

# A gluten-free diet lowers NKG2D and ligand expression in BALB/c and non-obese diabetic (NOD) mice

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## Summary

The interplay between diet and immune parameters which could affect type 1 diabetes (T1D) pathogenesis is not sufficiently clarified. Intestinal up-regulation of the activating receptor natural killer group 2D (NKG2D) (CD314) and its ligands is a hallmark of coeliac disease. However, the direct effect of gluten on NKG2D expression is not known. We studied, by fluorescence activated cell sorter (lymphoid tissues) and reverse transcription–quantitative polymerase chain reaction (intestine and pancreatic islets), if a gluten-free diet (GF diet) from 4 weeks of age or a gluten-free diet introduced in breeding pairs (SGF diet), induced changes in NKG2D expression on DX5<sup>+</sup>(CD49b) natural killer (NK) cells, CD8<sup>+</sup> T cells and in intestinal and islet levels of NKG2D and ligands in BALB/c and non-obese diabetic (NOD) mice. Gluten-free NOD mice had lower insulinitis ( $P < 0.0001$ ); reduced expression of NKG2D on DX5<sup>+</sup> NK cells in spleen and auricular lymph nodes ( $P < 0.05$ ); and on CD8<sup>+</sup> T cells in pancreas-associated lymph nodes ( $P = 0.04$ ). Moreover, the level of CD71 on DX5<sup>+</sup> NK cells and CD8<sup>+</sup> T cells ( $P < 0.005$ ) was markedly reduced. GF and SGF mice had reduced expression of NKG2D and DX5 mRNA in intestine ( $P < 0.05$ ). Differences in intestinal mRNA expression were found in mice at 8, 13 and 20 weeks. Intestinal expression of NKG2D ligands was reduced in SGF mice with lower expression of all ligands. In isolated islets, a SGF diet induced a higher expression of specific NKG2D ligands. Our data show that a gluten-free diet reduces the level of NKG2D and the expression of NKG2D ligands. These immunological changes may contribute to the lower T1D incidence associated with a gluten-free diet.

**Keywords:** coeliac disease, gluten, gluten-free diet, NKG2D, type 1 diabetes

## Introduction

Type 1 diabetes (T1D) and coeliac disease (CD) are both autoimmune diseases, and there is a high prevalence of patients with both diseases. Studies have revealed an average prevalence of CD among children with T1D on 2–12% and patients with CD have earlier diabetes onset [1,2]. Intake of gluten is known to affect the disease process leading to T1D. Thus, the highest incidence of disease in animal models of T1D [biobreeding (BB) rats and non-obese diabetic (NOD) mice] is found in those animals which are on a diet based on cereals [3–5], and a gluten-free (GF) diet prevents diabetes development in NOD mice [6]. The T1D incidence of

NOD mice is further reduced in animals that have never been in contact with gluten, even during fetal development [7]. Human studies have further supported the effect of a gluten-free diet on T1D development [8,9], and T1D patients without CD are found to have an abnormal immune reactivity to gluten [10,11] independent of CD-associated haplotype, which could indicate that the response to wheat proteins is diabetes-specific [12]. The potential of gluten to affect the diabetogenic process seems to be dependent upon the time of gluten introduction, both in animals [13,14] and humans [15,16].

In addition to diet, the importance of intestinal microflora on the development of type 1 diabetes has

become evident, and it has been found that gluten intake directly affects the composition of the intestinal microflora: NOD mice fed a gluten-free diet have reduced numbers of caecal bacteria and Gram-positive bacteria compared to mice fed a wheat-containing standard diet [17], and a gluten-containing diet specifically increased intestinal species of *Bifidobacterium*, *Tannerella* and *Barnesiella*, whereas *Akkermansia* was increased in microflora of NOD-mice fed a gluten-free diet [18]. Therefore, it cannot be excluded that diet-induced changes in intestinal immunity could be influenced by corresponding microbiota changes. However, Patrick *et al.* showed that a diet based on cereals was a stronger promoter of type 1 diabetes than gut microbes and more significant protection from disease was observed by a hydrolyzed casein-based diet than could be obtained by altering the microbiota environment (germ-free conditions) [19].

The intestinal immune system plays an important role in the pathogenesis of T1D, as diabetogenic T cells are activated in the intestinal compartment before infiltrating the pancreas [20,21]. However, the effect of gluten on different cell populations of the immune system has not been completely clarified. We have shown recently that gluten intake influences the proportion of multiple regulatory T cell subsets as well as T helper type 17 (Th17) cells in lymphoid tissues [22] and modify the cytokine pattern of both forkhead box protein 3 (FoxP3)<sup>-</sup> T cells and FoxP3<sup>+</sup> regulatory T cells towards a more inflammatory cytokine profile [23].

Natural killer group 2D (NKG2D) (CD314) is an activating receptor on NK cells, CD8<sup>+</sup> T cells, NKT cells and  $\gamma\delta$ <sup>+</sup> T cells [24,25]. Interleukin (IL)-15-induced up-regulation of NKG2D on intraepithelial cytotoxic T cells (IELs) and NKG2D ligands on enterocytes is a hallmark in CD and is responsible for epithelial cell destruction and subsequent villous atrophy [25]. NKG2D is also known to be involved in the development of T1D and seems to be essential for disease progression, as blockade of NKG2D prevents diabetes in NOD mice [26,27]. Furthermore, patients with T1D have aberrant signalling through the NKG2D receptor [28,29]. In NK cells, NKG2D acts as an activating receptor to mediate target cell killing, and in CD8<sup>+</sup> T cells the ligation of NKG2D delivers a co-stimulatory signal to the T cell. Normally, T cell receptor (TCR) ligation triggers the armed CD8<sup>+</sup> T cells to mediate target cell killing. Simultaneous ligation of TCR and NKG2D increases the cytolytic response [30] and the cytotoxic IELs become lymphokine-activated killer (LAK) cells with NK-like cytolytic function, i.e. the ability to kill target cells independent of TCR specificity [31].

The NKG2D receptor can bind to a number of ligands that are all distantly related to major histocompatibility complex (MHC) class I. In humans, MICA and MICB (MHC class I chain-related) are well-described ligands [32,33]. MICA and MICB have no mouse homologues, but

are related closely to the mouse proteins Mill1 and Mill2 [34]. Other ligands include the ULBP-family (UL16 binding protein) with the mouse homologue Mult1 [35], H-60 (minor histocompatibility antigen) [36] and the Rae-1 family [37]. All these ligands are selectively up-regulated in target cells by stress or infection, and act to induce NKG2D-mediated killing of the target cells [38].

Evidence is pointing towards a role for gluten in the development of T1D. In the multi-factorial pathogenesis of T1D, gluten could play a role in the complex interplay between predisposing genes, defective immune-regulation and inflammatory priming of the mucosal immune system. Thus, we wished to study the effect of a GF diet on specific immune populations, known to be involved in CD and T1D. The aim of the present study was to clarify the effect of a gluten-free (GF) diet on NKG2D levels on DX5<sup>+</sup> (CD49b) NK cells and CD8<sup>+</sup> T cells isolated from lymphoid tissues: spleen (S), pancreatic lymph nodes (PLN) and auricular lymph nodes (ALN) as control peripheral lymphoid tissue. These were compared to mice receiving the diabetes-promoting gluten-containing standard diet (STD). The experiments were performed in the NOD mice to study potential diabetes-related mechanisms as well as in fully immunocompetent BALB/c mice, to clarify the effect of gluten intake in normal healthy animals. Moreover, we studied diet-induced differences in NKG2D expression in intestine and isolated pancreatic islet and NKG2D ligand expression.

## Materials and methods

### Animals

Fluorescence activated cell sorter (FACS) studies were performed on 13-week-old BALB/c and NOD mice on a GF *versus* gluten-containing standard diet (STD) from 4 weeks of age: BALB/cJ BomTac were purchased from Taconic Europe A/S (Ry, Denmark) and NOD mice were delivered from Taconic US, purchased from Taconic Europe A/S. Both BALB/c mice and NOD mice arrived at 4 weeks of age and were divided equally in groups receiving the GF *versus* the STD diets upon arrival. Nine mice from each group were used for FACS studies and 11 mice from each group used for reversed transcription-quantitative polymerase chain reaction (RT-qPCR). These animals were also used in Larsen *et al.* ('Effects of dietary gluten on murine natural killer cell activity' 2014, unpublished data). To study if the effect of a GF diet on selected immune parameters could be influenced by the timing of gluten exposure, we bought breeding pairs of NOD mice (Taconic US, Taconic Europe A/S) and divided them into two groups receiving either GF or STD diet during breeding. Therefore, the pups were only exposed to one diet (either GF or STD) both *in-utero*, during weaning and after weaning. These groups of animals were named strictly gluten-free (SGF) *versus* strictly

standard diet (SSTD) and were used to study NKG2D and NKG2D-ligand expression in intestinal tissue and isolated islets by RT-qPCR and immunohistochemistry. Moreover, the animals were used to determine diet-induced differences in lymphocyte infiltration (insulinitis scoring) in 20-week-old mice.

First-generation female offspring (12 in each group) were used in the study when 8, 13, 20 weeks old to study if the effect of diet was in the prediabetic phase or later in the disease development.

The mice were kept in a specific pathogen-free (SPF) animal facility (temperature  $22 \pm 2^\circ\text{C}$ , 12-h light cycle, air changed 16 times per hour, humidity  $55 \pm 10\%$ ) with free access to water and food. The animal experiments were carried out with approval from The National Animal Experimentation Board (2012-15-2934-00086), and experiments were performed in accordance with international guidelines for the care and use of laboratory animals.

## Diets

The animals received either the STD, non-purified Altromin diet or a GF, modified Altromin diet (Altromin, Lage, Germany), shown previously to prevent diabetes development in NOD mice [6,7]. Both experimental diets were nutritionally adequate with a similar level of protein, amino acids, minerals, vitamins and trace elements. These two diets have been used previously at The Bartholin Institute to study the effect of a GF diet on diabetes incidence in NOD mice [6,7]. The exact composition of the STD and the GF diet is given in [6,7]. The diets were prepared to ensure the same content of milk and soya proteins, found previously to be diabetogenic. The overall protein contents of the STD and the GF diets were similar (22.9 *versus* 22.9%). The only component that differs between the two diets is that the gluten-containing proteins in the STD diet (6.9%) are replaced by more animal proteins in the GF diet. This results in a slightly increased level of animal proteins in the GF diet (15.3%) *versus* the STD diet (8.4%). With regard to this slight increase in animal proteins, it cannot be excluded that this could have an effect. However, it has been shown previously that meat meal as the protein source promotes development of T1D in NOD mice [39], suggesting an even stronger diabetes-protective effect of the gluten-free diet due to its higher level of animal proteins. The two diets had the same content of amino acids, minerals, vitamins and trace elements. The weight of the mice was monitored and both groups of animals displayed similar weight distribution.

## FACS antibodies

The following monoclonal antibodies (mAb) were purchased from BD Pharmingen (San Jose, CA, USA): allophycocyanin (APC)-H7-conjugated rat anti-mouse CD8a

mAb [immunoglobulin (Ig)G2a,  $\kappa$ ; catalogue number: 560182]; fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD71 mAb (IgG11,  $\kappa$ ; catalogue number: 553266). Phycoerythrin (PE)-conjugated rat anti-mouse CD49b (DX5), mAb (IgM,  $\kappa$ ; catalogue number: 48597182), PE-cyanin 7 (Cy7)-conjugated rat anti-mouse CD314 (NKG2D) and mAb (IgG21,  $\kappa$ ; catalogue number: 25588282) were purchased from eBioscience (San Diego, CA, USA).

## Cell purification and flow cytometry

Mice were killed and spleen (S), pancreas-draining lymph nodes (PLN) and auricular lymph nodes (ALN) were isolated from 13-week-old BALB/c and NOD mice on GF and STD diets. The ALN were chosen as control lymphoid organs to show whether or not the effect of gluten was strictly confined to PLN, or if gluten also has an effect on systemic immunity, as shown previously [22,23]. Cells from each organ were pooled and single-cell suspensions were prepared. Surface staining was initiated with use of the relevant mAb, and cells were incubated for 1/2 h. Fc block (CD16/CD32) was purchased from BD Pharmingen (2.4G2; IgG2b,  $\kappa$ ) and added to reduce Fc receptor-mediated binding. The cells were fixed and subsequently analysed by flow cytometry using a LSR-II (BD Bioscience, San Jose, CA, USA), and data were analysed with use of FACS diva software (BD Bioscience). Isotype control antibodies were used to determine the amount of non-specific binding and the AmCyan-conjugated LIVE/DEAD fixable aqua dead cell staining kit were purchased from Invitrogen (Carlsbad, CA, USA) (catalogue number: L34957) to exclude dead cells.

## Islet isolation

Islets of Langerhans were isolated from single BALB/c or NOD mice using collagenase digestion. Following injection of collagenase (754 U/ml; Sigma-Aldrich, St Louis, MO, USA) in RPMI-1640 into the pancreatic duct, the pancreas was removed and subjected to further collagenase digestion [40]. Islets were hand-sorted to obtain 90–95% pure islet isolates, estimated by visual inspection. Isolated islets were immediately put into Trizol (Invitrogen) for RNA extraction.

## RNA isolation

Intestinal sections were kept in RNA later until RNA extraction, where they were transferred to Trizol and homogenized mechanically using a Polytron (Kinetica, Lucerne, Switzerland). Isolated islets were not homogenized prior to RNA extraction. Total RNA was isolated from islets or intestinal sections using Trizol reagent (Invitrogen).

RNA yield and quality were assessed on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

### qRT-PCR

RNA was extracted from a number of tissues for qPCR analyses. Duodenum ( $n = 11$ ) and isolated islets ( $n = 4$ ) samples from the BALB/C and NOD mice introduced to gluten at 4 weeks were used for qPCR. Duodenum sections from mice of 8, 13 and 20 weeks of age ( $n = 12$ ,  $n = 8$ , and  $n = 11$ ) from NOD mice that were kept strictly gluten-free were used for qPCR along with isolated islets ( $n = 11$ ) from 13-week-old mice.

Approximately 1.0  $\mu\text{g}$  of total RNA was reverse-transcribed into cDNA using the qScript kit (Quanta Biosciences, Gaithersburg, MD, USA), as recommended by the manufacturer.

Specific mRNA levels were quantified on a Lightcycler II (Roche, Penzberg, Germany) using SYBR II qPCR mixture (Takara Bio, Otsu, Japan). Primers (see Supporting information) were designed for an anneal temperature of 61°C using Primer3 software [41] and synthesized by TAGCopenhagen (Copenhagen, Denmark). Diluted, purified and sequence-verified (GATC Biotech, Constance, Germany) PCR products were used to create a standard curve for each primer pair. Expression levels were calculated as absolute quantification in relation to the relevant standard curve, using Lightcycler Software version 4.05. Expression values were then normalized to the housekeeping gene beta actin. Cycling parameters were set up to obtain similar reaction efficiencies of between 1.9 and 2.0.

The parameters for qPCR cycling were: initial denaturation at 95°C followed by 45 cycles of 10 s denaturation at 95°C, 5 s anneal at primer-specific temperature (56–60°C) and 15 s extension at 72°C.

Following PCR, a melting curve analysis was conducted. Any reactions with a CP value above 40 or with a non-specific peak in the melting curve analysis were treated as negative reactions and given the value '0' for statistical analysis.

### Histology and immunohistology

NOD SSTD and SGF mice at 20 weeks of age were used to determine diet-induced differences in lymphocyte infiltration (insulinitis scoring). Haematoxylin and eosin-stained pancreas sections were evaluated for the insulinitis score using the following scale: (i) intact islets, (ii) peri-insulinitis, (iii) moderate insulinitis (50% of the islets infiltrated) and (iv) severe insulinitis (> 50% of the islets infiltrated) [7]. At least 20–25 islets from each mouse were scored blind.

Immunohistochemical staining was performed on snap-frozen sections of small intestine. Tissues were fixated in Stefanini, rinsed in phosphate-buffered saline (PBS) and incubated with 2.5% bovine serum albumin (BSA) in PBS for 1 h and then incubated with primary anti-NKG2D (Santa Cruz NKG2D s-c5494; Santa Cruz Biotechnology,

Santa Cruz, CA, USA) 1:50 and anti-CD3 (ab16669; Abcam, Cambridge, UK) 1:100 in 1% BSA for 2 h. Binding was visualized using donkey anti-goat Cy3 (705-166-147; Jackson ImmunoResearch, West Grove, PA, USA) (1 : 200) and donkey anti-rabbit Alexa Fluor 488 (711-546-152; Jackson ImmunoResearch) (1 : 200). Vectashield 4',6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei.

### Statistical analysis

Groups were compared pairwise by Student's *t*-test. The  $\chi^2$  method was used on the insulinitis score data.  $P < 0.05$  was considered significant. All data are shown as mean  $\pm$  standard error of the mean (s.e.m.). Statistical significance is indicated with one, two or three asterisks in the figures, signifying levels of 0.05, 0.01 and 0.001, respectively. The groups were only compared pairwise: GF *versus* STD and SGF *versus* SSTD, unless specified otherwise.

### Results

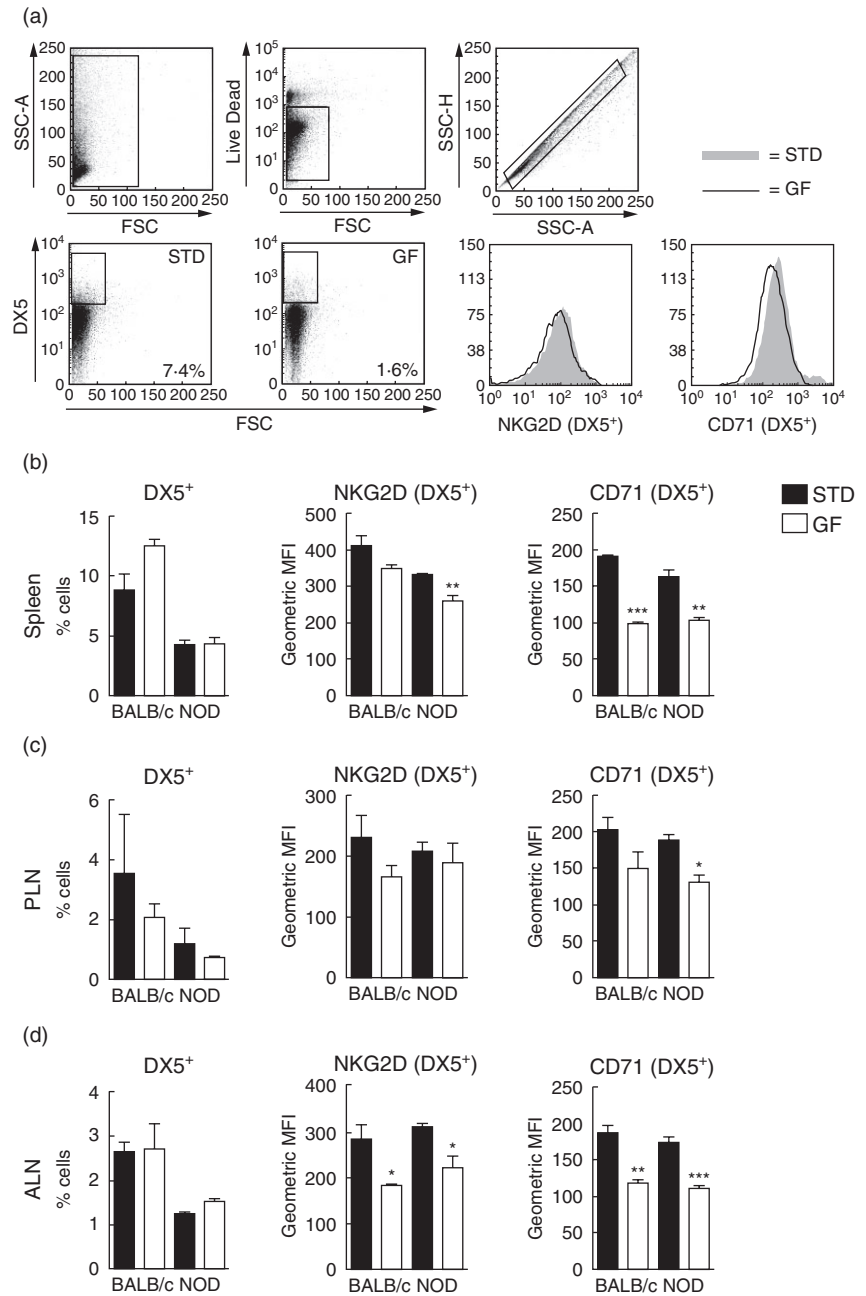
#### Decreased NKG2D expression in mice receiving GF diet on DX5<sup>+</sup> cells in S and ALN

Cell suspensions were prepared from isolated lymphoid organs (S, PLN and ALN) from 13-week-old mice on a GF *versus* STD diet and stained for DX5, CD8, NKG2D and CD71. Because BALB/c mice do not express the NK1.1 marker we used the anti-CD49b (clone DX5) pan-NK mAb, which has been shown previously to overlap with NK1.1 staining in C57Bl/6 mice as a marker for NK cells [42]. However, DX5 is also expressed on NKT cells [43,44]. By gating on DX5<sup>+</sup> cells (gating shown in Fig. 1a), we analysed the expression of NKG2D on DX5<sup>+</sup> cells. The mean fluorescence intensity (MFI) on NKG2D was decreased by 21.6% in the S of NOD mice receiving the GF diet (Fig. 1b,  $P = 0.0059$ ,  $n = 3$ ). No significant changes were found in PLN from BALB/c or NOD mice (Fig. 1c). In the ALN (Fig. 1d), NKG2D was decreased by 35.5% ( $P = 0.0371$ ,  $n = 3$ ) and in BALB/c by 28.7% ( $P = 0.0249$ ,  $n = 3$ ) in NOD receiving the GF diet.

We also analysed NKG2D expression on CD8<sup>+</sup> as a marker for cytotoxic T cells, and found a 29% ( $P = 0.0351$ ,  $n = 3$ ) increase in S (Fig. 2b) of BALB/c mice receiving the GF diet and an 8.8% ( $P = 0.0394$ ) decrease in the PLN (Fig. 2c) of NOD mice receiving the GF diet. No changes were seen in ALN.

#### The GF diet decreases proliferation marker, CD71, on DX5<sup>+</sup> and CD8<sup>+</sup> cells in S and PLN and ALN

To determine gluten-induced changes in the proliferation of DX5<sup>+</sup> NK cells and CD8<sup>+</sup> T cells, we gated on the two cell

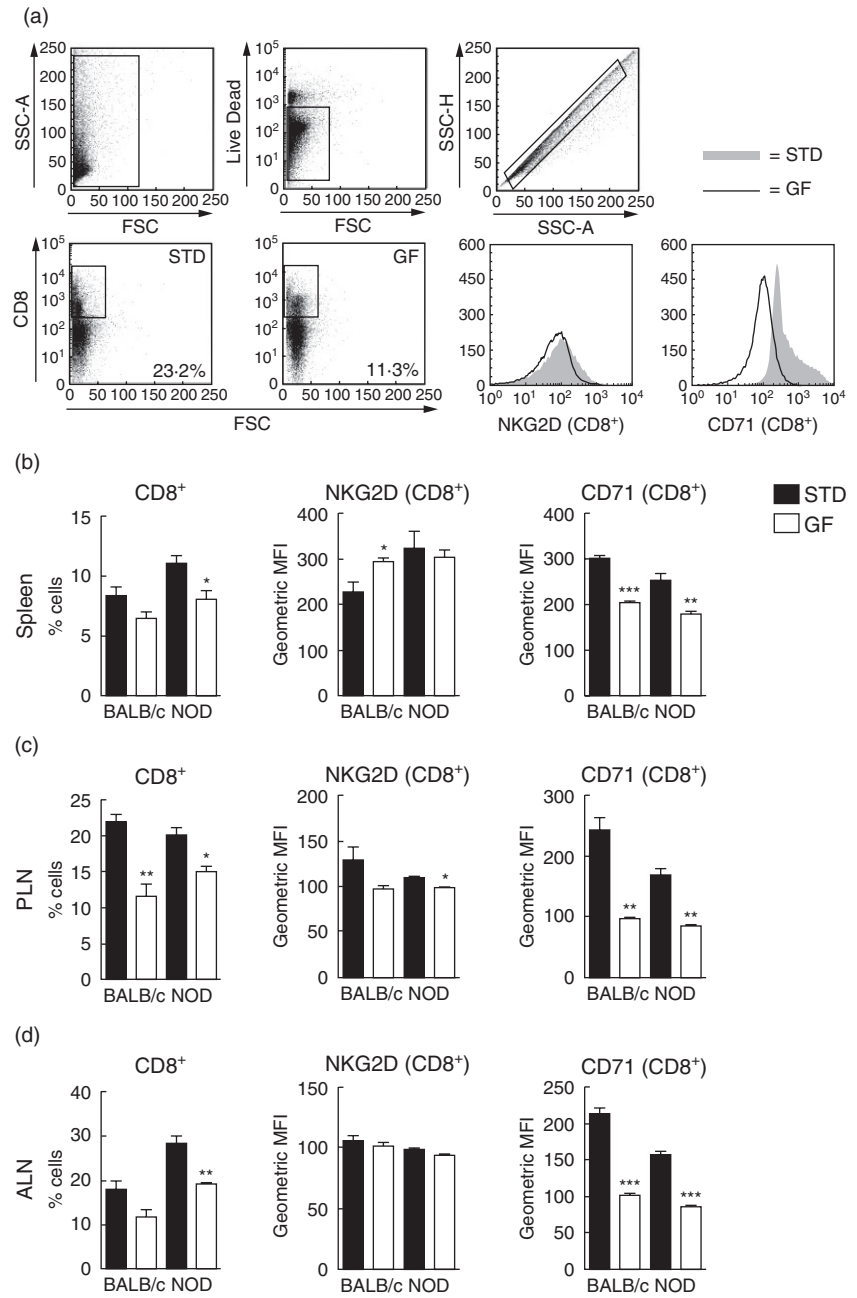


**Fig. 1.** Reduced level of CD71 and NKG2D expression on NK cells in mice on a gluten-free (GF) diet. (a) Representative plot of lymphocyte, singlet and live gate (upper panel). Examples of dot-plots showing the gating and percentages of DX5<sup>+</sup> cells in BALB/c standard diet (STD) and BALB/c GF mice and histograms showing expression of CD71 and NKG2D on DX5<sup>+</sup> cells. (b) Bars represent percentages of DX5<sup>+</sup> cells, and geometric mean fluorescence intensity (MFI) of CD71 and NKG2D gated on DX5<sup>+</sup> cells in BALB/c and non-obese diabetic (NOD) spleen (S). Data are represented as mean values  $\pm$  standard deviation of three independent experiments with three mice in each group. Black bars: STD diet; white bars: GF diet. (c) The same representation as in (b) in pancreatic lymph nodes (PLN); (d) the same representation as in (b) in auricular lymph nodes (ALN). \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

subsets and analysed the MFI of the transferrin receptor CD71. Transferrin receptor CD71 is a transmembrane homodimer glycoprotein, involved in uptake of iron and cell growth. The receptor expression is correlated with cellular proliferation and is thus expressed at greater levels on cells with a high proliferation rate [45–48]. We found a significant lower expression of CD71 on DX5<sup>+</sup> cells in S of BALB/c (48%,  $P < 0.0001$ ) and NOD (37%,  $P = 0.0036$ ) mice receiving the GF diet (Fig. 1b). We found a comparable decrease in the PLN of NOD mice (30%,  $P = 0.018$ ) receiving the GF diet (Fig. 1c). In ALN from both BALB/c and NOD mice we also found significant decreases (37%,

$P = 0.0028$  and 36%,  $P = 0.0009$ ) in mice receiving the GF diet (Fig. 1d).

When gating on CD8<sup>+</sup> cells we found a significant decrease of CD71 expression with the GF diet in both BALB/c and NOD mice in all organs analysed. In S (Fig. 2b) the decrease of CD71 was 32% ( $P = 0.0002$ ) for BALB/c mice and 29% ( $P = 0.0087$ ) for NOD mice. In PLN (Fig. 2c) the decrease of CD71 was 60% ( $P = 0.0023$ ) for BALB/c mice and 50% ( $P = 0.0073$ ) in NOD mice. Comparable effects were seen in ALN, where the decrease of CD71 was 52% ( $P = 0.0001$ ) for BALB/C mice and 45% ( $P = 0.0001$ ) in NOD mice.



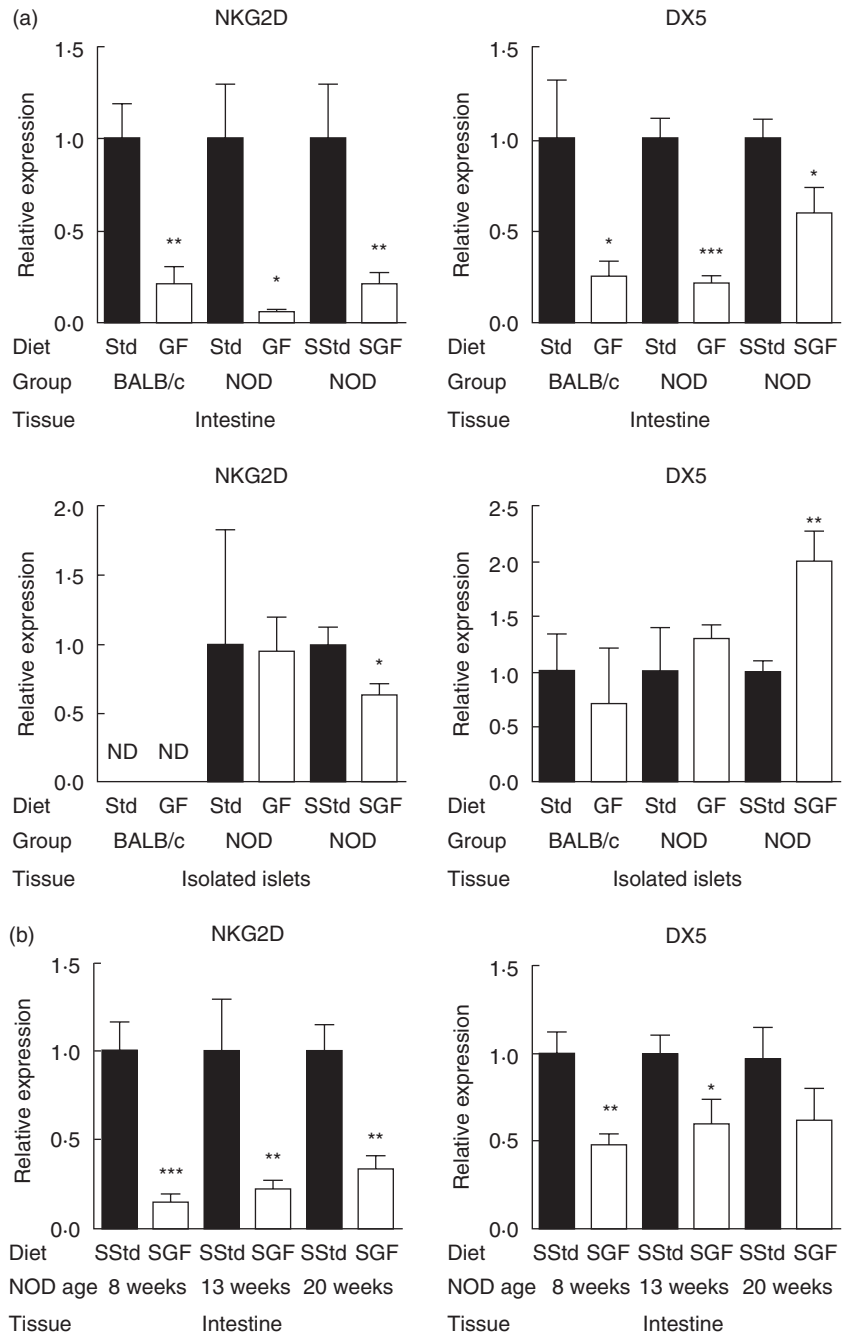
**Fig. 2.** Reduced level of CD71 expression and CD8<sup>+</sup> cells in mice on a gluten-free (GF) diet. (a) Representative plot of lymphocyte, singlet and live gate (upper panel). Examples of dot-plots showing the gating and percentages of CD8<sup>+</sup> cells in BALB/c standard diet (STD) and BALB/c GF mice and histograms showing expression of CD71 and NKG2D on CD8<sup>+</sup> cells. (b) Bars represent percentages of CD8<sup>+</sup> cells, and geometric mean fluorescence intensity (MFI) of CD71 and NKG2D gated on CD8<sup>+</sup> cells in BALB/c and non-obese diabetic (NOD) spleen (S). Data are represented as mean values ± standard deviation of three independent experiments with three mice in each group. Black bars: STD diet; white bars: GF diet. (c) The same representation as in (b) in pancreatic lymph nodes (PLN); (d) the same representation as in (b) in auricular lymph nodes (ALN). \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001.

**GF diet decreases CD8<sup>+</sup> cells in S, PLN and ALN of BALB/C and NOD mice**

We found a reduced proportion of CD8<sup>+</sup> cells in the S of NOD mice receiving the GF diet (33%, *P* = 0.0338) (Fig. 2b). In PLN we found reduced CD8<sup>+</sup> cells in BALB/c (47%, *P* = 0.0338) and NOD (25%, *P* = 0.0294) mice receiving the GF diet (Fig. 2c). Comparable effects were seen in ALN, where we found a reduction of 32% (*P* = 0.0046) in NOD mice receiving the GF diet (Fig. 2c).

**GF diet affects NKG2D and DX5 mRNA expression in intestine and islets**

We performed qRT-PCR on NKG2D and DX5 mRNA in intestines and isolated islets from 13-week-old mice, comparing GF or SGF diets with matched controls (STD/SSTD). Expression levels in intestinal tissue were markedly altered by diet (Fig. 3a). NKG2D and DX5 expression levels were reduced significantly in intestinal tissue in GF and SGF mice compared to controls. NKG2D levels were reduced to



**Fig. 3.** Reduced mRNA levels of NKG2D and DX5 in gluten-free (GF) mice. (a) Relative messenger RNA expression levels of NKG2D and DX5 in tissues from 13-week-old mice kept on a gluten free (GF), strictly gluten-free (SGF) or matched controls, standard diet (STD) and strictly standard diet (SSTD). Expression levels of NKG2D and DX5 in intestinal tissue (upper graphs). NKG2D and DX5 expression levels in islets (lower graphs). (b) Relative messenger RNA levels of NKG2D and DX5 in intestines from SGF-fed non-obese diabetic (NOD) mice at 8, 13 and 20 weeks of age. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

21, 6 and 22% in BALB/c, NOD and NOD SGF mice, respectively ( $P = 0.002$ ,  $P = 0.01$ ,  $P = 0.003$ ;  $n = 11$ ). DX5 levels were also reduced in GF and SGF mice of both BALB/c and NOD genetic backgrounds to 26, 22 and 60% of controls, respectively ( $P = 0.03$ ,  $P < 0.0001$ ,  $P = 0.002$ ;  $n = 11$ ).

In isolated islets (Fig. 3a), NKG2D and DX5 expression levels in islets only changed significantly in SGF animals where NKG2D expression was reduced to 64% ( $P = 0.04$ ;  $n = 8$ ), and DX5 expression was doubled compared to animals fed the SSTD diet ( $P = 0.003$ ;  $n = 8$ ). No changes were observed in GF animals.

### Prediabetic mRNA expression of NKG2D and DX5 in mouse tissues

To investigate the effect of a GF diet on NKG2D and DX5 down-regulation at different time-points, qRT-PCR was performed on intestines from SGF NOD mice of different ages.

NKG2D and DX5 expression levels were consistently lower in intestinal tissue of SGF mice compared to SSTD-fed controls (Fig. 3b). This effect was seen for animals of 8, 13 and 20 weeks of age. The SGF NOD mice had significantly lower expression of NKG2D at all measured time-points,

amounting to 15, 22 and 34% of SSTD at 8, 13 and 20 weeks, respectively ( $P < 0.0001$ ,  $P = 0.003$  and  $P = 0.005$ ). DX5 expression was only significantly lower at 8 and 13 weeks, amounting to 48, 60 and 61% of SSTD at 8, 13 and 20 weeks, respectively ( $P = 0.002$ ,  $P = 0.03$  and  $P = 0.20$ ). NKG2D expression was also compared between different age groups (Supporting information, Fig. S1). The NKG2D expression level of the SSTD groups was lower at 20 weeks than at both 8 and 13 weeks (38% of 8 weeks,  $P = 0.004$ ). In the SGF groups, NKG2D expression was higher at 13 weeks compared to 8 and 20 weeks (260% of 8 weeks,  $P = 0.01$ ).

DX5 expression levels decreased with mouse age in both SSTD and SGF groups (Supporting information, Fig. S1). The decrease was significant for all SSTD groups, with expression levels at 13 and 20 weeks that amounted to 43 ( $P = 0.0009$ ) and 25% ( $P < 0.0001$ ), respectively. The decrease was less prominent in SGF groups, where only the expression at 20 weeks was significantly lower than at 8 weeks (33%,  $P = 0.004$ ).

### GF diet affects the expression of NKG2D ligands

To study a direct effect of gluten on NKG2D ligand expression, we performed qPCR analyses of a number of known NKG2D ligands in mouse intestines and isolated islets.

The intestinal expression pattern of NKG2D ligands (Fig. 4a) did not change greatly in BALB/c mice fed a GF diet compared to a STD diet. Only the ligand H60a was reduced significantly to 42% of STD controls ( $P = 0.01$ ;  $n = 11$ ).

In NOD mice fed a GF diet, the expression level of Mill1 was increased 4.9-fold ( $P = 0.0001$ ;  $n = 11$ ), the level of Mill2 decreased to 20% ( $P = 0.02$ ;  $n = 11$ ) and the level of Mult1 increased threefold ( $P = 0.03$ ;  $n = 11$ ) compared to STD mice.

The expression levels of all NKG2D ligands were reduced in SGF NOD mice compared to SSTD controls. Thus, Mill1, Mill2, Mult1, Rae-1 and H60a was expressed at levels corresponding to 11 ( $P = 0.0004$ ), 35 ( $P = 0.01$ ), 45 ( $P = 0.003$ ), 27 ( $P = 0.001$ ) and 52% ( $P = 0.26$ ) of controls, respectively (all  $n = 12$ ).

A different pattern of NKG2D ligand expression was observed in isolated islets (Fig. 4b). None of the investigated ligands showed a significant change in expression level in GF BALB/c mice (upper graph) or NOD mice (middle graph) ( $n = 4$ ). In the SGF NOD mice (lower graph), the expression of Mill1, Mill2 and Mult1 was increased significantly compared to SSTD controls, with non-significant increases observed in the expression levels of the remaining ligands. The increased expression amounted to 23-fold ( $P = 0.01$ ), fivefold ( $P = 0.05$ ) and 1.7-fold ( $P = 0.02$ ) for Mill1, Mill2 and Mult1, respectively (all  $n = 11$ ).

The NKG2D ligands Mult1 (top) and Rae-1 (bottom) showed interesting expression patterns in intestines (Fig. 5c). Thus, while Mult1 expression was increased three-

fold ( $P = 0.03$ ;  $n = 11$ ) in NOD GF mice, it was consistently lower in SGF mice at 8, 13 and 20 weeks, corresponding to a reduction to 54 ( $P = 0.001$ ;  $n = 12$ ), 45 ( $P = 0.003$ ;  $n = 12$ ) and 70% ( $P = 0.15$ ;  $n = 9$ ), respectively.

Rae-1 expression was also reduced for all GF and SGF groups, with the only significant effects for SGF mice. The GF diet reduced Rae-1 expression to 81% in BALB/c mice ( $P = 0.83$ ,  $n = 11$ ), 54% in NOD GF mice ( $P = 0.25$ ,  $n = 11$ ), 31% in 8-week SGF mice ( $P = 0.01$ ,  $n = 12$ ), 27% in 13-week SGF mice ( $P = 0.001$ ,  $n = 12$ ) and to 31% of controls in 20-week SGF mice ( $P = 0.006$ ,  $n = 8$ ).

### Decreased islet infiltration in animals receiving SGF diet

Haematoxylin and eosin-stained (H&E) pancreatic sections were evaluated for islet infiltration. The insulinitis score was  $2.1 \pm 0.05$  for the SGF NOD mice and  $2.5 \pm 0.05$  for the SSTD NOD mice ( $P < 0.0001$ ). Severe insulinitis (>50% of the islet infiltrated) was rarely observed in the SGF NOD mice, and only few intact islets were found in the SSTD NOD mice. Using immunohistochemical staining we showed the presence of NKG2D-positive cells in intestinal tissue of NOD mice (Fig. 5).

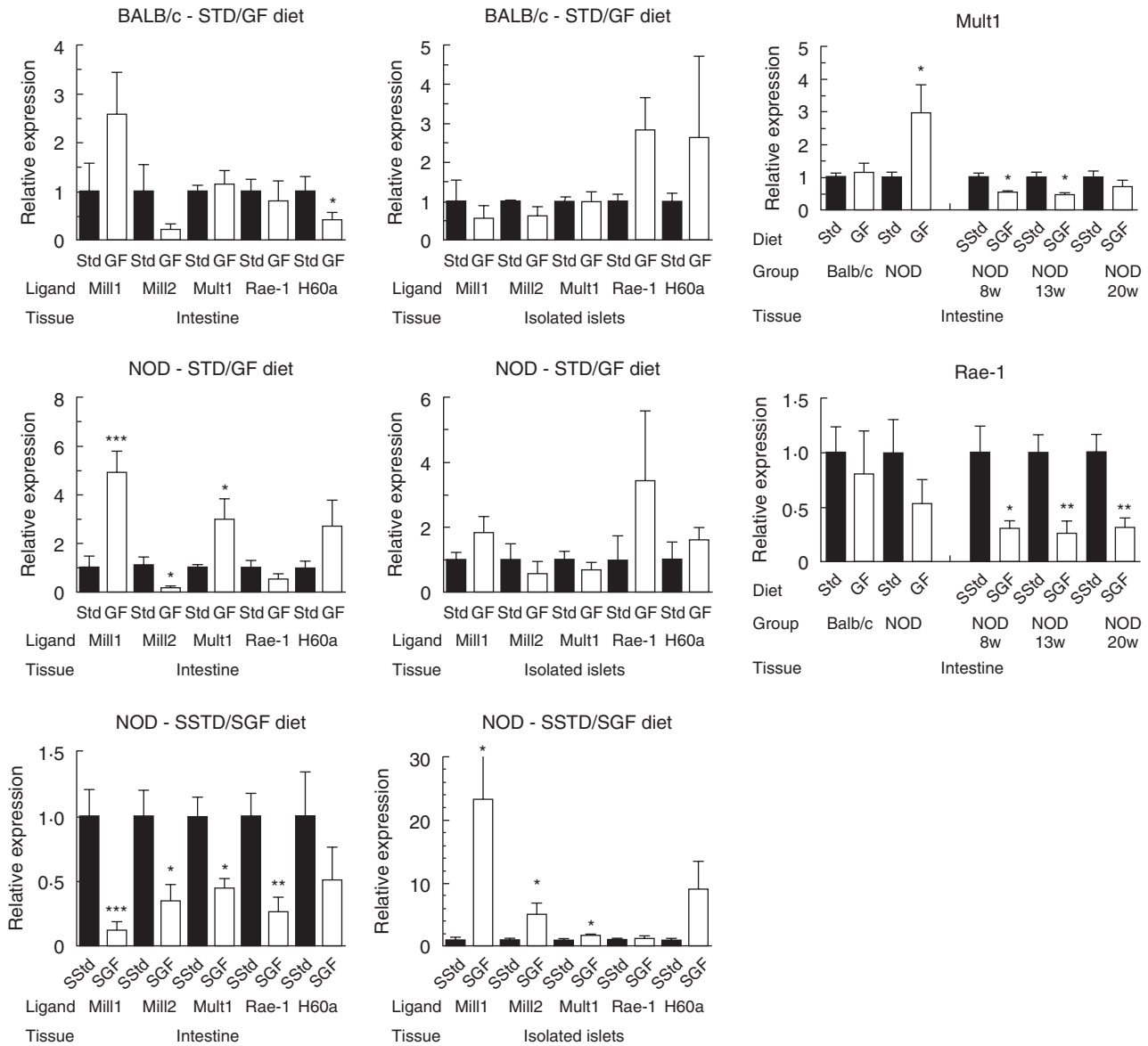
### Discussion

This study provides new evidence concerning the effect of dietary gluten on immune parameters associated with disease development, and may help to understand the effect of a GF diet observed both in animal models [6,7] of disease as well as in humans [9,15,16]. We studied the effect of a GF diet in both fully immune-competent BALB/c mice and in NOD mice. This allowed us to study the effect of gluten both in healthy animals and in those predisposed to disease.

We have shown that the GF diet reduces the expression of NKG2D on DX5<sup>+</sup> NK cells in S and ALN, and on CD8<sup>+</sup> T cells in PLN of NOD mice. Furthermore, a GF and SGF diet diminishes the intestinal expression of NKG2D in both BALB/c and NOD mice, and a SGF diet reduces NKG2D expression in isolated islets from NOD mice. The SGF diet induced a significantly lower expression of NKG2D ligands in intestine from NOD mice. Interestingly, a GF diet markedly reduced the expression of the proliferation marker (CD71) in both DX5<sup>+</sup> NK cells and CD8<sup>+</sup> T cells. The reduced proliferation was found in all lymphoid organs tested and was profound both in BALB/c mice and in NOD mice.

Our study shows that a diet without gluten reduces the level of NKG2D on DX5<sup>+</sup> NK cells and CD8<sup>+</sup> T cells and we have consistently found lower NKG2D mRNA expression in tissues from mice on GF or SGF diets. That this effect is most prominent in intestinal tissue is not surprising, given that this is the tissue in direct contact with gluten digestion



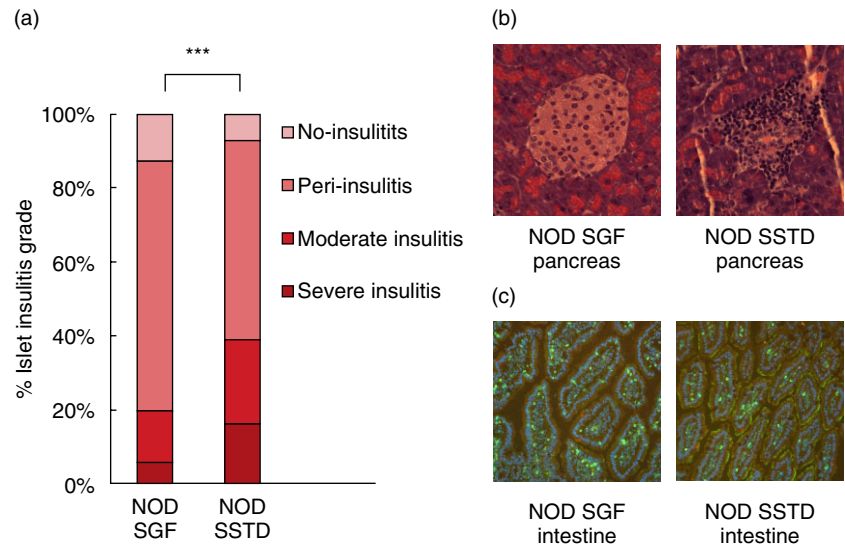


**Fig. 4.** Expression patterns of NKG2D ligand mRNAs in intestine and islets from BALB/c and non-obese diabetic (NOD) mice. Relative messenger RNA levels of known NKG2D ligands were quantified in intestine and islets. (a) The expression pattern of NKG2D ligands in intestinal tissue from 13-week-old BALB/c, NOD and NOD strictly gluten-free (SGF) mice. (b) Expression levels of NKG2D ligand in isolated islets of Langerhans from the same mice. (c) The expression levels of NKG2D ligands Mult1 (top) and Rae1 (bottom) in intestine, from BALB/c and NOD 13-week-old mice and from NOD SGF mice at 8, 13 and 20 weeks of age. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

products. However, we studied only changes in duodenum, and therefore we did not investigate possible important immunological changes in other parts of both the small and large intestines. Such differences could be interesting, because of the existence of distinctive functional differences in the lymphocyte populations residing at different intestinal compartments [49]. In the lymphoid tissues, we observed the main diet-induced differences in NKG2D and DX5 in S compared to PLN. This supports a systemic effect of gluten intake on immune activation, which is supported by earlier studies of gluten-induced effects on both intesti-

nal and systemic immunity [22,23]. It is surprising that a SGF diet also correlates with lower NKG2D expression in islets, especially as the same tissue shows increased DX5 expression. It is not clear whether the increase in DX5 expression reflects an increase in NK cells or if this reflects an increased level of islet-infiltrating NKT cells (expressing DX5) [44], which was found previously to prevent the development of T1D [50]. An increased level of NKT cells directly in the islets could be one mechanism behind the protective role of a GF diet in the prevention of T1D in NOD mice. Gluten-induced expression of NKG2D is of

**Fig. 5.** Islet insulinitis score, NKG2D and CD3 staining in non-obese diabetic (NOD) mice. (a) Scoring of histological grade of insulinitis was performed on pancreatic section stained with haematoxylin and eosin (H&E) from 20-week-old NOD mice receiving strictly gluten-free (SGF) ( $n = 11$ ) and strictly standard diet (SSTD) ( $n = 9$ ) diet. Islets were scored for no-insulinitis, peri-insulinitis, moderate insulinitis and severe insulinitis. \* $P < 0.0001$ . (b) Histological pancreatic sections stained with H&E from NOD SGF and NOD (SSTD). (c) Section of intestine from NOD SGF and NOD SSTD. The red indicates cells positive for NKG2D; the green cells are positive for CD3; and blue shows cell nuclei.



particular interest because studies indicate that NKG2D plays a key role in immune-mediated diseases, including CD and T1D [25,26]. NKG2D could serve as an immune activator that can tip the balance in favour of autoimmunity and chronic inflammation. The observation that NKG2D can mediate cytotoxicity independently of TCR ligation [31] in recently activated T cells is particularly relevant in this context, because it suggests that gluten intake alone can establish a self-perpetuating loop of immune activation.

Untreated CD patients have a decreased intestinal level of NK cells that remain low, even when the patients are treated with a GF diet [51], implying that a GF diet does not influence the level of NK cells in CD patients. We did not find any diet-induced effect on the level of DX5<sup>+</sup> NK cells in isolated lymphoid tissue (S, PLN or ALN) in either BALB/c or NOD mice, which was described previously [22]. Interestingly, we found that a GF and a SGF diet reduced the intestinal expression of DX5 mRNA, both in BALB/c and in NOD mice, and that this diet-induced difference was sustained at all measured time-points and was already present in NOD mice at the age of 8 weeks. Thus, a GF diet was found to have an effect on the intestinal level of NK cells, both in normal healthy intestinal tissue (BALB/c mice) as well as in NOD mice. Moreover, we found that a SGF diet induced a higher expression of NK cells in isolated islets. Therefore, it seems that the effect of gluten on the expression of DX5 is mainly directly in the tissues and not in lymphoid organs, suggesting an innate activation by gluten directly in the tissue.

Both T1D and CD [52] are found to be CD4<sup>+</sup> T cell-mediated diseases, but the activation of CD8<sup>+</sup> T cells is also central in controlling the immune response that causes immunopathology in both diseases [53–55]. We found diet-induced differences in the level of CD8<sup>+</sup> T cells in all tested lymphoid organs. A GF diet diminished the level of CD8<sup>+</sup> T

cells in NOD mice, both in S and ALN and in PLN. In BALB/c mice a GF diet was also found to reduce the level of CD8<sup>+</sup> T cells in PLN.

Interestingly, we found a consistent diet-induced effect on the level of CD71 on DX5<sup>+</sup> and CD8<sup>+</sup> cells in both BALB/c and NOD mice, where a GF diet reduced the expression on both NK cells and CD8<sup>+</sup> T cells in all tested lymphoid organs. Increased CD71 receptor levels are found on immature proliferating cells and upon antigen stimulation of T cells, as well as cells with a high proliferation rate [45–48]. This implies that a GF diet diminishes the proliferation or activation of both NK cells and CD8<sup>+</sup> T cells.

Interestingly, we found that the intestinal expression of NKG2D ligands was changed according to diet in NOD mice. The NOD mice on a GF diet from 4 weeks of age had an increased intestinal expression of Mill1 and Mult1. However, in SGF animals that had never been exposed to gluten we found a significantly reduced intestinal expression of almost all NKG2D ligands. This correlates well with the reduced intestinal expression of NKG2D and DX5. This resembles the transition from low levels of NKG2D ligand expression in healthy gut epithelium [25] that increases in patients with CD, where distressed intestinal epithelial cells up-regulate the stress-inducible non-classical MHC class I MIC (MICA and MICB) [56] upon exposure to gluten peptides. Lowered levels of NKG2D along with lowered levels of Mult1 and Rae-1 could be involved in the beneficial effects of a SGF diet compared to GF diet. Thus, it seems that a SGF diet can lower NKG2D-mediated immunity in intestines both through direct down-regulation of the NKG2D receptor as well as through target tissue ligand expression, which shows clearly that there is an effect of the timing of gluten exposure.

Isolated islets from SGF NOD mice had a higher expression of Mult1, Mill1 and Mill2 than matched SSTD

controls. This was found neither in BALB/c mice nor the GF NOD mice. The observed pattern of ligand expression could indicate that NKG2D ligands may perform different roles, but the details of such NKG2D ligand interplay remain to be established. Perhaps NKG2D ligand up-regulation could protect the islets from NKG2D-mediated cytotoxicity, because it has been shown that NK cells may be desensitized by continuous exposure to NKG2D ligand [57,58]. However, other studies have found that the expression of NKG2D ligands decreases MHC class I expression [59], which leads to increased NK cell killing, implying a complex relationship between NKG2D ligand expression and MHC I. In concordance with a study by Maier *et al.* [60], but in contrast to the study by Ogasawara *et al.* [26], we observed no dramatic difference in Rae-1 expression levels between NOD and BALB/c mice, either in intestine, isolated islets or with age. The reason for the difference between our results and those of Ogasawara *et al.* [26] is unknown, but it could be due to an effect of different housing conditions or to dietary differences.

These results help to clarify how the immune status is affected by dietary gluten, which has been reported as an environmental factor in the development of T1D [61] as well as in healthy individuals and individuals reporting to be 'gluten-sensitive' [62]. Our finding that gluten affects immunity both in BALB/c mice and in NOD mice supports the idea that gluten is able to induce cellular changes both in animals predisposed to disease as well as in healthy animals. The effect of gluten on NKG2D and NKG2D ligand expression, as observed in CD patients, is not disease-specific but also present in NOD and BALB/c mice.

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### Disclosures

The authors declare that there are no conflicts of interest.

### Author contributions

J. C. A. designed, performed, analysed the experiments and wrote the paper. C. W. designed, performed, analysed and wrote about the qPCR experiments. E. A. performed and analysed the histology. J. L. analysed and wrote about the FACS experiments. K. E. participated in the performance of the FACS experiments. D. A. and K. B. supervised and edited the paper.

### References

- Collin P, Kaukinen K, Valimaki M, Salmi J. Endocrinological disorders and celiac disease. *Endocr Rev* 2002; **23**:464–83.

- Hansen D, Brock-Jacobsen B, Lund E *et al.* Clinical benefit of a gluten-free diet in type 1 diabetic children with screening-detected celiac disease: a population-based screening study with 2 years' follow-up. *Diabetes Care* 2006; **29**:2452–6.
- Coleman DL, Kuzava JE, Leiter EH. Effect of diet on incidence of diabetes in nonobese diabetic mice. *Diabetes* 1990; **39**:432–6.
- Hoorfar J, Scott FW, Cloutier HE. Dietary plant materials and development of diabetes in the BB rat. *J Nutr* 1991; **121**:908–16.
- Scott FW. Food-induced type 1 diabetes in the BB rat. *Diabetes Metab Rev* 1996; **12**:341–59.
- Funda DP, Kaas A, Bock T, Tlaskalova-Hogenova H, Buschard K. Gluten-free diet prevents diabetes in NOD mice. *Diabetes Metab Res Rev* 1999; **15**:323–7.
- Funda DP, Kaas A, Tlaskalova-Hogenova H, Buschard K. Gluten-free but also gluten-enriched (gluten+) diet prevent diabetes in NOD mice; the gluten enigma in type 1 diabetes. *Diabetes Metab Res Rev* 2008; **24**:59–63.
- Pastore MR, Bazzigaluppi E, Belloni C, Arcovio C, Bonifacio E, Bosi E. Six months of gluten-free diet do not influence autoantibody titers, but improve insulin secretion in subjects at high risk for type 1 diabetes. *J Clin Endocrinol Metab* 2003; **88**:162–5.
- Sildorf SM, Fredheim S, Svensson J, Buschard K. Remission without insulin therapy on gluten-free diet in a 6-year old boy with type 1 diabetes mellitus. *BMJ Case Rep* 2012; doi:10.1136/bcr.02.2012.5878.
- Troncone R, Franzese A, Mazzarella G *et al.* Gluten sensitivity in a subset of children with insulin dependent diabetes mellitus. *Am J Gastroenterol* 2003; **98**:590–5.
- Auricchio R, Paparo F, Maglio M *et al.* *In vitro*-deranged intestinal immune response to gliadin in type 1 diabetes. *Diabetes* 2004; **53**:1680–3.
- Mojibian M, Chakir H, Lefebvre DE *et al.* Diabetes-specific HLA-DR-restricted proinflammatory T-cell response to wheat polypeptides in tissue transglutaminase antibody-negative patients with type 1 diabetes. *Diabetes* 2009; **58**:1789–96.
- Scott FW, Rowsell P, Wang GS, Burghardt K, Kolb H, Flohe S. Oral exposure to diabetes-promoting food or immunomodulators in neonates alters gut cytokines and diabetes. *Diabetes* 2002; **51**:73–8.
- Schmid S, Koczwara K, Schwinghammer S, Lampasona V, Ziegler AG, Bonifacio E. Delayed exposure to wheat and barley proteins reduces diabetes incidence in non-obese diabetic mice. *Clin Immunol* 2004; **111**:108–18.
- Norris JM, Barriga K, Klingensmith G *et al.* Timing of initial cereal exposure in infancy and risk of islet autoimmunity. *JAMA* 2003; **290**:1713–20.
- Ziegler AG, Schmid S, Huber D, Hummel M, Bonifacio E. Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. *JAMA* 2003; **290**:1721–8.
- Hansen AK, Ling F, Kaas A, Funda DP, Farlov H, Buschard K. Diabetes preventive gluten-free diet decreases the number of caecal bacteria in non-obese diabetic mice. *Diabetes Metab Res Rev* 2006; **22**:220–5.
- Marietta EV, Gomez AM, Yeoman C *et al.* Low incidence of spontaneous type 1 diabetes in non-obese diabetic mice raised on gluten-free diets is associated with changes in the intestinal microbiome. *PLOS ONE* 2013; **8**:e78687.
- Patrick C, Wang GS, Lefebvre DE *et al.* Promotion of autoimmune diabetes by cereal diet in the presence or absence of microbes associated with gut immune activation, regulatory imbalance, and

- altered cathelicidin antimicrobial peptide. *Diabetes* 2013; **62**:2036–47.
- 20 Hanninen A, Jaakkola I, Jalkanen S. Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. *J Immunol* 1998; **160**:6018–25.
  - 21 Hanninen A, Salmi M, Simell O, Jalkanen S. Mucosa-associated (beta 7-integrinhigh) lymphocytes accumulate early in the pancreas of NOD mice and show aberrant recirculation behavior. *Diabetes* 1996; **45**:1173–80.
  - 22 Antvorskov JC, Fundova P, Buschard K, Funda DP. Impact of dietary gluten on regulatory T cells and Th17 cells in BALB/c mice. *PLOS ONE* 2012; **7**:e33315.
  - 23 Antvorskov JC, Fundova P, Buschard K, Funda DP. Dietary gluten alters the balance of pro-inflammatory and anti-inflammatory cytokines in T cells of BALB/c mice. *Immunology* 2013; **138**:23–33.
  - 24 Guy-Grand D, Cuenod-Jabri B, Malassis-Seris M, Selz F, Vassalli P. Complexity of the mouse gut T cell immune system: identification of two distinct natural killer T cell intraepithelial lineages. *Eur J Immunol* 1996; **26**:2248–56.
  - 25 Abadie V, Discepolo V, Jabri B. Intraepithelial lymphocytes in celiac disease immunopathology. *Semin Immunopathol* 2012; **34**:551–66.
  - 26 Ogasawara K, Hamerman JA, Ehrlich LR *et al.* NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity* 2004; **20**:757–67.
  - 27 Van Belle TL, Ling E, Haase C, Bresson D, Urso B, von Herrath MG. NKG2D blockade facilitates diabetes prevention by antigen-specific Tregs in a virus-induced model of diabetes. *J Autoimmun* 2013; **40**:66–73.
  - 28 Qin H, Lee IF, Panagiotopoulos C *et al.* Natural killer cells from children with type 1 diabetes have defects in NKG2D-dependent function and signaling. *Diabetes* 2011; **60**:857–66.
  - 29 Rodacki M, Svoren B, Butty V *et al.* Altered natural killer cells in type 1 diabetic patients. *Diabetes* 2007; **56**:177–85.
  - 30 Roberts AL, Lee L, Schwarz E *et al.* NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. *J Immunol* 2001; **167**:5527–30.
  - 31 Meresse B, Chen Z, Ciszewski C *et al.* Coordinated induction by IL15 of a TCR-independent NKG2D signaling pathway converts CTL into lymphokine-activated killer cells in celiac disease. *Immunity* 2004; **21**:357–66.
  - 32 Bahram S, Bresnahan M, Geraghty DE, Spies T. A second lineage of mammalian major histocompatibility complex class I genes. *Proc Natl Acad Sci USA* 1994; **91**:6259–63.
  - 33 Stephens HA. MICA and MICB genes: can the enigma of their polymorphism be resolved? *Trends Immunol* 2001; **22**:378–85.
  - 34 Kasahara M, Watanabe Y, Sumasu M, Nagata T. A family of MHC class I-like genes located in the vicinity of the mouse leukocyte receptor complex. *Proc Natl Acad Sci USA* 2002; **99**:13687–92.
  - 35 Carayannopoulos LN, Naidenko OV, Fremont DH, Yokoyama WM. Cutting edge: murine UL16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. *J Immunol* 2002; **169**:4079–83.
  - 36 Malarkannan S, Shih PP, Eden PA *et al.* The molecular and functional characterization of a dominant minor H antigen, H60. *J Immunol* 1998; **161**:3501–9.
  - 37 Zou Z, Nomura M, Takihara Y, Yasunaga T, Shimada K. Isolation and characterization of retinoic acid-inducible cDNA clones in F9 cells: a novel cDNA family encodes cell surface proteins sharing partial homology with MHC class I molecules. *J Biochem* 1996; **119**:319–28.
  - 38 Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. *Nat Rev Immunol* 2001; **1**:41–9.
  - 39 Elliott RB, Reddy SN, Bibby NJ, Kida K. Dietary prevention of diabetes in the non-obese diabetic mouse. *Diabetologia* 1988; **31**:62–4.
  - 40 Aaen K, Rygaard J, Josefsen K *et al.* Dependence of antigen expression on functional state of beta-cells. *Diabetes* 1990; **39**:697–701.
  - 41 Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000; **132**: 365–86.
  - 42 Arase H, Saito T, Phillips JH, Lanier LL. Cutting edge: the mouse NK cell-associated antigen recognized by DX5 monoclonal antibody is CD49b (alpha 2 integrin, very late antigen-2). *J Immunol* 2001; **167**:1141–4.
  - 43 Pellicci DG, Hammond KJ, Coquet J *et al.* DX5/CD49b-positive T cells are not synonymous with CD1d-dependent NKT cells. *J Immunol* 2005; **175**:4416–25.
  - 44 Werner JM, Busl E, Farkas SA, Schlitt HJ, Geissler EK, Hornung M. DX5+NKT cells display phenotypical and functional differences between spleen and liver as well as NK1.1-Balb/c and NK1.1+ C57Bl/6 mice. *BMC Immunol* 2011; **12**:26.
  - 45 Franco A, Paroli M, Testa U *et al.* Transferrin receptor mediates uptake and presentation of hepatitis B envelope antigen by T lymphocytes. *J Exp Med* 1992; **175**:1195–205.
  - 46 Judd W, Poodry CA, Strominger JL. Novel surface antigen expressed on dividing cells but absent from nondividing cells. *J Exp Med* 1980; **152**:1430–5.
  - 47 Shipkova M, Wieland E. Surface markers of lymphocyte activation and markers of cell proliferation. *Clin Chim Acta* 2012; **413**:1338–49.
  - 48 Trowbridge IS, Omary MB. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc Natl Acad Sci USA* 1981; **78**:3039–43.
  - 49 Resendiz-Albor AA, Esquivel R, Lopez-Revilla R, Verdin L, Moreno-Fierros L. Striking phenotypic and functional differences in lamina propria lymphocytes from the large and small intestine of mice. *Life Sci* 2005; **76**:2783–803.
  - 50 Ghazarian L, Simoni Y, Pingris K, Beaudoin L, Lehuen A. [Regulatory role of NKT cells in the prevention of type 1 diabetes]. *Med Sci (Paris)* 2013; **29**:722–8.
  - 51 Calleja S, Vivas S, Santiuste M *et al.* Dynamics of non-conventional intraepithelial lymphocytes-NK, NKT, and gammadelta T in celiac disease: relationship with age, diet, and histopathology. *Dig Dis Sci* 2011; **56**:2042–9.
  - 52 Sollid LM. Molecular basis of celiac disease. *Annu Rev Immunol* 2000; **18**:53–81.
  - 53 Halstensen TS, Brandtzaeg P. TCR gamma/delta + and CD8+TCR alpha/beta + intraepithelial lymphocytes (IEL) express proliferation marker (Ki-67) in the coeliac lesion. *Adv Exp Med Biol* 1995; **371B**:1333–8.
  - 54 Gravano DM, Hoyer KK. Promotion and prevention of autoimmune disease by CD8+ T cells. *J Autoimmun* 2013; **45**:68–79.
  - 55 Roep BO. Islet autoreactive CD8 T-cells in type 1 diabetes: licensed to kill? *Diabetes* 2008; **57**:1156–57.
  - 56 Hue S, Mention JJ, Monteiro RC *et al.* A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. *Immunity* 2004; **21**:367–77.

- 57 Ogasawara K, Hamerman JA, Hsin H *et al.* Impairment of NK cell function by NKG2D modulation in NOD mice. *Immunity* 2003; **18**:41–51.
- 58 Champsaur M, Lanier LL. Effect of NKG2D ligand expression on host immune responses. *Immunol Rev* 2010; **235**:267–85.
- 59 Cheney EE, Wise EL, Bui JD *et al.* A dual function of NKG2D ligands in NK-cell activation. *Eur J Immunol* 2012; **42**:2452–8.
- 60 Maier LM, Howlett SK, Rainbow KM *et al.* NKG2D-RAE-1 receptor-ligand variation does not account for the NK cell defect in nonobese diabetic mice. *J Immunol* 2008; **181**:7073–80.
- 61 Buschard K. What causes type 1 diabetes? Lessons from animal models. *APMIS Suppl* 2011; **132**:1–19.
- 62 Catassi C, Bai JC, Bonaz B *et al.* Non-celiac gluten sensitivity: the new frontier of gluten related disorders. *Nutrients* 2013; **5**:3839–53.

### Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Natural killer group 2D (NKG2D) and DX5 expression intestinal expression levels at different ages. Expression levels of NKG2D (upper panel) and DX5 (lower panel) normalized to beta actin in intestinal tissue of non-obese diabetic (NOD) mice at 8, 13 and 20 weeks. Asterisks signify significance levels in comparison to expression at 8 weeks, unless directly compared by dotted line.