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Moderate zinc restriction affects intestinal health and immune function in lipopolysaccharide-challenged mice☆

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Abstract

Zinc (Zn) is an essential nutrient that affects immune function, especially within the digestive system, although the underlying mechanisms are not well understood. This study examined the effects of short-term moderate Zn restriction on intestinal health and immune function in lipopolysaccharide (LPS)-challenged mice through plasma cytokine profiling and histological evaluation of intestinal tissue sections. Adult male mice were fed with a Zn-adequate (40 ppm) or a Zn-marginal (4 ppm) diet for 4 weeks, and then a bacterial challenge was simulated by intraperitoneal injection of LPS (10 μg/g body weight [BW]) or saline (control). BW was recorded weekly, and feed intake was recorded daily over the last week. Voluntary locomotor activity was assessed 6 and 24 h after the challenge. Plasma and tissues were collected 0, 6 or 24 h after the challenge for analysis. Histological analysis of intestinal samples included evaluation of villi length and width, lamina propria (LP) width, crypt depth and intraepithelial as well as LP leukocyte numbers. Plasma was analyzed for IL-1β, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, interferon gamma and tumor necrosis factor α. Diet did not affect BW and feed intake. The LPS challenge led to decreased voluntary locomotor activity ($P<.05$). Moderate Zn restriction led to greater leukocyte infiltration in the LP after the LPS challenge ($P<.05$) and higher plasma IL-6 and IL-10 levels 24 h after the LPS challenge ($P<.01$). Results indicate that Zn status impacts intestinal responses to LPS through modulation of the cytokine response and leukocyte recruitment, and this impact is evident even with short-term (4 weeks) moderate Zn restriction.
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Keywords: Mice; Zinc; Immune response; Cytokines; Intestine

1. Introduction

Zinc (Zn) is a nutritionally essential trace element that functions in numerous metabolic pathways. It plays a critical role in immune function (reviewed in Reference [1]) as moderate Zn deficiency and severe Zn deficiency are known to affect immunity in human and nonhuman models [2,3]. Zn status affects numerous lymphocyte functions including mitogenesis, antibody synthesis and other facets of cell-mediated immunity, which are negatively impacted by Zn deficiency (reviewed in Reference [4]). Humoral immunity is also affected by Zn deficiency, which induces B-lymphocyte apoptosis and reduces antibody responses (reviewed in Reference [5]), although Zn deficiency does not affect the production of IL-4, IL-6 and IL-10 [6] cytokine expression in either nonchallenged or immune-challenged cells and animals [7,8]. Zn deficiency decreases IL-2 [6,9,10] and interferon gamma (IFNγ; [6,7,10]) production by peripheral blood mononuclear cells (PBMCs) but increases tumor necrosis factor α (TNFα) production by endothelial cells [11] and innate immune cells [8,10]. Similarly, moderate Zn restriction increases IL-1β production by PBMCs [6,8,10] and IL-8 messenger RNA expression [10].

Zn is also implicated in diarrhea due to its effects on intestinal mucosal permeability [12] and the ability of dietary

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Zn to prevent or alleviate intestinal diseases [13]. At pharmacological levels, this effect may be due to the antimicrobial properties of Zn (e.g., [14]) and disruption of bacterial-enterocyte binding as well as subsequent bacterial translocation [15]. However, at physiological levels, it is more likely that Zn affects intestinal physiology and immune function through its effects on cytokine production, as mentioned above.

The level of dietary Zn examined is a critical component of any investigation of the effect of Zn status on intestinal immune function and systemic immune function. Immune modulation by Zn has typically been studied in the context of frank Zn deficiency, although moderate Zn deficiency is of interest because this nutritional condition is difficult to diagnose — yet classical studies have demonstrated increased growth of Zn-supplemented children even from middle to upper socioeconomic classes in the United States and Canada [16,17]. This trial compared Zn adequacy with moderate Zn restriction in adult animals in order to assess the primary effects of Zn restriction in the absence of secondary effects such as impaired growth and decreased intake.

Intestinal immune function is often studied in the context of oral administration of a pathogen or an immunogenic compound. However, the use of live pathogens is of concern due to limitations on quantification of the exact number of organisms and their proliferative capacity after the challenge. Therefore, this trial examined a non-oral challenge that elicited intestinal and systemic immune responses.

The objectives of the present study were to determine the ability of an intraperitoneal challenge to elicit intestinal and systemic immune responses and to examine the effects of moderate Zn restriction on these intestinal responses as well as on systemic cytokine concentrations in adult male mice.

2. Materials and methods

2.1. Animals and treatments

All procedures were approved by the California Polytechnic State University Institutional Animal Care and Use Committee. Male BALB/c mice (n=96, 6 weeks old, mean body weight (BW)=20.05 g; Harlan, Indianapolis, IN, USA) were housed in eight cages (n=12/cage), and each cage was supplied with either a Zn-adequate (40.1±4.2 ppm) or a Zn-marginal (3.9±1.8 ppm) diet (Research Diets, New Brunswick, NJ, USA) for 4 weeks. Zn content of feed was assessed using flame atomic absorption spectroscopy (Perkin Elmer 2380, Perkin Elmer, Norwalk, CT, USA). Feed (~1.0 g of each diet) samples were diluted eightfold with 5% nitric acid (trace metal grade, Fisher Scientific, Pittsburgh, PA, USA). Zn standards, prepared from a reference solution (Fisher Scientific) in 5% nitric acid, were used as internal control. All analyses were conducted in acid-washed glassware. Recovery tests were performed to confirm the accuracy of the abovementioned method, and the recovery of Zn was 108±1.1% (n=5, CV=2.3%). In order to avoid environmental exposure to Zn, animals were allowed free access to deionized water at all times and were housed in plastic cages. BW was recorded weekly throughout the study, and feed intake was recorded daily for the last week of the study.

After 4 weeks of the dietary treatments, half of the animals in each cage (n=6) were exposed to intraperitoneal injection of saline (0.2 ml, control) and the other half (n=6) were injected with lipopolysaccharide (LPS; 10 μg/g BW in 0.2 ml) to simulate a bacterial challenge. LPS (intraperitoneal route) has previously been shown to induce monocyte adhesion and infiltration into intestinal tissues of mice [18]; thus, it was predicted that LPS would induce intestinal distress, similar to other diarrhea models. Four animals from each cage (two control and two LPS) were euthanized by CO_2 asphyxiation for sample collection 0, 6 and 24 h after the intraperitoneal challenge.

2.2. Behavioral analysis

Prior to sample collection, animals were assessed for voluntary locomotor activity by a method adapted from the work of Cravatt et al. [19]. Briefly, each mouse was placed in the center of a 78×56-cm cage marked with 7-cm^2 grid lines on the bottom, and the number of squares traversed in 1 min was recorded.

2.3. Tissue sample collection

Immediately after euthanasia, blood was collected into heparinized tubes and plasma was isolated and frozen at −20°C for cytokine analysis. Subsequently, intestinal samples were isolated from animals 24 h after the challenge. Jejunum samples were isolated from the intestine immediately distal to the pancreatic loop. Ileum samples were isolated from the midpoint between the jejunum and the ileocecal junction. Each intestinal sample was immediately washed with cold saline and then rinsed with 10% formalin, recut to ~2-cm sections and placed in 10% formalin jars for embedding and hematoxylin–eosin staining by a commercial laboratory (IDEXX, West Sacramento, CA, USA).

2.4. Plasma cytokine analysis

Plasma cytokine analysis was performed using Bio-Plex mouse cytokine assay panels (Bio-Rad Laboratories, Hercules, CA, USA) analyzed using a Luminex 100 analyzer (Luminex, Austin, TX, USA). Cytokines analyzed were IFNγ, IL-1β IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70 and TNFα.

2.5. Histological analysis of intestinal sections

For each intestinal section (per animal), three villi were randomly selected to measure villus length, villus width, lamina propria (LP) width and crypt depth as well as to count leukocytes in the LP and intraepithelial regions. Using a 5× field, villus length was evaluated as the distance from the apical region to the base of the villus and villus width was evaluated as the distance from one side of the brush border

Table 1
Feed intake and BW of animals fed with a Zn-adequate (Ad-Zn) diet and those fed with a Zn-marginal (Lo-Zn) diet

	Lo-Zn diet	Ad-Zn diet	*P*
Intake (g/day)	2.59±0.06	2.56±0.05	.52
Initial BW (g)	20.34±0.33	19.76±0.12	.12
Final BW (g)	24.32±0.26	23.65±0.23	.07

Intake was measured daily during Week 4, and BW was measured weekly across the 4-week experiment (n=4 cages/diet, 12 animals/cage). Values are expressed as mean±S.E.M.

membrane to the other side of the brush border membrane [20]. Using a 10× field, LP width was determined by measuring the width of the vascular region in the center of the villus [21,22] and crypt depth was measured as the depth of the invaginations located at the base of each villus. Leukocytes were identified and counted as described by Bjerregaard [23].

2.6. Statistical analysis

All dependent variables were analyzed using a general linear model (JMP, SAS, Cary, NC, USA) with analysis of variance to determine the main effects of diet, challenge, time after the challenge (not included for intestinal histology) and their interactions. When interactions were not significant (P>.20), they were removed from the model. Tukey's HSD (honestly significant difference) was used to assess differences among all pairwise comparisons. Cage was initially included in the statistical analysis, but it was found to be nonsignificant in all cases and subsequently dropped from the model. Differences were considered significant at P<.05.

3. Results

Consumption of the Zn-marginal diet for 4 weeks did not affect feed intake over the last week of the study, BW change over the course of the study, and initial or final BW (Table 1).

Visual observation revealed that challenge with LPS resulted in severe diarrhea 6 and 24 h after the challenge. Voluntary locomotor activity was significantly affected by the LPS challenge; saline-challenged mice had greater activity than did LPS-challenged animals (26.4 traversed squares vs. 4.5 traversed squares, as pooled means for both time points and both diet groups, P<.05; Table 2).

The immune challenge also affected plasma cytokine concentrations (Table 3). Specifically, LPS increased plasma IL-6, IL-10, IL-12p40, IFNγ and TNFα (P<.05 for each) but decreased plasma IL-4 and IL-12p70 (P<.05 for each). The Zn-marginal diet led to an overall increase (across all time points and for LPS and saline groups combined) in IL-6 (P<.05), with a similar trend for IL-10 (P=.07). Of greatest interest is the interaction of diet and LPS challenge on plasma IL-6 and IL-10 (P<.05 for each), for which concentrations of each cytokine were elevated 6 h after the LPS challenge for mice fed with either diet but remained elevated 24 h after the injection only for mice fed with the Zn-marginal diet (Fig. 1).

Intestinal histology was affected by diet and the LPS challenge. In the jejunum 24 h after the challenge, LPS resulted in decreased villus length and increased crypt depth (P<.05 for each; Fig. 2A) as well as increased numbers of leukocytes in the intraepithelial and LP regions (P<.05;

Table 2
Voluntary locomotor activity of mice 6 or 24 h after challenge by intraperitoneal injection of LPS or saline after 4-week treatment with either an Ad-Zn or an Lo-Zn diet (n=4 cages/diet, 6 animals/cage for each challenge, 12 animals/cage in total)

Time after challenge (h)	Saline		LPS	
	Lo-Zn	Ad-Zn	Lo-Zn	Ad-Zn
6	28.3±4.9[a]	21.0±4.5[a]	6.5±2.3[b]	3.5±0.9[b]
24	26.0±6.7[a]	30.5±9.7[a]	3.6±1.3[b]	4.6±2.6[b]

Activity was assessed by placing animals in a 78×56-cm cage marked with 7-cm^2 grid lines and by counting the number of squares traversed in 1 min. Values are expressed as mean±S.E.M. Differing superscripts within a row indicate significant differences (P<.05).

Table 3
Mean plasma cytokine concentrations (pg/ml) of mice 0, 6 or 24 h after challenge by intraperitoneal injection of LPS or saline after 4-week treatment with either an Ad-Zn or an Lo-Zn diet[a]

	LPS			Saline			S.E.M.	Effect
	0 h	6 h	24 h	0 h	6 h	24 h		
IL-1β								
Ad-Zn	18.1	87.4	13.9	20.5	10.7	21.6	15.2	NS
Lo-Zn	31.2	194.7	42.4	35.6	9.7	ND		
IL-4								
Ad-Zn	22.0	6.9	6.1	34.9	15.8	34.7	2.0	C, T
Lo-Zn	28.2	8.0	2.9	25.5	8.1	ND		
IL-5								
Ad-Zn	0	18.3	7.5	5.9	13.5	0	1.6	T
Lo-Zn	0	27.2	12.6	1.2	16.1	ND		
IL-6								
Ad-Zn	1.5	4865.6	1009.5	2.2	38.7	2.2	320.0	D, C, T,
Lo-Zn	2.3	4540.6	5066.2	2.1	1.6	ND		C×T, D×T
IL-10								
Ad-Zn	0	108.8	68.9	0	2.0	9.1	13.6	D, C, T,
Lo-Zn	0	152.4	221.8	0	0	ND		C×T, D×T
IL-12p40								
Ad-Zn	187.5	481.1	143.7	219.7	250.6	284.5	24.6	T, C×T
Lo-Zn	271.9	508.5	108.9	227.5	170.6	ND		
IL-12p70								
Ad-Zn	158.4	29.9	12.1	179.0	96.4	228.7	15.4	C×T
Lo-Zn	234.5	35.3	16.1	193.5	22.3	ND		
IFNγ								
Ad-Zn	458.0	892.1	252.0	577.6	438.7	750.7	73.9	C×T
Lo-Zn	643.2	1297.9	297.7	530.5	267.2	ND		
TNFα								
Ad-Zn	0	175.3	25.5	0.8	0.2	0	14.7	D, C, T
Lo-Zn	0.4	215.1	127.7	0	0.3	ND		

[a] Standard error represents the pooled S.E.M. for each cytokine. The effect of diet (D), challenge (C), time (T) or interactions was considered significant at P<.05 and is listed in the last column. ND indicates not determined; NS, not significant.

Fig. 2B). Zn status did not significantly affect surface area measurements in the jejunum ($P>.1$ for all; Fig. 3A), but mice fed with the Zn-marginal diet had significantly greater leukocyte numbers in the intraepithelial region ($P<.05$; Fig. 3B). Additionally, an interaction between diet and challenge demonstrated that mice fed with a Zn-marginal diet and challenged with LPS had greater numbers of intraepithelial leukocytes in the jejunum as compared with Zn-adequate animals (Diet×Challenge interaction, $P<.05$; Fig. 4).

In the ileum 24 h after the challenge, LPS increased crypt depth ($P<.05$; Fig. 5A) and led to greater numbers of leukocytes in the intraepithelial region as well as a significant increase in LP leukocyte numbers ($P<.05$; Fig. 5B). Diet also affected ileum histology; animals fed with the Zn-marginal diet had greater villus length and crypt depth as compared with the Zn-adequate animals ($P<.05$; Fig. 6A). Finally, the Zn-marginal diet led to a significantly greater number of leukocytes in the LP region as compared with animals fed with the Zn-adequate diet ($P<.05$; Fig. 6B).

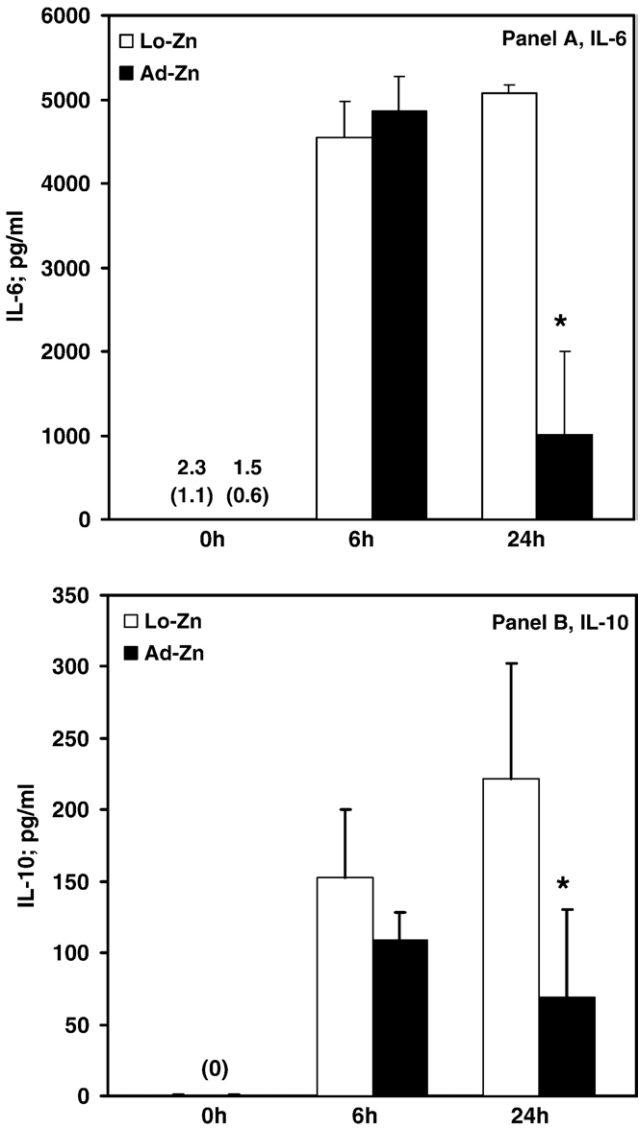

Fig. 1. IL-6 (A) and IL-10 (B) are induced 6 h after the immune challenge and remain elevated at 24 h in Zn-marginal (Lo-Zn) mice but decline in Zn-adequate (Ad-Zn) mice. Mice were challenged with intraperitoneal injection of LPS and then sacrificed 6 and 24 h after the challenge. Serum was collected, and serum IL-6 and IL-10 protein concentrations were determined. IL-6 values 0 h after the challenge are presented as mean (S.E.M.); IL-10 was not detected 0 h after the challenge. Asterisks indicate significant difference between the Lo-Zn and Ad-Zn animals ($P<.05$); error bars represent S.E.M.

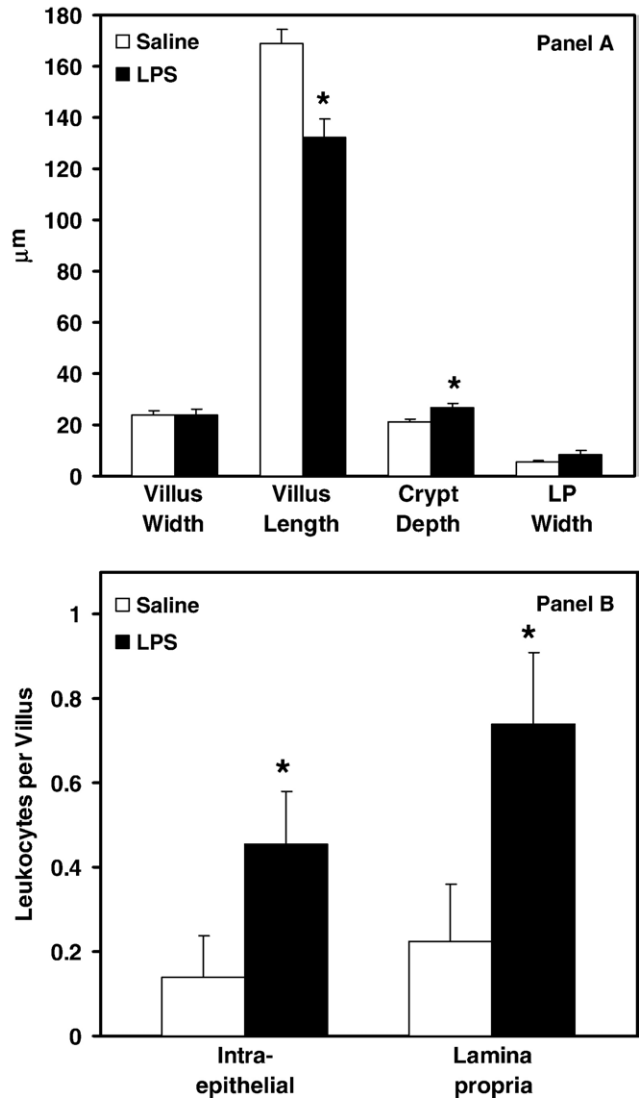

Fig. 2. Effects of immune challenge (white bars indicate saline control; black bars, LPS) on intestinal morphology (A) and leukocyte recruitment (B) in the jejunum of Ad-Zn and Lo-Zn animals. Significant differences due to the challenge are noted by an asterisk ($P<.05$); error bars represent S.E.M.

4. Discussion

This experiment demonstrates that an intraperitoneal LPS challenge is sufficient to induce intestinal inflammation. In this experiment, immune challenge generally increased crypt depth and the number of leukocytes in the jejunum and

ileum, all of which are responses typical of intestinal inflammation. In response to cytokines and other chemical mediators induced by an enteric challenge, leukocyte infiltration occurs via interaction of leukocyte integrins and endothelial cell surface adhesion molecules such as intracellular adhesion molecule [18]. As a result of leukocyte infiltration to the intestine, crypt abscess formation may be induced, as seen in the current trial in the form of increased crypt depth. Additionally, excessive inflammation in the intestine may reduce enterocyte barrier function, thus allowing additional bacterial and inflammatory challenges to occur (reviewed in Reference [24]).

In addition to intestinal inflammation, systemic inflammation was induced, as evidenced by changes in plasma cytokines as well as behavior. In terms of behavioral responses, LPS-challenged mice had substantially reduced exploratory activity. Previous trials have shown that an LPS challenge reduces the frequency of typical behaviors, particularly in dominant animals [25], and this response is likely due to cytokine action on the brain; LPS has been shown to induce TNFα, IFNγ and IL-10 in a similar mouse enteric challenge model [26] as well as IL-6, IL-10 and TNFα in a cecal ligation model of sepsis [27]. Similarly, the LPS challenge in the current trial increased secretion of IL-6, IL-10, IFNγ and TNFα.

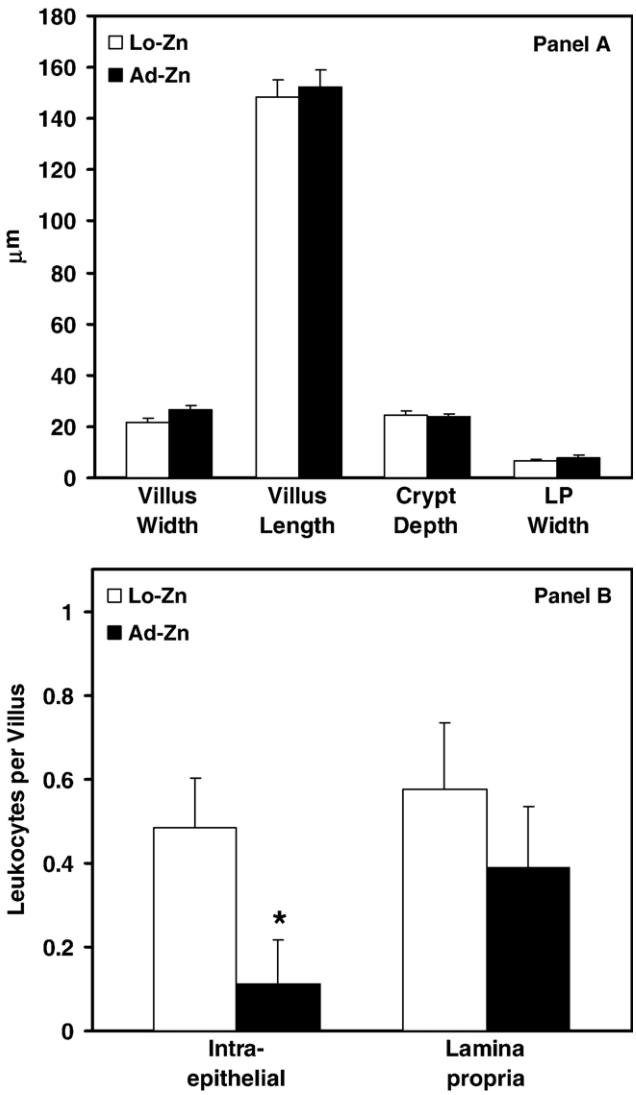

Fig. 3. Effects of diet (white bars indicate Lo-Zn mice; black bars, Ad-Zn mice) on intestinal morphology (A) and leukocyte recruitment (B) in the jejunum of LPS-challenged and nonchallenged animals combined. Significant differences due to diet are noted by an asterisk ($P<.05$); error bars represent S.E.M.

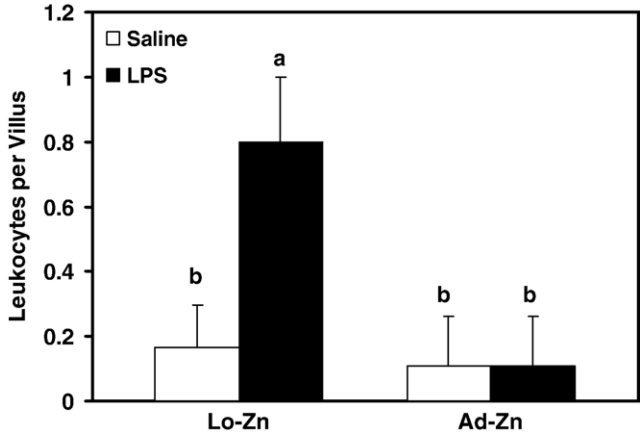

Fig. 4. Interaction of immune challenge with LPS or saline (control) and Zn status on leukocyte recruitment in the intraepithelial region of the jejunum. Different letters indicate significant differences ($P<.05$); error bars represent S.E.M.

Dietary Zn status also affected cytokine secretion and intestinal leukocyte numbers. The LPS challenge elicited a similar and pronounced immune response in all animals, but the response in the Zn-restricted mice was prolonged as compared with that in the mice fed with adequate Zn in terms of IL-6 and IL-10 and in terms of intestinal leukocyte numbers. IL-6 is generally considered to be pro-inflammatory (e.g., [28]), while IL-10 is anti-inflammatory [29] — in addition, its levels are generally correlated with those of IL-6 during bacterial infection [30]. In this trial, inflammatory responses appeared to overwhelm anti-inflammatory parameters in Zn-restricted mice since enhanced intestinal leukocyte infiltration was observed after the LPS challenge in these animals. The implications of prolonged inflammation and leukocyte infiltration in the intestine are substantial; resolution of intestinal inflammation is critical for host survival due to alterations in intestinal permeability and loss of nutrients and water, increased bacterial translocation and other detrimental effects on host physiology (reviewed in References [24,31]). It has been shown that adequate Zn status prevents barrier disruption in the enterocyte monolayer as well as bacterial adhesion and internalization [15], which result in intestinal immune activation [32]. Further experimentation will be required to elucidate the mechanistic basis for the observed interaction between diet and LPS challenge on IL-6, IL-10 and leukocyte recruitment.

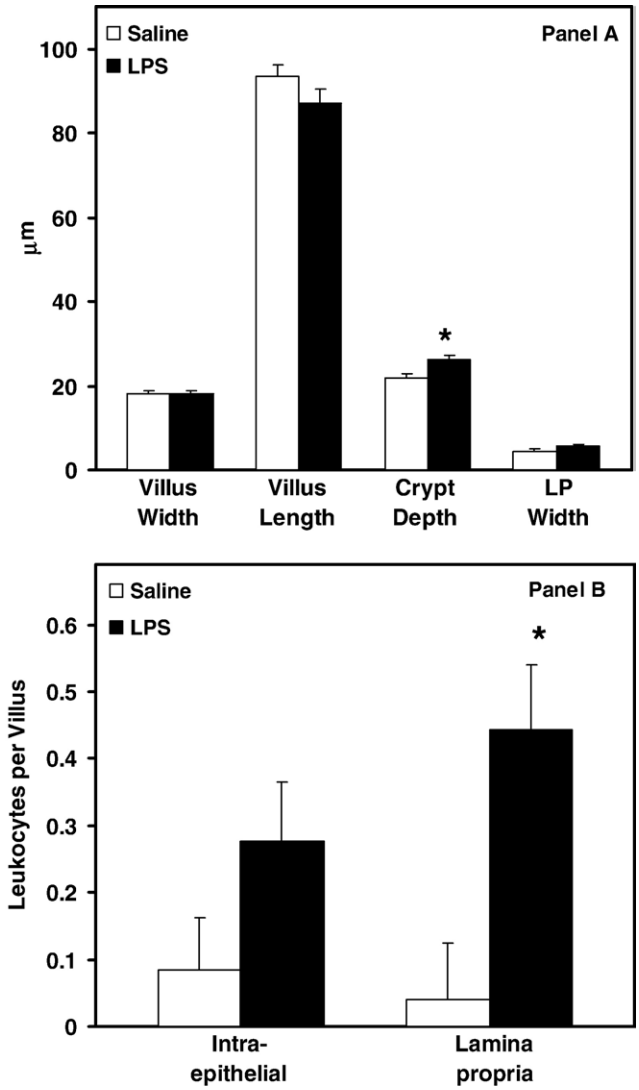

Fig. 5. Effects of immune challenge (white bars indicate saline control; black bars, LPS) on intestinal morphology (A) and leukocyte recruitment (B) in the ileum of Ad-Zn and Lo-Zn animals combined. Significant differences due to the challenge are noted by an asterisk ($P<.05$); error bars represent S.E.M.

Interestingly, previous trials have not shown effects of Zn status on IL-10, although Zn deficiency has been associated with increased production of IL-2 and IFNγ by T helper cells [10]. The mechanism by which Zn status may have affected IL-10 and IL-6 production in the current trial may be via effects on nuclear factor-kappa B (NF-κB) and peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ is known to suppress expression of inflammatory cytokines [33], and Zn deficiency has been shown to reduce PPARγ expression and increase NF-κB activation in endothelial cells [34]. Regardless of the mechanism, enhancement of IL-10 is negatively correlated with survival during bacterial challenges in humans [30].

In contrast to its effects on immune responses, moderate Zn restriction in adult mice for 4 weeks had little effect on feed intake and BW. Given that mice were at maintenance and that the level of Zn deficiency was moderate — and for a relatively short period, this response is not surprising. In growing animals, similar levels of dietary Zn have been shown to result in reduced growth rate and feed intake due to direct effects on appetite-related gene expression in the pituitary [35]. The lack of effect on BW and intake in the present study despite a 10-fold difference in dietary Zn content supports the notion that the Zn deficiency that these animals experienced was only moderate.

In summary, this trial demonstrates that an intraperitoneal challenge with LPS is sufficient to induce intestinal inflammation and systemic inflammation. Moderate Zn restriction in adult mice altered the response to LPS and generally resulted in prolonged systemic cytokine levels and increased leukocyte infiltration in the small intestine. These changes may contribute to the etiology of diarrhea and other

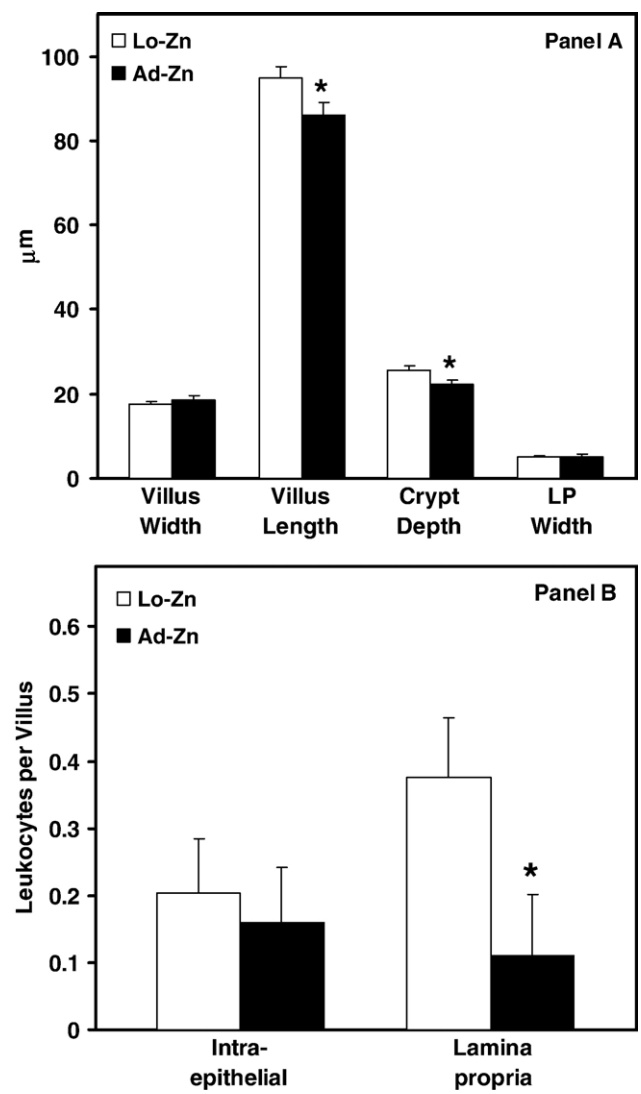

Fig. 6. Effects of diet (white bars indicate Lo-Zn mice; black bars, Ad-Zn mice) on intestinal morphology (A) and leukocyte recruitment (B) in the ileum of LPS-challenged and nonchallenged animals combined. Significant differences due to diet are noted by an asterisk ($P<.05$); error bars represent S.E.M.

issues associated with immunocompetence observed in Zn deficiency, and this study demonstrates that even moderate Zn restriction for a short period may affect immune function.

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