Title

Daily Preventive Zinc Supplementation Decreases Lymphocyte and Eosinophil Concentrations in Rural Laotian Children from Communities with a High Prevalence of Zinc Deficiency: Results of a Randomized Controlled Trial

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ABSTRACT

Background: Zinc deficiency impairs immune function and is common among children in South-East Asia.

Objectives: The effect of zinc supplementation on immune function in young Laotian children was investigated.

Methods: Children ($n = 512$) aged 6–23 mo received daily preventive zinc tablets (PZ; 7 mg Zn/d), daily multiple micronutrient powder (MNP; 10 mg Zn/d, 6 mg Fe/d, plus 13 other micronutrients), therapeutic dispersible zinc tablets only in association with diarrhea episodes (TZ; 20 mg Zn/d for 10 d after an episode), or daily placebo powder (control). These interventions continued for 9 mo. Cytokine production from whole blood cultures, the concentrations of T-cell populations, and a complete blood count with differential leukocyte count were measured at baseline and endline. Endline means were compared via ANCOVA, controlling for the baseline value of the outcome, child age and sex, district, month of enrollment, and baseline zinc status (below, or above or equal to, the median plasma zinc concentration).

Results: T-cell cytokines (IL-2, IFN-γ, IL-13, IL-17), LPS-stimulated cytokines (IL-1β, IL-6, TNF-α, and IL-10), and T-cell concentrations at endline did not differ between intervention groups, nor was there an interaction with baseline zinc status. However, mean $\pm$ SE endline lymphocyte concentrations were significantly lower in the PZ than in the control group (5018 $\pm$ 158 compared with 5640 $\pm$ 160 cells/μL, $P = 0.032$). Interactions with baseline zinc status were seen for eosinophils ($P_{ixn} = 0.0036$), basophils ($P_{ixn} = 0.023$), and monocytes ($P = 0.086$) but a significant subgroup difference was seen only for eosinophils, where concentrations were significantly lower in the PZ than in the control group among children with baseline plasma zinc concentrations below the overall median (524 $\pm$ 44 compared with 600 $\pm$ 41 cells/μL, $P = 0.012$).

Conclusions: Zinc supplementation of rural Laotian children had no effect on cytokines or T-cell concentrations, although zinc supplementation affected lymphocyte and eosinophil concentrations. These cell subsets may be useful as indicators of response to zinc supplementation. This trial was registered at clinicaltrials.gov as NCT02428647. *J Nutr* 2020;00:1–10.

Keywords: zinc supplementation, children, cytokine production, T-cell concentration, complete blood count

Introduction

Zinc is an important nutrient for maintaining homeostasis of the immune system and zinc deficiency can impair immune function [1]. Zinc deficiency is associated with both an increased risk and an increased severity of common childhood infections such as diarrhea and pneumonia in lower-income countries around the world [2], perhaps as a result of impaired immune

function. Zinc is a transition metal that plays an important role in the function of many proteins (e.g., as a cofactor in the active site in enzymes, or as a constituent of zinc-finger motifs in regulatory proteins that bind DNA). The observation that ~10% of the proteins encoded in the human genome bind zinc highlights the important role of zinc in many aspects of protein function (3). In addition, intracellular zinc transporters may allow rapid intracellular signaling in immune cells by facilitating a flux of zinc ions between intracellular compartments and the cytoplasm (4).

Previous studies in both murine and human primary mono-cytes showed that zinc deficiency impairs Toll-like receptor (TLR)-4-mediated signaling and thus diminishes the response to bacterial LPS which generally stimulates phagocytic activity and production of cytokines such as IL-1β, TNF-α, IL-6, and IL-10 (5, 6). Moreover, zinc deficiency causes thymic atrophy with a substantial reduction of T-cell counts (7). Zinc deficiency also impairs T-cell function, impairing T-cell receptor (TCR) signaling (8) and thus decreasing T-cell proliferation and cytokine production in response to signaling through the TCR (9). Studies in humans have found that zinc deficiency impairs the production of T-helper (Th) 1 cytokines (IL-2, TNF-α, and IFN-γ) while showing a lesser effect on the production of Th2 cytokines (IL-4, IL-6, and IL-10) (10), suggesting that zinc affects Th1/Th2 balance. Several studies have reported that these dysregulations associated with low zinc concentration are restored by zinc supplementation (10, 11). In some cases, zinc treatment in vivo and in vitro shows regulatory activity, dampening Th-cell-mediated immune responses (e.g., Th2 allergic responses and Th17 autoimmune responses), and increases the number and activity of regulatory T (Treg) cells (12, 13). These effects of zinc deficiency on immune functions may be responsible, at least in part, for the increased risk of infection (14).

Inadequate zinc status and zinc deficiency are estimated to range from 7.5% in high-income regions to 30% in South Asia (15). Undernutrition, including fetal growth restriction, suboptimum breastfeeding, stunting, wasting, and deficiencies of vitamin A and zinc, are an underlying cause of 45% of child deaths, resulting in 3.1 million deaths annually (16); 25% of these deaths are among infants and children <5 y old (16). Multiple systematic reviews have reported that preventive zinc supplementation is associated with a decrease in diarrhea and pneumonia morbidity and mortality in children in lower-income countries (17–19). The present study was conducted as a substudy of immune function nested within a randomized controlled trial examining the optimal zinc supplementation strategy for improving growth, diarrheal morbidity, and hematologic and micronutrient status in rural Laotian children at risk of zinc deficiency (20). The intervention groups were supplementation with daily preventive zinc tablets (PZ), daily multiple micronutrient powder (MNP) that included zinc, use of supplementation with therapeutic dispersible zinc tablets (TZ) for 10 d as part of treatment of diarrhea, and a daily placebo (control). Children from 6–23 mo of age were randomly assigned to receive these treatments for ~9 mo in a community-based intervention trial. As previously reported, the parent study found no impact on growth and overall diarrhea burden (21) but PZ and MNP increased plasma zinc relative to the control and TZ groups (21).

In the present study, we hypothesized that innate and adaptive immune defenses of these Laotian children would be altered by zinc supplementation. To investigate the effect of the interventions, we measured cytokine production from whole blood cultures stimulated with bacterial LPS (an activator of the innate immune system) or with TCR activation (to stimulate these adaptive immune cells) at baseline and endline. In addition, we measured the concentrations of total and memory CD4 and CD8 T-cells, and Treg cells from whole blood at the same time points. Finally, in a secondary analysis, we performed a complete blood count (CBC) with an auto-differential to measure lymphocyte, monocyte, neutrophil, eosinophil, basophil, and platelet concentrations at baseline and endline.

Methods

Study design and recruitment

This study is a substudy within a randomized, masked, community-based trial in rural Lao People's Democratic Republic (20, 21). Briefly, this parent study aimed to determine the effects of 2 daily zinc supplementation regimens—a therapeutic zinc supplementation regimen for diarrhea, and a placebo intervention—on physical growth and morbidity, including the risk of episodes of diarrhea, in young children. In total 3407 children 6–23 mo of age were enrolled and randomly assigned to 1 of 4 treatment groups. The children received either 1) daily PZ supplements (7 mg Zn), 2) daily MNP (containing 10 mg Zn, 6 mg Fe, and 13 other vitamins and minerals), 3) 10 d of TZ (containing 20 mg Zn) for episodes of diarrhea, or 4) daily placebo (control) dispersible tablets as the negative control group. All children not in the TZ group received placebo capsules in association with diarrhea episodes rather than the zinc received in the TZ group. The supplement regimen for the preventive supplements (PZ and MNP) was daily for 9 mo, and therapeutic supplements (TZ) were provided to be taken along with oral rehydration solution starting on the first day of diarrhea for 10 d for episodes of diarrhea. The enrolled children remained under observation for ~36 wk, so the children's ages ranged from 15 to 32 mo at endline. Children were excluded if they met any of the following criteria at baseline: weight-for-length z score (WLZ) < −3 SD, presence of bipedal edema, severe illness, congenital abnormalities, chronic medical condition (e.g., malignancy), known HIV infection of index child or child's mother, severe anemia, current consumption of zinc supplements, or current participation in any other clinical trial.

Infants included in the present substudy lived in 2 of the districts closest to the laboratory used for blood processing (which allowed reliable, same-day transport of samples from the field site to the laboratory) and were enrolled on Monday through Thursday to facilitate the studies of immune function. Enrollment of infants at baseline was conducted between September and December 2015 and the endline follow-up occurred from May to August 2016. At baseline, samples from a total of 636 subjects were received at Nakonphanom Hospital; CBC analysis was performed on 574 of these samples, whole

blood cultures on 506, and flow cytometry analysis on 492, depending on the volume of the blood sample available. Approximately 82% of participants were re-examined at the follow-up visit. The present analysis included only subjects for whom we had both baseline and endline data.

The trial was approved by the National Ethics Committee for Health Research, Ministry of Health, Lao People's Democratic Republic (040/2014, 069/2015, 039/2016); the University of California, Davis Institutional Review Board (626187); and the Khon Kaen University, Thailand Ethics Committee in Health Research (HE572312).

Anthropometry

Anthropometric assessments were recorded by trained data collectors using standardized procedures at baseline and endline. Measurements included weight to the nearest 0.02 kg and recumbent length to the nearest 0.1 cm and means were determined from 2 measurements. Length-for-age z scores (LAZs), weight-for-age z scores (WAZs), and WLZs were calculated [22] and used to identify study participants with stunting (LAZ < −2 SD) and underweight (WAZ < −2 SD). Children who were severely wasted (WLZ < −3 SD) at baseline were excluded from participation and referred to the nearest health center or hospital. Maternal weights were measured to 0.05 kg precision and maternal heights to 0.1 cm precision at baseline.

Blood collection, transport, and sample size

At baseline and endline blood was collected at the field site from children using an antecubital, dorsal metacarpal, or saphenous vein using techniques recommended by the International Zinc Nutrition Consultative Group [23]. Blood was first collected into trace element–free 7.5-mL polyethylene blood collection tubes containing lithium heparin (Sarstedt AG & Co; ref. 01.1604.400) for immune assays and measurement of plasma zinc, C-reactive protein (CRP), and α-1-acid glycoprotein (AGP) concentrations. Blood was then collected into a trace element–free 1.2-mL polyethylene blood collection tube containing EDTA (Sarstedt; ref. 06.1666.100), which was used for a CBC with differential. Two-milliliter aliquots of heparinized whole blood were immediately removed from the blood collection tube and stored at 20–25°C; EDTA-containing blood collection tubes were stored at 4–8°C; both samples were transported to the laboratory at Nakonphanom Hospital in Thailand, where the laboratory tests on peripheral blood were completed the same day (i.e., CBC with 5-part automated differential, flow cytometry analysis of T-cell subsets, and whole blood cultures to stimulate production of cytokines). The remaining whole blood in the lithium heparin tube was stored at 4–8°C until centrifugation at 4°C for 10 min at 1,000 × g to collect plasma. Plasma aliquots were stored at −20°C before laboratory analyses (i.e., plasma zinc, CRP, and AGP concentrations).

Blood cultures for cytokine production

Whole blood treated with heparin was used to set up whole blood cultures in 96-well plates. The external wells contained 200 mL sterile PBS to prevent excessive evaporation of experimental wells during the culture. Blood was diluted 10-fold in RPMI 1640 cell-culture medium (Gibco) and then 200 μL/well was treated with *1)* 3 μg/mL plate-coated anti-CD3 antibody plus anti-CD28 antibody in solution (eBioscience) to stimulate T-cell cytokine production or *2)* the same volume of isotype control antibodies (eBioscience). Undiluted blood (final blood dilution 1:2) was treated with *1)* LPS from *Escherichia coli* 0111:B4 (List Biological Laboratories) at a final concentration of 20 μg/mL [in pyrogen-free water (Sigma-Aldrich)] or only *2)* RPMI, the diluent for the working solution of LPS. All cells were cultured for 48 h at 37°C in a 5% CO_2 atmosphere. Supernatants were collected and kept frozen at −80°C for later shipment on dry ice to the Western Human Nutrition Research Center in Davis, CA, for cytokine analysis.

T-cell staining and flow cytometry analysis

In addition to preparing the blood cultures, surface staining of lymphocytes in whole blood was performed using directly conjugated antibodies, including PerCP anti-CD3, APC anti-CD4, FITC anti-CD8,

PE anti-CD45RO, FITC anti-CD25, and PE anti-CD127 (BD), and AccuCount Fluorescence Beads (Spherotech) to determine absolute T-cell concentrations. Two staining tubes were used. Tube 1 identified total ($CD3^+$) T-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, and memory ($CD45RO^+$) CD4 and CD8 T-cells. Tube 2 was used to identify $CD4^+$ Treg cells ($CD3^+CD4^+CD25^+CD127_{low}$). The staining was performed in Nakhonphanom. After fixation with paraformaldehyde, stained samples were stored at 4°C for ≤3 d before transportation at 4–10°C to Khon Kaen University (303 km over good, asphalt roads) for flow cytometric analysis using a FACSCalibur flow cytometer (BD). Gating was performed using FlowJo software (BD).

Measuring cytokines in supernatants from whole blood cultures

Supernatants from whole blood cultures stimulated to activate T-cells (and negative control supernatants) were analyzed for IL-2, IL-10, IL-13, IL-17A, and IFN-γ, whereas LPS-stimulated (and negative control) supernatants were analyzed for IL-1β, IL-6, IL-10, and TNF-α. Cytokines were assayed using an electrochemiluminescence-based detection platform with multiplexed immunoassays using the U-PLEX system from MSD (Mesoscale Discovery, LLC). U-PLEX plates were coated overnight at 4°C with a complex of specific linker for the corresponding spots and biotinylated Capture Antibody under light shaking (600–800 rpm) overnight and kept for ≤7 d at 4°C. Samples (final dilution 1:2 in Diluent 43) were also incubated overnight at 4°C (600–800 rpm). The next day, plates were incubated after washing with 1× detection antibodies for 2 h at room temperature under light shaking (600–800 rpm) and subsequently washed. After adding 2× Readbuffer the plates were analyzed on an MSD instrument (MESO QuickPlex SQ120, 4-parameter logistic curve).

CBC with 5-part differential

This analysis was performed following Nakhonphanom Hospital laboratory protocols by an automated hematology analyzer (Sysmex, XT-1800i). The analyzer provides a CBC and leukocyte 5-part differential on whole blood (including lymphocytes, monocytes, neutrophils, eosinophils, and basophils). We were able to measure these variables because the clinical laboratory in which we worked made these measurements routinely.

Plasma analysis

Plasma zinc was analyzed by inductively coupled plasma optical emission spectrophotometry (5100 ICP-OES SVDV, Agilent) at the Children's Hospital of Oakland Research Institute as described previously [20, 21]. CRP and AGP were measured using a combined sandwich ELISA technique at the VitMin Laboratory (Willstaett, Germany), and used to adjust plasma zinc concentrations for inflammation, as described previously [21].

Statistical analysis

A statistical analysis plan was developed before analysis. Analyses were conducted with SAS for Windows release 9.4 (SAS Institute). Adjusted cytokine concentrations (i.e., adjusted concentration equals concentration for the stimulated whole blood culture minus the concentration for the negative control culture of the same sample) were used for statistical comparisons. Nearly all adjusted cytokine concentrations were positive (as expected) but 6 (5 for IL-10 at baseline and 1 for IL-13 at endline) yielded a small negative number for the adjusted value and these were changed to small positive numbers (0.1 for IL-10 and 1.0 for IL-13) for statistical analysis to allow for retaining rank order and for ease of transformation, if needed. Descriptive statistics were calculated for all variables, and extreme outliers were identified and examined for possible removal. Such outliers were only found with the flow cytometry data, where some samples at endline ($n = 5$ for the T-cell collection tube, $n = 17$ for the Treg collection tube, and $n = 7$ for both) had implausibly high counts of beads (>20,000 per collection) or of CD3 T-cells (>10,000 cells/μL; usually resulting from implausibly low bead counts of <1000 per collection), presumably as a result of technical problems. These samples were

TABLE 1 Baseline characteristics of total study population (All) and each treatment group[1]

Characteristic	All	Control	TZ	MNP	PZ	P value
Age, mo	15.6 ± 5.11	15.6 ± 5.12	15.6 ± 5.11	16.1 ± 4.97	15.3 ± 5.29	0.41
Males	52.3 (268)	55.2 (74)	50.0 (60)	53.7 (73)	50.0 (61)	0.78
LAZ[2]	−1.67 ± 1.07	−1.36 ± 1.06ᵃ	−1.79 ± 0.97ᵇ	−1.78 ± 1.04ᵇ	−1.76 ± 1.17ᵇ	0.0018
Stunting (LAZ < −2 SD)[2]	36.8 (188)	28.4 (38)	38.3 (46)	39.7 (54)	41.3 (50)	0.13
WAZ[2]	−1.39 ± 0.99	−1.11 ± 0.96ᵃ	−1.46 ± 0.96ᵇ	−1.47 ± 0.98 ᵇ	−1.53 ± 1.01ᵇ	0.0023
Underweight (WAZ < −2 SD)[2]	25.6 (131)	14.9 (20)ᵃ	26.7 (32)ᵃ,ᵇ	29.4 (40)ᵇ	32.2 (39)ᵇ	0.0096
WLZ[2]	−0.73 ± 0.88	−0.57 ± 0.84	−0.74 ± 0.90	−0.76 ± 0.84	−0.8 ± 0.93	0.054
Wasting (WLZ < −2 SD)[2]	6.8 (35)	3.0 (4)	5.8 (7)	7.4 (10)	11.6 (14)	0.073
Maternal age,[3] y	26.8 ± 6.07	26.5 ± 5.82	26.7 ± 6.03	27.7 ± 6.35	26.2 ± 6.02	0.29
Maternal BMI,[4] kg/m²	21.8 ± 3.24	22.1 ± 3.75	21.5 ± 3.04	22.2 ± 3.12	21.5 ± 2.86	0.30
Plasma zinc,[5] mg/dL	57.4 ± 11.9	56.6 ± 11.6	58.0 ± 11.1	58.4 ± 11.3	56.6 ± 13.4	0.26
Plasma zinc below median[5]	51.1 (261)	48.5 (65)	49.6 (59)	46.3 (63)	60.7 (74)	0.11
Plasma zinc < 65 mg/dL[5]	77.5 (396)	82.1 (110)	79.0 (94)	72.8 (99)	76.2 (93)	0.31

[1]Values are means ± SDs or % (n). Continuous variables were compared by van der Waerden's nonparametric test and categorical variables by chi-square test. When the overall P value was <0.05, post hoc comparisons between groups were performed. Means in the same row without a common letter differ (P < 0.05 with the Tukey–Kramer adjustment). Control, placebo; LAZ, length-for-age z score; MNP, daily multiple micronutrient powder; PZ, daily preventive zinc tablets; TZ, therapeutic dispersible zinc tablets; WAZ, weight-for-age z score; WLZ, weight-for-length z score.
[2]Anthropometry measurements: n for PZ = 121 (All = 511).
[3]Maternal age: n for control = 130, TZ = 115, MNP = 133, and PZ = 117 (All = 495).
[4]Maternal BMI: n for control = 112, TZ = 93, MNP = 96, and PZ = 96 (All = 397).
[5]Plasma zinc adjusted for inflammation using plasma C-reactive protein and α-1-acid glycoprotein as described in reference 21.

dropped from analysis. All variables were then assessed for conformance to the Gaussian distribution and transformed if needed for analysis. For use in figures, untransformed group means were estimated by back-transforming the group means of transformed variables. Means of endline values were compared with ANCOVA (SAS GLM procedure), controlling for the baseline value of the outcome, child age and sex, district, month of enrollment, and zinc status [below, or above or equal to, the median baseline plasma zinc concentration (54.2 mg/dL) from all study participants, or CRP- and AGP-adjusted plasma zinc (21) (56.0 mg/dL), considered in separate models]. Pairwise comparisons of all group means to one another were adjusted with the Tukey–Kramer test. Zinc status (i.e., baseline plasma zinc concentration below the median), child sex, and child age at enrollment were tested individually as possible effect modifiers by including interaction terms in the model, and subgroup means were estimated when the P value for the interaction term was <0.20.

Results

Demographic characteristics and nutritional status
The mean ± SD age of infants was 15.6 ± 5.1 mo at baseline and 52.3% of participants were male (Table 1). The prevalence of stunting and underweight was high, at 36.8% and 25.6%, respectively, whereas the prevalence of wasting was 6.8%. The mean ± SD plasma zinc concentration (not adjusted for

inflammation) at baseline was 57.4 ± 11.9 μg/dL, with 77.5% of participants being categorized as deficient using the cutoff of 65 μg/dL (24). Plasma zinc concentration did not differ by treatment group at baseline. The PZ and MNP treatments caused a significant increase in plasma zinc concentration by the end of the study, with mean plasma zinc at endline being significantly greater in these 2 groups than in the control and TZ groups (Table 2). Similarly, the percentages of infants with zinc deficiency at endline were lower in the PZ and MNP groups than in the control and TZ groups.

T-cell cytokines
The concentration of IL-2 was measured as a key growth cytokine for T-cells, IL-10 was measured as an important regulatory cytokine, and IFN-γ, IL-13, and IL-17 were measured as markers for Th1, Th2, and Th17 CD4 T-cell subsets, respectively. No differences were seen between the treatment groups for any of the T-cell cytokines at endline, nor was any interaction seen between treatment group and baseline zinc status (Figure 1). **Supplemental Table 1** shows unadjusted baseline and endline concentrations of these cytokines. The concentrations of IL-2 and IL-17 did not differ from baseline to endline, whereas the concentrations of the other 3 cytokines decreased (without adjustment for covariates).

TABLE 2 Endline characteristics of total study population (All) and each treatment group[1]

Characteristic	All	Control	TZ	MNP	PZ	P value
Length-for-age z score	−1.83 ± 1.05	−1.55 ± 1.02	−2.00 ± 1.00	−1.90 ± 1.01	−1.90 ± 1.14	0.30
Weight-for-age z score	−1.58 ± 0.98	−1.32 ± 0.94	−1.69 ± 1.05	−1.67 ± 0.95	−1.66 ± 0.94	0.54
Weight-for-length z score	−0.86 ± 0.88	−0.73 ± 0.81	−0.87 ± 0.96	−0.90 ± 0.93	−0.94 ± 0.83	0.97
Plasma zinc, mg/dL[2]	61 ± 16	56.8 ± 11.9ᵃ	58.4 ± 14.5ᵃ	63.6 ± 15.9ᵇ	67.2 ± 18.1ᵇ	<0.0001
Plasma zinc < 65 mg/dL[2]	68.6 (349)	80.6 (108)ᵃ	78.3 (94)ᵃ	61.5 (83)ᵇ	53.3 (64)ᵇ	<0.0001

[1]Values are means ± SDs or % (n). The number of study participants is indicated in Table 1. The P values indicate statistical significance for differences between treatment group means. The analysis was adjusted for baseline value of each variable, sex, district, age, season, and baseline plasma zinc (above/below median) as described in the Methods. Percentages were compared by chi-square test. When the overall P value was <0.05, post hoc comparisons between groups were performed. Means in the same row without a common letter differ (P < 0.05 with the Tukey–Kramer adjustment). Control, placebo; MNP, daily multiple micronutrient powder; PZ, daily preventive zinc tablets; TZ, therapeutic dispersible zinc tablets.
[2]Plasma zinc adjusted for inflammation using plasma C-reactive protein and α-1-acid glycoprotein as described in reference 21.
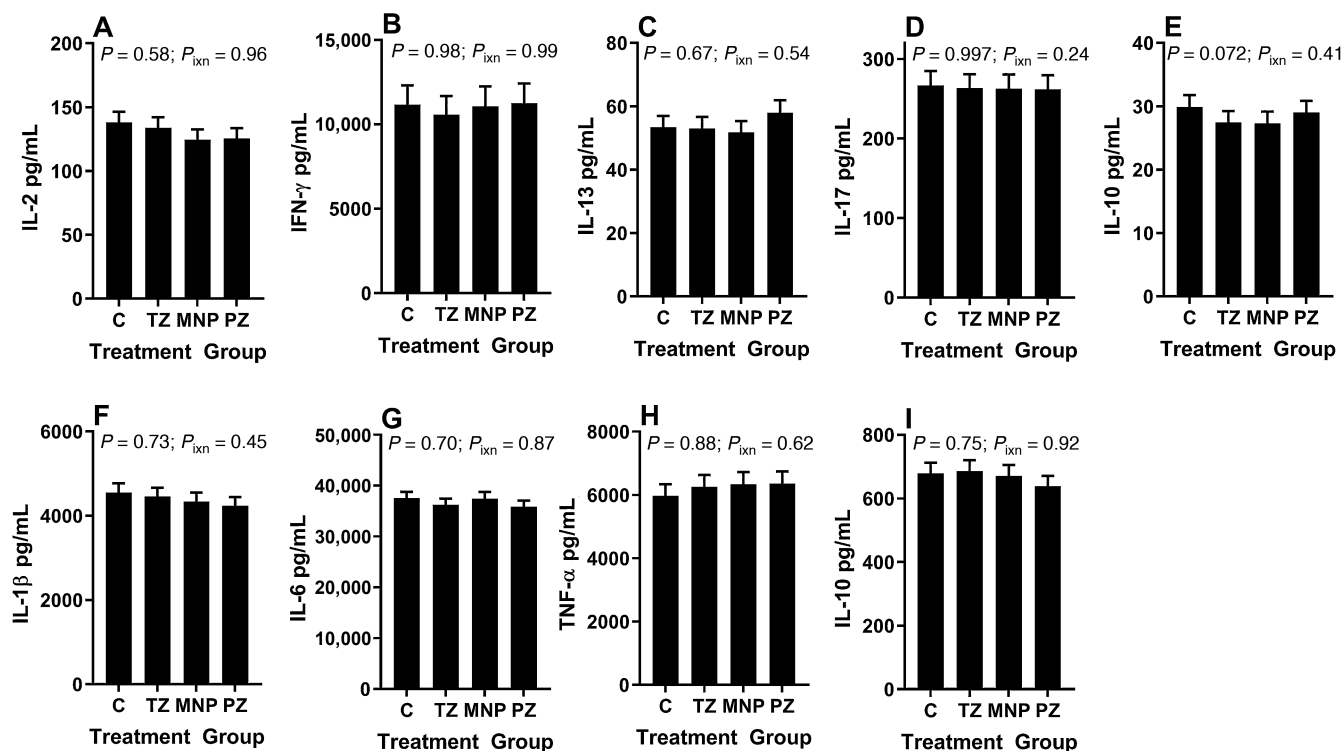
FIGURE 1 Endline cytokine concentrations of 4 treatment groups. Whole blood from each treatment group (C, *n* = 134; TZ, *n* = 120; MNP, *n* = 136; PZ, *n* = 122) was stimulated with anti-CD3 plus anti-CD28 antibodies (A–E) or with bacterial LPS (F–I). Data are represented as back-transformed means and estimated SEs. Means are adjusted for baseline value of each variable, sex, district, age, season, and baseline plasma zinc (above or equal to, and below, the median). *P* values indicate statistical significance for differences between treatment group means (*P*) and significance of interaction with categorical plasma zinc status (P_{ixn}). C, control; MNP, daily multiple micronutrient powder; PZ, daily preventive zinc tablets; TZ, therapeutic dispersible zinc tablets.

LPS-stimulated cytokines

The concentration of IL-10 was measured as a regulatory cytokine that can be produced by innate immune cells (e.g., monocytes) that would be stimulated by LPS, whereas IL-1β, IL-6, and TNF-α were measured as key proinflammatory cytokines. There was no treatment effect on these cytokines, nor any interaction with baseline zinc status (Figure 1). **Supplemental Table 2** shows the unadjusted baseline and endline concentrations. The concentration of IL-1β decreased over time, whereas the concentrations of the other cytokines increased.

T-cell concentrations

Concentrations of total and memory CD4 T-cells, total and memory CD8 T-cells, and Treg cells were measured by flow cytometry (**Supplemental Figure 1**). There was no treatment effect on these cell types, nor any interactions with baseline zinc status (Figure 2). **Supplemental Table 3** shows the unadjusted baseline and endline concentrations. The concentrations of total CD4 and CD8 T-cells decreased over time, as did the concentration of memory CD8, but not CD4, T-cells. The concentration and percentage of Treg cells both decreased over time.

Leukocyte and platelet concentrations

Lymphocyte, monocyte, neutrophil, eosinophil, basophil, and platelet concentrations were measured and regression analysis found a significant treatment effect on lymphocyte concentrations (*P* = 0.032) but not for the other leukocyte variables (Figure 3). Endline lymphocyte concentrations were significantly lower in the PZ group than in the control group, whereas the other 2 treatment groups were intermediary and did not differ significantly from either the PZ or the control group. A very similar, but nonsignificant, trend was seen for total lymphocytes (the sum of lymphocytes and atypical lymphocytes, which were present at very low concentrations) (*P* = 0.081) (Figure 3A, B). Significant interactions of treatment with baseline zinc status were seen for monocytes, eosinophils, and basophils (Figure 3C, F, G). Stratified post hoc comparisons found significant differences in eosinophil concentrations between the control and PZ groups, as well as between the TZ and PZ groups, for infants with baseline plasma zinc below (but not above) the median concentration (Figure 4). No significant differences were seen between means in such stratified analyses for basophils or monocytes (Figure 4). **Supplemental Table 4** shows unadjusted baseline and endline concentrations of leukocytes and platelets, whereas **Supplemental Table 5** shows unadjusted concentrations of eosinophils, basophils, and monocytes divided by baseline zinc status. Eosinophil concentrations were somewhat higher than expected for healthy children, with 33% (135 of 410) of children having mild eosinophilia, using a cutoff of $\geq$500 eosinophils/μL, whereas 6.8% (28 of 410) had moderate eosinophilia ($\geq$1500 eosinophils/μL), a concentration that would warrant clinical evaluation if it were chronic (25). Neutrophil concentrations increased from baseline to endline, whereas platelets and all other leukocyte variables decreased over time.
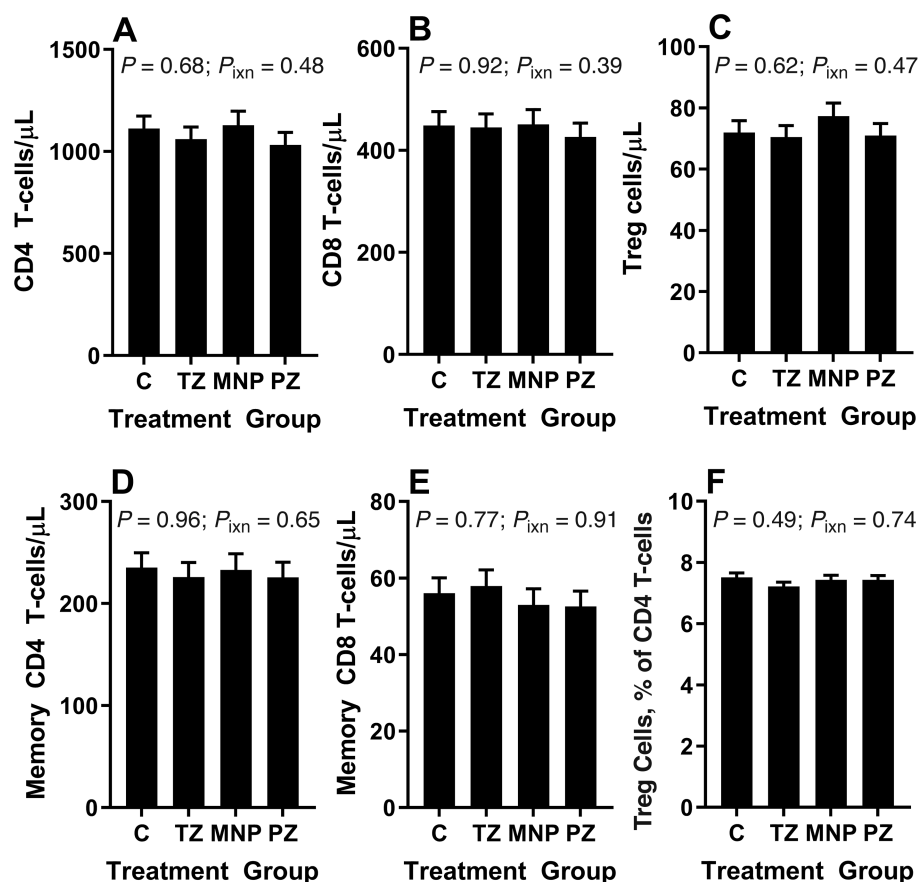
FIGURE 2 Endline T-cell concentrations of 4 treatment groups. Peripheral blood from each treatment group (C, $n = 134$; TZ, $n = 120$; MNP, $n = 136$; PZ, $n = 122$) was stained for T-cell surface markers and analyzed data are represented as back-transformed means and estimated SEs for total (A, B) and memory (D, E) CD4 and CD8 T-cell concentrations and for Treg cell concentration (C) and percentage (F). Means are adjusted for baseline value of each variable, sex, district, age, season, and baseline plasma zinc (above or equal to, and below, the median). P values indicate statistical significance for differences between treatment group means (P) and significance of interaction with categorical plasma zinc status (P_{ixn}). C, control; MNP, daily multiple micronutrient powder; PZ, daily preventive zinc tablets; TZ, therapeutic dispersible zinc tablets.

Adjusting for baseline anthropometric status

In an additional, exploratory, analysis for all immunology variables, baseline anthropometric variables (WLZ and LAZ) were included in the regression model to adjust for group imbalance and, as a result, a significant overall treatment effect was seen for platelets ($P = 0.032$) and the TZ group was found to be significantly lower than the control group ($P = 0.017$), with the other 2 groups being intermediate. The adjusted, back-transformed mean ± SE values at endline for the control, TZ, MNP, and PZ groups were 422 ± 10, 387 ± 10, 408 ± 10, and 419 ± 11/mL, respectively.

Discussion

The primary dependent variables in the present study were cytokines produced after LPS stimulation as an index of the responsiveness of innate immune cells, T-cell cytokines as an index of T-cell responsiveness, and concentrations of T-cells in peripheral blood. No effect was seen of the different zinc supplementation regimens on these endpoints, relative to the control intervention. Our primary focus was on the 2 daily zinc supplementation regimens, PZ and MNP, which both increased plasma zinc and decreased the prevalence of zinc deficiency over the course of the study in this subset of study participants, as was

also reported by the parent study using a slightly larger sample size (21). Thus, our finding of no effect of these interventions on the selected immunologic endpoints demonstrates that these measures of innate and adaptive immunity did not improve over a 9-mo period during which zinc status was improved. Because the children in this study had a high prevalence of zinc deficiency, the failure to see a response is likely not due to an absence of zinc deficiency at baseline. In addition, we included an interaction term to evaluate the effect of the interventions on children stratified by baseline zinc status and saw no differential effect, strengthening our conclusion that these indicators are not responsive to improvements of zinc status. However, at the end of the study >50% of participants in the TZ and MNP groups were still zinc deficient, using the cutoff of <65 μg/dL for plasma zinc, which leaves open the possibility that a further improvement in zinc status might have produced a change in these variables.

Previous randomized trials of zinc supplementation to children have examined effects on T-cell concentration or function. Treatment of marasmic infants in Chile with zinc ($n = 16$, 2 mg · kg^{-1} · d^{-1}; $n = 16$ placebo) for 90 d increased the T-cell proliferative response to the mitogen phytohemagglutinin (PHA), suggesting increased T-cell concentrations or proliferative ability (26). This study in severe malnutrition (marasmus) is not directly comparable with ours, which excluded severe
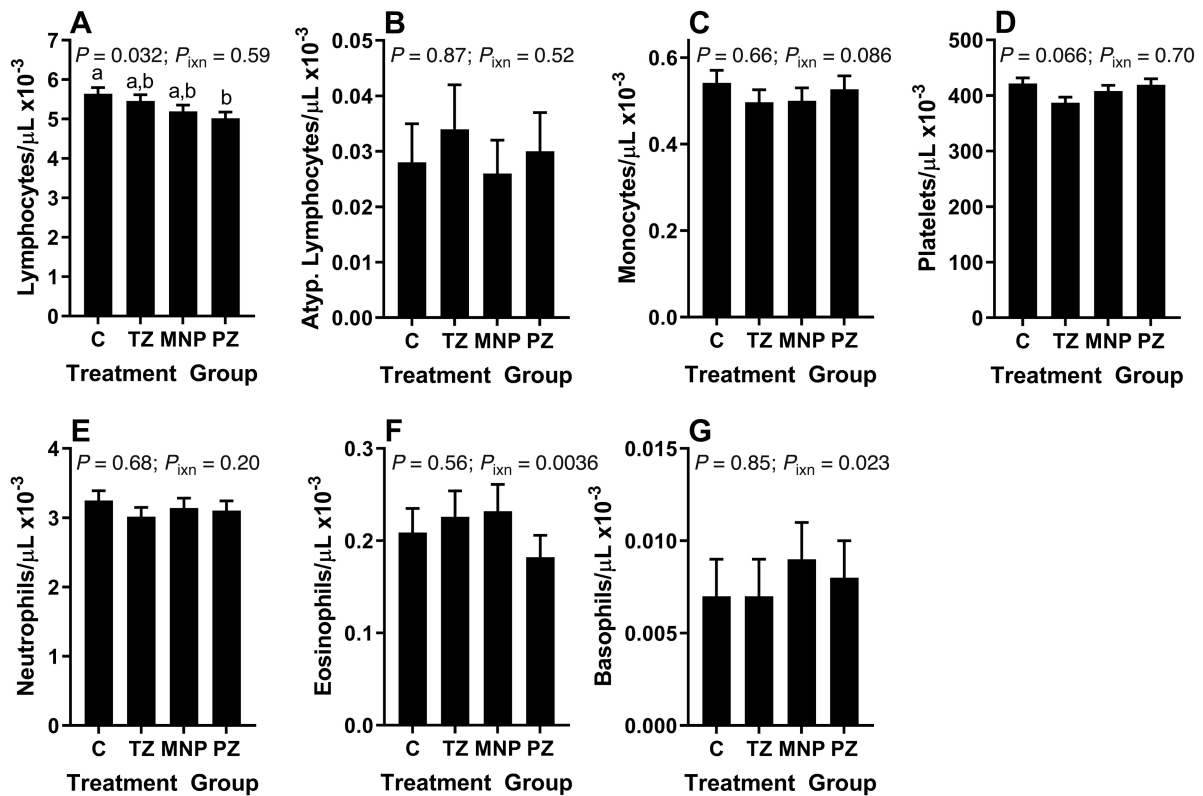
FIGURE 3 Endline leukocyte and platelet concentrations of 4 treatment groups. Complete blood count data were obtained from peripheral blood of each treatment group (C, $n = 134$; TZ, $n = 120$; MNP, $n = 136$; PZ, $n = 122$) and analyzed data are represented as back-transformed means and estimated SEs for lymphocytes (A), atypical lymphocytes (B), monocytes (C), platelets (D), neutrophils (E), eosinophils (F), and basophils (G). Means are adjusted for baseline value of each variable, sex, district, age, season, and baseline plasma zinc (above or equal to, and below, the median). P values indicate statistical significance for differences between treatment group means (P) and significance of interaction with categorical plasma zinc status (P_{ixn}). Means with different letters have significantly different concentrations ($P < 0.05$ with Tukey–Kramer adjustment for multiple comparisons). C, control; MNP, daily multiple micronutrient powder; PZ, daily preventive zinc tablets; TZ, therapeutic dispersible zinc tablets.

malnutrition (WLZ and WAZ < 3 z scores). A community-based trial in India compared 10 mg Zn daily (20 mg during diarrhea episodes; $n = 38$) with placebo ($n = 48$) for 120 d and found a relative increase in the percentage of CD4 (but not CD8) T-cells, but with no results reported for T-cell concentrations (27). A short treatment study of Bangladeshi children with *Shigella* dysentery used 20 mg Zn/d plus multivitamins for 2 wk ($n = 28$) compared with placebo ($n = 28$) and found an increase in the T-cell proliferative response to PHA with no effect on production of IFN-γ from the same cultures (28), although this study differs from ours in its short duration and treatment of children with dysentery during recovery. A longer study (29) began with treatment of Bangladeshi children with diarrhea due to enterotoxigenic *E. coli* with 20 mg Zn/d for all children, but continued after recovery with random assignment of children to 10 mg Zn/d ($n = 74$) or placebo for 3 mo ($n = 74$). The study examined the ratio of naïve (CD45RA$^+$) to memory (CD45RO$^+$) total or CD4 T-cells at baseline and endline, finding no effect (29). An intervention study in Mexican-American children supplemented with 20 mg Zn plus multiple micronutrients 5 d/wk for 10 wk ($n = 27$), as compared with placebo recipients ($n = 27$), saw a relative increase in the ratio of naïve to memory CD4 T-cells in the zinc group, as well as an increased production of IFN-γ by PHA-stimulated peripheral blood mononuclear cell cultures, and a decrease in IL-10 from the same cultures (30). In summary,

these studies showed inconsistent effects on measures of T-cell concentration and function. Where significant differences were seen between the placebo and zinc intervention (typically 10 or 20 mg/d), the tendency was to see an increase in naïve or total CD4 T-cell concentrations, or an increase in T-cell proliferation or production of the T-cell cytokine IFN-γ (in 1 study). The latter 2 results could both result from increased T-cell concentrations in peripheral blood, because the assays were not standardized for T-cell concentrations. We predicted similar increases in total CD4 T-cell concentrations and in CD4 Treg cell concentrations in the present study, but found no differences. Our daily doses of zinc (7 mg/d in the PZ group and 10 mg/d in the MNP group) were similar to those of these previous studies, whereas our sample size per group ($\sim$100) was larger and the duration of our study (9 mo) was longer than these previous studies. We thus feel that the null outcome of our study for these T-cell endpoints is not due to an inadequate treatment dose, a small sample size, or inadequate study duration, but is likely due to a lack of effect of treating zinc deficiency on this aspect of immune function in this population. We were not able to examine T-cell proliferation in the present study owing to the small volume of blood that was available and thus we do not know if the measure of T-cell function may have been affected.

In addition to these comparisons, we also examined the effect of these zinc interventions on peripheral blood lymphocyte,
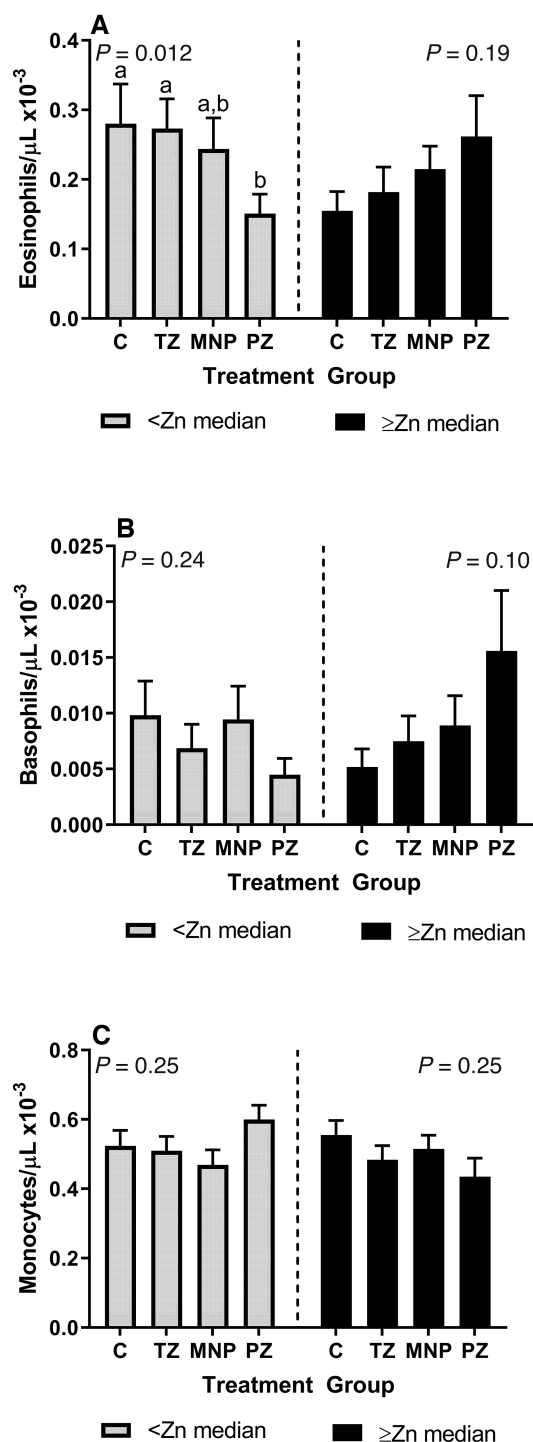
FIGURE 4 Endline eosinophil (A), basophil (B), and monocyte (C) concentrations of 4 treatment groups by baseline zinc status (above/below the median plasma zinc concentration). Complete blood count data of these cell types from peripheral blood were analyzed for a significant interaction of zinc status with treatment group (C, $n = 134$; TZ, $n = 120$; MNP, $n = 136$; PZ, $n = 122$). The data are represented as back-transformed means and estimated SEs. Means are adjusted for baseline value of each variable, sex, district, age, season, and baseline plasma zinc (above or equal to, and below, the median). P values indicate statistical significance for differences between treatment group means within zinc-status subgroups (P). Means with different letters have significantly different concentrations ($P < 0.05$ with Tukey–Kramer adjustment for multiple comparisons). C, control; MNP, daily multiple micronutrient powder; PZ, daily preventive zinc tablets; TZ, therapeutic dispersible zinc tablets.

monocyte, neutrophil, eosinophil, basophil, and platelet concentrations. Among these secondary endpoints, we saw a significant decrease in lymphocyte concentration (which includes both T and B cells) in the PZ group relative to the control group in the overall study population. The mean total lymphocyte concentrations in our study subjects at baseline (6–23 mo of age) and endline (∼9 mo later) are similar to recent reports for healthy Chinese children at these ages, a period over which a decline of ∼10–20% would be expected (31), as was seen in the present study. The health implication, if any, for the relatively greater decrease in the PZ than in the control group is uncertain. Because T-cell concentrations did not decrease in response to zinc treatment, one can speculate that B-cell concentrations did decrease in response to PZ supplementation, but we have no way to assess that retrospectively. Two recent studies have identified 2 different zinc transporters as having key roles in B-cell biology (32, 33), suggesting that further examination of this cell type is warranted. Another interpretation of this result is that zinc may have dampened a mild lymphocytosis in this population, perhaps resulting from undiagnosed infections, but this is also speculation.

In addition to a decrease in lymphocytes, we saw a decrease in eosinophil concentration in the PZ group relative to the control group in children with low zinc status (below the overall median plasma zinc concentration) at baseline. This finding is similar to results seen in 2 rodent studies which found that zinc supplementation decreased pulmonary eosinophilia in models of allergic asthma (34, 35). A human study of relevance has also been reported: infants with chronic wheeze, a condition consistent with a risk of atopic asthma, were found to have poor zinc status (as indicated by low hair zinc concentrations) relative to other children, suggesting an association of zinc deficiency with atopic disease, although no association was seen with eosinophil concentrations (36). Helminth infections also induce eosinophilia and in a rodent model of *Heligmosomoides polygyrus* infection, investigators found that zinc deficiency was associated with lower rather than higher blood eosinophil concentrations, although the effect of intervening with zinc supplements was not examined (37, 38). Thus, the literature does not show a consistent association of zinc status with eosinophilic inflammation, but the present study suggests that future work on this topic may be of interest.

Children in our study had an unexpectedly high prevalence of eosinophilia, with 33% showing evidence of at least mild eosinophilia, even though they were apparently healthy at the time of blood collection (i.e., absence of reported diarrhea or measured fever within 24 h of the blood sample collection). Eosinophilia is relatively uncommon but can result from intestinal helminth infections (39) as well as other conditions (40). Even asymptomatic intestinal helminth infections can cause eosinophilia, as shown by a recent study among asymptomatic adults with *Strongyloides stercoralis* infection in south India where higher eosinophil concentrations were seen in asymptomatic, infected individuals (geometric mean: 550 eosinophils/μL) than in asymptomatic, uninfected controls (geometric mean: 281 eosinophils/μL) (41). Although we do not have diagnostic information on helminth infections for the children in our study population, a recent study in another rural area of Laos found high prevalence rates of intestinal helminths in a community-based study of children and adults (≥ 2 y; $n = 574$), with infection rates of 87% for hookworm, 33% for *Trichuris trichiura*, and 10% for *Ascaris lumbricoides*. These infections can cause eosinophilia (39) and if such infections were common in our study

population this could explain the high levels of eosinophilia. If this were true, one could then speculate that the zinc treatment might have reduced eosinophil concentrations by helping to clear or reducing the severity of intestinal helminth infections. Although the aforementioned rodent studies (37, 38) indicate that zinc deficiency decreases eosinophilia in *H. polygyrus* infection, the study did not examine effects of zinc supplementation on resolution of infection or eosinophilia, leaving open the possibility that zinc may be beneficial for intestinal helminth infections in humans. In addition, helminth infections themselves can affect both innate and adaptive immune function (42) and results of a zinc intervention trial such as this might differ between children with and without such infections.

Strengths of the present study include its large sample size and its setting in a population with a high prevalence of zinc deficiency and close proximity to laboratory facilities suitable for analysis of immune function. A shortcoming of the study is that a majority of the participants remained zinc-deficient at the end of the trial, raising the question of whether a higher dose of zinc might have produced different results. The apparent effect of preventive zinc (PZ group), given daily as a 7-mg supplement, on total lymphocytes (perhaps on B cells) and on eosinophils (possibilities that were discussed above), is interesting and suggests that further examination of the effect of zinc treatment on these cell subsets may prove to be fruitful in identifying an immunologic indicator that is responsive to zinc supplementation.

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