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The activating receptor NKp46 is essential for the development of type 1 diabetes

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The mechanism of action of natural killer (NK) cells in type 1 diabetes is still unknown. Here we show that the activating receptor NKp46 recognizes mouse and human ligands on pancreatic beta cells. NK cells appeared in the pancreas when insulitis progressed to type 1 diabetes, and NKp46 engagement by beta cells led to degranulation of NK cells. NKp46-deficient mice had less development of type 1 diabetes induced by injection of a low dose of streptozotocin. Injection of soluble NKp46 proteins into nonobese diabetic mice during the early phase of insulitis and the prediabetic stage prevented the development of type 1 diabetes. Our findings demonstrate that NKp46 is essential for the development of type 1 diabetes and highlight potential new therapeutic modalities for this disease.

Type 1 diabetes mellitus is a multifactorial autoimmune disease in which insulin-producing beta cells in pancreatic islets are destroyed by autoreactive T cells. The most intensively studied model of autoimmune type 1 diabetes is the nonobese diabetic (NOD) mouse, which develops diabetes spontaneously after a variable period of insulitis, similar to human type 1 diabetes. Inflammation of pancreatic islets (insulitis) is observed in 4- to 5-week-old NOD mice, but diabetes does not develop until 10–20 weeks later, even with massive infiltration of the pancreatic islets by T cells[1,2]. An additional accepted model of experimental autoimmune diabetes in mice is the induction of diabetes by multiple injections of a low dose of streptozotocin (LDST)[3,4]. Streptozotocin causes diabetes by direct beta cell cytotoxicity as well as by initiation of T cell–mediated autoimmune attack of beta cells[4,5]. Indeed, adoptive transfer of activated splenocytes from LDST-treated mice induces diabetes in untreated healthy mice[5].

Although type 1 diabetes is considered a T cell–mediated disease, several studies have proposed a role for the innate immune system in the pathogenesis of this disease. Some data indicate an essential contribution of macrophages to the onset of type 1 diabetes[6], and other researchers have found that natural killer (NK) cells infiltrate the islets of NOD mice[7]. Islet inflammation mediated mainly by NK cells has also been reported in human type 1 diabetes[8], and alterations in the NK cell compartments of patients with type 1 diabetes at the onset of the disease or after long-term hyperglycemia have been observed[9]. The proportion of NK cells, their numbers and the timing of their entry into the pancreas correlate with the severity of type 1 diabetes in transgenic NOD mice[10]. In addition, depletion of NK cells in transgenic NOD mouse models of accelerated type 1 diabetes substantially inhibits diabetes development[10,11]. However, the molecular mechanisms of the involvement of NK cells in type 1 diabetes are still unknown.

NK cells have been detected in target organs of patients suffering from autoimmune diseases[12] and are able to attack autologous cells[13–15]. NK cells recognize target cells via a diverse array of activating and inhibitory receptors, and a delicate balance between inhibitory and activating signals tightly regulates NK cell activation[16]. Activating receptors, including the natural cytotoxicity receptors (NCRs) and NKG2D, promote NK cell activation[16]. The NCRs, which include NKp30, NKp44 and NKp46, are expressed almost exclusively on NK cells, whereas NKG2D is also expressed on other lymphocytes, such as $CD8^+$ T cells[16,17]. NKp46 is considered the most specific NK cell marker for which an orthologous protein (NCR1) has been found in mice[18,19]. Indeed, even cells that are not cytotoxic and secrete interleukin 22 (IL-22) are considered an NK cell subset because of the presence of NKp46 (ref. 20).

Whether NK cell activating receptors are involved in type 1 diabetes is still an open question. Although blockade of NKG2D has been shown to prevent diabetes development in NOD mice[21], this effect was attributed to $CD8^+$ T cells and the exact mechanism of NKG2D action is still controversial[17,21,22]. NKp46, in contrast, is important in the defense against pathogens[18,23]; however, the role of NKp46 and NCR1 in autoimmune diseases and the cellular ligands recognized by this receptor are still unknown.

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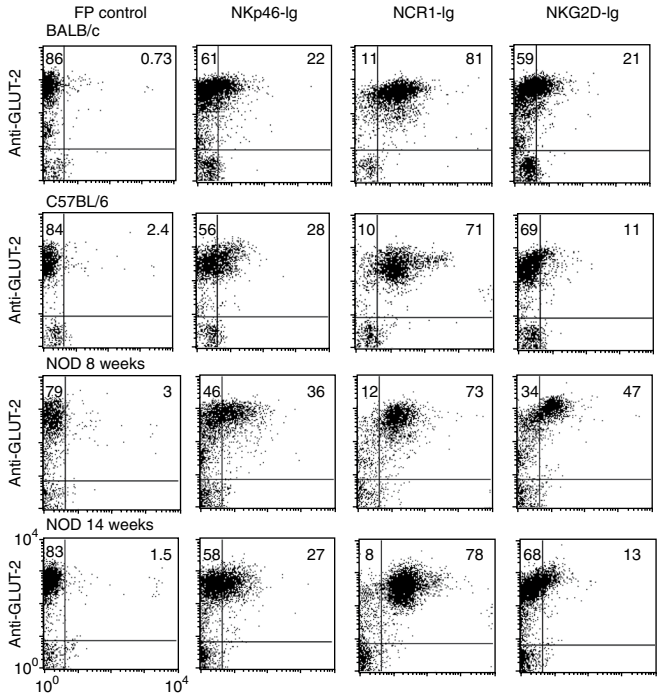

Figure 1 NKp46 recognizes a ligand (or ligands) on beta cells. Identification of beta cells isolated from BALB/c mice, C57BL/6 mice and prediabetic female NOD mice at 8 or 14 weeks of age (n = 5–7 mice), stained with CEA-Ig (fusion protein (FP) control), NKp46-Ig, NCR1-Ig or NKG2D-Ig and identified by staining with anti-GLUT-2. Numbers in quadrants indicate percent positive cells in each. Data are representative of three independent experiments (summary of all experiments, **Supplementary Fig. 1**).

Here we demonstrate that NKp46 and NCR1 specifically recognize human and mouse pancreatic beta cells and salivary glands and that NK cells degranulate after interaction with mouse beta cells in an NKp46-dependent manner. Diabetes development was impaired in the absence of NKp46, and we observed the highest percentage of NK cells in the pancreas at the time when insulitis develops into diabetes (the prediabetic stage). Injection of soluble NKp46 variants into female NOD mice both in the early stage of insulitis and in the late prediabetic stage prevented the development of diabetes almost entirely. Thus, our results demonstrate that NKp46 has a critical role in diabetes progression.

RESULTS

Pancreatic beta cells express NKp46 ligand(s)

As NCR1 is the only NCR expressed in mice, we investigated its involvement in mouse models of type 1 diabetes. Because the cellular ligands for NCR1 and NKp46 are still unknown, we tested whether NKp46 and NCR1 would recognize ligand(s) on pancreatic beta cells by using fusion proteins of NKp46 and NCR1 with immunoglobulin (NKp46-Ig and NCR1-Ig, respectively)[18,24]. As negative controls, we used a truncated extracellular portion of NKp46 lacking the ligand-binding domain (NKp46D1-Ig)[25] and another irrelevant fusion protein containing the protein carcinoembryonic antigen (CEA-Ig). As a positive control, we used the fusion protein NKG2D-Ig. We isolated beta cells from female BALB/c, C57BL/6 and NOD mice and double-stained the cells with mouse antibody to GLUT-2 (anti–GLUT-2; this specifically marks beta cells) and the various immunoglobulin fusion proteins. Beta cells derived from all mouse strains tested were recognized by the mouse NCR1-Ig and NKp46-Ig fusion proteins and, as expected because of species specificity, the most efficient binding was obtained with mouse NCR1-Ig (**Fig. 1** and **Supplementary Fig. 1**). The staining of beta cells with NKp46-Ig and NCR1-Ig was specific, as we found no staining with the control fusion protein CEA-Ig (**Fig. 1**) or NKp46D1-Ig (fusion protein lacking the NKp46 binding domain; data not shown). In agreement with published results demonstrating that NKG2D on $CD8^+$ T cells is involved in beta-cell recognition[17,21], beta cells were recognized by NKG2D-Ig. However, not all beta cells expressed NKG2D ligands and the expression of the NKG2D ligands decreased substantially during the progression of diabetes (**Fig. 1** and **Supplementary Fig. 1**). Notably, most of the beta cells derived from all mice strains were recognized by NCR1-Ig, and expression of the NCR1 ligand remained constant during diabetes development (**Fig. 1**). Indeed, we found four subpopulations of beta cells expressing different amounts of GLUT-2 and NCR1 ligands (**Supplementary Fig. 1**).

To further confirm that NKp46 recognizes specific beta-cell ligands, we used a published reporter assay of BW mouse thymoma cells[24]. In this system, the extracellular portion of NKp46 is fused to the transmembrane and tail domains of mouse CD3ζ (NKp46-CD3ζ; **Supplementary Fig. 2a**) and thus ligand recognition leads to secretion of mouse IL-2. We found substantial secretion of IL-2 in NKp46-CD3ζ–transfected BW cells incubated with beta cells derived from all mouse strains (**Supplementary Fig. 2b**). Small amounts of IL-2 were produced by parental BW cells incubated with beta cells and by NKp46-CD3ζ–transfected BW cells incubated with HeLa human cervix carcinoma cells (**Supplementary Fig. 2b**), pancreatic exocrine tissue–derived cells or peripheral blood lymphocytes (data not shown).

To demonstrate that ligands for NKp46 also exist on human beta cells and that specific staining can be observed in the endocrine tissue of the whole pancreas, we used immunohistochemical staining. We found intense NKp46-Ig staining of beta cells in islets of pancreatic tissues derived from humans, female NOD mice and BALB/c mice (**Fig. 2a**). In female NOD mice, we found insulitis manifested by mononuclear cell infiltration, and the residual pancreatic islet cells were stained by NKp46-Ig.

To investigate the importance of the NKp46 ligand in the pathogenesis of type 1 diabetes, we did immunohistochemical staining of NOD islets before and after the development of insulitis, including the embryonic period. We did not detect NKp46 ligands in islets at embryonic day 20 (E20; **Fig. 2b**). However, NKp46 ligands gradually appeared during the postnatal stage and were present before the development of insulitis (week 2) and throughout the progression of diabetes (weeks 6 and 14; **Fig. 2b**). Insulitis in the type 1 diabetes of NOD mice is accompanied by autoimmune sialitis, and NKp46 ligands were also expressed in salivary glands of female NOD and BALB/c mice (**Fig. 2c**). We found mononuclear cell infiltration of salivary glands in NOD mice (**Fig. 2c**). No other tissues examined in female NOD and BALB/c mice were recognized by NKp46-Ig (**Fig. 2d**).

To support the observation that the ligand for NKp46 in the pancreatic tissue is specifically expressed on insulin-producing beta cells and not on other islet cells (for example, glucagon- or somatostatin-producing cells), we did double- and triple-immunofluorescence staining of pancreatic tissue derived from BALB/c mice (**Fig. 2e**), NOD mice (**Fig. 2f**) and humans (**Fig. 2g**). We detected substantial overlap of staining with NKp46-Ig and anti-insulin, which indicated that NKp46 uniquely stains beta cells. We found no staining with the control fusion protein NKp46D1-Ig (**Fig. 2e–g**) or CEA-Ig (data not shown), and there was no overlap for staining with anti-somatostatin

or anti-glucagon and NKp46-Ig (**Fig. 2h**). Other normal tissues examined were not recognized by NKp46-Ig (**Fig. 2i**). Together, the four different methods indicate that a specific ligand for NKp46 is expressed on beta cells in human and mice.

Beta cells induce NKp46-dependent degranulation

The two main functions of NK cells are cytotoxicity and the secretion of cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor. Our next aim, therefore, was to determine whether NKp46 signaling was activated by beta cells. To measure the induction of cytokine secretion, we used NK cells from *Ncr1*$^{gfp/gfp}$ and *Ncr1*$^{+/gfp}$ mice[18]. In these mice, a reporter gene encoding green fluorescent protein (GFP) is inserted into the *Ncr1* locus and thus *Ncr1* is knocked out and all NK cells are green; *Ncr1*$^{+/gfp}$ mice have NK cell function similar to that of wild-type mice[18]. To assay cytokine secretion from NK cells, we isolated GFP-expressing NK cells from the splenocytes of *Ncr1*$^{+/gfp}$ and *Ncr1*$^{gfp/gfp}$ mice and incubated them together with beta cells derived from NOD, BALB/c and C57BL/6 mice. Neither IFN-γ (**Fig. 3a**) nor tumor necrosis factor (data not shown) was secreted regardless of whether NK cells expressed NCR1 or not. However, *Ncr1*$^{gfp/gfp}$ NK cells secreted less IFN-γ than did *Ncr1*$^{+/gfp}$ NK cells when incubated together with a mouse rhabdomyosarcoma cell line. Thus, we concluded that the interaction of NKp46 with its ligand on beta cells does not lead to cytokine secretion.

Next, we measured NK degranulation[26,27] (which indicates mobilization of CD107a to the cell surface), rather than direct cytotoxicity, because mouse beta cells do not proliferate much at all and thus it is almost impossible to label them with radioactive isotopes. We incubated NK cells from the spleens of *Ncr1*$^{+/gfp}$ and *Ncr1*$^{gfp/gfp}$ mice (**Supplementary Fig. 3a**) with beta cells derived from BALB/c and NOD mice. We found significantly less degranulation in the *Ncr1*$^{gfp/gfp}$ cells incubated with beta cells derived from each mouse strain (**Fig. 3b** and **Supplementary Fig. 3b**). In contrast, *Ncr1*$^{+/gfp}$ and *Ncr1*$^{gfp/gfp}$ NK cells showed similar degranulation in response to mouse YAC-1 lymphoma cells (which are killed in an NCR1-independent manner[18]; **Fig. 3b** and **Supplementary Fig. 3b**). We found minimal NK cell degranulation in response to the negative control human HeLa cells.

Finally, we examined the *in vivo* degranulation state of pathogenic pancreatic NK cells, which are present in the islets of NOD mice

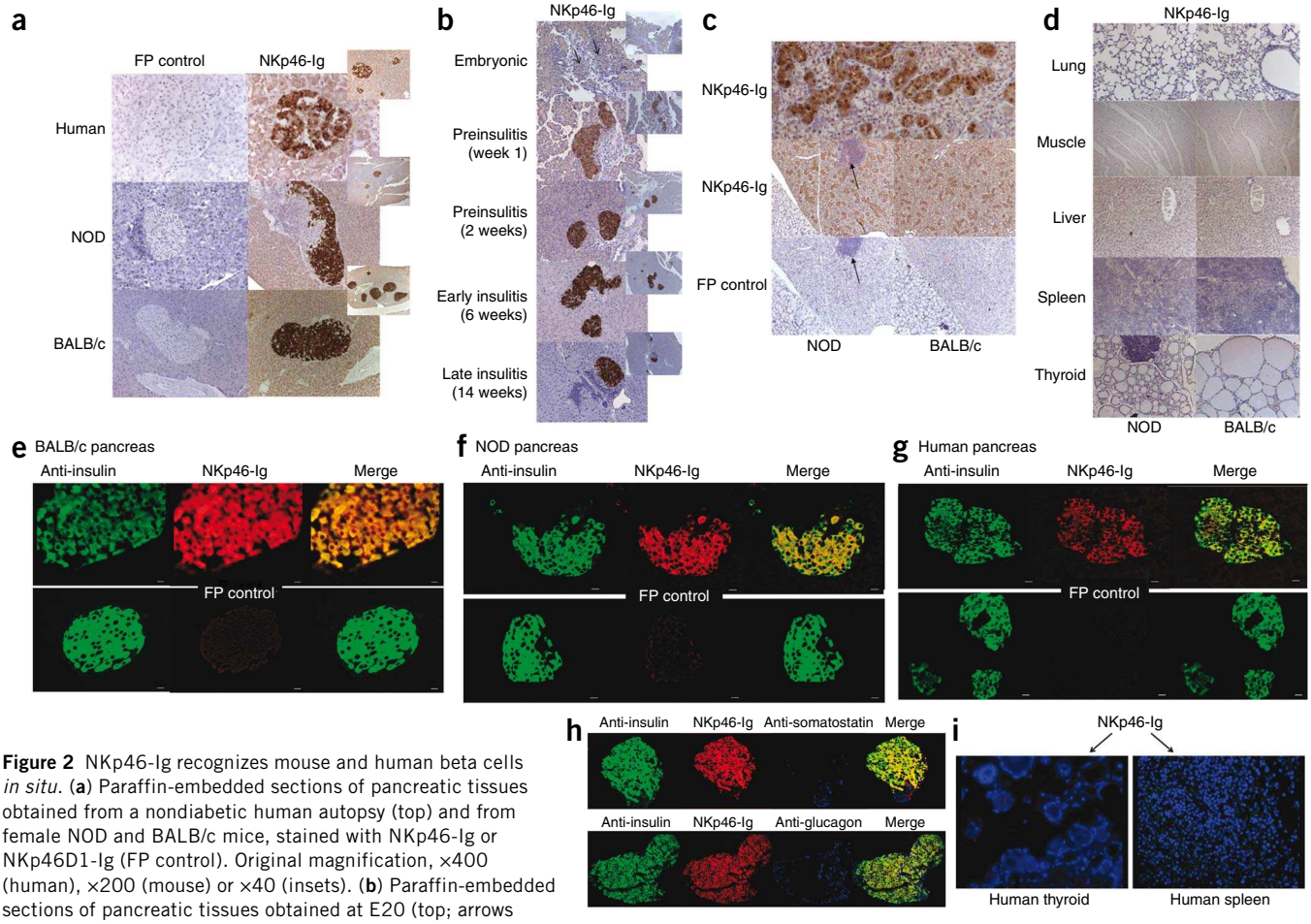

Figure 2 NKp46-Ig recognizes mouse and human beta cells *in situ*. (**a**) Paraffin-embedded sections of pancreatic tissues obtained from a nondiabetic human autopsy (top) and from female NOD and BALB/c mice, stained with NKp46-Ig or NKp46D1-Ig (FP control). Original magnification, ×400 (human), ×200 (mouse) or ×40 (insets). (**b**) Paraffin-embedded sections of pancreatic tissues obtained at E20 (top; arrows indicate islets) and from female NOD mice during the course of diabetes development. Original magnification, ×100 (main images) or ×40 (insets). (**c**) Salivary glands derived from female NOD and BALB/c mice and stained with NKp46-Ig or NKp46D1-Ig (FP control). Original magnification, ×400 (top) or × 40 (middle and bottom). (**d**) Tissues derived from female NOD and BALB/c mice and stained with NKp46-Ig. Original magnification, ×100 (lung, liver and thyroid) or ×40 (muscle and spleen). (**e**–**g**) Paraffin-embedded sections of pancreatic tissues derived from BALB/c mice (**e**), female NOD mice (**f**) and a nondiabetic human autopsy (**g**) and incubated with anti-insulin (green) and NKp46-Ig or NKp46D1-Ig (FP control; red). Scale bars, 10 μm. (**h**) Paraffin-embedded sections of pancreatic tissue derived from BALB/c mice and incubated with anti-insulin (green), NKp46-Ig (red) and anti-somatostatin or anti-glucagon (blue). (**i**) Thyroid and spleen samples from a nondiabetic human autopsy, stained with NKp46-Ig. Blue, nuclear staining with the DNA-intercalating dye DAPI (4,6-diamidino-2-phenylindole). Results are representative of six (**a**), four (**b**,**e**–**g**), three (**c**,**d**,**i**) or two (**h**) independent experiments.

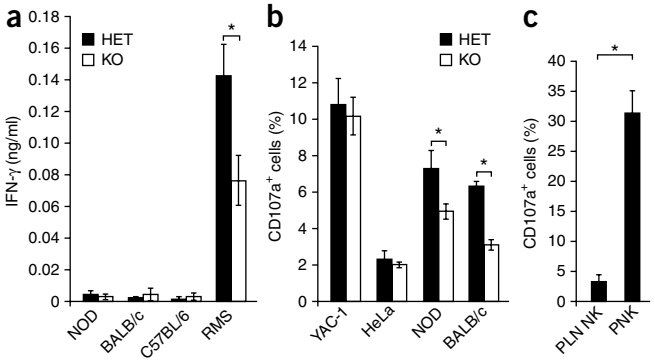

Figure 3 NKp46-mediated killing of beta cells. (**a**) Enzyme-linked immunosorbent assay of IFN-γ production by purified NK cells from *Ncr1*$^{gfp/gfp}$ mice (KO) or *Ncr1*$^{+/gfp}$ mice (HET) activated with polyinosinic-polycytidylic acid and incubated for 48 h with beta cells derived from C57BL/6, BALB/c or prediabetic female NOD mice. RMS, methylcholanthrene-induced rhabdomyosarcoma cell line (positive control). *$P < 0.05$ (Student's *t*-test). Data are representative of two independent experiments. (**b**) Flow cytometry analysis of splenic NK cells cultured together with beta cells from NOD or BALB/c mice or with YAC-1 or HeLa cells (effector/target ratio, 1:1), then stained with allophycocyanin-conjugated anti-CD107a, gated on NK cells (GFP$^+$CD3$^-$). CD107a$^+$ cells are presented as percent of total NK cells. *$P < 0.05$ (Student's *t*-test). Data are representative of three independent experiments. (**c**) NK cells obtained from pancreatic islets (PNK) or the pancreatic lymph nodes (PLN NK) of 12-week-old prediabetic female NOD mice ($n = 6–7$) and stained with allophycocyanin-conjugated anti-CD107a; plots are gated on NCR1$^+$ cells. CD107a$^+$ cells are presented as percent of total NK cells. *$P < 0.0015$ (Student's *t*-test). Data are representative of two independent experiments.

during diabetes development (discussed below). We isolated NK cells from the pancreatic lymph nodes and beta cell islets of prediabetic female NOD mice (12 weeks old) and stained them for CD107a expression. We detected substantial derganulation of the pathogenic pancreatic islets NK cells but little or no degranulation of pancreatic lymph node NK cells (**Fig. 3c**). In agreement with the results reported above, we found no IFN-γ secretion from the pathogenic pancreatic islet NK cells (data not shown). Thus, the pathogenic NK cells present *in vivo* in the pancreatic islets had degranulated.

Impaired diabetes development in the absence of NCR1

Our next goal was to test the function of NKp46 in the development of type 1 diabetes *in vivo*. We injected sex- and age-matched *Ncr1*$^{gfp/gfp}$ and wild-type mice intraperitoneally for 5 consecutive days with streptozotocin and measured blood glucose concentrations from day 7 up to 45 d after injection. In the absence of NCR1, diabetes development was significantly impaired (**Fig. 4a**). Hyperglycemia, defined as a non-fasting blood glucose concentration of >250 mg/dl in two sequential measurements, was less severe in the *Ncr1*$^{gfp/gfp}$ diabetic mice (**Fig. 4b**). NCR1-deficient mice also showed less-severe insulitis, as determined by a published pathological insulitis scale[28] (**Supplementary Fig. 4**). These results indicate that NKp46 is important for diabetes development and islet destruction in the LDST model.

Appearance of NK cells in the pancreas during type 1 diabetes

As the unknown ligand for NKp46 is expressed on normal beta cells, why does diabetes not develop in every individual? Our hypothesis was that NK cells and T cells that are normally not found in the pancreas appear in this organ after diabetes development. To test our hypothesis, we monitored the appearance of NK cells in the pancreatic tissues in two mouse models of type 1 diabetes.

We tracked NK cells in the pancreas of NOD mice during the embryonic period (E20), preinsulitis (3–4 weeks of age), early insulitis (6–8 weeks of age), prediabetes (12–14 weeks of age; mice with normal fasting glucose concentrations but a pathological intraperitoneal glucose tolerance test) and late overt diabetes (2–3 weeks after the diagnosis of diabetes). The highest percentage (around 4%) of NK cells expressing NCR1 in the pancreas was in the prediabetic stage (**Fig. 5**). We also monitored NK cell appearance (by GFP expression) in the pancreatic tissues of LDST-injected *Ncr1*$^{gfp/gfp}$ mice during the embryonic period (E20), preinsulitis (day 0; the day of LDST injection), early insulitis (day 7 after LDST injection), prediabetes (day 9 after LDST injection) and late overt diabetes (day 45 after LDST injection). We found the most NK cells in the pancreas in the prediabetic stage, on day 9 after the injection, the same day at which the transition from insulitis to diabetes usually starts in this model (**Figs. 4b** and **5**). The few NK cells observed in the pancreas during the embryonic period and the preinsulitis stage in both models were probably contaminating lymphocytes. By monitoring CD3 expression, we found that like NK cells, T cells were normally not found in the pancreas and that they appeared in the pancreas concomitantly with NK cells (data not shown). Thus, in normal conditions, despite the fact that pancreatic beta cells of both human and mice express ligands for NKp46, diabetes does not develop, probably because NK cells and T cells are absent from the pancreas.

NKp46 proteins prevent type 1 diabetes when injected early

Our next aim was to demonstrate that NKp46 is indeed involved in diabetes development in the NOD mouse model and, in parallel, to develop a new therapeutic tool for the treatment of type 1 diabetes. Treatment of NOD mice with nondepleting NKG2D-specific monoclonal antibodies attenuates the development of diabetes by impairing the function and migration of autoreactive CD8$^+$ T cells[21]. Unfortunately, however, no blocking, nondepleting antibody directed against mouse NKp46 is available. However, injection of the NKp46-Ig fusion protein results in the production of NKp46-specific antibodies that block the killing of virus-infected cells[24]. Therefore, we decided to induce blocking NKp46-specific antibodies in NOD mice by repeated injection of the NKp46-Ig fusion protein. We used NKp46-Ig and NCR1-Ig and, to prevent nonspecific binding, we also used an additional version of the mouse NCR1 receptor that lacks the complement- and Fc-binding sites (NCR1-t).

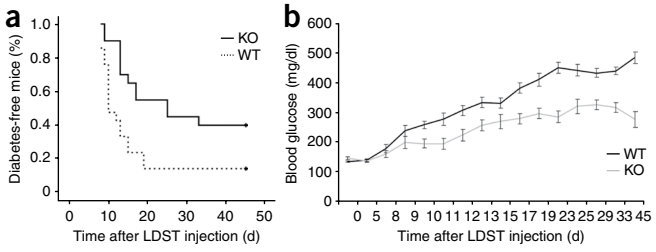

Figure 4 Impaired diabetes development in the absence of NKp46. (**a**) Kaplan-Meier analysis of the development of diabetes in *Ncr1*$^{gfp/gfp}$ mice (KO) and *Ncr1*$^{+/+}$ mice (WT) after streptozotocin injection (LDST). $P < 0.010$ (log-rank test). Data are representative of three independent experiments. (**b**) Blood glucose concentrations up to 45 d after the first streptozotocin injection. Data are representative of three independent experiments (error bars, s.e.m.).

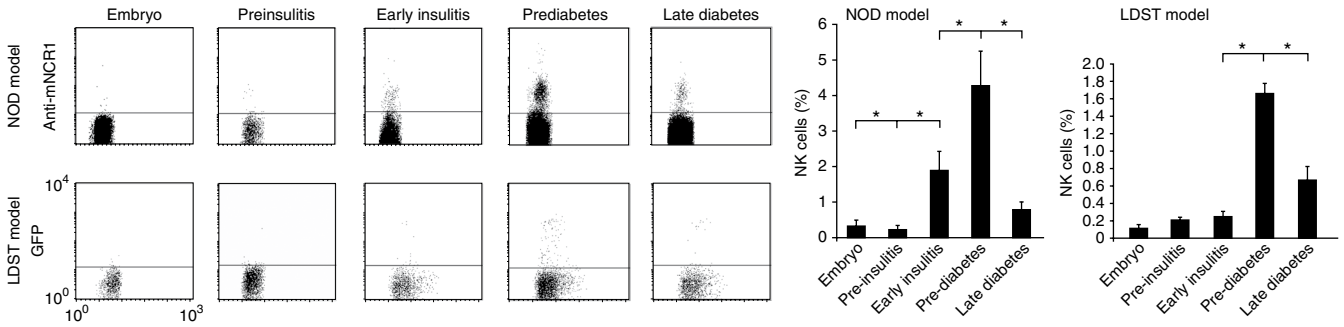

Figure 5 Appearance of NK cells in the pancreas during the development of diabetes. NK cells in the pancreas of female NOD mice (NOD model) or *Ncr1*$^{gfp/gfp}$ mice injected with streptozotocin (LDST model), identified by staining with anti–mouse NCR1 (NOD model) or as GFP$^+$ cells (LDST model). Cells were stained during several stages of insulitis and diabetes development; for each stage, lymphocytes were purified from two to three pancreatic tissues derived from female NOD mice and *Ncr1*$^{gfp/gfp}$ mice, except at the embryonic and the preinsulitis stages, for which eight to ten pancreatic tissues were used. $*P < 0.05$ (Student's *t*-test). Data are from three independent experiments (mean and s.d.).

We injected female NOD mice intraperitoneally with the various fusion proteins (at a dose of 0.005 g per kg body weight) or PBS twice a week, starting from 6 weeks of age. Mice treated with PBS alone began to develop diabetes at 10 weeks of age, and 67% were diabetic by 24 weeks, as expected in this model (**Fig. 6a**). At this point, we stopped the immunoglobulin treatment. We did not find diabetes in any NOD mice treated with the fusion proteins during the first 19 weeks of treatment (**Fig. 6a**). Most of the fusion protein–treated NOD mice remained vital (**Supplementary Movies 1** and **2**) and disease-free up to 36 weeks of age. In contrast, 89% of the PBS-treated mice became diabetic, and most of them died before 33 weeks of age (**Fig. 6a**). Hematoxylin and eosin staining of pancreatic tissue derived from all fusion protein–treated mice showed many residual islets with usually only mild insulitis, a situation similar to that of pancreatic tissue derived from healthy, 30-week-old, untreated nondiabetic female NOD mice (**Fig. 6b**). In contrast, we detected no pancreatic islets in mice injected with PBS (**Fig. 6b**). We noted no side effects in the treated mice either by gross examination or by histological analysis (**Fig. 6c**).

To investigate the mechanism responsible for the protective effect mediated by the NKp46 fusion proteins, we collected serum from the various mouse groups during the course of the experiment. As predicted, injection of the various NKp46-Ig variants resulted in the generation of specific antibodies directed against NKp46 and NCR1, but injection of PBS did not (**Fig. 7a**). The NKp46- and NCR1-specific antibodies were mostly of the IgM isotype, were present in the serum starting at 2 and 4 weeks, respectively, after injection of the soluble fusion protein, and remained in the serum for up to 36 weeks of age (end of experiment; data not shown).

To gain insight into the mechanism by which the NKp46 fusion proteins inhibited diabetes development, we first excluded the possibility that these fusion proteins acted by depleting NK cells or by suppressing the appearance of NK cells in the pancreas (data not shown). That finding is in contrast to the observation of lower CD8$^+$ T cell percentages in the healthy pancreas of mice treated with an NKG2D-specific antibody[21]. In addition, beta cells derived from NOD mice treated with PBS or NKp46 fusion proteins expressed similar amounts of the unknown NKp46 ligand (data not shown). We also ruled out the possibility of potential involvement of the Fc portion because NCR1-t (the truncated version of the fusion protein lacking the complement- and Fc-binding domain) was almost as effective as the other fusion proteins in suppressing diabetes (**Fig. 6a**).

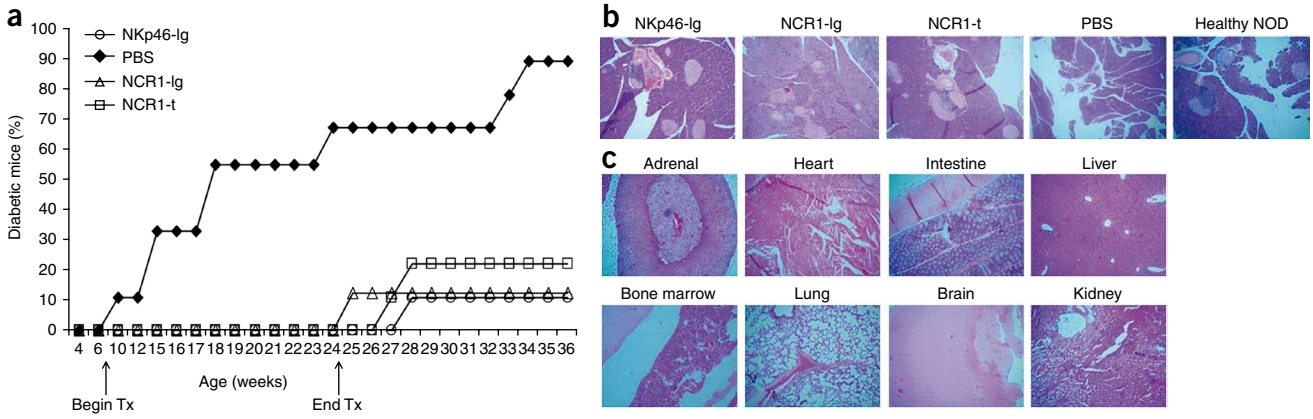

Figure 6 Treatment with NKp46-Ig fusion proteins prevents the development of diabetes in NOD mice. (**a**) Development of diabetes (blood glucose concentration >250 mg/dl in two consecutive measurements) in female NOD mice treated with NKp46-Ig, NCR1-Ig or NCR1-t (0.005 g per kg body weight, injected intraperitoneally twice weekly) or PBS starting from 6 weeks of age and continuing up to 24 weeks of age (treatment (Tx), upward arrows). $P < 0.0002$, fusion protein versus PBS (Kaplan-Meyer analysis, log-rank test). Data are representative of two independent experiments with eight to nine mice per group. (**b**) Hematoxylin and eosin staining of pancreatic tissues derived from the treated groups in **a** (36 weeks of age) and from an untreated 30-week-old nondiabetic female NOD mouse (far right). Original magnification, ×40. Data are representative of two experiments with eight to nine mice. (**c**) Hematoxylin and eosin staining of various tissues derived from NKp46-Ig-treated mice. Original magnification, ×40. Data are representative of two experiments with eight to nine mice.

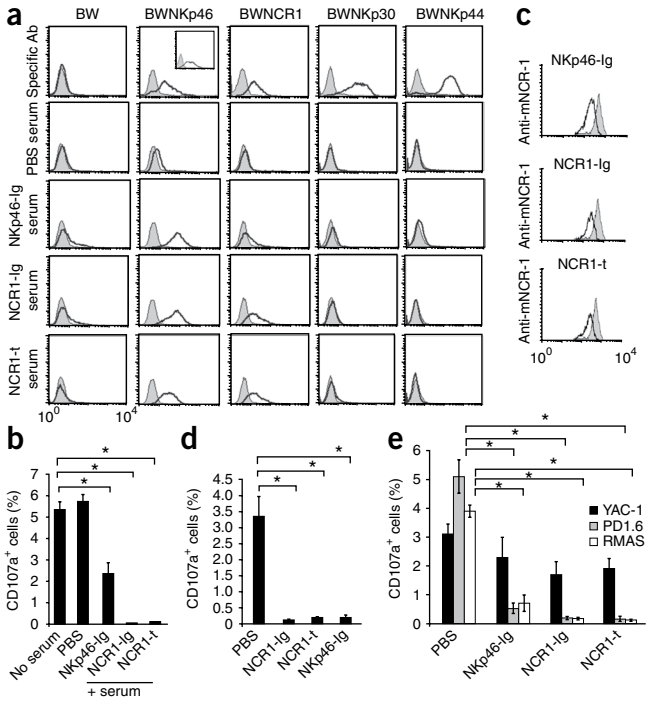

Figure 7 Impaired NKp46 function. (**a**) Flow cytometry of BW transfectants stained with specific antibodies (Ab; top row) or with serum derived from 30-week-old treated mice. Black lines, specific staining; gray histograms, secondary antibody staining. Inset (top row), staining of BWNKp46 cells with anti-NCR1. Data are representative of three independent experiments. (**b**) CD107a expression on NK cells isolated from NOD splenocytes preincubated with serum (horizontal axis) and then incubated with beta cells at an effector/target ratio of 1:1. NK cells were identified by NCR1 expression. $CD107a^+$ cells are presented as percent of total NK cells. $^*P < 0.05$ (Student's *t*-test). Data are representative of two independent experiments (error bars, s.d.). (**c**) Flow cytometry of NK cells derived from splenocytes of fusion protein–treated mice (black lines) or PBS-treated mice (gray filled histograms) at 36 weeks of age and stained with anti-NCR1. Data are representative of two independent experiments. (**d**,**e**) $CD107a^+$ cells among NK cells derived from the splenocytes of 36-week-old PBS- or fusion protein–treated treated mice, incubated with beta cells (**d**) or other target cells (**e**) at an effector/target ratio of 1:1 and stained with allophycocyanin-conjugated anti-CD107a; plots are gated on $NCR1^+$ cells. $CD107a^+$ cells are presented as percent of total NK cells. $^*P < 0.001$ (Student's *t*-test). Data are representative of two independent experiments (error bars, s.d.).

We also determined whether the NKp46-specific antibodies generated after injection of fusion proteins could block the degranulation of NK cells. For this, we cultured NK cells derived from the spleens of 8-week-old nondiabetic female NOD mice with beta cells obtained from prediabetic female NOD mice and did the CD107a-mobilization assay. Degranulation of NK cells was significantly lower after preincubation with serum derived from mice treated with the NKp46 fusion proteins, but normal serum or serum derived from PBS-treated mice did not alter NK cell degranulation (**Fig. 7b**).

As injection of anti-NKG2D, or a soluble NKG2D ligand, induces internalization of NKG2D[17,29], we next determined whether NCR1 was also downregulated because of the generation of NKp46-specific antibodies in the fusion protein–treated mice. We noted lower expression of NCR1 on splenic NK cells derived from mice treated with the fusion proteins than on those from the PBS-treated group (**Fig. 7c**). The NCR1 reduction was systemic, as we found similarly lower expression on NK cells derived from the blood (discussed below) or the pancreas (data not shown). Staining with anti–mouse IgG was similar on NK cells from PBS- and fusion protein–treated mice (data not shown), which indicated that the NK cells were not simply coated with the mouse NKp46–specific antibodies.

The NCR1 downregulation affected the degranulation of NK cells, as shown by CD107a staining of NK cells isolated from splenocytes of 36-week-old mice (12 weeks after the end of treatment with PBS or fusion proteins) and incubated with beta cells *in vitro*. PBS-treated mice were already diabetic and had a low but still significant percentage of $CD107a^+$ NK cells (**Fig. 7d**). In contrast, NK cells derived from healthy mice treated with NKp46 fusion proteins failed to express CD107a after interaction with beta cells (**Fig. 7d**). The relatively low function of NK cells derived from the PBS-treated group compared with that of cells from the healthy control 8-week-old mice (**Fig. 7b**) could have been due to the effect of hyperglycemia.

Finally, to demonstrate that the impairment in NK cell activity was specific to NKp46, we measured the degranulation ability of NK cells derived from PBS- or fusion protein–treated mice after incubation with NKp46-dependent target cells (such as PD1.6 and RMAS cells[18,30]) or NKp46-independent, NKG2D-dependent target cells (such as YAC-1 cells[18]). All target cells whose killing was NKp46 dependent induced minimal CD107a expression on NK cells derived from mice treated with the various fusion proteins but induced substantial CD107a expression on NK cells derived from PBS-treated mice (**Fig. 7e**). In contrast, NK cells derived from all groups expressed CD107a after incubation with YAC-1 cells (**Fig. 7e**).

Late injection of NKp46 fusion proteins prevents type 1 diabetes

To investigate whether our treatment modality was able to prevent or delay the onset of diabetes even in the late prediabetic stage and to demonstrate that injection of the immunoglobulin fusion proteins did not result in a nonspecific effect, we injected 11- to 12-week-old nondiabetic female NOD mice with PBS, NKp46-Ig, NCR1-Ig or the irrelevant fusion protein CEA-Ig. As expected, all mice developed antibodies directed against the injected fusion protein, including the control CEA-Ig (**Fig. 8a**). We detected NCR1-specific antibodies in the serum at around 4 weeks after the first injection (probably because tolerance had to be broken), and in the other groups, we detected fusion protein–specific antibodies as early as 2 weeks after the injection. Most antibodies were of the IgM isotype (data not shown).

We injected NOD mice with the various fusion proteins or PBS from 11–12 weeks of age until 20 weeks of age. The diabetes diagnosis and schedule and dose of fusion protein injection were the same as in the early-injection experiment (**Fig. 6a**). CEA-Ig did not prevent diabetes development and even caused a slight acceleration in the disease relative to that of the PBS-treated group (**Fig. 8b**). However, 67% of the NCR1-Ig–treated mice and all of the NKp46-Ig–treated mice remained diabetes-free until 20 weeks of age and during the first 6 weeks after therapy was halted (**Fig. 8b**). Antibody generation resulted in the specific downregulation of NCR1 at 2 or 4 weeks after injection of NKp46-Ig or NCR1-Ig, respectively (**Fig. 8c**). In addition, although around 30% of the pathogenic pancreatic NK cells derived from groups treated with CEA-Ig or PBS expressed CD107a after incubation with beta cells, less than 15% of the NK cells from mice treated with NCR1-Ig or NKp46-Ig had substantial expression of CD107a (**Fig. 8d**). Together, these findings indicate that NKp46 is critical for the development of diabetes and that NKp46 therapy might be used to prevent the development of diabetes at the late prediabetic stage, when beta cells face immediate destruction by NK cells.

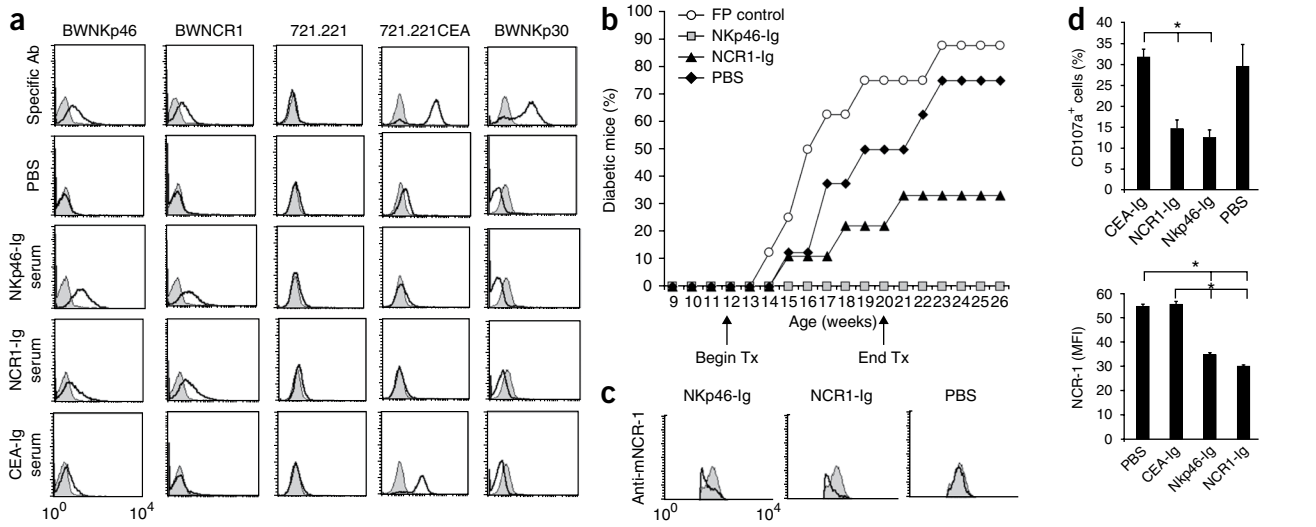

Figure 8 Treatment with NKp46 at a late prediabetic stage prevents the development of diabetes. (**a**) Flow cytometry of BW transfectants and 721.221 transfectants (Epstein-Barr virus–transfected B cell line) stained with specific antibodies (top row) or with serum obtained from 16-week-old treated mice. Black lines, specific staining; gray histograms, secondary antibody staining. Data are representative of two independent experiments. (**b**) Development of diabetes in NOD mice treated with NKp46-Ig, NCR1-Ig, CEA-Ig (FP control) or PBS starting at 11–12 weeks of age and continuing to 20 weeks of age ($n = 8$–9 mice per group). $P < 0.001$, NKp46-Ig and NCR1-Ig versus FP control and PBS (Kaplan-Meyer analysis, log-rank test). Data are representative of two independent experiments. (**c**) NK cells isolated from peripheral blood lymphocytes of 16-week-old treated mice (treatment, above plots) and stained with goat anti–mouse NCR1. Gray filled histograms, CEA-Ig (FP control); black lines, NKp46-Ig or NCR1-Ig. Right, mean fluorescent intensity (MFI) of NCR1 staining. $*P < 0.05$ (Student's *t*-test). Data are representative of two independent experiments (error bars, s.d.). (**d**) $CD107a^+$ cells among pathogenic NK cells derived from the islets of treated mice and stained with CD107a; plots are gated on $NCR1^+$ cells. $CD107a^+$ cells are presented as percent of total NK cells. $*P < 0.03$, CEA-Ig versus NCR1-Ig, or $P < 0.007$, CEA-Ig versus NKp46-Ig (Student's *t*-test). Data are representative of two independent experiments (error bars, s.d.).

DISCUSSION

Although it is known that NK cells have a crucial role in diabetes development[10,11], the mechanisms that control NK cell function in type 1 diabetes remain unknown. Here we have demonstrated that NKp46 is critically involved in diabetes development. In the absence of NKp46, or when the function of NKp46 was blocked, diabetes was prevented in most cases.

NK cells derived from all mouse strains tested degranulated after engagement with beta cells in an NKp46-dependent manner. Furthermore, pathogenic pancreatic NK cells derived from the islet cells of NOD mice degranulated *in vivo*, but those from other organs did not. In normal conditions, NK cells do not infiltrate the healthy pancreas, so NK cell–mediated killing of beta cells is prevented. NK cells appear in the pancreas concomitantly with T cells when diabetes starts to develop. We suggest that NKp46 is crucial at this point for islet cell destruction and, consequently, for diabetes progression. In support of those observations, several studies have shown that activated NK cells can kill autologous beta cells[14,31,32].

Notably, both normal and pathological pancreatic tissues and salivary glands, but no other normal human or mouse tissues examined, expressed a ligand for NKp46. It is still unknown why NKp46 ligands are expressed on healthy beta cells. Islets derived from pancreatic tissues of embryonic NOD mice do not express ligands for NCR1 and contain no infiltrating NK cells. Thus, it seems that NK cells do not have a critical role during the embryonic period of normal pancreatic tissue development.

Ligands for other stress receptors such as NKG2D are also present in the pancreas (as presented here and in refs. 17,21). However, the expression of NKG2D ligands was restricted to a subpopulation of beta cells and diminished as diabetes progressed, which suggests that some of the NKG2D-Ig–positive beta cells were killed during diabetes development or that the NKG2D ligands were downregulated. In contrast, almost all beta cells expressed the NCR1 ligand and its expression remained constant during diabetes progression.

The NKp46 ligand appeared at the postnatal stage, and we still do not know its identity or why normal beta cells express a ligand with the potential to cause harm. The function of the NKp46 ligand in beta cells is probably not related to NK cell activity, because NK cells are absent from the healthy pancreas. It is possible that the NKp46 ligands in the pancreas resemble foreign ligands recognized by NKp46 and that NK cells attack beta cells unintentionally because of this molecular mimicry. Indeed, infectious diseases have been suggested as potential triggers for many autoimmune diseases, and a link between viruses and type 1 diabetes has been demonstrated[33,34]. Expression of the NCR1 ligand is also apparent in the salivary glands. The salivary glands of NOD mice also serve as a target organ in the autoimmune process, and insulitis is usually accompanied by sialitis[35]. Notably, progenitor cells in the salivary glands of several animal strains can differentiate into functional beta cells after injury[36]. Although NK cells degranulated after interaction with beta cells in an NKp46-dependent manner and NK cells present in the beta cells of the pancreas express CD107a, it is possible that NK cells also affect diabetes development indirectly, for example, through cytokine secretion. However, we did not detect cytokine secretion by NK cells cultured together with beta cells.

Notably, injection of NKp46 variants into NOD mice, either at an early stage of insulitis or in the late prediabetic stage, blocked diabetes development almost entirely. Our model to explain these observations is that NKp46-specific antibodies (which contain blocking antibodies) are generated and that these antibodies cause systemic downregulation of NKp46. Such downregulation is the main cause of the NK cell dysfunction.

These results highlight areas for potential specific new therapeutic modalities for type 1 diabetes. An NKp46-dependent pharmacological intervention during the 'honeymoon' period of diabetes development might slow down or arrest the ongoing destruction of the remaining beta cells. We anticipate two possible scenarios for such treatment, each with its own advantages and disadvantages. The first is a passive vaccination approach in which patients would be treated with anti-NKp46 to block NKp46 function; this would bypass the need to induce antibodies, which, as shown here, might be time consuming. The main disadvantage of such treatment is the relatively rapid generation of neutralizing antibodies to the therapeutically injected monoclonal antibodies, as noted with the anti–tumor necrosis factor therapies used in autoimmune diseases[37]. Alternatively, it might be possible to actively vaccinate patients with NKp46 protein to generate long lasting NKp46-specific antibodies. The main disadvantages of this approach are that antibody generation takes time and that the NKp46 dysfunction might affect not only diabetes development but also NKp46-mediated activity against tumors and viruses. Nevertheless, our study has provided new insight into the relationship between NK cells and diabetes and may aid the development of therapeutic and imaging approaches based on the unique interaction of the NKp46 ligand and the activating receptor NKp46.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

C.G. designed all experiments, did all experiments, analyzed the data and wrote the manuscript; A.P. made the initial observation that NKp46 recognizes beta cells and supervised the project; M.E., R.G., S.M., N.S.-G., H.A., H.G., T.N., O.H. and M.M. contributed reagents; Y.D. provided guidance and reagents; V.D. helped in the immunohistochemical experiments and in determining the insulitis scoring; Y.N. supervised the project and contributed reagents; and O.M. supervised the entire project and analyzed the data, and all experiments were done in the O.M. laboratory under the guidance of O.M.

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ONLINE METHODS

Mice. The generation of *Ncr1*$^{gfp/gfp}$ mice has been described[18]. All experiments were done in a specific, pathogen-free unit of the Hadassah Medical School (Ein-Karem, Jerusalem) in accordance with the guidelines of the local ethical committee.

Cells and fusion proteins. Cell lines used in this study were as follows: HeLa, BW (and BW transfectants), RMA-s, PD1.6, YAC-1, RMS and 721.221 (and the 721.221 CEA transfectant). NCR1-Ig, NKp46-Ig, NCR1-t, NKG2D-Ig, CEA-Ig and NKp46D1-Ig fusion proteins were generated in COS-7 monkey kidney cells and were purified on a protein G column as described[25].

Immunohistochemical and immunofluorescence staining. Paraffin-embedded sections of pancreatic tissues were prepared from a nondiabetic human autopsy, from NOD mice embryos (E20) and from 1- to 14-week-old female NOD mice and 8- to 12-week-old female BALB/c mice. After antigen retrieval, sections were incubated for 2 h with fusion proteins. Sections were then incubated with polyclonal biotin-labeled rabbit antibodies directed against the human Fcγ part of the fusion proteins (309-065-082; Jackson ImmunoResearch). For immunohistochemical staining, the EnVision+ system (K4002; Dako) was used; this is based on a horseradish peroxidase–labeled polymer conjugated to anti-rabbit. After 30 min of incubation with anti-rabbit, slide staining was completed by 1–3 min of incubation with DAB+ Chromogen (3,3′-diaminobenzidine) and then counterstaining with hematoxylin. For immunofluorescence staining, in addition to being incubated with fusion proteins, tissues were also incubated with polyclonal anti–mouse insulin (A0564; DakoCytomation,), anti-somatostatin (som-018; Beta Cell Biology Consortium) and anti-glucagon (glu-001; Beta Cell Biology Consortium), followed by incubation with a mixture of the following three fluorochrome-conjugated secondary polyclonal antibodies (all from Jackson ImmunoResearch): indocarbocyanine-conjugated anti–guinea pig (706-165-148), carbocyanine-conjugated anti-mouse (715-225-150) and indodicarbocyanine-conjugated anti-rabbit (711-176-152). As a control, samples were stained with each fusion protein and reagent (primary and secondary antibodies) individually. For both immunohistochemical and the immunofluorescence staining, an NKp46-Ig fusion protein containing the binding D2 domain and the stalk region only was used[25], which gave a better staining than intact NKp46-Ig.

Isolation of beta cells. Pancreatic islets from normal and NOD mice were prepared with a solution of collagenase XI (Sigma) diluted in Hank's balanced-salt solution (Biological Industries Kibbutz Beit Haemek) at a concentration of 1 mg/ml. The solution was first injected into the pancreatic duct before removal of the pancreas, followed by digestion for 15–23 min at 37 °C. Individual islets were selected by hand with a microscope and then were separated into single cells.

CD107a mobilization and BW reporter assay. Analysis of cell surface–mobilized CD107a has been described[27]. In some experiments, NK cells were preincubated for 1 h with serum derived from a pool from each group of treated mice, diluted to a titer of 1:10,000. For measurement of CD107a *in vivo*, NK cells derived from the islets were stained for CD107a expression. For flow cytometry staining, beta cells were stained with 1 μg biotin-conjugated anti–mouse GLUT-2 (205115; R&D Systems) and 5 μg fusion protein. The generation and use of BW cells expressing NKp46 fused to the transmembrane and tail domains of mouse CD3ζ have been described[24].

Streptozotocin-induced diabetes. For the multiple-LDST model, ten to twelve sex- and age-matched mice 8–10 weeks of age were injected intraperitoneally for 5 consecutive days with streptozotocin (Sigma) dissolved in citrate buffer, pH 4.5, at a concentration of 50 mg per kg body weight. Day 0 was defined as the first day of injection of streptozotocin. Blood glucose concentrations were measured with a glucometer (Accu-Check; Roche Diagnostics) at day 7 and up to 45 d after first injection. Statistical analysis of multiple LDST experiments was done by Kaplan-Meier analysis with the log-rank test for comparison of survival curves of the two groups and by analysis of variance with repeated measures model for assessment the time effect, the group effect and the interaction between time and group during the development of diabetes.

Flow cytometry antibodies and enzyme-linked immunosorbent assay. Before pancreatic lymphocytes were isolated, pancreatic lymph nodes were removed to avoid lymphocyte contamination. Next, pancreatic tissues were cut into pieces 1 mm^3 and were digested with 1.5 mg type I DNAse and 15 mg type IV collagenase (Sigma). Supernatants were passed through a 40-μm cell strainer and then were loaded on a Ficoll density gradient for purification of the lymphocyte population. Peripheral blood was obtained from the tail vein. A monoclonal antibody specific for CD16 and CD32 (93; Biolegend) was used for blockade of Fc receptors before staining. NK cells derived from NOD mice were detected by staining with phycoerythrin-conjugated polyclonal goat antibody to mouse NCR1 and NKp46 (FAB2225P; R&D systems). For staining of BW cells transfected with NKp46, NKp30 or NKp44, 721.221 cells, and 721.221 cells transfected with CEA, specific antibodies to NKp46 (9E2; BioLegend), NKp30 (210845; BioLegend), NKp44 (253415; R&D Systems) and CEA (ASL-32; BioLegend) were used. For staining of BW cells transfected with NCR1, phycoerythrin-conjugated goat polyclonal antibody to mouse NCR1 and NKp46 (FAB2225P; R&D Systems) was used. For measurement of the secretion of mouse IL-2 from the BW transfectants or IFN-γ from mouse NK cells, a standard enzyme-linked immunosorbent assay was used with pairs of antibodies to mouse IL-2 (ges65h4 and ges61a12; BioLegend) or IFN-γ (R4-6A2 and XMG1.2; BD Pharmingen).