Long-chain inulin increases dendritic cells in the Peyer's patches and increases *ex vivo* cytokine secretion in the spleen and mesenteric lymph nodes of growing female rats, independent of zinc status

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Prebiotics may increase Zn absorption, a mineral known to play a central role in the immune system. Zn-deficient states are characterised by suppressed immune function, while prebiotics may improve both gut and cell-mediated immunity. Our objective was to determine if inulin alters the number and proportion of immune cells in the spleen, mesenteric lymph nodes (MLN) and Peyer's patches (PP), *ex vivo* cytokine secretion, intestinal permeability and Zn status in healthy as well as Zn-deficient rats. Weanling female rats were fed diets supplemented with 5 % cellulose (CEL) or 5 % inulin (PRE) for 4 weeks. The rats received the CEL or PRE diet *ad libitum* (ZN) or in restricted amounts (DR), or deficient in Zn (ZD) for another 4 weeks. The PRE-fed rats had a higher number and proportion of dendritic cells in PP, and greater *ex vivo* secretion of IL-2, IL-10 and interferon-γ from spleen and MLN cells compared with CEL-fed rats. PRE reduced the number and proportion of T cell receptor (TCR)-αβ⁺CD8⁺ cells in spleen and CD45RA⁺ cells in MLN compared with CEL. ZD rats had lower serum IgG2a and T cell numbers in MLN compared with ZN and DR rats. TCRγδ⁺ cell numbers in PP were higher in ZD-PRE rats compared with ZD-CEL rats. Femur Zn concentrations of DR-PRE rats were higher than those of DR-CEL rats. Intestinal permeability was unchanged. The higher proportion and number of dendritic cells in the PP of inulin-fed rats indicates a need for further research on how prebiotics and their metabolites affect immune function possibly through intestinal dendritic cells.

Inulin: Zinc deficiency: Cytokines: Dendritic cells: Rats

Inulin-type fructans, including inulin, oligofructose and synthetic fructo-oligosaccharides (FOS) are considered prebiotics, which are defined as non-digestible food ingredients that selectively stimulate the growth, composition and/or activity of health-promoting bacteria already present in the colon[1]. In rodent models, prebiotics have been shown to enhance the thickness of the epithelial mucus layer[2], and increase the proportion of lymphocytes and increase *ex vivo* cytokine secretion in the gut-associated lymphoid tissue[3−6]. Furthermore, prebiotics have been shown to increase the proportion of dendritic cells in the lamina propria of patients with Crohn's disease[7].

Dendritic cells play an important link between the innate and acquired immune systems. The major function of dendritic cells is to process and present antigens to CD4⁺T helper cells (Th) in a stimulatory or tolerogenic fashion[8]. Activated Th can differentiate into Th1 or Th2 cells depending on the type of antigen and cytokines that they encounter[9]. Viruses and bacteria influence naive T cells to become Th1 cells, which drive cell-mediated immunity. Th1 cells secrete the

cytokines IL-2 and interferon-γ (IFN-γ), which activate CD8⁺ cytotoxic T cells and induce antibody-class switching to IgG2a[10]. Allergens and parasites favour the Th2 pathway, which is essential for humoral immunity and promoting antibody-class switching to IgG1[9]. Tolerogenic responses are attributed to regulatory T-cells (T_reg) which are important in the presence of dietary antigens and commensal bacteria[8]. T_reg secrete cytokines such as IL-10, which suppress the activation of Th1 and Th2 cells[11].

Initial studies show that prebiotics may increase the absorption of Zn[12,13], a mineral known to play a central role in the immune system. Clinical manifestations of Zn deficiency include: growth retardation, reduced appetite, and immunodeficiency[14]; the suppression of cytotoxic T cell activity[15]; a reduction in the number of B cells and their precursors[16]; alteration of cytokine secretion[17−20]. Most Zn research has focused on systemic immunity; the effect of Zn deficiency on the gut-associated lymphoid tissue is unclear. Villous atrophy, oedema, mucosal ulceration, necrosis and inflammation have all been observed in the small intestine of Zn-deficient

rat models[21,22]. Furthermore, Zn may play a role in intestinal permeability as demonstrated by the positive effects of Zn supplementation on gut permeability in rats with acute colitis[23] and patients with Crohn's disease[24]. Since Zn deficiency has been shown to damage the intestinal tract, perhaps Zn deficiency may also increase intestinal permeability and alter the immune cells in the gut.

To date, no research has explored the effects of prebiotics on gut and immune function using a Zn-deficient model. This research may be important for diseases such as HIV, AIDS and inflammatory bowel diseases, where Zn deficiency is common and can exacerbate symptoms[25–27]. Prebiotics could be advantageous for ameliorating intestinal damage caused by Zn deficiency based on previous research with probiotics and Zn deficiency. Mengheri *et al.*[22] found that the probiotic *Bifidobacterium animalis* reduced intestinal damage in Zn-deficient rats. Unlike prebiotics, probiotics are live bacteria that provide health benefits when consumed. Since prebiotics have been shown to increase bifidobacteria in the colon[28], these findings suggest that prebiotics could indirectly protect the intestine against damage caused by Zn deficiency. Prebiotics may improve both gut and cell-mediated immune function; therefore, we hypothesised that inulin might be beneficial during Zn deficiency. Thus, the objective of the present research was to determine if inulin alters the number and proportion of immune cells in the spleen, mesenteric lymph nodes (MLN) and Peyer's patches (PP), and *ex vivo* cytokine secretion as well as intestinal permeability, an indicator of gut barrier function, in growing female rats. An additional objective was to determine if inulin modulates these immune parameters and Zn status in Zn-adequate *v.* Zn-deficient states.

Materials and methods

Animals and diets

Sixty female Sprague–Dawley rats, aged 3 weeks (Charles River Laboratories, St Constant, QC, Canada), weighing about 50–70 g were acclimatised for 7 d and then randomly assigned to a Zn normal (ZN) control diet (30 mg Zn/kg diet, 5 % cellulose) supplemented with either cellulose (CEL; 5 % cellulose; *n* 30) or inulin (PRE; 5 % inulin, Beneo™HP; *n* 30) for 4 weeks. Beneo™HP is a long-chain inulin with an average degree of polymerisation > 23, and was provided by the Orafti Group (Tienen, Belgium). For an additional 4 weeks, ten rats remained in their original groups (ZN-CEL, *n* 10; ZN-PRE, *n* 10). The remaining forty rats were fed a Zn-deficient diet (ZD; 1 mg Zn per kg diet) supplemented with cellulose (ZD-CEL; *n* 10) or inulin (ZD-PRE; *n* 10) or fed the Zn normal cellulose diet in restricted amounts (DR-CEL; *n* 10) or Zn normal inulin diet in restricted amounts (DR-PRE; *n* 10). These latter two groups were designated as diet-restricted (DR) groups. The DR rats were individually pair-fed to the ZD rats, and all other groups were provided diet *ad libitum*. A consequence of Zn deficiency is a reduction in feed intake and growth, thus a DR group was required to differentiate the effects of malnutrition *v.* Zn deficiency *per se*. The 4-week Zn deficiency period was based on a preliminary study which demonstrated that serum and femur Zn concentrations were reduced by 76 and 30 %, respectively, in male Sprague–Dawley rats, aged 9 weeks, after consuming a 1 mg Zn/kg diet for 3 weeks (NR Ryz and CG Taylor, unpublished results). Female rats were chosen for the present study because Zn deficiency is more prevalent in the female population[29] and there is a lack of experimental Zn research using females as the animal model. The diet formulations, based on the AIN-93G diet, are shown in Table 1. The PRE diet (5 % inulin + 5 % cellulose) was designed to have a sufficient amount of prebiotic to affect immune responses while minimising gastrointestinal side effects[6,30] and to include a source of insoluble fibre for faecal bulking. The rats were maintained in an environment of controlled temperature (21–23°C), humidity (55 %) and light cycle (14 h light − 10 h dark). Feed intake was determined daily and body weights were determined weekly. The protocol for animal care procedures received ethics approval from the University of Manitoba Protocol Management and Review Committee.

Table 1. Diet formulations*

Ingredient (g/kg diet)	CEL	ZD-CEL	PRE	ZD-PRE
Inulin	0	0	50	50
Cellulose	100	100	50	50
Maize starch	312·59	322·59	312·59	322·59
Maltodextrin	132	132	132	132
Sucrose	100	100	100	100
Egg white	212·5	212·5	212·5	212·5
Biotin mix†	10	10	10	10
Mineral mix (AIN-93M-MX, Zn-free)	35	35	35	35
Zn premix‡	10	0	10	0
Vitamin mix (AIN-93-VX)	10	10	10	10
Potassium phosphate§	5·4	5·4	5·4	5·4
Choline	2·5	2·5	2·5	2·5
Tert-butylhydroquinone	0·014	0·014	0·014	0·014
Soyabean oil	70	70	70	70

CEL, cellulose; ZD-CEL, Zn-deficient cellulose; PRE, prebiotic inulin; ZD-PRE, Zn-deficient prebiotic inulin.
* All ingredients were from Harlan Teklad (Madison, WI, USA) except potassium phosphate (Fisher Scientific, Mississauga, ON, Canada), *tert*-butylhydroquinone (Aldrich Chemical Company Inc., Milwaukee, WI, USA) and inulin (Orafti Group, Tienen, Belgium).
† Biotin mix was 200 mg biotin/kg cerelose.
‡ Zn premix was 5·775 g zinc carbonate per kg cerelose.
§ Additional potassium phosphate was added to make the AIN-93M-MX Zn-free mineral mix equivalent to the AIN-93G-MX.

Intestinal permeability

Animals were tested for intestinal permeability using sucrose, lactulose and mannitol probes as previously described[31,32]. Elevated concentrations of these intact probes in the urine represent increased permeability in the stomach (sucrose) and small intestine (lactulose:mannitol ratio)[32]. At 5 d before the end of the study, rats were fasted (no food or water) for 4 h, then given a 2 ml solution containing 1 g sucrose, 120 mg lactulose and 80 mg mannitol by oral administration. Animals were placed in individual metabolism cages for 2 h with no food or water, and then allowed free access to water for the next 22 h. During this time, urine was collected into plastic tubes containing 10 % thymol as an antibacterial agent and 100 μl mineral oil to prevent evaporation. The HPLC methods to determine sucrose, lactulose and mannitol have been previously reported[31].

Tissue collection

Rats were euthanised by CO_2 asphyxiation and decapitation. Trunk blood was collected and stored on ice until centrifuged to obtain serum and stored at $-80°C$. The spleen and MLN were removed aseptically, weighed and processed as previously described[33]. The small intestine was flushed with 30 ml cold, sterile Hanks' balanced salt solution with 4 mM-dithiothreitol, weighed and measured, and PP were removed and immediately processed as previously described[34]. To determine total viable cell numbers per tissue, cells were counted using a haemocytometer and trypan blue to distinguish viable from non-viable cells.

Fluorescence staining of lymphocyte subpopulations

Single-cell suspensions (1×10^6 mononuclear cells/tube) from spleen, MLN and PP were incubated with monoclonal antibodies (obtained, unless stated otherwise, from BD Pharminigen, Mississauga, ON, Canada) for T cell receptor (TCR)-αβ (R-phycoerythrin label, R73 clone), TCRγδ (fluorescein isothiocyanate label, V65 clone), CD4 (allophycocyanin label, OX-35 clone), CD8a (peridinin–chlorophyll–protein complex label, OX-8 clone), CD45RA (phycoerythrin-Cy5 label, OX-33 clone, which recognises a cell-surface glycoprotein expressed by mature B-cells in the rat) and OX62 (R-phycoerythrin label, MRC OX-62 clone; Serotec, MorphoSyS, Raleigh, NC, USA). OX62 recognises an integrin expressed on both dendritic cells and γδ T cells in the rat[35]; thus, in the present study, dendritic cells were defined as $OX62^+$ $TCRγδ^-$. The sample combinations for dual-laser analysis were tube 1, TCRαβ, TCRγδ, CD4 and CD8 or their respective isotype controls, and tube 2, CD45RA, OX-62 and TCRγδ or their respective isotype controls. Flow cytometry analysis was performed on a BD FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA) using the BD FACStation data management system software provided with the instrument. Fluorochrome-isotype matched controls were prepared to assess autofluorescence and non-specific binding, and single-colour samples were employed to adjust colour compensation. Phenotype results were expressed as both proportion and as total number of cells per total tissue collected (calculated by multiplying the percentage for each phenotype by cell counts obtained using the haemocytometer).

Cytokines

Single-cell suspensions (1×10^6 mononuclear cells/tube) from spleen and MLN were stimulated with 100 μl concanavalin A (2·5 μg/ml; MP Biomedicals, Solon, OH, USA) for 48 h at 37°C, 5 % CO_2 and 95 % humidity. Concentrations of IL-2, IFN-γ and IL-10 in the supernatant fractions were measured by a rat LINCOplex kit (Linco Research Inc., St Charles, MO, USA) following the manufacturer's instructions and analysed with Luminex technology (Lincoplex 200; Luminex Corporation, Austin, TX, USA). Samples were analysed in duplicate and agreement was ≥ 80 %.

IgG1 and IgG2a

A sandwich ELISA method was used to measure IgG1 and IgG2a in the serum. All antibodies and buffers were obtained from BD Pharminigen, Mississauga, Canada unless otherwise noted. Maxisorb ninety-six-well microtiter plates (NUNC, Roskilde, Denmark) were coated with 100 μl of appropriate coating antibody (mouse anti-rat IgG1 or IgG2a monoclonal antibody diluted 0·20 or 0·50 μg/ml, respectively, in PBS) and incubated for 1 h at room temperature on a shaker. After incubating with 200 μl of blocking buffer (PBS containing 1 % (w/v) bovine serum albumin) for 30 min and washing once (PBS, containing 0·05 % (v/v) Tween 20), 100 μl of blanks (blocking buffer), standards (rat reference serum diluted over a range of about 2–1000 ng/ml; Bethyl Laboratories, Montgomery, TX, USA) and samples (diluted 40 000 times with blocking buffer) were added and incubated for 1 h at room temperature on a shaker. After washing three times, 100 μl of appropriate detection antibody (biotin-conjugated mouse anti-rat IgG1 or IgG2a monoclonal antibody diluted 0·20 or 0·50 μg/ml, respectively, in blocking buffer) was added and incubated for 1 h at room temperature on a shaker. Plates were washed six times and avidin–horseradish peroxidase conjugate diluted 1:1000 in blocking buffer was added and incubated for 30 min at room temperature on a shaker. Plates were washed six times, and 100 μl tetramethylbenzidine substrate was added, incubated for 20 min at room temperature on a shaker, followed by the addition of 1·0 M-phosphoric acid (50 μl) to stop the reaction. Absorbance was read at 450–570 nm using a microplate reader (SpectraMax 340; Molecular Devices Corp., Sunnyville, CA, USA) and analysed with a 4-parametric logistic curve fit using SOFTmax PRO Software (version 1.2.0; Molecular Devices Corp.). Samples were analysed in triplicate and agreement was ≥ 85 %.

Mineral analysis

Right femurs were excised and thoroughly cleaned of soft tissue. Whole femurs and diet samples were wet-ashed using trace element-grade nitric acid[36] in disposable DigiPrep tubes (SCP Science, Baie d'Urfé, QC, Canada) over 6–7 h at 85°C in a DigiPrep HP heater (SCP Science). Acid digests and serum were diluted appropriately with double-deionised water before the analysis of Zn by atomic absorption spectrometry (Varian Canada, Georgetown, ON, Canada).

Statistical methods

Data were analysed by two-way ANOVA (SAS, version 9.1; SAS Institute, Cary, NC, USA) for main effects: fibre (PRE v. CEL), diet (ZN, ZD or DR) and fibre × diet interaction, while feed efficiency was analysed using two-way ANOVA with repeated measures. A significant main effect for fibre determined that pooled means for fibre (CEL (n 30) represents ZN-CEL, ZD-CEL and DR-CEL; PRE (n 30) represents ZN-PRE, ZD-PRE and DR-PRE) were significantly different. When main effects for diet were significant, Duncan's multiple-range test was used to determine significant differences among pooled means for diet (ZN (n 20) represents ZN-CEL and ZN-PRE; ZD (n 20) represents ZD-CEL and ZD-PRE; DR (n 20) represents DR-CEL and DR-PRE). When the fibre × diet interaction was significant, contrast statements were used to determine differences among the six study groups. When necessary, data were normalised by log transformation but non-transformed means are reported. The level of significance was set at $P < 0.05$. All data are reported as mean values with their standard errors.

Results

In the case of no significant interaction (i.e. the effect of fibre (CEL or PRE) was similar regardless of diet (ZN, ZD or DR)), the results are presented for the main effect of fibre and the main effect of diet.

Zinc status and lymphoid organs

After 8 weeks, the total feed intake of PRE rats was about 7% lower compared with CEL rats, but there was no difference in final body weight (Table 2) or feed efficiency (Fig. 1 (A)) throughout the study. ZD and DR rats consumed 6% less total feed and had a 10% lower final body weight compared with ZN rats. There was no difference in total feed intake or final body weight between ZD and DR rats; however, the feed efficiency of DR rats was higher than ZD rats at week

6 and week 7 (Fig. 1 (B)). There was no difference in serum Zn or femur Zn between CEL and PRE rats. In ZD rats, serum Zn and femur Zn were reduced by 85% and about 30%, respectively, compared with both ZN and DR rats. There was a significant fibre × diet interaction such that femur Zn concentrations in DR-PRE rats were 21% higher than DR-CEL rats (Fig. 2) but there was no difference between ZN-CEL and ZN-PRE or between ZD-CEL and ZD-PRE rats. There was no difference in the Zn content of the ZN-CEL compared with the ZN-PRE diet (35·3 (SEM 2·3) and 34·3 (SEM 1·0) mg Zn/kg diet, respectively; $P < 0.05$) or the ZD-CEL compared with the ZD-PRE diet (1·17 (SEM 0·02) and 1·12 (SEM 0·29) mg Zn/kg diet, respectively; $P < 0.05$). There was no difference in spleen weight among ZN, ZD or DR animals. However, when adjusted for body weight, the spleen of ZD and DR rats weighed 10% more than those of ZN rats. The small intestine weight and length of PRE rats were about 5% greater than those of CEL rats.

Intestinal permeability

There was no difference in sucrose permeability or the lactulose:mannitol ratio between PRE and CEL or among ZN, ZD or DR animals (Table 3).

Immune cell phenotyping

Spleen. Total cell numbers in the spleen were 17% lower in PRE compared with CEL rats (Table 4). PRE rats had a 4% higher proportion of TCRαβ$^+$CD4$^+$ cells in the spleen compared with CEL rats (74·0 (SEM 0·8) and 71·3 (SEM 0·9) % of cells, respectively; $P = 0.02$). PRE rats had 24% fewer TCRαβ$^+$CD8$^+$ cell numbers compared with CEL rats and a 10% lower proportion of TCRαβ$^+$CD8$^+$ cells (PRE: 22·4 (SEM 0·8) % of cells, CEL: 25·0 (SEM 0·8) % of cells; $P = 0.02$). TCRγδ$^+$ cell numbers were 22% lower in the spleen of PRE rats compared with those of CEL rats. ZD and DR rats had a 7% lower proportion of CD45RA$^+$ cells

Table 2. Body weight, feed intake, serum zinc and lymphoid organs*

(Mean values with their standard errors)

	Fibre effect (n 60)					Diet effect (n 60)						
	CEL (n 30)†		PRE (n 30)†			ZN (n 20)‡		ZD (n 20)‡		DR (n 20)‡		
	Mean	SEM	Mean	SEM	P	Mean	SEM	Mean	SEM	Mean	SEM	P
Body weight (g)	254	4	250	6	NS	271^a	5	242^b	5	244^b	6	0·0006
Total feed intake (g)	907	13	846	14	0·0006	924^a	18	872^b	16	834^b	13	0·0003
Serum Zn (μmol/l)	14·6	1·7	14·9	1·6	NS	20·7^a	0·9	3·1^b	0·5	20·6^a	0·8	< 0·0001
Spleen (mg)	545	15	551	13	NS	551	14	540	20	552	17	NS
Spleen (g/g body weight)	0·22	0·01	0·22	0·01	NS	0·20^b	0·01	0·22^a	0·01	0·23^a	0·01	0·02
Small intestine (g/g body weight × 100)	20·6	0·3	21·9	0·4	0·01	21·3	0·5	21·8	0·4	20·5	0·5	NS
Small intestine (cm)	104	1	108	1	0·003	104	1	105	1	108	1	NS

CEL, cellulose; PRE, prebiotic inulin; ZN, Zn normal control diet; ZD, Zn-deficient diet; DR, Zn normal control diet provided in restricted amounts.
^{a,b} For the diet effect, mean values within a row with unlike superscript letters were significantly different ($P \leq 0.05$).
* As there were no interactions between fibre (CEL or PRE) and diet (ZN, ZD, DR), but there were significant main effects of fibre and significant main effects of diet, data were pooled to show pooled means for the main effects only.
† CEL (n 30) represents ZN-CEL, ZD-CEL and DR-CEL groups and PRE (n 30) represents ZN-PRE, ZD-PRE and DR-PRE groups; for details, see Animals and diets.
‡ ZN (n 20) represents ZN-CEL and ZN-PRE groups, ZD (n 20) represents ZD-CEL and ZD-PRE groups and DR (n 20) represents DR-CEL and DR-PRE groups; for details, see Animals and diets.
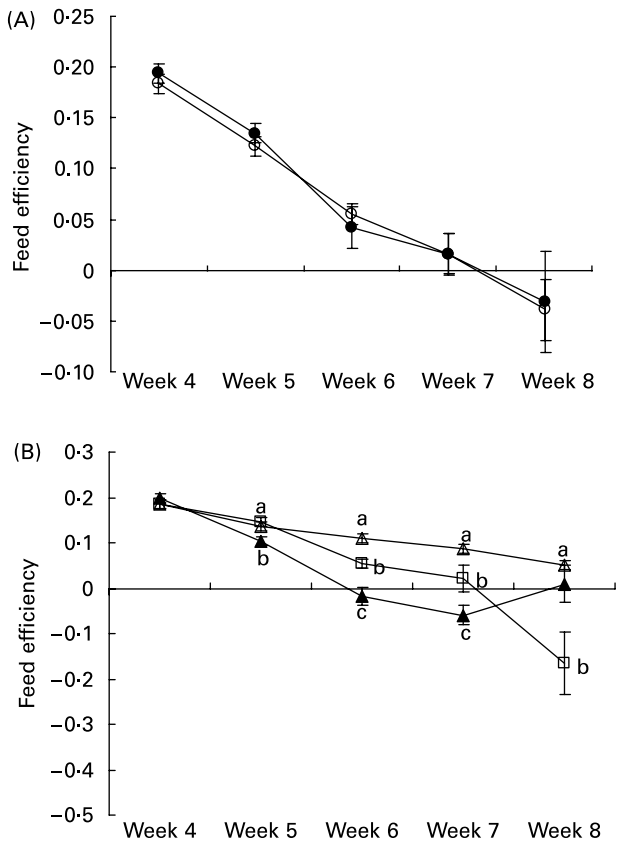
Fig. 1. Feed efficiency over time (calculated as g weight gain per week/g feed intake per week). (A) Fibre effect, (B) diet effect. (○), Cellulose; (●), prebiotic inulin; (△), Zn normal control diet; (▲), Zn-deficient diet; (□), Zn normal control diet provided in restricted amounts. Values are means, with standard errors represented by vertical bars. For details of groups, see Table 2. Data were analysed by two-way ANOVA with repeated measures. There was a significant main effect for diet ($P < 0.0001$). There were no significant main effects for fibre or fibre × diet interaction. [a,b,c] Mean values with unlike letters were significantly different ($P \leq 0.05$).
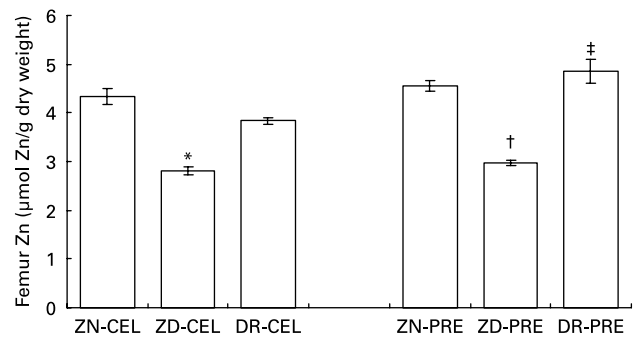

Fig. 2. Femur Zn concentration. ZN-CEL, Zn normal cellulose; ZD-CEL, Zn-deficient cellulose; DR-CEL, diet restricted cellulose; ZN-PRE, Zn normal prebiotic inulin; ZD-PRE, Zn-deficient prebiotic inulin; DR-PRE, diet restricted prebiotic inulin. Values are means, with standard errors represented by vertical bars. Significant main effects were fibre ($P = 0.0001$), diet ($P < 0.0001$) and fibre × diet interaction ($P = 0.0042$). Since there was a significant interaction by two-way ANOVA, individual study groups are shown. *Mean value was significantly different from that of the ZN-CEL and DR-CEL groups ($P \leq 0.05$). † Mean value was significantly different from that of the ZN-PRE and DR-PRE groups ($P \leq 0.05$). ‡ Mean value was significantly different from that of the DR-CEL group ($P \leq 0.05$).

in the spleen compared with ZN rats (54·5 (SEM 1·4), 54·3 (SEM 1·1) and 58·4 (SEM 0·9) % of cells, respectively; $P = 0.02$). There were no differences in TCRαβ^{+}, TCRγδ^{+}CD4^{+}, TCRγδ^{+}CD8^{+}, or OX62^{+}TCRγδ^{−} cell numbers or proportions of these cells in the spleen between PRE and CEL or among ZN, ZD or DR rats.

Mesenteric lymph nodes. Total cell numbers in the MLN were 16 % lower in PRE compared with CEL rats, and numbers in ZD rats were 29 and 59 % lower compared with ZN

and DR rats, respectively (Table 4). TCRαβ^{+}CD8^{+} cell numbers were 15 % lower in the MLN of PRE rats compared with CEL rats. PRE rats had 22 % fewer CD45RA^{+} cell numbers compared with CEL rats and an 8 % lower proportion of CD45RA^{+} cells (PRE: 32·9 (SEM 0·9) % of cells; CEL: 35·8 (SEM 1·0) % of cells; $P = 0.03$). TCRαβ^{+}, TCRαβ^{+}CD4^{+} and TCRγδ^{+} cell numbers in ZD rats were about 30 % and about 50 % lower compared with ZN and DR rats, respectively. TCRαβ^{+}CD8^{+} and TCRγδ^{+}CD8^{+} cell numbers in ZD rats were about 50 % lower compared with both ZN and DR rats. DR rats had about 36 % higher CD45RA^{+} cell numbers compared with ZN and ZD rats. There were no differences in TCRγδ^{+}CD4^{+} or OX62^{+}TCRγδ^{−} cell numbers or proportions of these cells in the MLN between PRE and CEL rats or among ZN, ZD or DR rats.

Peyer's patches. Total cell numbers in the PP had a significant fibre × diet interaction ($P = 0.01$); however, contrast statements did not reveal any significant differences among study groups (Table 5). There was another significant fibre × diet interaction in the PP, such that TCRγδ[+] cell numbers were 53 % higher in ZD-PRE rats compared with ZD-CEL rats. The proportion of TCRαβ[+]CD8 cells was 10 % lower in PP of PRE rats compared with CEL rats (14·1 (SEM 0·5) and 16·2 (SEM 0·6) % of cells, respectively; $P = 0.005$). PRE rats had a 50 % higher OX62^{+}TCRγδ[−] cell number

Table 3. Intestinal permeability*

(Mean values with their standard errors)

| | Fibre effect | | | | Diet effect | | | | | |
| | CEL | | PRE | | ZN | | ZD | | DR | |
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Sucrose	0·007	0·001	0·007	0·001	0·007	0·001	0·006	0·001	0·008	0·001
Lactulose:mannitol	0·31	0·01	0·33	0·02	0·34	0·02	0·30	0·02	0·32	0·02

CEL, cellulose; PRE, prebiotic inulin; ZN, Zn normal control diet; ZD, Zn-deficient diet; DR, Zn normal control diet provided in restricted amounts.
* Results are expressed as fractional excretion (fraction of the orally administered dose recovered in the urine sample) or the ratio of fractional excretion (lactulose:mannitol). Data were analysed by two-way ANOVA, but there were no significant main effects.

N. R. Ryz *et al.*

Table 4. Immune cells in the spleen, mesenteric lymph nodes and Peyer's patches*

(Mean values with their standard errors)

| | Fibre effect | | | | | Diet effect | | | | | | |
| | CEL | | PRE | | | ZN | | ZD | | DR | | |
Cell numbers($\times 10^4$)	Mean	SEM	Mean	SEM	P	Mean	SEM	Mean	SEM	Mean	SEM	P
Spleen												
Total cells	1206	68	1006	62	0·03	1197	81	1039	97	1082	66	NS
TCRαβ⁺CD8⁺ cells	98	7	75	5	0·01	95	9	83	9	81	6	NS
TCRγδ⁺ cells	18·9	1·4	14·7	1·5	0·05	18·8	1·7	15·4	1·8	16·2	2·0	NS
Mesenteric lymph nodes												
Total cells	335	26	282	25	0·05	299[b]	23	213[c]	18	418[a]	34	<0·0001
TCRαβ⁺ cells	194	15	171	15	NS	181[b]	15	124[c]	11	244[a]	19	<0·0001
TCRαβ⁺CD4⁺ cells	153	12	137	12	NS	142[b]	11	100[c]	9	195[a]	15	<0·0001
TCRαβ⁺CD8⁺ cells	34	3	29	3	0·01	33[a]	3	20[b]	2	41[a]	4	<0·0001
TCRγδ⁺ cells	2·69	0·33	2·22	0·3	NS	2·53[b]	0·29	1·42[c]	0·15	3·47[a]	0·43	0·0002
TCRγδ⁺CD8⁺ cells	2·44	0·31	1·98	0·27	NS	2·27[a]	0·26	1·26[b]	0·14	3·17[a]	0·40	0·0001
CD45RA⁺ cells	121	11	94	8	0·02	97[b]	8	79[b]	8	148[a]	14	0·0001
Peyer's patches												
TCRαβ⁺CD8⁺ cells	12·9	0·8	12·0	0·7	NS	11·3[b]	0·9	11·9[b]	0·8	14·5[a]	1·0	0·04

CEL, cellulose; PRE, prebiotic inulin; ZN, Zn normal control diet; ZD, Zn-deficient diet; DR, Zn normal control diet provided in restricted amounts.

[a,b,c] For the diet effect, mean values within a row with unlike superscript letters were significantly different ($P \leq 0.05$).

* Data are shown for total cells or subpopulations of immune cells when there was a significant main effect of fibre and/or diet. Cell numbers are expressed $\times 10^4$. Total cells are per total tissue collected (spleen, mesenteric lymph nodes or Peyer's patches). TCRαβ⁺, TCRγδ⁺ and CD45RA⁺ cells are the number of respective cells in each tissue. TCRαβ⁺CD4⁺ and TCRαβ⁺CD8⁺ cells are the number of the respective subsets calculated from the total number of TCRαβ⁺ cells. Similarly, TCRγδ⁺CD8⁺ cells are calculated from the total number of TCRγδ⁺ cells.

and a 31 % higher proportion of OX62⁺TCRγδ⁻ cells in the PP compared with CEL rats (Fig. 3). The proportion of TCRαβ⁺ cells was 15 % higher in DR animals compared with ZN and ZD animals (17·5 (SEM 0·9), 14·9 (SEM 0·6) and 15·1 (SEM 0·9) % of cells, respectively; $P = 0.04$). DR rats had a 10 % higher proportion of TCRαβ⁺CD8⁺ cells compared with ZN and ZD rats (16·6 (SEM 0·7), 14·5 (SEM 0·8) and 14·3 (SEM 0·6) % of cells, respectively; $P = 0.02$). The proportion of CD45RA⁺ cells was 4 % higher in both ZN and ZD rats compared with DR rats (77·4 (SEM 1·0), 77·9 (SEM 0·9) and 74·6 (SEM 1·0) % of cells, respectively; $P = 0.04$). There were no differences in TCRαβ⁺CD4⁺, TCRγδ⁺CD4⁺ or TCRγδ⁺CD8⁺ cell numbers or proportion of these cells in the PP between PRE and CEL rats or among ZN, ZD or DR rats (data not shown).

Cytokines

Concanavalin A-stimulated *ex vivo* secretion of IL-2, IL-10 and IFN-γ in both the spleen and MLN was 40–70 % higher in PRE

rats compared with CEL rats (Fig. 4). Neither ZD nor DR had an effect on cytokine secretion in the spleen or MLN.

IgG1 and IgG2a

There was no difference in serum IgG1 or IgG2a between PRE and CEL rats. There was no difference in serum IgG1 among ZD, ZN or DR animals (Fig. 5 (A)). Serum IgG2a of ZD rats was about 35 % lower than both ZN and DR rats (Fig. 5 (B)).

Discussion

This is the first study to show that inulin can increase the proportion and number of dendritic cells (OX62⁺TCRγδ⁻) in the PP. It has previously been shown that the percentage of IL-10 positive dendritic cells is increased in the lamina propria following oral consumption of FOS in patients with moderately active Crohn's disease[7]. An increase in the numbers of dendritic cells has been associated with

Table 5. Immune cells in Peyer's patches*

(Mean values with their standard errors)

| | Cellulose | | | | | | Prebiotic | | | | | | |
| | ZN-CEL | | ZD-CEL | | DR-CEL | | ZN-PRE | | ZD-PRE | | DR-PRE | | |
Cell numbers ($\times 10^4$)	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Main effects (P)
Total cells	71	8	70	6	101	7	86	1	104	8	81	11	Fibre×diet 0·01
TCRγδ⁺	0·21	0·03	0·18	0·03	0·33	0·05	0·30	0·05	0·38†	0·04	0·29	0·06	Fibre 0·03
													Fibre × diet 0·05

ZN-CEL, Zn normal cellulose; ZD-CEL, Zn-deficient cellulose; DR-CEL, diet-restricted cellulose; ZN-PRE, Zn normal prebiotic inulin; ZD-PRE, Zn-deficient prebiotic inulin; DR-PRE, diet-restricted prebiotic inulin.

* Since there was a significant interaction by two-way ANOVA, individual groups are shown. Although there was a significant fibre × diet interaction for total cells, contrast statements did not reveal any significant differences among means for the six study groups. Total cells and TCRγδ⁺ cells are per total tissue collected.

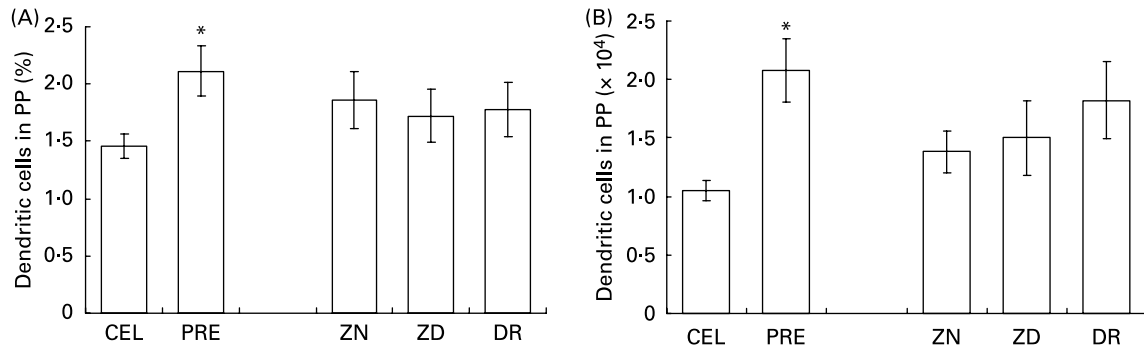† Mean value was significantly different from that of the ZD-CEL group ($P \leq 0.05$).

Fig. 3. Dendritic cells in Peyer's patches. (A) Proportion of dendritic cells (OX62$^+$TCRγδ$^-$ as a percentage of total cells). CEL, cellulose; PRE, prebiotic inulin; ZN, Zn normal control diet; ZD, Zn-deficient diet; DR, Zn normal control diet provided in restricted amounts. Values are means, with standard errors represented by vertical bars. For details of groups and analysis, see Table 2. *There was a significant main effect of fibre ($P = 0.01$). (B) Dendritic cell numbers (OX62$^+$TCRγδ$^-$ cells × 10^4). *There was a significant main effect of fibre ($P = 0.0001$). There were no significant main effects for diet or for fibre × diet interaction.
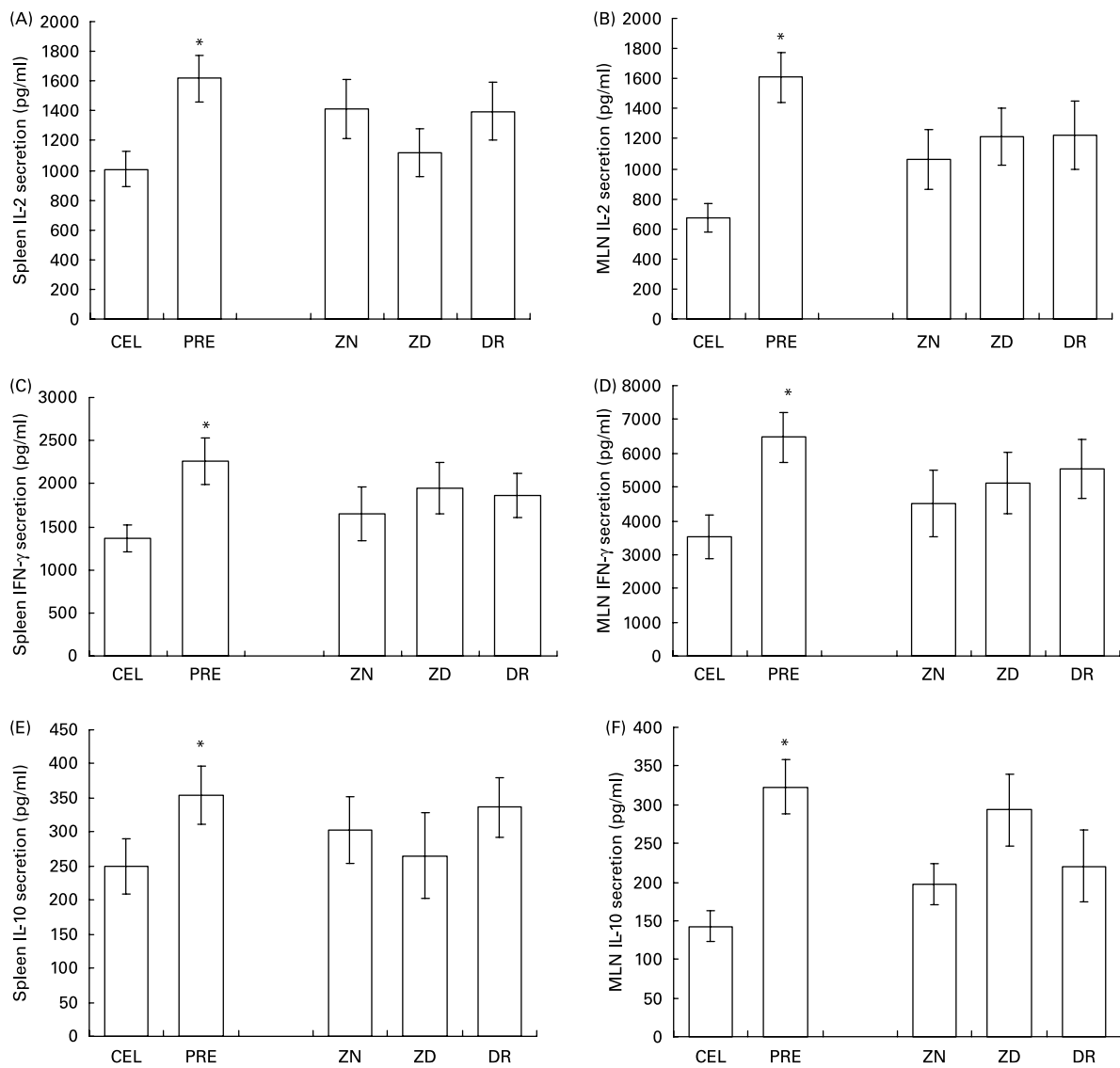

Fig. 4. *Ex-vivo* cytokine secretion of immune cells from the spleen and mesenteric lymph nodes (MLN). (A) IL-2 secretion from spleen cells, (B) IL-2 secretion from MLN cells, (C) interferon (IFN)-γ secretion from spleen cells, (D) IFN-γ secretion from MLN cells, (E) IL-10 secretion from spleen cells, (F) IL-10 secretion from MLN cells. CEL, cellulose; PRE, prebiotic inulin; ZN, Zn normal control diet; ZD, Zn-deficient diet; DR, Zn normal control diet provided in restricted amounts. Values are means, with standard errors represented by vertical bars. For details of groups and analysis, see Table 2. *There were significant main effects for fibre: IL-2 from spleen ($P = 0.006$); IL-2 from MLN ($P < 0.0001$); IFN-γ from spleen ($P = 0.008$); IFN-γ from MLN ($P = 0.005$); IL-10 from spleen ($P = 0.01$); IL-10 from MLN ($P < 0.0001$). There were no significant main effects for diet or fibre × diet interaction.
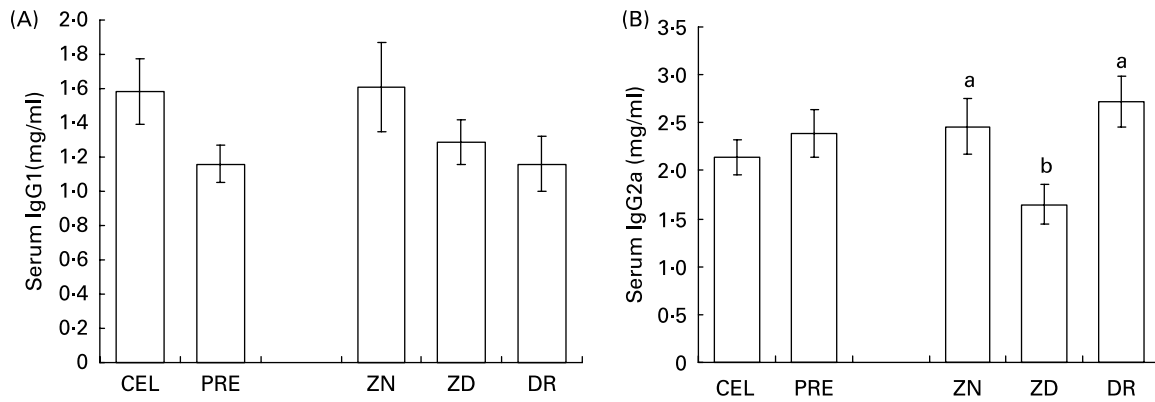
Fig. 5. Serum IgG1 (A) and IgG2a (B) concentrations. CEL, cellulose; PRE, prebiotic inulin; ZN, Zn normal control diet; ZD, Zn-deficient diet; DR, Zn normal control diet provided in restricted amounts. Values are means, with standard errors represented by vertical bars. For details of groups and analysis, see Table 2. For serum IgG2a there was a significant main effect for diet ($P = 0.002$). ^{a,b} Mean values with unlike letters were significantly different ($P \leq 0.05$). There were no significant main effects for fibre or fibre × diet interaction.

increased immunological tolerance[37], which is the immune unresponsiveness to dietary antigens and indigenous bacterial antigens[38]. Prebiotics may increase dendritic cells indirectly through the modulation of indigenous bacteria. For instance, Smits et al. [39] have demonstrated that the probiotics Lactobacillus reuteri and L. casei were able to prime dendritic cells to promote the development of regulatory T cells, which mediate tolerance by inhibiting the proliferation and cytokine production of effector T cells. Further research is required to determine if prebiotics can alter the function of dendritic cells.

Most prebiotic studies are 4–6 weeks long[3,5,40], which may not be long enough to affect systemic immunity; however, the present 8-week study with long-chain inulin (Beneo™HP; average degree of polymerisation >23) was sufficient to alter the proportion and number of immune cells in the spleen and gut. The changes in the proportion of T and B cells between CEL and PRE rats were small, but statistically significant. Other studies found no difference in the proportion of T and B cells in the spleen or MLN of mice fed 10 % oligofructose or inulin for 6 weeks compared with 10 % cellulose[40], or rats supplemented with a combination of inulin and oligofructose (Synergy1) for 4 weeks[3]. Manhart et al. [5] found that both healthy and endotoxaemic female mice supplemented with 10 % FOS for 16 d had an increased proportion of B cells in PP, while T cells were only increased in the PP of endotoxaemic mice fed the FOS diet.

Interestingly, there was a fibre × diet interaction in the PP, such that ZD-PRE rats had 53 % higher TCRγδ$^+$ cell numbers compared with ZD-CEL rats, which may indicate a protective function of inulin during Zn deficiency. To our knowledge, this is the first study to examine the effects of prebiotic and Zn deficiency on TCRγδ$^+$ cells in the gut. Splenic TCRγδ$^+$ cells were unaltered by Zn deficiency in agreement with Hosea et al. [41]. T cells expressing the γδ TCR are a minor T-cell population in the circulation and lymphoid organs, but they represent a major subset within the epithelia of intestine and skin. The functions of γδ T cells are not well understood, but they seem to play a key role in oral tolerance and have anti-inflammatory and immunosuppressive activities[42]. In murine models, intestinal γδ intra-epithelial lymphocytes have been shown to play a

protective role in disease conditions associated with tissue damage, such as inflammatory bowel disease[42].

In the present study, PRE rats had greater ex vivo secretion of cytokines (IL-2, IL-10 and IFN-γ) in the spleen and MLN. Prebiotic studies with an experimental period of 4–6 weeks have found that shorter-chain prebiotics, such as FOS, or a combination of long- and short-chain prebiotics (Synergy1) increased ex vivo secretion of IL-10 and IFN-γ in the PP, but had fewer effects in the spleen or MLN of rats[3,5,6]. In a long-term study, Roller et al. [4] found that rats fed Synergy1 for 33 weeks had increased ex vivo secretion of IL-10 in both PP and MLN, but no effects in the spleen.

There are three potential mechanisms through which prebiotics may affect the immune system. First, prebiotics stimulate the proliferation of beneficial bacteria, including Bifidobacterium and Lactobacillus[43], which can interact with the host. Cells of the innate immune system, including dendritic cells, express toll-like receptors, which recognise specific pathogen-associated molecular patterns, including bacterial DNA. Toll-like receptor signalling can trigger signal transduction cascades, such as the NF-κB system, resulting in the secretion of cytokines[44]. Bifidobacteria and Lactobacillus have also been shown to increase the proportion of CD4$^+$T cells in the spleen[45] and the gut[46]. Second, bacteria in the large intestine can completely degrade prebiotics to yield SCFA, including butyrate, acetate and propionate[47]. Butyrate has been shown to increase ex vivo secretion of IL-10, while inhibiting IL-2 and IFN-γ[48–50], while acetate and propionate have been shown to increase ex vivo secretion of IFN-γ[48]. In addition, butyrate has been shown to inhibit T cell activation and B cell function[51–54]. SCFA also support the epithelial cell mass[55], which may be reflected by the increase in length and weight of the small intestine as observed in the present study and others[56,57]. The third mechanism may involve the interaction of prebiotics with carbohydrate receptors on immune cells. For instance, a β-glucan dectin-1 receptor has been identified on neutrophils, monocytes and macrophages[58,59]. It is presently unknown if fructose receptors exist on immune cells[60].

Prebiotics and their metabolites may play a role in increasing intestinal Zn absorption[61] and potentially improving Zn status as assessed by femur Zn concentrations. Interestingly,

inulin helped to conserve femur Zn concentrations during diet restriction, when dietary Zn was available despite reduced energy intake, but not during dietary Zn deficiency when availability of Zn was severely limited. However, bone Zn concentrations were not altered by inulin consumption in healthy *ad libitum*-fed rats with adequate dietary Zn intake (present study and Coudray *et al.* [61]). Raschka & Daniel[13] found that femur Zn was increased by 8 % in male adolescent rats provided with a mixture of short- and long-chain prebiotics for 15 d, suggesting that shorter-chain prebiotics may be effective for increasing Zn absorption.

ZD rats had about a 35 % drop in serum IgG2a, which has previously been demonstrated in mice[62]. The IgG2a subclass reflects a Th1 immune response and since Zn deficiency is known to inhibit type 1 responses[63], the present results appear logical. Very little is known about the impact of Zn deficiency and diet restriction on mucosal immunity. Interestingly, diet restriction altered the proportion of immune cells in the PP; DR rats had a 10–15 % higher proportion of TCR$\alpha\beta^+$ and TCR$\alpha\beta^+$CD8$^+$ cells, and a 4 % lower proportion of CD45RA$^+$ cells in the PP compared with both ZN and ZD rats. In the MLN, ZD rats had lower T cell numbers (TCR$\alpha\beta^+$, TCR$\alpha\beta^+$CD4$^+$, TCR$\alpha\beta^+$CD8$^+$, TCR$\gamma\delta^+$, TCR$\gamma\delta^+$CD8$^+$) compared with ZN and DR rats. However, in the present study, Zn deficiency did not alter immune markers in the spleen. It appears that Zn deficiency in young adult female rats is not as detrimental to systemic immunity as compared with Zn deficiency in male weanling rats[64,65], which may be attributed to the degree of Zn deficiency. For instance, based on experiences in our laboratory with the same Zn-deficient diet and experimental conditions, femur Zn concentrations were about 70 % lower in weanling male rats[41,66,67] compared with 35 % lower in older female rats (present study), even though serum Zn was reduced by about 80 % in all studies.

We postulated that prebiotic ingestion may help normalise the gastrointestinal barrier dysfunction in Zn-deficient rats; however, gut permeability was not altered by Zn deficiency or prebiotic intake in young adult female rats. Zn deficiency has been previously shown to alter intestinal morphology[21,22], while having no effect on epithelial tight junctions[68] in otherwise healthy rats. On the other hand, Zn supplementation has been shown to improve gut permeability in malnutrition, diarrhoea and inflammatory bowel disease[23,24,69,70]. The present study also demonstrated that long-chain inulin had no effect on gut permeability in healthy rats. Other studies have found an improvement in gut permeability in models of disease with the use of probiotics or symbiotics (mixture of prebiotic and probiotic bacteria) but not prebiotics[71–74]. In healthy rats, FOS has been shown to increase intestinal permeability, a marker of mucosal damage[75]; however, FOS did not alter intestinal permeability in healthy men[76].

In summary, future research should investigate both systemic (blood, spleen) and gut (PP, MLN) levels of immunity as they may respond differently to dietary treatments. For instance, PRE elevated the proportion of TCR$\alpha\beta^+$CD4$^+$ cells in spleen and the *ex vivo* secretion of IL-2, IL-10 and IFN-γ from both spleen and MLN cells, while reducing the number and proportion of TCR$\alpha\beta^+$CD8$^+$ cells in both spleen and PP, and CD45RA$^+$ cells in the MLN. Furthermore, ZD had no effect on immune cells in the spleen, while reducing T cell numbers in the MLN. The present study also

found that ZD lowers TCR$\alpha\beta^+$CD4$^+$ and TCR$\gamma\delta^+$ cell numbers in the gut and that inulin may counteract these effects of ZD. Since TCR$\gamma\delta^+$ cells are found in abundance in the epithelia of the mucosa, future studies should analyse intra-epithelial lymphocytes. Last, the novel finding of an increased proportion and number of dendritic cells in the PP of inulin-fed rats indicates a need for further research on how prebiotics and their metabolites affect immune function possibly through intestinal dendritic cells.

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