

Gut immune deficits in LEW.1AR1-*iddm* rats partially overcome by feeding a diabetes-protective diet

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doi:10.1111/imm.12457

Received 31 October 2014; revised 27

January 2015; accepted 17 February 2015.

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Summary

The gut immune system and its modification by diet have been implicated in the pathogenesis of type 1 diabetes (T1D). Therefore, we investigated gut immune status in non-diabetes-prone LEW.1AR1 and diabetes-prone LEW.1AR1-*iddm* rats and evaluated the effect of a low antigen, hydrolysed casein (HC)-based diet on gut immunity and T1D. Rats were weaned onto a cereal-based or HC-based diet and monitored for T1D. Strain and dietary effects on immune homeostasis were assessed in non-diabetic rats (50–60 days old) and rats with recent-onset diabetes using flow cytometry and immunohistochemistry. Immune gene expression was analysed in mesenteric lymph nodes (MLN) and jejunum using quantitative RT-PCR and PCR arrays. T1D was prevented in LEW.1AR1-*iddm* rats by feeding an HC diet. Diabetic LEW.1AR1-*iddm* rats had fewer lymphoid tissue T cells compared with LEW.1AR1 rats. The percentage of CD4⁺ Foxp3⁺ regulatory T (Treg) cells was decreased in pancreatic lymph nodes (PLN) of diabetic rats. The jejunum of 50-day LEW.1AR1-*iddm* rats contained fewer CD3⁺ T cells, CD163⁺ M2 macrophages and Foxp3⁺ Treg cells. *Ifng* expression was increased in MLN and *Foxp3* expression was decreased in the jejunum of LEW.1AR1-*iddm* rats; *Ifng/Il4* was decreased in jejunum of LEW.1AR1-*iddm* rats fed HC. PCR arrays revealed decreased expression of M2-associated macrophage factors in 50-day LEW.1AR1-*iddm* rats. Wheat peptides stimulated T-cell proliferation and activation in MLN and PLN cells from diabetic LEW.1AR1-*iddm* rats. LEW.1AR1-*iddm* rats displayed gut immune cell deficits and decreased immunoregulatory capacity, which were partially corrected in animals fed a low antigen, protective HC diet consistent with other models of T1D.

Keywords: diet; gut immunity; M2 macrophage; regulatory T cell; type 1 diabetes.

Introduction

Type 1 diabetes (T1D) results from a heterogeneous disease process caused by poorly understood interactions among several risk genes and environmental factors.¹ Onset occurs between 6 months and > 40 years in humans, reflecting the likelihood that the disease develops via many different pathways. There are several animal models of spontaneous T1D, including the diabetes-prone

BioBreeding (BBdp) rat, Komeda (KDP) rat, LEW.1AR1-*iddm* rat and the most studied model, the NOD mouse. Because these animals are highly inbred with a limited number of pathways to T1D,^{2,3} the relevance to human T1D has been questioned. Translational potential would be increased if outcomes could be demonstrated in more than one animal model.^{4,5}

The LEW.1AR1-*iddm* rat arose from a spontaneous mutation in LEW.1AR1 rats in Hannover, Germany.⁶

Abbreviations: 1AR1 control, non-diabetes-prone LEW.1AR1 rat, cereal-fed; BBdp, diabetes-prone BioBreeding rat; CAI, Cell Activation Index; CDI, Cell Division Index; d, recent onset diabetes; HC, hydrolysed casein; *iddm* (d-CER), LEW.1AR1-*iddm* diabetic rat, cereal-fed; *iddm* (nd-CER), LEW.1AR1-*iddm* non-diabetic (50 days old), cereal-fed rat; *iddm* (nd-HC), LEW.1AR1-*iddm* non-diabetic (50 days old), HC-fed rat; IEL, intraepithelial lymphocytes; LPL, lamina propria lymphocytes; MLN, mesenteric lymph node; nd, non-diabetic; NOD, non-obese diabetic mouse; PLN, pancreatic lymph node; Treg, regulatory T cells

These animals are distinct from the well-characterized NOD model of T1D in that diabetes develops with equal frequency in both male and female animals and unlike the BBdp rat, LEW.1AR1-*iddm* rats are not severely lymphopenic, although recent reports have demonstrated a mild T-cell deficit associated with diabetes onset.^{7,8} Similarities and differences among the various models were recently compared.⁵ Diabetes develops between 60 and 90 days and is characterized by a rapidly progressing insulinitis, which leads to extensive β -cell destruction.⁹ Diabetes can be induced by transferring autoreactive T cells¹⁰ and genetic analyses have identified multiple susceptibility loci.¹¹ In addition to the diabetes-related MHC class II (*Iddm1*) susceptibility locus, *Iddm8* was identified recently as the site of mutation(s) contributing to disease susceptibility in the LEW.1AR1 strain.¹²

Many studies have linked agents first encountered in the gastrointestinal tract to diabetes development, including enteroviruses and wheat proteins.¹ Additional studies have demonstrated increased intestinal permeability in humans and rodents with T1D,^{13–19} suggesting that the gut barrier is impaired. These data point to the gastrointestinal tract as a critical and perhaps global factor in T1D development. Indeed, there is presently a strong emphasis on the role of gut microbiota in T1D and this has re-focused attention on the gut immune system. However, because diet is the main determinant of gut microbiota composition,²⁰ it is important to examine not just gut microbes but also to understand the role of diet as a primary link between gut microbiota, gut immunity and development of T1D.^{1,21,22}

It has been demonstrated that BBdp rats and NOD mice fed low antigen, hydrolysed casein (HC)-based diets (or other non-diabetogenic amino acid sources²³) are protected from T1D compared with animals fed cereal-based, mainly wheat-containing diets.^{1,21,22,24} We have also demonstrated that mesenteric lymph node (MLN) T cells (but not splenic T cells) from BBdp rats proliferate in response to wheat peptides and secrete pro-inflammatory cytokines such as interferon- γ whereas cells from control animals do not,²⁵ demonstrating impaired oral tolerance. Similarly, peripheral blood mononuclear cells from T1D patients have increased proliferation and secretion of T helper type 1 and T helper type 17 cytokines in response to wheat peptides.²⁶ In the current study, we investigated differences in gut immune homeostasis and oral tolerance between the LEW.1AR1 parental strain and the diabetes-prone LEW.1AR1-*iddm* strain. We asked whether feeding a low-antigen HC diet affected T1D development in the LEW.1AR1-*iddm* model. We provide evidence that LEW.1AR1-*iddm* rats have defects in oral tolerance and immunoregulation in the gut and associated immune tissues compared with the parental strain. Furthermore, the HC diet suppressed diabetes development and modified immune cell distribution in LEW.1AR1-*iddm* rats.

Materials and methods

Animals

The LEW.1AR1-*iddm* rat model arose following a spontaneous mutation associated with the telomeric region of rat chromosome 1 (*Iddm8*) in a colony of LEW.1AR1 rats at the Hannover Medical School.¹² They originated from a single breeding cage and have been maintained as a separate strain since the identification of the mutation.⁶ LEW.1AR1-*iddm* rats (abbreviated as '*iddm*' in the figures) and the LEW.1AR1 non-diabetic control strain (abbreviated as '1AR1' in the figures) were obtained from Drs S. Lenzen and D. Wedekind (Hannover Medical School, Hannover, Germany), and were maintained at the Ottawa Hospital Research Institute under specific pathogen-free conditions. The colony was screened quarterly using the Prevalent PCR Rodent Infectious Agent (PRIA) Panel (Charles River, Montreal, QC, Canada) and annually using the Surveillance PRIA Panel (Charles River) and tested negative for the duration of the study. Animals were given free access to water and a standard, cereal-based rodent diet (Teklad Global 18% Protein Rodent diet; Harlan, Montreal, QC, Canada) or an iso-nitrogenous, semi-purified HC diet.²⁷ Some animals were removed at 50 days or 60 days of age before development of T1D and are designated as non-diabetic (nd). Rats were monitored biweekly for glucosuria and diabetes onset was confirmed when fasting blood glucose exceeded 11.1 mmol/mM. All diabetic animals (abbreviated as 'd') were killed within 48 hr of diagnosis. Experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care. Studies were approved by the Ottawa Hospital Research Institute Animal Care Committee.

Flow cytometry

Immune cell phenotyping was performed in diabetic rats within 48 hr of onset, as well as 50-day-old normoglycaemic diabetes-prone rats or age-matched controls. Splenocyte, MLN and pancreatic lymph node (PLN) cell suspensions were washed in flow cytometry buffer (Iso-Flow™ sheath fluid; Beckman Coulter, Mississauga, ON, Canada) supplemented with 1% BSA. Aliquots of 5×10^5 cells were then incubated with combinations of the following rat-specific antibodies for 30 min at 4° in the dark: FITC-conjugated (-FITC) or phycoerythrin-conjugated (-PE) $\alpha\beta$ TCR, CD8 α -PE (Cedarlane Laboratories, Burlington, ON, Canada), anti- κ chain-FITC, CD4-PE-cyanine 5 (-PC5), CD86-PE (BD Biosciences, Mississauga, ON, Canada), CD11c-FITC, CD103-biotin, CD68-FITC and CD163-PE (AbD Serotec, Raleigh, NC). Analysis of regulatory T (Treg) cells was performed using the PE-conjugated anti-mouse/rat/human Foxp3 Flow Kit (Biolegend, San Diego, CA) and anti-CD4-PC5 antibodies. Phenotyping and cell proliferation analyses were

performed using a Beckman Coulter FC500 flow cytometer equipped with CXP software.

Immunohistochemistry

Morphometric analysis was performed on Bouin's-fixed tissues as described previously.²² Briefly, paraffin-embedded jejunum sections from 50-day-old LEW.1AR1-*iddm* and LEW.1AR1 rats were incubated with anti-CD3 (Abcam, Toronto, ON, Canada), anti-CD8 α (BD Biosciences, Mississauga, ON, Canada), anti-Foxp3 (eBioscience, San Diego, CA) or anti-CD163 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies before the addition of appropriate biotinylated secondary antibodies. Antibody labelling was detected using VECTASTAIN[®] ABC reagent (Vector Laboratories Canada Inc.), diaminobenzidine and H₂O₂. Sections were counterstained with haematoxylin. Analysis of coded sections was performed using a Zeiss Axioplan2 microscope and NORTHERN ECLIPSE morphometry software (Empix Imaging, Mississauga, ON, Canada).²⁸ Small intestine immune cell populations were quantified as the number of lamina propria lymphocytes per mm² mucosal area (CD163, CD3 and Foxp3) or as the number of intraepithelial lymphocytes per 100 epithelial cells (CD8 α).

Insulinitis was evaluated using coded haematoxylin & eosin-stained sections from 50-day-old cereal-fed and HC-fed LEW.1AR1-*iddm* rats by assessing immune cell infiltration per islet and islet number per cm² section area as described previously.²⁹

Gene expression assays

Jejunum and MLN from 50-day-old rats were snap-frozen in liquid N₂ and stored at -80°. RNA was extracted using TRIzol (Life Technologies, Burlington, ON, Canada). Synthesis of cDNA was performed using M-MLV Reverse Transcriptase (Life Technologies). The reaction was incubated at 42° for 90 min and then inactivated for 5 min at 90° in a thermocycler. Quantitative RT-PCR analysis was performed using an ABI Prism[®] 7000 Sequence Detection System (Life Technologies) and TaqMan[®] Gene Expression Assays (Life Technologies) for the following genes: *Foxp3*, *Ctla4*, *Ifng*, *Il4*, *Il23*, *Il10*, *Il17a*, *Tgfb1* and *Tnf*.

PCR array analysis

Total RNA was isolated from jejunum of 50-day-old cereal-fed LEW.1AR1 (CER, *n* = 3), normoglycaemic cereal-fed LEW.1AR1-*iddm* (nd-CER, *n* = 4) and normoglycaemic HC-fed LEW.1AR1-*iddm* (nd-HC, *n* = 4) rats using the NucleoSpin[®] RNA II Kit (Macherey-Nagel, Bethlehem, PA). RNA quantity and integrity were evaluated on a 6000 Nano LabChip[®] using an Agilent 2100 Bioanalyzer (OHRI Stemcore Laboratories, Ottawa, ON,

Canada). RNA was transcribed to cDNA using the RT² First Strand cDNA synthesis kit (Qiagen, Toronto, ON, Canada). Expression of a focused panel of immune-related genes was measured using the Innate and Adaptive Immune Responses RT² Profiler[™] PCR Array System (Qiagen).

T-cell proliferation assays

Single-cell suspensions were prepared from spleen and MLN of 60- to 90-day-old diabetic LEW.1AR1-*iddm* (d-CER) rats or age-matched controls. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Sigma, Oakville, ON, Canada), 25 mM HEPES, 2.0 mM/l L-glutamine, 50 μ M β -mercaptoethanol, 100 U/ml penicillin and 100 μ g/ml streptomycin in the presence of medium alone, 1 μ M ovalbumin, 2.7 Lf/ml tetanus toxoid, 1.5 μ g/ml concanavalin A (ConA; Sigma-Aldrich, Oakville, ON, Canada) or chymotrypsin-treated wheat gluten proteins at 37° and 5% CO₂ for 7 days. Cell cultures with ConA were divided as required and supplemented with 10 U/ml interleukin-2 (IL-2; Peprotech, Dollard des Ormeaux, QC, Canada). In some cases, cells were labelled with 5 μ M carboxyfluoresceinsuccinimidyl ester (CFSE; Life Technologies) for 20 min at 37° in PBS before antigen stimulation. T-cell proliferation was measured by determining the Cell Division Index (CDI).²⁶ Briefly, the number of CFSE^{dim} events was normalized to 5000 CFSE^{bright} events for each condition, and the CDI was calculated according to the following formula for each antigen tested:

CDI =

$$\frac{\text{Number of } \alpha\beta\text{TCR}^+\text{CD4}^+\text{CFSE}^{\text{dim}} \text{ cells with antigen}}{\text{Number of } \alpha\beta\text{TCR}^+\text{CD4}^+\text{CFSE}^{\text{dim}} \text{ cells without antigen}}$$

PLN cells were not labelled with CFSE because of the scarcity of this population. Instead, a Cell Activation Index (CAI) was calculated by measuring increased expression of CD25 in $\alpha\beta\text{TCR}^+\text{CD4}^+$ cells incubated with antigen compared with cells incubated without antigen from 60-day-old LEW.1AR1 (CER) and LEW.1AR1-*iddm* (nd-CER) rats according to the formula (number of CD25⁻ events was normalized to 5000 CD25⁺ events):

CAI =

$$\frac{\text{Number of } \alpha\beta\text{TCR}^+\text{CD4}^+\text{CD25}^+ \text{ cells with antigen}}{\text{Number of } \alpha\beta\text{TCR}^+\text{CD4}^+\text{CD25}^+ \text{ cells without antigen}}$$

Statistics

The Kaplan–Meier survival curve was analysed using the log-rank test in PRISM statistical software (GraphPad, La Jolla, CA). Age of diabetes onset in LEW.1AR1-*iddm*

(CER) and LEW.1AR1-*iddm* (HC) rats was compared using *t*-test (STATISTICA; StatSoft, Tulsa, OK). PCR Array data were analysed using the RT² PROFILER™ PCR Array Data Analysis software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>). CDI and CAI data were analysed by factorial analysis of variance with Scheffé post-hoc analysis. Other data were analysed by analysis of variance using STATISTICA with Scheffé post-hoc analysis. Data are presented as mean ± standard deviation (SD) unless noted; *P* < 0.05 was considered to be statistically significant.

Results

Fewer diabetes cases in HC-fed LEW.1AR1-*iddm* rats

Fewer animals fed the HC diet developed diabetes compared with those fed the cereal-based diet [incidence 38.5% (10/26) versus 62% (44/71); *P* = 0.017] and disease onset (defined as the initial detection of fasting hyperglycaemia) was delayed [mean age of 66 ± 7 days (*n* = 10) versus mean age of 59 ± 7 days (*n* = 44), *P* = 0.009, Fig. 1]. No differences in insulinitis were observed (Table 1).

Cereal-fed diabetic LEW.1AR1-*iddm* rats display deficits in T-cell numbers compared with cereal-fed LEW.1AR1 controls

In spleen, MLN and PLN cells from cereal-fed diabetic LEW.1AR1-*iddm* rats, the proportion of αβTCR⁺ T cells was smaller than LEW.1AR1 controls (Fig. 2a). T-cell deficiencies were particularly striking in the αβTCR⁺ CD4⁺ cell population (Fig. 2b) whereas αβTCR⁺ CD8⁺ cells were deficient in spleen cells, but not MLN and PLN cells (Fig. 2c). No significant differences were observed for markers of dendritic cells (CD86, CD11c, CD103) and macrophages (CD68, CD163) in spleen, MLN and PLN, but significantly higher numbers of κ-chain⁺ cells were observed in the spleen of cereal-fed diabetic LEW.1AR1-*iddm* rats (data not shown).

Because Foxp3⁺ Treg cells could be deficient and/or defective in T1D,^{30–33} we quantified Treg cells in spleen, MLN and PLN of cereal-fed diabetic LEW.1AR1-*iddm* rats. CD4⁺ Foxp3⁺ cells were significantly decreased in PLN (Fig. 3a,b). Because rats fed an HC diet were protected from diabetes (Fig. 1), we investigated potential diet-induced differences in the CD4⁺ Foxp3⁺ cells in normoglycaemic 50-day LEW.1AR1-*iddm* rats fed cereal (nd-CER) and LEW.1AR1-*iddm* rats fed HC (nd-HC). HC-fed 50-day rats had significantly more CD4⁺ Foxp3⁺ MLN cells compared with cereal-fed LEW.1AR1-*iddm* rats. Though not significantly different from cereal-fed LEW.1AR1-*iddm* rats (*P* = 0.07), the number of CD4⁺ Foxp3⁺ cells in PLN of HC-fed LEW.1AR1-*iddm*

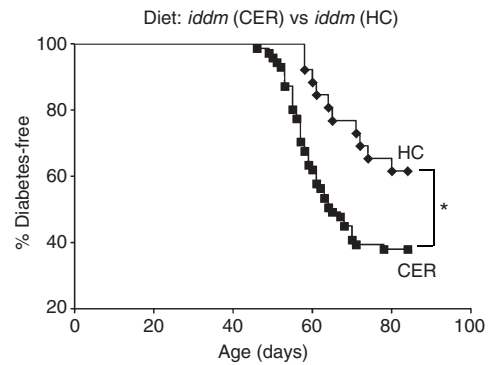


Figure 1. Diabetes incidence in LEW.1AR1-*iddm* rats modified by diet. Fewer hydrolysed casein (HC)-fed LEW.1AR1-*iddm* rats developed diabetes (10/26, diamonds) compared with cereal-fed animals (44/71, squares; 38.5% versus 62%, *P* = 0.017). Kaplan–Meier survival curve was analysed by log-rank test.

Table 1. Insulinitis was evaluated by assessing immune cell infiltration per islet and islet number per cm² section area

Diet	Number	Age/Status	Insulinitis (±SD)
Cereal	9	50 days (non-diabetic)	1.28 (±0.36)
Cereal	24	60–90 days (diabetic)	4.48 (±0.62)
Cereal	10	90 days (asymptomatic)	2.00 (±1.33)
HC	8	50 (non-diabetic)	1.06 (±0.18)
HC	10	60–90 days (diabetic)	4.40 (±0.70)
HC	15	90 days (asymptomatic)	1.23 (±0.53)

No significant difference in insulinitis scores was observed among age-matched cereal-fed and hydrolysed casein (HC)-fed LEW.1AR1-*iddm* rats.

rats was increased to levels similar to control LEW.1AR1 rats, suggesting a rationale for the protective effects of the HC diet (Fig. 3c,d).

Fewer Foxp3⁺ cells and M2 macrophages in jejunum of LEW.1AR1-*iddm* rats

Consistent with results from MLN, analysis of the pan-T-cell marker CD3 showed that 50-day non-diabetic LEW.1AR1-*iddm* rats had fewer T cells in the lamina propria compartment than controls (Fig. 4a); this was also reflected in the intraepithelial CD8α⁺ T-cell subset (Fig. 4b). Regardless of diet, 50-day non-diabetic LEW.1AR1-*iddm* rats had fewer Foxp3⁺ cells in the lamina propria (Fig. 4c). HC-fed LEW.1AR1-*iddm* rats had fewer CD3⁺ and CD8α⁺ cells than cereal-fed LEW.1AR1-*iddm* rats (Fig. 4a,b) and the Foxp3⁺/CD3⁺ ratio was higher (Fig. 4d). These data suggest that the HC diet decreased effector T cells and increased the proportion of Treg cells in the lamina propria, consistent with flow cytometry analyses of MLN.

We also analysed the number of CD163⁺ cells in the lamina propria (Fig. 4e). CD163 is a scavenger receptor

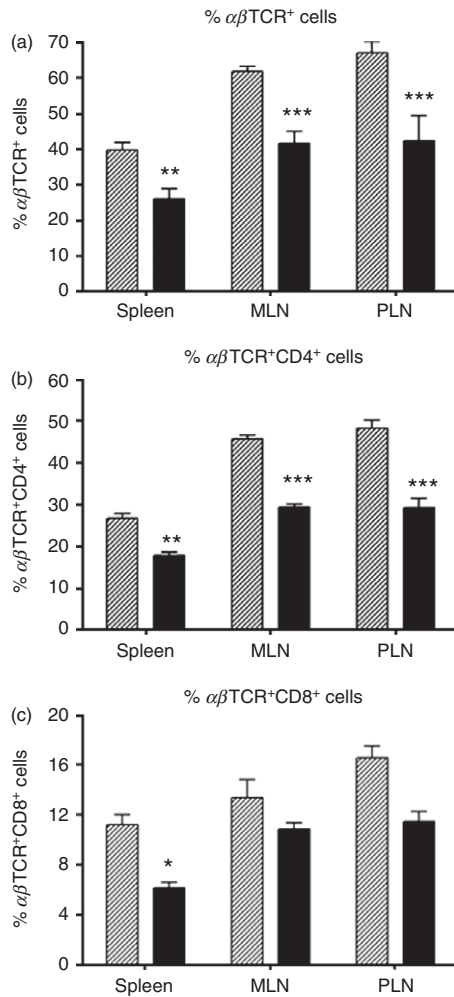


Figure 2. T-cell deficits in diabetic LEW.1AR1-*iddm* rat spleen, mesenteric lymph node (MLN) and pancreatic lymph node (PLN). (a) Diabetic LEW.1AR1-*iddm* rats (solid bars, $n = 5-6$) had fewer $\alpha\beta$ TCR⁺ cells compared with control LEW.1AR1 rats (hatched bars, $n = 7$ or 8) in all three tissues; (b) diabetic rats were also deficient in $\alpha\beta$ TCR⁺ CD4⁺ cells and (c) a significant decrease in $\alpha\beta$ TCR⁺ CD8⁺ cells was observed in the spleen. Data are mean \pm SD, analysis of variance followed by Scheffé post-hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

expressed by tissue-resident macrophages, which have anti-inflammatory activity and are associated with the M2 phenotype.³⁴ Regardless of diet, 50-day diabetes-prone rats had fewer CD163⁺ cells compared with the control strain, suggesting that M2 macrophages are deficient in LEW.1AR1-*iddm* rats similar to the BBdp rat.²²

Increased pro-inflammatory cytokines in jejunum and MLN of 50-day LEW.1AR1-*iddm* rats

Previous studies in the BBdp rat have demonstrated a pro-inflammatory cytokine bias in the gut and associated lymphoid tissues.²⁵ We hypothesized that a similar

situation prevails in the gut-associated lymphoid tissues of the LEW.1AR1-*iddm* rat. We investigated cytokine mRNA expression in MLN and jejunum of 50-day non-diabetic rats using quantitative RT-PCR.

Expression of *Il10*, *Il4*, *Il17a*, *Il23*, *Tgfb1*, *Tnf* and *Ifng* in MLN was compared among cereal-fed (control) LEW.1AR1, LEW.1AR1-*iddm* and HC-fed LEW.1AR1-*iddm* rats. Expression of *Ifng* was significantly increased in 50-day cereal-fed LEW.1AR1-*iddm* rats compared with controls (Fig. 5a). No differences were observed for other cytokines (*Tnf*, *Il23*, *Il17a*, *Tgfb1*, *Il10*) or *Il4* expression (Fig. 5b). Cereal-fed LEW.1AR1-*iddm* rats had an increased *Ifng/Il4* ratio^{25,35} in MLN compared with the parental cereal-fed LEW.1AR1 strain (Fig. 5c), similar to the T helper type 1 bias we observed in BBdp rats.²⁵ *Foxp3* (Fig. 5d) and *Ctla4* (Fig. 5e) gene expression was significantly decreased in the jejunum of 50-day cereal-fed LEW.1AR1-*iddm* rats compared with the control strain. The decreased ratio of *Ifng/Il4* in jejunum of young 50-day HC-fed diabetes-prone rats (Fig. 4f) suggested that the protective HC diet decreased inflammatory responses in the lamina propria.

Next, we used RT² ProfilerTM PCR Arrays to investigate the expression of a panel of genes associated with innate and adaptive immune responses in the jejunum of cereal-fed control LEW.1AR1 rats ($n = 3$), cereal-fed diabetes-prone LEW.1AR1-*iddm* rats ($n = 4$), as well as HC-fed LEW.1AR1-*iddm* rats ($n = 4$ /group). Cereal-fed LEW.1AR1-*iddm* rats had significantly decreased expression of *Fn1* and *Stab1* (Fig. 6a), both of which are associated with the M2 macrophage phenotype. This is consistent with the immunohistochemistry data showing fewer CD163⁺ cells in the lamina propria (Fig. 4e). *Cxcr4* and *Casp1* were also down-regulated in diabetes-prone rats (Fig. 6a). In contrast, comparison of gene expression in the jejunum of cereal-fed and HC-fed LEW.1AR1-*iddm* rats demonstrated that the cereal diet decreased expression of the anti-inflammatory genes *Nfkb1a* and *Il1f10* (Fig. 6b).

Cereal-fed LEW.1AR1-*iddm* rats have increased T-cell proliferation in MLN and increased CD25 expression in PLN in response to wheat peptides

Wheat peptides induce T-cell proliferation and pro-inflammatory cytokine production in subjects with T1D.^{25,26} Therefore, we incubated CFSE-labelled splenocytes and MLN cells with hydrolysed wheat gluten or control antigens for 7 days and measured $\alpha\beta$ TCR⁺ CD4⁺ T-cell proliferation by flow cytometry in cereal-fed diabetic LEW.1AR1-*iddm* (d-CER, $n = 6$) and age-matched cereal-fed LEW.1AR1 rats ($n = 4$). MLN cells from diabetic rats displayed increased proliferation to wheat peptides compared with control animals and control antigens

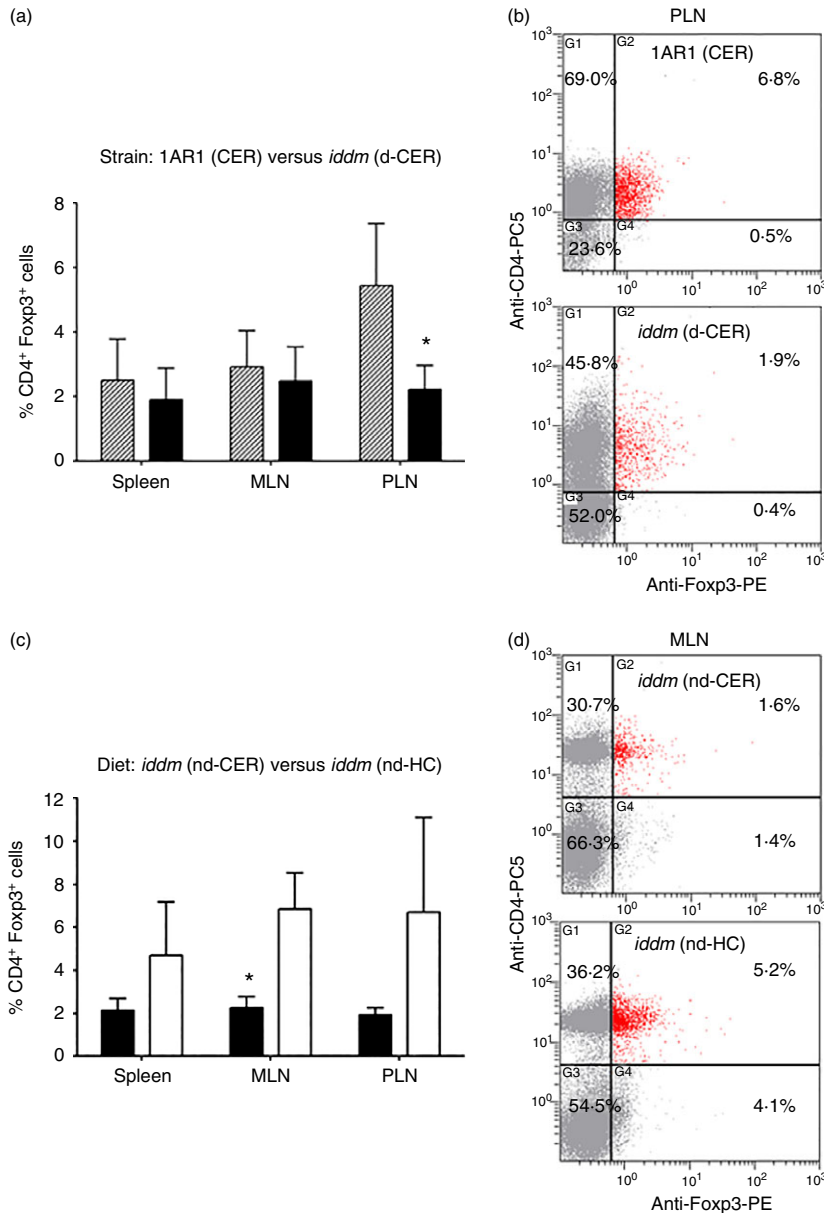


Figure 3. Cereal-fed LEW.1AR1-*iddm* rats have fewer Foxp3⁺ CD4⁺ regulatory T (Treg) cells in pancreatic lymph nodes (PLN) – hydrolysed casein (HC) diet increases mesenteric lymph node (MLN) Treg cells. (a) Diabetic LEW.1AR1-*iddm* (d-CER) rats (solid bars, *n* = 4 or 5) had fewer Treg cells in PLN compared with control LEW.1AR1 (CER) animals (hatched bars, *n* = 7 or 8); (b) representative scatterplot from PLN cells; (c) 50-day LEW.1AR1-*iddm* (nd-HC) rats (open bars, *n* = 7 or 8) had increased Treg cells in MLN compared with age-matched LEW.1AR1-*iddm* (nd-CER) rats (filled bars, *n* = 5 or 7); (d) representative scatterplot from MLN. Data are mean ± SD, analysis of variance followed by Scheffé post-hoc test. **P* < 0.05.

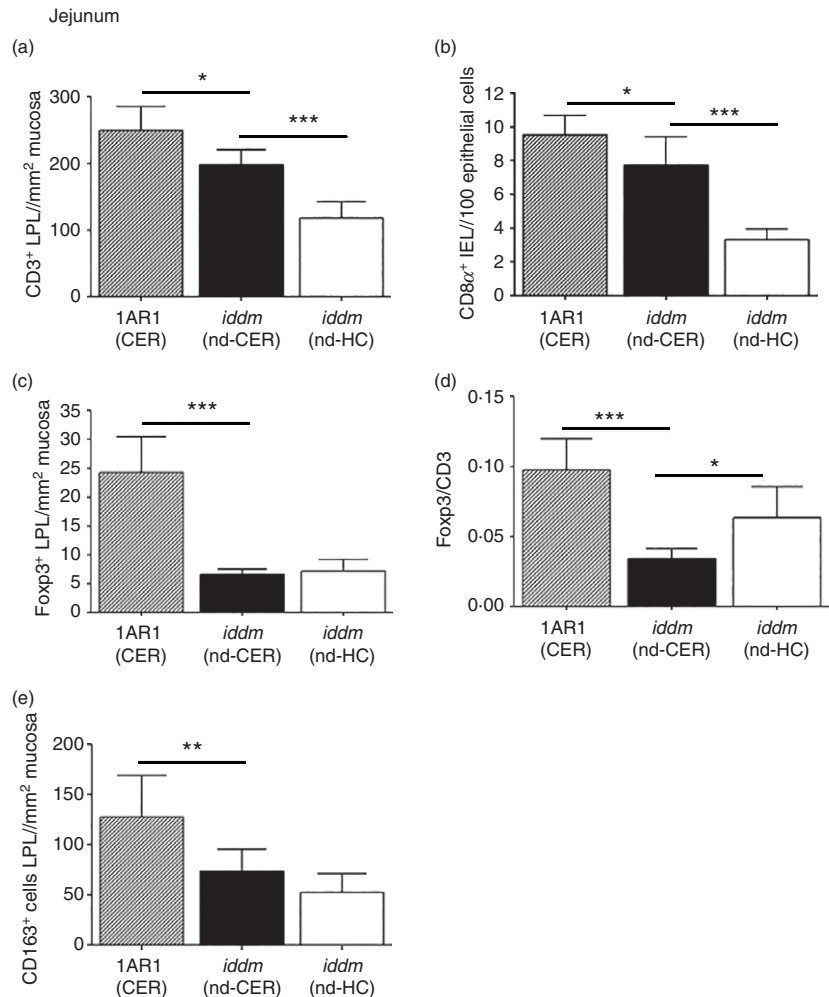
(Fig. 7a). Splenocytes did not respond to wheat peptide stimulation in either strain.

We also measured T-cell activation in response to wheat peptides in MLN and PLN cells from normoglycaemic 60-day cereal-fed LEW.1AR1-*iddm* and LEW.1AR1 rats (*n* = 6/group). After 7 days in culture, the cells were labelled with CD25-FITC, $\alpha\beta$ TCR⁺-PE and CD4-PC5 antibodies and analysed by flow cytometry. CD25 expression was increased in LEW.1AR1-*iddm* MLN cells in response to wheat peptides but not to control ovalbumin (Fig. 7b) and tetanus toxoid (data not shown) antigens as observed in the cell proliferation assays (Fig. 7a); splenocytes did not show increased expression of CD25. Interestingly, PLN also showed increased CD25 expression in response to wheat peptides, suggesting that wheat-specific T cells traffic to the PLN³⁶ (Fig. 7c).

Discussion

Current evidence suggests that both genetic predisposition and environmental factors act in concert to break self-tolerance and induce T1D. We recently demonstrated²² that antigen-rich cereal diets provoke pro-inflammatory immune responses in diabetes-prone rats with underlying defects in gut immune regulation, which can be corrected by feeding a low antigen hydrolysed casein diet. Studies in humans have shown that T1D is associated with abnormal responses to dietary antigens.^{37,38} In particular, we demonstrated that peripheral blood mononuclear cells from ~50% of human patients displayed pro-inflammatory responses to wheat peptides and other dietary proteins *in vitro*.²⁶ In the current study, cereal-fed LEW.1AR1-*iddm* rats had fewer Foxp3⁺ Treg

Figure 4. Lamina propria lymphocytes (LPL) and macrophages in jejunum of 50-day LEW.1AR1 and LEW.1AR1-*iddm* rats fed a cereal or hydrolysed casein (HC) diet. Immunohistochemical analysis of LPL populations in 50-day LEW.1AR1 (CER) (hatched bars, $n = 7$), LEW.1AR1-*iddm* (nd-CER) (solid bars, $n = 7$) and LEW.1AR1-*iddm* (nd-HC) rats (open bars, $n = 8$). (a) The number of CD3⁺ LPL was larger in cereal-fed LEW.1AR1(CER) rats compared with LEW.1AR1-*iddm* (nd-CER) rats, which had more CD3⁺ cells than HC-fed LEW.1AR1-*iddm* (nd-HC) rats as also seen in (b) the number of CD8 α ⁺ intraepithelial lymphocytes; (c) LEW.1AR1 (CER) had more Foxp3⁺ LPL than LEW.1AR1-*iddm* (nd-CER) rats; (d) LEW.1AR1-*iddm* (nd-CER) rats had the lowest Foxp3/CD3⁺ ratio compared with LEW.1AR1 (CER) rats and LEW.1AR1-*iddm* (nd-HC) fed rats; (e) LEW.1AR1 (CER) rats had more CD163⁺ M2 macrophages than LEW.1AR1-*iddm* (nd-CER) rats. Data are mean \pm SD, analysis of variance followed by Scheffé post-hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



cells and CD163⁺ M2 cells in the jejunum associated with an increased *Ifng/Il4* ratio in the MLN and associated immune tissues. Additionally, we demonstrated increased T-cell proliferation and activation in response to wheat peptides, characteristic of impaired oral tolerance. We also observed increased T-cell activation in the PLN where β -cell-specific effector T cells are activated, suggesting these PLN-resident, wheat-specific cells participate in the autoimmune attack. Furthermore, the low antigen HC diet protected a subset of animals from diabetes development in association with increased CD3⁺ Foxp3⁺ cells in the lamina propria and MLN and a decreased *Ifng/Il4* ratio in the jejunum, indicating that a substantial portion of diabetes in the LEW.1AR1-*iddm* rat is gut-associated and linked with exposure to dietary antigens. Diabetes development in human patients³⁹ and other rodent models^{13,15,16,40} can be associated with enteropathy and increased gut permeability, and the HC diet ameliorated the permeability defects in the BBdp rat.¹⁸ Whereas histological evidence of overt enteropathy was not observed in this study, it would be beneficial

to investigate gut permeability in this model in future studies.

Gene expression analysis of jejunum from cereal-fed and HC-fed LEW.1AR1-*iddm* rats demonstrated that cereal-fed rats had decreased expression of *Nfkbia* and *Il1f10*. *Nfkbia* encodes nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α protein, which inhibits nuclear factor- κ B activity, and has been linked to inflammation and autoimmunity.⁴¹ Interleukin-1f10 (also known as IL-38) negatively regulates IL-17 and IL-22 production through IL-36 receptor.⁴² This suggests that the anti-inflammatory effects of the low antigen HC diet are mediated through suppression of the nuclear factor- κ B and Th17/Th22 pathways, combined with an increase in the proportion of Treg cells.

We observed decreased T-cell numbers in both the MLN and lamina propria immune compartments, in agreement with recent reports of decreased CD3⁺ and CD4⁺ cells in the periphery of (cereal-fed) LEW.1AR1-*iddm* rats.^{7,8} Arndt *et al.* demonstrated that the variability in T-cell frequency was associated with T1D development

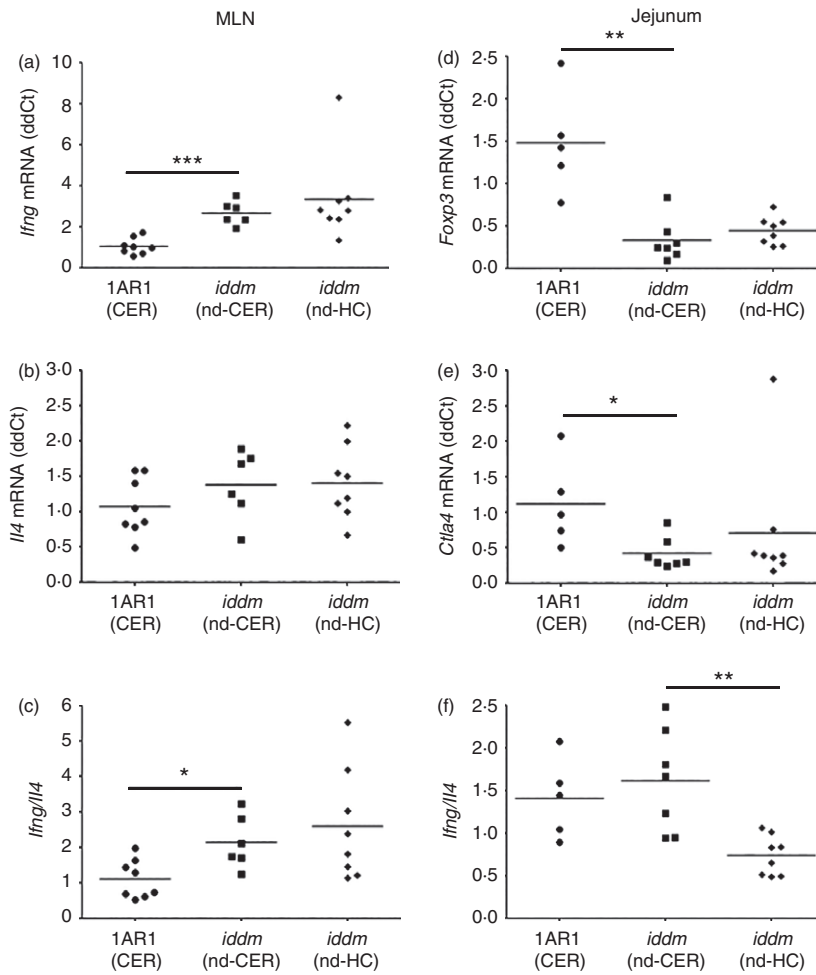


Figure 5. Cereal-fed 50-day LEW.1AR1-*iddm* rats have a pro-inflammatory bias in the mesenteric lymph nodes (MLN) and jejunum. Quantitative RT-PCR analyses of 50-day rat MLN (a–c) and jejunum (d–f) in LEW.1AR1 (CER) (filled circles, $n = 5–8$), LEW.1AR1-*iddm* (nd-CER) (filled squares, $n = 6–7$) and LEW.1AR1-*iddm* (nd-HC) (filled diamonds, $n = 8$) rats: (a) increased expression of *Ifng* in LEW.1AR1-*iddm* (nd-CER) versus LEW.1AR1 (CER) MLN; (b) no difference in *Il4* expression in MLN; (c) increased *Ifng/Il4* ratio in the LEW.1AR1-*iddm* (nd-CER) MLN compared with controls; (d) high levels of *Foxp3* and (e) *Ctla4* in LEW.1AR1 (CER) rat jejunum compared with LEW.1AR1-*iddm* (nd-CER) rats; (f) ratio of *Ifng/Il4* increased in LEW.1AR1-*iddm* (nd-CER) compared with LEW.1AR1-*iddm* (nd-HC) rats. Mean \pm SD, analysis of variance followed by Scheffé post-hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

and linked to the *Iddm8* susceptibility locus.^{7,8} This is similar to the mild T-cell deficits observed in the NOD mouse model,⁴³ and reminiscent of the more severe lymphopenia observed in the BBdp rat.⁴⁴ T-cell homeostatic expansion under lymphopenic conditions promotes the development of autoreactive T cells^{45–47} particularly in the absence of a robust regulatory immune system, possibly because expansion of effector cells outpaces Treg cell proliferation in a lymphopenic environment.⁴⁸ Imbalances between regulatory and effector T cells in diabetes have been reported,^{22,46,49} and Treg deficiencies have effects on other regulatory populations, further biasing the immune response to a pro-inflammatory phenotype.^{50,51} Additionally, we observed decreased expression of the chemokine receptor CXCR4, which participates in T-cell recruitment in partnership with CXCL12, in diabetes-prone rats. Blockade of the CXCR4/CXCL12 pathway causes T1D in the NOD mouse,⁵² and may be particularly relevant for the maintenance of the PLN Treg population.⁵³

We recently reported that CD163⁺ macrophages are deficient in cereal-fed BBdp rats²² and observed putative

autoantibodies to anti-inflammatory CD163⁺ intestinal macrophages in a patient with T1D and coeliac disease.⁵⁴ We observed decreased numbers of CD163⁺ M2 macrophages in the lamina propria of LEW.1AR1-*iddm* rats and analysis of gene expression by PCR array demonstrated that the CD163⁺ cell deficit was associated with decreased expression of the M2-macrophage-associated genes *Stab1* and *Fn1*. Fibronectin (*Fn1*) is an extracellular matrix protein associated with the wound-healing process. Stabilin-1 (*Stab1*) is a phagocytic receptor on M2 macrophages^{55,56} that has been reported to mediate remodelling of the extracellular-matrix via endocytosis of SPARC (secreted protein acidic and rich in cysteine).⁵⁷ In addition, endothelial expression of stabilin-1 supports leucocyte extravasation, and has been shown to support preferential migration of Treg cells into sites of inflammation.⁵⁸ While the HC diet did not correct the CD163⁺ cell deficit, it did significantly decrease the *Ifng/Il4* ratio, suggesting that the HC diet impacted M1/M2-associated cytokine balance.

In summary, combined defects in adaptive and innate immune regulation result in a persistent inflammatory

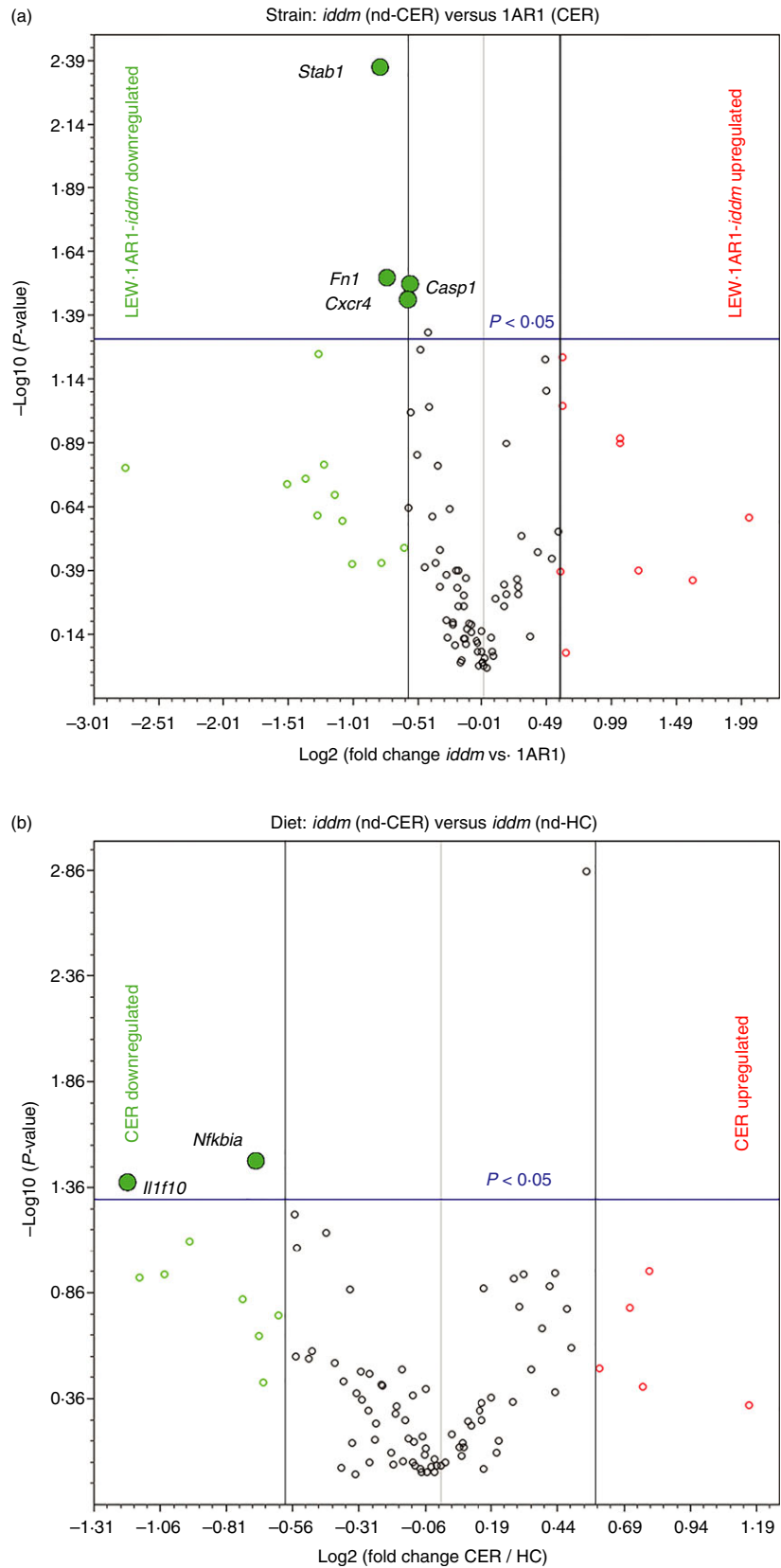


Figure 6. Strain- and diet-related changes in pro-inflammatory and regulatory gene expression in LEW.1AR1-*iddm* jejunum. Young 50-day cereal-fed LEW.1AR1 rats, LEW.1AR1-*iddm* (nd-CER) rats, and LEW.1AR1-*iddm* (nd-HC) rats were profiled by PCR array for intestinal expression of a panel of innate and adaptive immune genes. (a) Strain comparison: LEW.1AR1-*iddm* (nd-CER) ($n = 4$) versus LEW.1AR1 (CER) ($n = 3$); results presented as a volcano plot of gene expression in LEW.1AR1-*iddm* rats relative to LEW.1AR1 rats. (b) Diet comparison: volcano plot shows gene expression in LEW.1AR1-*iddm* (nd-CER) versus LEW.1AR1-*iddm* (nd-HC) ($n = 4$) rats, vertical (black line) boundaries were set at ± 1.5 -fold change and horizontal (blue line) denotes $P = 0.05$ significance cut-off.

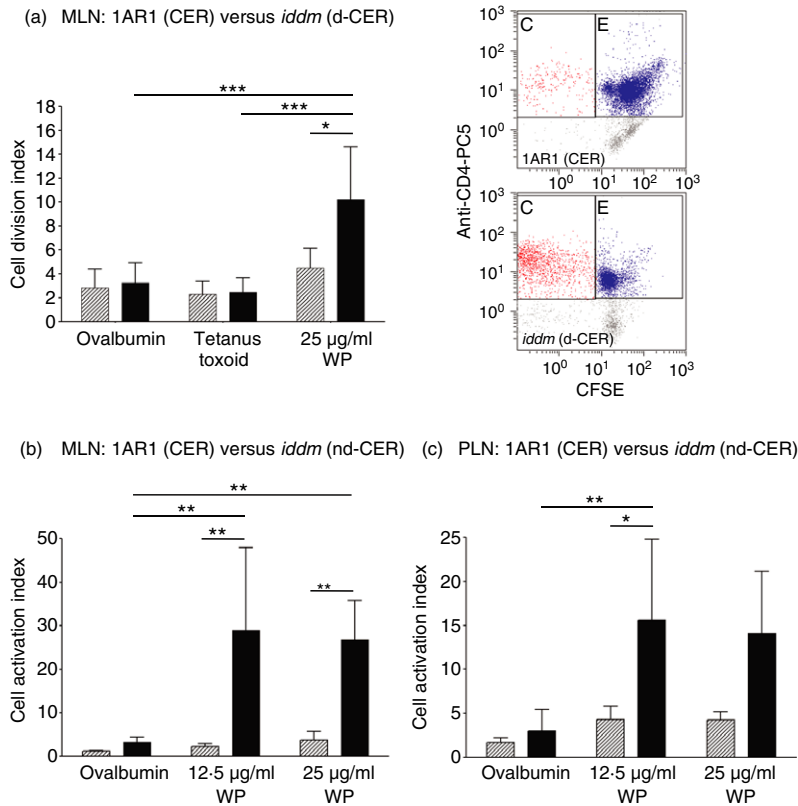


Figure 7. Mesenteric lymph node (MLN) and pancreatic lymph node (PLN) T-cell reactivity to wheat peptides. (a) CFSE-labelled, $\alpha\beta\text{TCR}^+ \text{CD4}^+$ MLN cells from cereal-fed diabetic LEW.1AR1-*iddm* rats ($n = 6$, solid bars) incubated with wheat peptides (WP) displayed increased proliferation compared with cereal-fed LEW.1AR1 rats ($n = 4$, hatched bars) and with cells incubated with control antigens (ovalbumin and tetanus toxoid); representative dot plots are shown; (b) increased numbers of $\text{CD25}^+ \text{CD4}^+ \alpha\beta\text{TCR}^+$ MLN cells were observed in cereal-fed normoglycaemic 60-day LEW.1AR1-*iddm* rats ($n = 6$, solid bars) versus cereal-fed LEW.1AR1 control rats ($n = 6$, hatched bars) when stimulated with 12.5 µg/ml WP and 25 µg/ml WP and compared with ovalbumin; (c) number of $\text{CD25}^+ \text{CD4}^+ \alpha\beta\text{TCR}^+$ PLN cells was increased in response to 12.5 µg/ml WP in diabetes-prone rats compared with controls and ovalbumin. Data are mean \pm SD, factorial analysis of variance followed by Scheffé post-hoc test. Representative dot plots are shown. * $P < 0.05$ (except in (a) where $P = 0.050$), ** $P < 0.01$, *** $P < 0.001$.

bias in the gut of LEW.1AR1-*iddm* rats fed a cereal-based diet, leading to a loss of tolerance towards dietary antigens. These deficits were partially corrected by feeding a low antigen, hydrolysed casein diet, in association with protection from diabetes. A defect in M2 macrophage function would impair anti-inflammatory responses such as the clearance of apoptotic cellular debris,⁵⁹ predisposing individuals to chronic inflammation, particularly in a lymphopenic environment that favours the development of autoimmunity.^{45–47} These combined risk factors could be critical for T1D development, as suggested by our recent studies, which demonstrate innate immunoregulatory defects in the BBdp rat,²² and a study in which T1D was prevented in NOD mice by adoptive transfer of M2 macrophages.⁶⁰

Acknowledgements

This work was supported by the Canadian Institutes of Health Research and the Canadian Diabetes Association. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

C. Patrick was supported by the Queen Elizabeth II Graduate Scholarship in Science and Technology, Ontario Graduate Scholarship program and Ontario Graduate Scholarships in Science and Technology; J.A. Noel was

supported by the Natural Sciences and Engineering Research Council and by the Ontario Graduate Scholarship program.

JAC, CP and GSW performed the experiments, analysed the data and wrote the manuscript. JAN performed the experiments and analysed the data. FWS conceived and designed the study, analysed the data, and wrote the manuscript. FWS is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Parts of this work were presented at the 70th Scientific Sessions of the American Diabetes Association (Orlando, USA, *Diabetes* 2010;59 (Suppl. 1):OR-333), and at the 2nd International Conference on Immune Tolerance (Amsterdam, the Netherlands, 2011).

We are grateful to Dr S. Lenzen and Dr D. Wedekind (Hannover Medical School) for the kind gift of LEW.1AR1 and LEW.1AR1-*iddm* rats. We thank Dr C. Kennedy, A. Côté, A. Hendin, G. Rodger and P. Bradley (OHRI) for technical assistance.

Disclosures

No potential conflicts of interest relevant to this article were reported.

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