous T1D in both early-preonset and late-preonset models of NOD mice. NOD mice were i.v. injected with cells ( $1 \times 10^7$  cells per mouse) at either the late-preonset age (A; 10 wk) or the early-preonset age (B; 6 wk). The mice were monitored up to 30 wk of age. (C) Histological analyses of islets from NOD recipient mice. H&E staining of frozen pancreas sections obtained from 21-wk-old NOD mice treated with 2D2-Scrm cells or 2D2-shKIR cells. Images are representative of sections from at least three mice per group. (D) Cumulative percentage of different types of insulinitis detected in NOD mice treated with either 2D2-Scrm or 2D2-shKIR cells. At least 30 sections were examined.

## Materials and Methods

**Mice and Cells.** NOD mice were purchased from the Jackson Laboratory. BDC2.5 T-cell receptor (TCR) transgenic NOD (BDC) mice were a gift from D. Mathis and C. Benoist (Joslin Diabetes Center/Harvard Medical School, Boston, MA) (40). All animals were housed in a specific pathogen-free animal facility at The Beckman Research Institute of the City of Hope. About 75% of female NOD mice develop diabetes by the age of 6 mo. 2D2 cells are a Treg clone derived from the previously described N206 Treg line (9). C1i cells are a Treg clone derived from the NR206 Treg line (11). The previously described N79 cells, recognizing a mimotope p79 peptide, function neither as Tregs nor as Tpaths and are used as functionally neutral control cells (41).  $CD4^+$  BDC cells, isolated from the BDC mice, were cultured and activated three times by the p79 peptide in vitro and were used as the Tpaths in the gene array assays. These BDC cells did not express Foxp3 and were able to induce an aggressive form of T1D when transferred to NOD/SCID mice.

**Microarray Analysis.** For microarray analyses, total RNA was extracted from each cell line using an RNeasy Miniprep kit (Qiagen). The Affymetrix GeneChip Mouse Gene 1.0-ST array was used to determine gene expression profiles. Synthesis and labeling of cDNA targets as well as hybridization and scanning of GeneChips were carried out by the Microarray Core Facility at the City of Hope. Raw intensity measurements of all probe sets were background-corrected, normalized, and converted into expression measurements using the Affymetrix Expression Console v1.1.1. The Bioconductor "ArrayTools" package was then used to identify the genes differentially expressed between the 2D2, C1i, BDC, and N79 cells. The corresponding *P* values were adjusted using the false discovery rate method. Significant genes were selected with a cutoff of adjusted *P* < 0.05 and  $\log_2$  ratio of 1.7.

**Lentivirus Production and T-Cell Transduction.** Lentivirus-based vectors containing shRNA against the KIR3DL1 (target sequence CCTTCCTCTTGATTC-TACAA) or KIR3DL2 (target sequence CAATAAACAGACAGCTTCTTA) were purchased from Sigma-Aldrich. A scrambled shRNA was used as a control. VSV-G (G protein of vesicular stomatitis virus) pseudotyped lentiviral vectors were produced using a four-plasmid transfection system as described (42). Supernatant containing recombinant lentivirus was collected 3 d after transfection, filtered, and concentrated by ultracentrifugation. Viral pellets were resuspended in culture medium and frozen at  $-80^\circ\text{C}$  until use. After sequential concentration, lentiviral titers were determined using HT1080 cells and reached  $1.5\text{--}3 \times 10^8$  TU/mL. For KIR knockdown, 2D2 cells were infected overnight with recombinant lentivirus at multiplicity of infection of 10 in the presence of  $8 \mu\text{g/mL}$  polybrene and selected with puromycin ( $18 \mu\text{g/mL}$ ) for 28 d. Cells showing effective suppression of KIR3DL1 or KIR3DL2 expression were identified using real-time PCR. Separate transduction experiments were done to obtain at least two independent cell lines for each construct.

**Real-Time RT-PCR.** For KIR3DL1 gene expression analyses, total RNA was prepared using the RNeasy Miniprep kit (Qiagen) and converted to cDNA using SuperScript III First-strand Synthesis System (Invitrogen) according to the manufacturer's protocols. Real-time PCR was performed by SYBR Green Gene Expression Assays (Applied Biosystems). All reactions were performed in triplicate for 40 cycles. The PCR primers were as follows: KIR3DL1, 5'-ACA-CAGAAGCAGATACCAAAACAA-3' and 5'-GTCTTCAAGTCTGTGTG-3', and  $\beta$ -actin, 5'-GTATGGAATCCTGTGGCATCCATG-3' and 5'-GGACTCATCGTACTC-TGCTTGCT-3'. Equal amounts of RNA from cell samples were used as PCR templates to obtain the threshold cycle (Ct), and the Ct was normalized using the known Ct from  $\beta$ -actin RNAs. For RT-PCR analysis, KIR3DL1 transcripts were amplified using the following primers with an annealing temperature of  $56^\circ\text{C}$ : 5'-CACTCCCGGTCATTTTCATAATTTCCATGTACAC-3' and 5'-TATAC-TCGAGAGCCATGTGCTCTGGTTCCTC-3'.

**Cell Proliferation Assays.** The 2D2, 2D2-Scrm, or 2D2-shKIR cells ( $5 \times 10^4$  per well) were stimulated with soluble anti-mouse CD3/CD28 (Invitrogen) or GAD p206 peptide in the presence of antigen-presenting cells (APCs; irradiated NOD mouse splenocytes) in 96-well plates for 72 h. A total of  $0.5 \mu\text{Ci}$  of  $^3\text{H}$ -thymidine (Amersham Biosciences) was added per well during the last 24 h of culture. The cells were harvested onto glass fiber filter mats, and the incorporated radioactivity was measured.

**Analyses of Cell Death.** Cells ( $1 \times 10^5$  per well) were activated in 96-well plates coated with the anti-TCR Ab H57 ( $25 \mu\text{g/mL}$ ) plus soluble anti-CD28 ( $5 \mu\text{g/mL}$ ) for 24 h. The percentage of apoptotic cells was measured by Annexin V (BD PharMingen) staining. Specific cell death was calculated as follows: specific cell death = (% of activated cell death - % of nonactivated cell death) / (100 - % of nonactivated cell death)  $\times$  100.

**Analyses of Cytokine Production.** Cell culture supernatant was harvested after activating cells with anti-CD3/CD28 or GAD p206 and irradiated APCs for 24 h. Mouse IFN- $\gamma$ , IL-4, and IL-10 OptEIA ELISA kits (BD PharMingen) were used to measure the amount of cytokines according to the manufacturer's instruction.

**In Vitro Inhibition Assays.** These assays have been described (9–12).  $CD4^+$  T cells from BDC mouse splenocytes were purified by negative selection using magnetic beads (Miltenyi Biotec). The purified  $CD4^+$  BDC cells (the target cells) cultured in the absence of Tregs with or without stimulation with p79 peptide ( $0.1 \mu\text{g/mL}$ ) were used as controls. To measure inhibition, the  $CD4^+$  T cells were cultured with p79 ( $0.1 \mu\text{g/mL}$ ) plus irradiated ( $2,000 \text{ rad}$ ) 2D2, 2D2-Scrm, or 2D2-shKIR cells. Irradiated  $CD4^+$  T cell-depleted splenocytes were used as APCs. The cells were cultured in RPMI medium 1640 containing 5% FCS (vol/vol) for 4 d in 96-well plates and pulsed with  $0.5 \mu\text{Ci}$  of  $^3\text{H}$ -thymidine during the last 24 h of culture.

To determine the effect of different cytokines on target cell proliferation, anticytokine antibodies were added to the cell cultures of in vitro inhibition assays. In these cytokine blockade assays, a saturating amount of antibodies against IL-10, IL-4, or IFN- $\gamma$  (24  $\mu$ g/mL) was added to the culture and the cells were incubated for 4 d before being harvested for analyses.

**Adoptive Transfer and Histology.** For adoptive transfer experiments, either 6- or 10-wk-old female NOD mice received a single i.v. injection of  $1 \times 10^7$  cells of 2D2-Scrm or 2D2-shKIR Tregs. Recipient mice were monitored for up to 30 wk of age and were considered diabetic after 2 consecutive weeks of glycosuria  $\geq 2\%$  and a blood glucose level  $\geq 250$  mg/dL. At 21 wk of age, pancreases were removed from treated mice for histological analyses. Pancreatic tissues were fixed in 10% formalin/PBS (vol/vol), followed by 70–100% eth-

anol and xylene, and were then paraffin-embedded and sectioned. Sections were stained with H&E to detect islet-infiltrating leukocytes.

**Statistical Analysis.** Kaplan–Meier survival analysis was used to compare cumulative diabetes incidence. The Student's *t* test was used for data analyses of all other studies. Significance was set at  $P \leq 0.05$ .

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