

## Postnatal nutritional restriction affects growth and immune function of piglets with intra-uterine growth restriction

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### Abstract

Postnatal rapid growth by excess intake of nutrients has been associated with an increased susceptibility to diseases in neonates with intra-uterine growth restricted (IUGR). The aim of the present study was to determine whether postnatal nutritional restriction could improve intestinal development and immune function of neonates with IUGR using piglets as model. A total of twelve pairs of normal-birth weight (NBW) and IUGR piglets (7 d old) were randomly assigned to receive adequate nutrient intake or restricted nutrient intake (RNI) by artificially liquid feeding for a period of 21 d. Blood samples and intestinal tissues were collected at necropsy and were analysed for morphology, digestive enzyme activities, immune cells and expression of innate immunity-related genes. The results indicated that both IUGR and postnatal nutritional restriction delayed the growth rate during the sucking period. Irrespective of nutrient intake, piglets with IUGR had a significantly lower villous height and crypt depth in the ileum than the NBW piglets. Moreover, IUGR decreased alkaline phosphatase activity while enhanced lactase activity in the jejunum and mRNA expressions of Toll-like receptor 9 (*TLR-9*) and DNA methyltransferase 1 (*DNMT1*) in the ileum of piglets. Irrespective of body weight, RNI significantly decreased the number and/or percentage of peripheral leucocytes, lymphocytes and monocytes of piglets, whereas the percentage of neutrophils and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> were increased. Furthermore, RNI markedly enhanced the mRNA expression of *TLR-9* and *DNMT1*, but decreased the expression of *NOD2* and *TRAF-6* in the ileum of piglets. In summary, postnatal nutritional restriction led to abnormal cellular and innate immune response, as well as delayed the growth and intestinal development of IUGR piglets.

**Key words:** Birth weight: Nutritional intake: Innate immunity: Intestine

Intra-uterine growth restriction (IUGR) is defined as impaired growth and development of the embryo and/or its organs during gestation<sup>(1)</sup>. Approximately 5–10% of human neonates and 15–20% of piglets suffer from IUGR, which has been a common problem in human health and animal production<sup>(1,2)</sup>. Neonates with IUGR have been found to have increased morbidity and mortality during the early life period, and have been associated with long-term effects on the risk of adult-onset diseases<sup>(3,4)</sup>. Because of IUGR, the development and function of immune system is impaired in neonates<sup>(5)</sup>. Also, IUGR has been found to cause immunodeficiency and increased vulnerability to infectious diseases in later life<sup>(6–8)</sup>. In animal models, poor proliferation of lymphocytes in thymus and decreasing cytokine levels in peripheral blood had been observed in rats and sheep with IUGR<sup>(9,10)</sup>.

Neonates with IUGR are generally fed high nutritional diet to achieve rapid growth, which predisposes to postnatal

metabolic syndrome such as obesity, diabetes and CVD<sup>(11–13)</sup>. Furthermore, the recent study has demonstrated that high nutrient intake could impair systematic and intestinal innate immunity of piglets with IUGR<sup>(14)</sup>. In contrast, postnatal milk restriction by cross-fostering feeding was able to protect rats with IUGR from the metabolic syndrome<sup>(15,16)</sup>. In addition, the low nutrient density has been shown to be beneficial for the immune system of broilers<sup>(17)</sup> and primates<sup>(18)</sup>, probably by enhancing antioxidant capability and T-lymphocyte response<sup>(19)</sup>.

Gastrointestinal tract is the major location for digestion and absorption of nutrients, and the gut-associated lymphoid tissue is the largest immune organ in the body<sup>(20)</sup>. In the present study, therefore, we investigated the effects of postnatal nutritional restriction on systematic and intestinal immune function of piglets with IUGR. Because of the structural and physiological similarities of gastrointestinal tract between pigs and human beings, pigs have been recognised as an

**Abbreviations:** ANI, adequate nutrient intake; AP, alkaline phosphatase; BW, body weight; IUGR, intra-uterine growth restriction; NBW, normal-birth weight; RNI, restricted nutrient intake; VCR, villi: crypt ratio.

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ideal model for the study of clinical nutrition<sup>(21,22)</sup>. As a multi-fetal domestic animal, pigs have exhibited severe naturally occurring IUGR due to utero-placental insufficiency<sup>(21)</sup>.

## Materials and methods

### Animal care and formula milk

The experiment followed the actual law of animal protection and was approved by the Animal Care and Use Committee of the Sichuan Agricultural University, and was performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The basic formula milk powder (Table 1) was formulated according to our previous studies<sup>(14)</sup>. The basic nutrient-level formula milk was prepared by mixing 1 kg of formula milk powder (DM 87.5%) with 4 litres of water to a milk solution, which was prepared to supply the same amount of nutrient as sow milk.

### Postnatal nutritional restriction model

A total of twelve pairs of newborn boars (Pig Improvement Company 327 × 1050) of normal-birth weight (NBW) with a body weight (BW) of 1.56 (SD 0.05) kg and IUGR with a BW of 0.91 (SD 0.03) kg from twelve healthy sows with the same litter size (ten piglets/litter) were chosen according to the previous studies<sup>(23)</sup>. All piglets were weaned at 7 d of age, and were moved to be individually fed with formula milk per

meal every 3 h by bottle feeding between 06.00 and 24.00 hours in nursing cages (0.8 m × 0.7 m × 0.4 m). For nutritional treatments, six pairs of NBW and IUGR piglets were randomly assigned to have adequate nutrient intake (ANI) and the other six pairs were allocated to have restricted nutrient intake (RNI) in pairs. Thus, in total, four groups (birth weight-nutrient intake) of male piglets were created and studied: NBW-ANI; IUGR-ANI; NBW-RNI; IUGR-RNI (*n* 6). Among them, NBW-ANI and IUGR-ANI piglets had formula milk *ad libitum*, NBW-RNI piglets were provided the same amount of formula milk as IUGR-ANI piglets, while IUGR-RNI piglets were provided approximately 70% of formula milk intake by IUGR-ANI pigs (Table 3). All piglets had free access to drinking-water. Room temperature was maintained at approximately 30°C, and humidity was controlled between 50 and 60%. BW and formula milk intake of piglets were recorded daily. The average daily DM intake was calculated via multiplying the average daily intake of formula milk by its DM content (%), while formula milk intake was calculated as the difference between the offered amounts and the refusals.

### Blood sampling and analyses

Blood samples were collected by venepuncture on the morning (08.00 hours) of day 21 after an overnight fast, and were injected into two vacuum tubes containing sodium heparin. The vacuum tubes were immediately placed on ice until they arrived at the veterinary hospital for the examination of leucocytes and flow cytometry analysis, respectively (within 2 h). Leucocyte examinations (neutrophil, lymphocyte and monocyte counts) were done through an automatic blood analyser. Total peripheral blood lymphocytes were separated from heparinised peripheral blood by separation medium, and were then stained with mouse anti-porcine CD3e-SPRD (PE-Cy5; catalogue no. 4510-13), CD4a-FITC (catalogue no. 4515-02) and CD8a-PE (catalogue no. 4520-09), which were purchased from Southern Biotechnology Associates. PBS (1 ×; Gibco) and 1.0% bovine serum albumin (ICN Biomedicals) were used as diluent and washing buffer. Flow cytometry analysis was performed on a FACSCalibur flow cytometer (Becton Dickinson) and was repeated for the same sample and compared for repeatability.

### Tissue sample collection

Piglets were weighed, and crown-rump length (CRL) was taken (the supine length of the piglet from the crown of its head to the base of its tail) at days 7 and 28. BMI (BW/CRL<sup>2</sup>) was calculated for each piglet. At day 28, all piglets were anaesthetised with an intravenous injection of sodium pentobarbital (15 mg/kg BW) and killed. The liver, spleen, kidney, heart and pancreas of each piglet were weighed immediately. The length and weight of the small intestine were measured after the removal of luminal contents. Duodenal, jejunal and ileal samples of approximately 2 cm in length were stored in 4% paraformaldehyde solution for histological analyses. The rest of the ileum was snap-frozen and then stored at -80°C until further analysis.

**Table 1.** Composition and nutrient level of the basal formula milk powder (87.5% DM basis, %)

Ingredients	%
Whole-milk powder (24% CP)	58.00
Whey protein concentrate (34% CP)	25.00
Casein	5.70
Coconut oil	10.00
CaH <sub>2</sub> PO <sub>4</sub>	0.10
Choline chloride (50%)	0.10
Vitamin premix*	0.10
Mineral premix†	0.50
L-Arg (98.5%)	0.06
DL-Met (98.5%)	0.06
L-Lys.HCl (78.5%)	
L-Thr (98%)	0.03
L-Trp (98%)	0.05
Total	100.00
Nutrient content	
Digestible energy (kJ/kg)	18390
CP (%)	25.30
Ca (%)	1.02
Total P (%)	0.81
Available P (%)	0.67
Digestible Lys (%)	1.93
Digestible Met (%)	0.63
Digestible Arg (%)	0.86

CP, crude protein.

\* Vitamin premix provided per kg powder diet: vitamin A, 0.94 mg; vitamin D<sub>3</sub>, 0.01 mg; vitamin E, 20 mg; vitamin K<sub>3</sub>, 1 mg; vitamin B<sub>12</sub>, 0.04 mg; riboflavin, 5 mg; niacin, 20 mg; pantothenic acid, 15 mg; folic acid, 1.5 mg; thiamin, 1.5 mg; pyridoxine, 2 mg; biotin, 0.1 mg.

† Mineral premix provided per kg powder diet: Zn, 90 mg; Mn, 4.0 mg; Fe, 90 mg; Cu, 6.0 mg; I, 0.2 mg; Se, 0.3 mg.

### Small intestinal morphology and goblet cell counting

The duodenal, jejunal and ileal samples were preserved in 4% paraformaldehyde solution and then embedded in paraffin. Each sample (duodenum, jejunum and ileum) was used to prepare five slides, and each slide had three sections (5 µm thickness), which were stained with eosin and haematoxylin for intestinal morphology measurement by twenty well-oriented villi and crypts each section (Optimus software version 6.5; Media Cybergenetics), and villi: crypt ratio (VCR) was calculated. The goblet cell number per villus was measured (NIS-Elements BR 2.3; Nikon France SAS). The values obtained from ten villi from each small intestinal segment per piglet were averaged.

### Enzyme analyses

After thawing, the frozen jejunal sample was weighed and homogenised (5 min) in the nine times volume of 50 mM-Tris-HCl buffer, pH 7.0, centrifuged (3000 g, 10 min), and the supernatant was collected and stored at -20°C for the enzyme assay. Total proteins were extracted, and their concentration was determined according to the procedure of bicinchoninic acid (Solarbio, Inc.), with bovine serum albumin as the standard. Disaccharidase (including maltase, sucrase and lactase) and alkaline phosphatase (AP) were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

The absorbance was determined with spectrophotometer (Beckman Coulter DU-800; Beckman Coulter, Inc.). The activities of disaccharidases were presented as U/mg protein, while the AP activity was presented as U/g protein. One unit (U) was defined as 1 nmol maltose, sucrose, lactose or AP as substrate for the enzymatic reaction.

### Total RNA extraction and real-time RT-PCR

Total RNA was extracted from frozen placenta using TRIzol Reagent (catalogue no. 15 596-026; Invitrogen) according to the manufacturer's instructions. The quality and purity of RNA samples were assessed by electrophoresis on 1.0% agarose gel and nucleic acid analyser (A260/A280, Beckman DU-800; Beckman Coulter, Inc.), respectively. Subsequently, the RNA was performed at 37°C for 15 min, followed by RT inactivation at 85°C for 5 s using PrimeScript™ RT reagent Kit (catalogue no. RR047A; Takara). A 1 µl portion of the RT products was used directly for real-time PCR. Real-time PCR was performed on ABI-7900HT instrument (Applied Biosystems). Oligonucleotide primers were used to detect the gene expressions of the target gene and the reference gene (*β-actin*) using the SYBR green system (catalogue no. RR820A; Takara). The sequences of primers and length of the products were presented in Table 2. The reaction mixture (10 µl) contained 5 µl of fresh SYBR® Premix Ex TaqII (Tli RNaseH Plus) and 0.2 µl ROX Reference Dye II (50 ×), 0.8 µl of the primers, 1 µl of RT products and

**Table 2.** Primer sequences of the target and reference genes

Genes	Primer sequence (5'–3')	Product (bp)	GenBank accession
<i>TLR-2</i>	Forward: TCGAAAAGAGCCAGAAAACCAT Reverse: CTTGCACCACTCGCTCTTCA	58	NM213761
<i>TLR-4</i>	Forward: AGAAAATATGGCAGAGGTGAAAGC Reverse: CTTGCTCCTGGCTGGAGTAGA	64	GQ304754
<i>TLR-9</i>	Forward: AATCCAGTCGGAGATGTTTGCT Reverse: GACCGCCTGGGAGATGCT	79	AY859728
<i>MyD88</i>	Forward: GTGCCGTCGGATGGTAGTG Reverse: TCTGGAAGTCACATTCCTTGCTT	65	NM001099923
<i>TRAF-6</i>	Forward: GCTGCATCTATGGCATTGAAG Reverse: CCACAGATAACATTTGCCAAAGG	70	AJ606305.1
<i>NF-κB1</i>	Forward: AGGATGGGATCTGCACTGTC Reverse: TCTGTCAATTCGTGCTTCCAG	121	DQ834921.1
<i>SIGIRR</i>	Forward: ACCTGGGCTCCGAAACTAC Reverse: GTCATCTTCTGACACCAGGCAAT	62	AK239384.1
<i>TOLLIP</i>	Forward: CCCGCGCTGGAATAAGG Reverse: CATCAAAGATCTCCAGGTAGAAGGA	74	AK239879.1
<i>IL-1β</i>	Forward: TCTGCCCTGTACCCCAACTG Reverse: CCAGGAAGACGGGCTTTTG	64	NM214055.1
<i>β-Actin</i>	Forward: GGCGCCAGCACGAT Reverse: CGATCCACACGGAGTACTTG	66	DQ845171.1
<i>IL-6</i>	Forward: GATGCTTCCAATCTGGGTTCA Reverse: CACAAGACCGGTGGTGATTCT	62	M80258.1
<i>NOD2</i>	Forward: CCGAGCGCATCCTCTTAA Reverse: GACGCTCGTGATCCGTGAA	70	AB195466.1
<i>DNMT1</i>	Forward: AGGTGAGGACATGCAGCTTT Reverse: AACTTGTTGCTCCTCCGTTGG	68	DQ060156.1
<i>DNMT3A</i>	Forward: TGCCAAAAGTGAAGAAGT Reverse: CAGCAGATGGTGCAGTAGGA	83	NM_001097437.1
<i>DNMT3B</i>	Forward: AATCGCAACAGGGTACTTGG Reverse: GGTTCACACAGCAATGGACT	97	NM_001162404.1

*TLR*, Toll-like receptor; *MyD88*, myeloid differentiation factor 88; *TRAF-6*, TNF receptor-associated factor 6; *SIGIRR*, single Ig IL-1-related receptor; *TOLLIP*, Toll-interacting protein; *NOD2*, nucleotide-binding oligomerisation domain 2; *DNMT*, DNA methyltransferase.

3  $\mu$ l of diethylpyrocarbonate-treated water. The following PCR protocol was used: one cycle (95°C 30 s); forty cycles (95°C 5 s, 60°C 31 s); one cycle (95°C 15 s, 60°C 1 min and 95°C 15 s). The standard curve of each gene was run in duplicate and three times for obtaining reliable amplification efficiency values as described previously<sup>(24)</sup>. The correlation coefficients (*r*) of all the standard curves were >0.99, and the amplification efficiency values were between 90 and 110%. At the end of amplification, melting curve analysis was performed to identify amplification specificity.  $\beta$ -Actin transcript was used to standardise the results by eliminating variations in mRNA and complementary DNA quantity and quality, and each mRNA level was expressed as its ratio to  $\beta$ -actin mRNA. The relative quantification of gene expression among the treatment groups was analysed by the  $2^{-\Delta\Delta C_t}$  method<sup>(25)</sup>.

### Statistical analysis

Results are presented as means with their standard errors. Data of intestinal morphology were analysed as repeated measures using the MIXED procedure of Statistical Product and Service Solutions 20.0 (SPSS, Inc.) according to the following model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + U_k + \omega_l + (\alpha\omega)_{il} + (\beta\omega)_{jl} + (\alpha\beta\omega)_{ijl} + \epsilon_{ijkl},$$

where  $Y_{ijkl}$  represents the dependent variable,  $\mu$  is the mean,  $\alpha_i$  is the effect of BW ( $i = \text{IUGR, NBW}$ ),  $\beta_j$  is the effect of NI ( $j = \text{ANI, RNI}$ ),  $(\alpha\beta)_{ij}$  is the interaction between BW and NI,  $U_k \sim N(0, \sigma^2)$  is the litter ( $k = 1, 2, \dots, 12$ ),  $\omega_l$  is the segment (duodenum, jejunum and ileum),  $(\alpha\omega)_{il}$  refers to the interaction between BW and segment,  $(\beta\omega)_{jl}$  refers to the

interaction between NI and segment,  $(\alpha\beta\omega)_{ijl}$  refers to the interaction between BW, NI and segment, and  $\epsilon_{ijkl}$  represents the error term. Data on growth performance, organ indices, blood leucocytes, lymphocyte percentages, enzyme activities and gene expressions were analysed according to the model, but omitting the effect of segment and the interaction between segment, BW and NI. Differences between groups were also analysed using general linear model procedure followed by Duncan's test.  $P < 0.05$  was considered as statistically significant.

## Results

### Growth performance

In the present study, IUGR piglets had lower BW ( $-33$ – $38\%$ ,  $P < 0.001$ ) and shorter CRL ( $-10$ – $17\%$ ,  $P < 0.001$ ) than NBW piglets, and these differences persisted until 4 weeks of age (Table 3). Regardless of BW, RNI markedly decreased the net weight gain ( $-25\%$ ,  $P = 0.002$ ), average daily gain ( $-26\%$ ,  $P = 0.002$ ) and average daily DM intake ( $-29\%$ ,  $P < 0.001$ ) of piglets; however, no difference in feed conversion ratio was observed. Throughout the experimental period, moreover, there was similar average daily gain and net weight gain between the IUGR-ANI and the NBW-RNI piglets due to their similar average daily DM intake and feed conversion ratio.

### Organ indices

Regardless of NI, weights of internal organs such as intestine, heart, liver, spleen, kidney, brain and pancreas were markedly

**Table 3.** Effects of the level of nutrient intake (NI) on the growth performance of intra-uterine growth restricted (IUGR) and normal-birth weight (NBW) neonates (Mean values with their standard errors)

Parameters	ANI		RNI		SEM	P		
	NBW	IUGR	NBW	IUGR		BW	NI	BW×NI
Initial weight (kg)	2.92 <sup>b</sup>	1.80 <sup>a</sup>	2.91 <sup>b</sup>	1.81 <sup>a</sup>	0.16	<0.001	0.976	0.904
Day 7 CRL (cm)	34 <sup>b</sup>	29 <sup>a</sup>	35 <sup>b</sup>	28 <sup>a</sup>	1	<0.001	0.874	0.084
Final weight (kg)	8.23 <sup>d</sup>	5.57 <sup>b</sup>	6.88 <sup>c</sup>	4.60 <sup>a</sup>	0.71	<0.001	0.006	0.720
Day 28 CRL (cm)	50 <sup>b</sup>	46 <sup>a</sup>	51 <sup>b</sup>	44 <sup>a</sup>	2	<0.001	0.663	0.178
Net weight gain (kg)	5.31 <sup>c</sup>	3.76 <sup>b</sup>	3.96 <sup>b</sup>	2.82 <sup>a</sup>	0.60	<0.001	0.002	0.713
ADG (g/d)								
Days 0–7	101 <sup>b</sup>	69 <sup>a</sup>	68 <sup>a</sup>	50 <sup>a</sup>	19	0.007	0.016	0.151
Days 7–14	275 <sup>b</sup>	160 <sup>a</sup>	171 <sup>a</sup>	115 <sup>a</sup>	47	<0.001	0.004	0.122
Days 14–21	361 <sup>c</sup>	294 <sup>b</sup>	309 <sup>b</sup>	222 <sup>a</sup>	56	0.005	0.058	0.745
Days 0–21	253 <sup>c</sup>	180 <sup>b</sup>	189 <sup>b</sup>	133 <sup>a</sup>	30	<0.001	0.002	0.352
ADMI (g/d)								
Days 0–7	97 <sup>c</sup>	60 <sup>a,b</sup>	76 <sup>b,c</sup>	48 <sup>a</sup>	13	<0.001	0.040	0.562
Days 7–14	212 <sup>b</sup>	130 <sup>a</sup>	131 <sup>a</sup>	86 <sup>a</sup>	25	<0.001	<0.001	0.228
Days 14–21	280 <sup>c</sup>	216 <sup>b</sup>	212 <sup>b</sup>	151 <sup>a</sup>	22	<0.001	<0.001	0.916
Days 0–21	201 <sup>c</sup>	139 <sup>b</sup>	143 <sup>b</sup>	97 <sup>a</sup>	17	<0.001	<0.001	0.398
FCR*								
Days 0–7	0.98	0.88	1.12	0.99	0.16	0.170	0.115	0.862
Days 7–14	0.78	0.83	0.79	0.77	0.11	0.746	0.697	0.490
Days 14–21	0.84	0.74	0.69	0.69	0.11	0.420	0.143	0.441
Days 0–21	0.80	0.78	0.76	0.74	0.05	0.416	0.139	0.979

ANI, adequate nutrient intake; RNI, restricted nutrient intake; BW, body weight; CRL, crown-rump length; ADG, average daily gain; ADMI, average daily DM intake; FCR, feed conversion ratio.

<sup>a,b,c,d</sup>Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* FCR was calculated by dividing the ADMI by its corresponding ADG.

decreased ( $-7-33\%$ ,  $P<0.010$ ) in IUGR relative to NBW piglets (Table 4). However, the relative intestinal length, heart and brain weights to BW were significantly higher ( $+21-41\%$ ,  $P<0.050$ ) in IUGR relative to NBW piglets. Meanwhile, BMI values at both days 7 and 28 were significantly decreased by IUGR ( $-11-19\%$ ,  $P<0.001$ ) relative to NBW piglets. Regardless of BW, moreover, the weights of heart ( $P=0.019$ ), liver ( $P=0.010$ ) and kidney ( $P=0.014$ ) were markedly reduced by RNI, while NI and BW had significant interaction on the weight of heart ( $P=0.003$ ).

#### Composition of peripheral leucocytes and lymphocyte percentages

In Fig. 1, regardless of BW, the counts of leucocytes, lymphocytes and monocytes were significantly decreased ( $-20-45\%$ ,  $P<0.010$ ) by RNI. Moreover, the percentage of lymphocytes decreased ( $P=0.028$ ) while the percentage of neutrophils increased ( $P=0.036$ ) by RNI. In addition, IUGR-RNI piglets had lower counts of leucocytes and lymphocytes ( $P<0.050$ ) than IUGR-ANI piglets, respectively. The percentage of CD8<sup>+</sup> T cells had the tendency to decrease ( $P=0.083$ ) by RNI, resulting in the increased ( $P=0.034$ ) ratio of CD4<sup>+</sup> to CD8<sup>+</sup> (Fig. 2(D)). Furthermore, the percentage of CD8<sup>+</sup> of IUGR-RNI piglets was lower ( $P<0.050$ ) than that in IUGR-ANI piglets (Fig. 2(C)).

#### Intestinal morphology and goblet cell density

BW and NI had a significant interaction effect on villous height ( $P=0.001$ ) and the VCR ( $P<0.001$ ). The villous height

( $P<0.001$ ), the crypt depth ( $P<0.001$ ) and the VCR ( $P<0.035$ ) were significantly affected by the segment in the small intestine, with the duodenum having the highest villous height and the deepest crypt depth and the jejunum having the highest VCR (Table 5). Moreover, the density of the total goblet cells per intestinal villus of the IUGR-RNI piglets was higher ( $P<0.050$ ) than that of the IUGR-ANI piglets in the small intestine (Table 6).

#### Jejunal enzyme activities

Irrespective of NI, the activity of jejunal AP was markedly lower ( $P=0.028$ ), while the activity of lactase was markedly higher ( $P=0.046$ ) in IUGR piglets relative to NBW piglets (Table 7). Furthermore, IUGR-RNI piglets had similar AP activity as NBW-RNI piglets, while it was much lower in IUGR-ANI piglets relative to NBW-ANI piglets ( $P<0.050$ ).

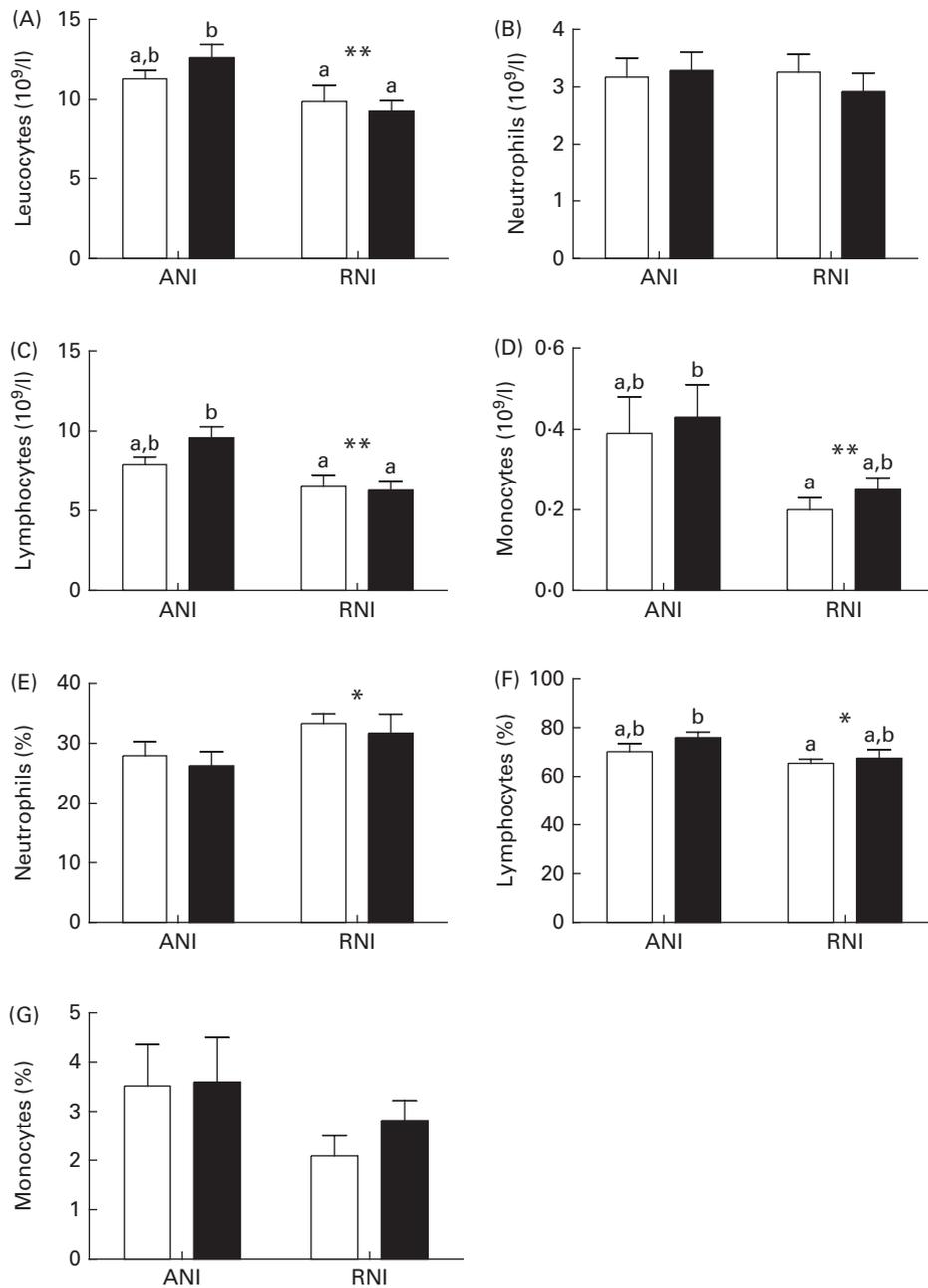
#### Gene expression in the ileum

Regardless of NI, the mRNA abundance of Toll-like receptor 9 (*TLR-9*) was markedly increased ( $P=0.030$ ), and DNA methyltransferase 1 (*DNMT1*) tended to increase ( $P=0.070$ ) in the ileum of IUGR piglets relative to NBW piglets. Regardless of BW, moreover, the mRNA abundance of *TLR-9* and *DNMT1* was significantly higher ( $P<0.010$ ), while the mRNA abundance of TNF receptor-associated factor 6 (*TRAF-6*) and nucleotide-binding oligomerisation domain 2 (*NOD2*) was significantly lower ( $P<0.010$ ) in the ileum tissues of piglets with RNI than that of piglets with ANI. In addition, the significant interaction between BW and NI was observed for the mRNA abundance of *DNMT1* ( $P=0.007$ ) in the ileum (Fig. 3).

**Table 4.** Effects of the level of nutrient intake (NI) on the organ indices of intra-uterine growth restricted (IUGR) and normal-birth weight (NBW) neonates (Mean values with their standard errors)

Parameters	ANI		RNI		SEM	P		
	NBW	IUGR	NBW	IUGR		BW	NI	BW×NI
Intestinal L (cm)	815 <sup>c</sup>	644 <sup>a,b</sup>	728 <sup>b,c</sup>	590 <sup>a</sup>	87	<0.001	0.074	0.670
Intestinal wt (g)	492 <sup>b</sup>	356 <sup>a</sup>	495 <sup>b</sup>	312 <sup>a</sup>	67	<0.001	0.523	0.457
Heart wt (g)	48 <sup>b</sup>	32 <sup>a</sup>	39 <sup>a</sup>	34 <sup>a</sup>	4	<0.001	0.019	0.003
Liver wt (g)	215 <sup>c</sup>	141 <sup>a</sup>	174 <sup>b</sup>	120 <sup>a</sup>	20	<0.001	0.010	0.359
Spleen wt (g)	14.9 <sup>b</sup>	9.8 <sup>a</sup>	13.1 <sup>b</sup>	9.3 <sup>a</sup>	1.8	<0.001	0.189	0.455
Kidney wt (g)	28 <sup>c</sup>	19 <sup>b</sup>	23 <sup>b</sup>	16 <sup>a</sup>	3	<0.001	0.014	0.573
Brain wt (g)	51 <sup>b</sup>	46 <sup>a</sup>	49 <sup>b</sup>	46 <sup>a</sup>	3	0.006	0.599	0.475
Pancreas wt (g)	13.5 <sup>c</sup>	9.9 <sup>a,b</sup>	11.4 <sup>b,c</sup>	8.8 <sup>a</sup>	1.7	0.001	0.132	0.792
Dissected organs wt (g)	817 <sup>b</sup>	619 <sup>a</sup>	803 <sup>b</sup>	544 <sup>a</sup>	69	<0.001	0.175	0.353
Intestinal wt:BW (%)	6.06	6.94	6.74	6.98	0.67	0.091	0.265	0.312
Intestinal L:BW (cm/kg)	102 <sup>a</sup>	124 <sup>b,c</sup>	108 <sup>a,b</sup>	131 <sup>c</sup>	11	<0.001	0.129	0.754
Liver wt:BW (%)	2.70	2.80	2.68	2.68	0.41	0.810	0.702	0.923
Spleen wt:BW (%)	0.19	0.20	0.19	0.21	0.02	0.244	0.401	0.866
Kidney wt:BW (%)	0.35	0.39	0.34	0.35	0.04	0.172	0.145	0.385
Heart wt:BW (%)	0.57 <sup>a</sup>	0.64 <sup>a,b</sup>	0.58 <sup>a</sup>	0.77 <sup>b</sup>	0.08	0.025	0.191	0.216
Pancreas wt:BW (%)	0.17	0.19	0.17	0.19	0.03	0.144	0.741	0.741
Brain wt:BW (%)	0.65 <sup>a</sup>	0.93 <sup>b</sup>	0.75 <sup>a</sup>	1.04 <sup>b</sup>	0.09	<0.001	0.033	0.944
Day 7 BMI (kg/m <sup>2</sup> )	26.3 <sup>c</sup>	21.1 <sup>a</sup>	24.0 <sup>b,c</sup>	23.7 <sup>b</sup>	1.5	0.001	0.782	0.002
Day 28 BMI (kg/m <sup>2</sup> )	32.3 <sup>b</sup>	24.5 <sup>a</sup>	26.3 <sup>a</sup>	23.0 <sup>a</sup>	2.6	<0.001	0.009	0.089

ANI, adequate nutrient intake; RNI, restricted nutrient intake; BW, body weight; L, length; wt, weight.  
<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different ( $P<0.05$ ).



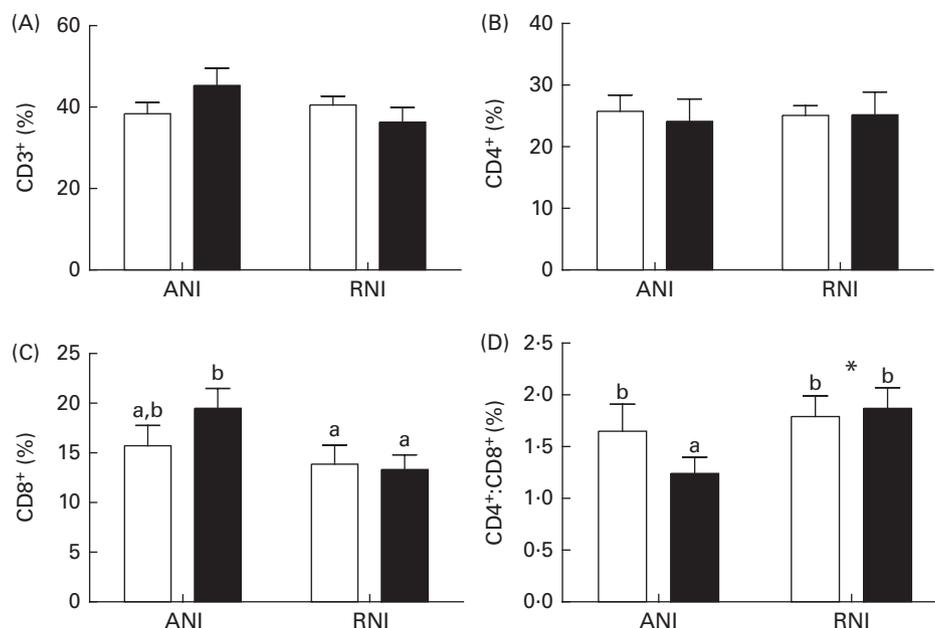
**Fig. 1.** Effects of the level of nutrient intake on the count and percentage of blood leucocytes (A), neutrophils (B and E), lymphocytes (C and F) and monocytes (D and G) in intra-uterine growth restricted (■) and normal-birth weight (□) neonates. Values are means, with their standard errors represented by vertical bars. <sup>a,b</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ). Mean values were significantly different from those of the adequate nutrient intake (ANI) group: \*  $P < 0.05$ , \*\*  $P < 0.01$  (significant effect of level of nutrient intake). There was no significant interaction between body weight and nutrient intake. RNI, restricted nutrient intake.

### Discussion

Neonates with IUGR have been shown to present immature immune system compared with their normal counterparts<sup>(26,27)</sup>. In the present study, we sought to elucidate the role of postnatal nutritional environment on growth performance and immune function of neonates with IUGR using piglets as model. Interestingly, the results of the present study demonstrated that postnatal nutritional restriction may delay the growth and development of the small intestine, as well

as immune response of IUGR piglets through altering intestinal morphology, enzyme activities, composition of peripheral leucocytes and innate immunity-related gene expressions. However, previous studies have reported the improved immune response in low nutrient intake of broilers and primates<sup>(17,18)</sup>; this differential response may be related to the degrees of nutritional restriction, restricted nutrients and species.

In agreement with previous reports<sup>(14)</sup>, piglets with IUGR had lighter BW than NBW piglets at 28d of birth, which



**Fig. 2.** Effects of the level of nutrient intake on the percentage of CD3<sup>+</sup> (A), CD4<sup>+</sup> (B), CD8<sup>+</sup> (C) T-lymphocytes and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> (D) in intra-uterine growth restricted (■) and normal-birth weight (□) neonates. Values are means, with their standard errors represented by vertical bars. <sup>a,b</sup> Mean values with unlike letters were significantly different ( $P < 0.05$ ). \* Mean values were significantly different from those of the adequate nutrient intake (ANI) group ( $P < 0.05$ ; significant effect of level of nutrient intake). There was no significant interaction between body weight and nutrient intake. RNI, restricted nutrient intake.

could be resulting from the lower intake of nutrients on day 21 of the suckling period, as indicated by the decreased intake of average daily DM intake in piglets with IUGR. However, the growth rate was similar between IUGR and NBW piglets once they received the same amount of nutrients; however, feed conversion ratio was not markedly different between IUGR and NBW piglets. This finding is consistent with the previous study, which demonstrated that piglets with IUGR had comparable growth response to a high protein diet as NBW piglets<sup>(28)</sup>. In fact, piglets with IUGR have physiological basis for catch-up growth when they are followed by *ad libitum* milk intake<sup>(15)</sup>.

Along with the smaller body size and weight, piglets with IUGR had lighter relative organ weights than NBW piglets. Consistent with previous results<sup>(29)</sup>, the relative weight of the intestine, heart and brain to BW was markedly increased in piglets with IUGR compared with NBW piglets. These phenotypic changes could be further explained by the programming of 'trade-offs'<sup>(30)</sup>, which selectively allocated maternal nutrition to optimise the growth of internal organs to adapt the chronic placental restriction<sup>(31)</sup>.

The small intestine histological analysis could reflect the renewal rate of intestinal epithelial cells. Piglets with IUGR have been shown to have longer and thinner small intestine

**Table 5.** Effects of the level of nutrient intake (NI) on the intestinal morphology of intra-uterine growth restricted (IUGR) and normal-birth weight (NBW) neonates (Mean values with their standard errors)

Parameters	ANI		RNI		SEM	P			Segment
	NBW	IUGR	NBW	IUGR		BW	NI	BW×NI	
Villous height (μm)									
Duodenum	516	445	423	493	72	0.978	0.333	0.001	<0.001
Jejunum	506	430	412	486	93				
Ileum	439 <sup>b</sup>	369 <sup>a,b</sup>	427 <sup>a,b</sup>	286 <sup>a</sup>	76				
Crypt depth (μm)									
Duodenum	216	217	208	213	26	0.505	0.573	0.607	<0.001
Jejunum	184	182	188	168	19				
Ileum	187	149	220	129	44				
VCR									
Duodenum	2.39 <sup>b</sup>	2.05 <sup>a</sup>	2.03 <sup>a</sup>	2.32 <sup>b</sup>	0.24	0.555	0.832	<0.001	0.035
Jejunum	2.76	2.34	2.19	2.92	0.50				
Ileum	2.34	2.52	2.05	2.23	0.36				

ANI, adequate nutrient intake; RNI, restricted nutrient intake; BW, body weight; VCR, villi: crypt ratio.

<sup>a,b</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ). There was no significant interaction between NI and Segment.

**Table 6.** Effects of the level of nutrient intake (NI) on the density of goblet cells in the small intestine of intra-uterine growth restricted (IUGR) and normal-birth weight (NBW) neonates

(Mean values with their standard errors)

Parameters	ANI		RNI		SEM	P			Segment
	NBW	IUGR	NBW	IUGR		BW	NI	BW×NI	
Number per villus									
Duodenum	15.4 <sup>a,b</sup>	13.6 <sup>a</sup>	14.5 <sup>a,b</sup>	18.6 <sup>b</sup>	2.2	0.898	0.740	0.476	0.056
Jejunum	12.9	13.2	12.7	13.0	2.7				
Ileum	14.9	15.0	16.4	14.8	2.3				

ANI, adequate nutrient intake; RNI, restricted nutrient intake; BW, body weight.

<sup>a,b</sup>Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ). There was no significant interaction between NI and Segment.

with reduced villous height relative to NBW piglets<sup>(32,33)</sup>. In the present study, consistently, BW affected intestinal morphology, as indicated by the lower ileum villous height of piglets with IUGR relative to NBW piglets was found. Moreover, there was an interactive effect on intestinal morphology between BW and NI; nutritional restriction to piglets with IUGR would increase jejunum villous height and VCR. When it comes to distal intestine (ileum), however, RNI tends to decrease villous height of piglets with IUGR. This segmental difference in intestinal morphology might be related to the amount of NI. The proximal intestine had priority to be nursed by nutrition, whereas the distal intestine might be starved with relatively less enteral nutrition in piglets with IUGR-RNI. It has been reported that inadequate enteral nutrition is detrimental for intestinal development<sup>(34)</sup>.

Regardless of NI, there were markedly lower villous height and crypt depth in the ileum of piglets with IUGR; accordingly, intestinal activities of AP, which is expressed exclusively in villus-associated enterocytes<sup>(35)</sup>, had been compromised in the jejunum of piglets with IUGR. Because AP has been shown to detoxify lipopolysaccharide and to prevent bacterial invasion across the gut mucosal barrier<sup>(35)</sup>, lower AP activity suggested that intestinal barrier functions may be impaired in the intestine of piglets with IUGR. In the present study, however, the relatively higher lactase activity in piglets with IUGR may be a compensatory response to lack of nutrition supply. The previous study has also shown that survived piglets with IUGR had an enhanced intestinal tropic response to feeding relative to NBW piglets<sup>(23)</sup>. Since lactose is the main component of carbohydrate in milk, higher lactase activity in piglets with IUGR indicated their priority to utilise lactose as energy under the shortage of nutrient<sup>(36)</sup>.

Intestinal epithelial cells could provide an immunological barrier to microbial invasion through both innate and adaptive immune response<sup>(37)</sup>. TLR and Nod-like receptors are two major forms of innate immune sensors<sup>(38)</sup>. TLR are a family of membrane-bound receptors in the activation of innate immunity, whereas Nod-like receptors reside within the cytoplasm to detect microbial motifs that enter into the host cell<sup>(39)</sup>. In the present study, the nutritional restriction to piglets with IUGR markedly up-regulated the intestinal gene expression of *TLR-9*, but inhibited the gene expression of *NOD2* and its downstream molecule (*TRAF-6*) relative to normal piglets. These findings suggest that nutritional restriction to piglets with IUGR may impair intestinal innate immune response. As a matched nutritional strategy, nutritional restriction has been widely proved to improve the metabolic syndrome of IUGR offspring<sup>(40)</sup>; however, it may not be true when it comes to the evaluation of immunological traits of piglets with IUGR receiving RNI. Moreover, the ileal mRNA level of DNA methyltransferase 1 in piglets with IUGR-RNI was markedly higher than piglets in other groups, which indicates that there may be abnormal DNA methylation, the genomic methylation status has been shown to influence gene expression and recognised as an epigenetic mechanism to link the intra-uterine environment to adult diseases<sup>(41)</sup>.

The immunotype of blood is an important tool in the diagnosis of immunological disorders<sup>(42)</sup>. The cellular immune response of piglets with IUGR to postnatal nutritional restriction is determined by immune cells and lymphocyte subpopulations. In the present study, RNI markedly decreased the number and/or percentage of lymphocytes, leucocytes and monocytes; particularly, RNI disturbed the balance of T-lymphocyte subsets of piglets with IUGR. Similarly, it has been

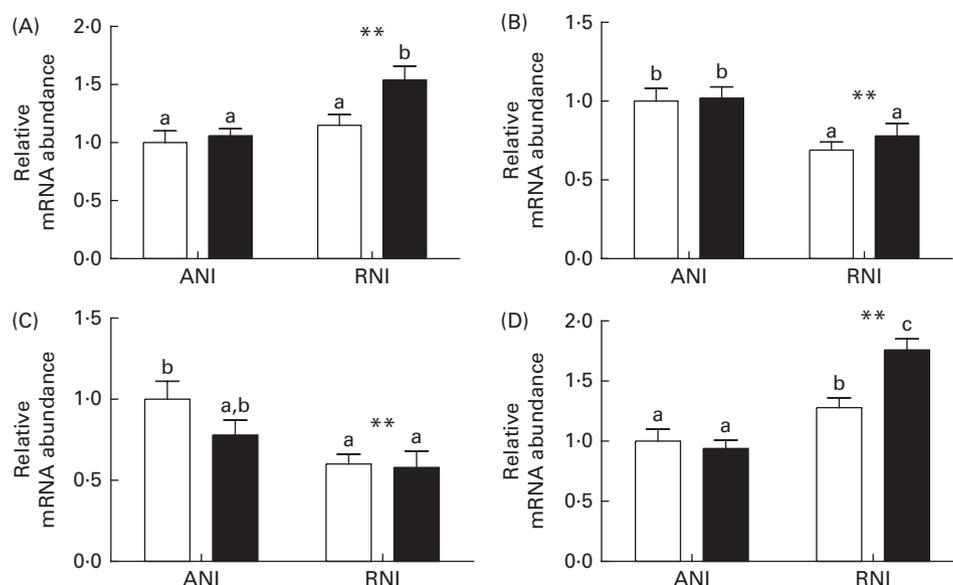
**Table 7.** Effects of the level of nutrient intake (NI) on enzyme activities in the jejunum of intra-uterine growth restricted (IUGR) and normal-birth weight (NBW) neonates

(Mean values with their standard errors)

Parameters	ANI		RNI		SEM	P		
	NBW	IUGR	NBW	IUGR		BW	NI	BW×NI
AP (U/g protein)	16.64 <sup>b</sup>	13.19 <sup>a</sup>	14.80 <sup>a,b</sup>	15.14 <sup>a,b</sup>	2.05	0.028	0.393	0.280
Lactase (U/mg protein)	30.44	39.43	26.41	44.33	13.80	0.046	0.835	0.734
Maltase (U/mg protein)	151.15	103.15	129.08	153.09	47.89	0.545	0.482	0.079
Sucrase (U/mg protein)	30.52	26.76	23.02	33.27	7.31	0.669	0.948	0.359

ANI, adequate nutrient intake; RNI, restricted nutrient intake; BW, body weight; AP, alkaline phosphatase.

<sup>a,b</sup>Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).



**Fig. 3.** Effects of the level of nutrient intake on the mRNA abundance of Toll-like receptor 9 (*TLR-9*) (A), TNF receptor-associated factor 6 (*TRAF-6*) (B), nucleotide-binding oligomerisation domain 2 (*NOD2*) (C) and DNA methyltransferase 1 (*DNMT1*) (D) in the ileum of intra-uterine growth restricted (■) and normal-birth weight (□) neonates. Values are means, with their standard errors represented by vertical bars. <sup>a,b,c</sup>Mean values with unlike letters were significantly different ( $P < 0.05$ ). \*\* Mean values were significantly different from those of the adequate nutrient intake (ANI) group ( $P < 0.01$ ; significant effect of level of nutrient intake). For *TLR-9* (A), there was a significant effect of body weight ( $P < 0.05$ ). There was a significant interaction between body weight and nutrient intake on the mRNA abundance of *DNMT1* ( $P < 0.05$ ). RNI, restricted nutrient intake.

demonstrated that IUGR leads to the lower lymphocyte counts and alterations in CD4<sup>+</sup> and CD8<sup>+</sup> populations of thymus and spleen in rats<sup>(9)</sup>. The ratio of CD4<sup>+</sup> to CD8<sup>+</sup> has been widely used to determine cellular immune status during disease, nutritional stress and autoimmune problem<sup>(43)</sup>. Neonates with IUGR have been shown to compromise immune system, which may be caused by inadequacy of cell-mediated immune response<sup>(5)</sup>.

In conclusion, postnatal nutritional restriction delayed growth and intestinal development in piglets with IUGR. Most importantly, the present study demonstrated that the immunological traits were abnormal in piglets with IUGR receiving postnatal nutritional restriction. Further investigation is required to determine whether this impact by an early nutrition intervention would persist in adult life.

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The authors declare that there are no conflicts of interest.

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