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Associations between Mucosal Innate and Adaptive Immune Responses and Resolution of Diarrheal Pathogen Infections▽

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The identification of immune response mechanisms that contribute to the control of diarrheal disease in developing countries remains an important priority. We addressed the role of fecal chemokines and cytokines in the resolution of diarrheal *Escherichia coli* and *Giardia lamblia* infections. Stools collected from 127 Mexican children 5 to 15 months of age enrolled in a randomized, double-blind, placebo-controlled, vitamin A supplementation trial were screened for enteropathogenic *Escherichia coli* (EPEC), enterotoxigenic *E. coli* (ETEC), and *Giardia lamblia*. Fecal concentrations of tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-4 (IL-4), IL-5, IL-6, IL-8, IL-10, and interferon-γ (IFN-γ) were determined. Hazard models incorporating cytokine variables were fit to durations of asymptomatic and symptomatic pathogen infections, controlling for treatment group. Increased levels of TNF-α and IL-6 were associated with decreased durations of EPEC infection and increased ETEC durations. Increased IL-4 and IFN-γ levels were associated with decreased and increased durations, respectively, of both EPEC and ETEC infections. Increased IL-10 levels were associated with increased and decreased durations of asymptomatic and symptomatic EPEC infections, respectively, and increased durations of both asymptomatic and symptomatic ETEC infections. Increased levels of MCP-1, IFN-γ, IL-4, and IL-5 were associated with increased *G. lamblia* infection duration, while increased IL-8 levels were associated with decreased durations. Differences in proinflammatory and Treg cytokine levels are associated with differences in the resolution of inflammatory and noninflammatory pathogen infections.

Pediatric diarrheal disease continues to be an important health problem in developing countries, with the disability-adjusted life year (DALY) for this disease estimated to be approximately 100 million, >95% of which is due to mortality (36). A broad range of gastrointestinal pathogens cause diarrhea among young children in these settings. Diarrheagenic *Escherichia coli* pathotypes (DEPs) represent a leading bacterial cause of diarrhea, with enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) representing the most important pathogens of this group (7, 41). The disease burden due to such gastrointestinal parasites as *Giardia lamblia* also continues to be an important health problem in countries such as Mexico (31).

We have addressed the efficacy of vitamin A supplementation in reducing the burden of pathogen-specific diarrhea by carrying out a randomized, placebo-controlled, double-blind trial among children living in peri-urban areas of Mexico City. Supplementation in this trial was associated with pathogen-specific effects on the fecal innate and adaptive cytokine responses and with divergent pathogen-specific clinical outcomes (30, 32). We now want to understand how the effect of vitamin A on these cytokine responses is associated with pathogen-specific outcomes.

Recent research has reported that distinct cytokine pathways are involved in the host responses to each of these enteropathogens (12, 21). These findings suggest that a specific cytokine response adequate for resolving one pathogen infection may be inappropriate against a different pathogen and so lead to prolonged infection and pathogenesis. An important first step in our overall analysis then is to determine how these different cytokine response pathways are associated with specific pathogen infection outcomes. These findings subsequently will allow us to determine how the modification of these responses by vitamin A changes these associations.

Accordingly, in this paper we examine whether distinct pathways in the gut are effective responses in resolving infections by ETEC, EPEC, and *G. lamblia*. These pathogens were chosen because each produces pathology through quite different mechanisms and induce quite different cytokine responses. EPEC expresses virulence factors that lead to the formation of attaching and effacing (A/E) lesions in the mucosa and subsequent onset of diarrhea (7). ETEC, in contrast, expresses the

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heat-labile and heat-stable toxins and multiple adhesive pili called colonization factors (CFs), which lead to the production of watery diarrhea (41). Finally, *G. lamblia* produces inflammation and villous atrophy after attaching to epithelial cell surfaces and so reduces the gut's absorptive capability (2). These differences allowed us to address the hypothesis that the distinct fecal cytokine profiles produced during infections by each of these pathogens will be associated with differences in pathogen infection duration.

MATERIALS AND METHODS

Study population and recruitment. A census was carried out of all children less than 2 years of age living within nine neighborhoods (*colonias*) that are part of the community of La Magdalena Atlicpac, which is located along the eastern perimeter of Mexico City. Mothers of all children from 5 to 15 months of age were invited to participate in the study as described previously (29, 32). Children were excluded if they had diseases causing immunosuppression or any congenital or acquired alteration of the digestive tract, such as chronic diarrhea, that could alter the absorption of micronutrients. Children who were taking vitamin supplements also were excluded. For the overall study, 200 children living in this community were identified and enrolled during a 10-month period after their parents consented to their participation.

Each child, once enrolled, was randomly assigned to receive vitamin A or a placebo every 2 months. Recruited children then were monitored prospectively for up to 15 months, during which time households were visited twice a week. At each visit mothers or child caretakers were interviewed to determine the presence of the following symptoms: diarrhea, the number and consistency of evacuations, the presence of blood and mucus in stools, fever, cough, and respiratory difficulty.

Sample collection and processing. A stool sample was collected twice a month among healthy children, and up to three stools were collected in the week following a diarrheal episode. Project supervisors accompanied approximately 5% of all household visits to ensure the quality of data collection. Children were referred to the study physician for diagnosis and treatment when the fieldworker or caregiver was concerned about the child's health status.

Examination of stools for parasites and bacteria. All stool samples were plated onto salmonella-shigella, MacConkey, and MacConkey-tellurite agars for the identification of *Salmonellae* spp., *Shigella* spp., and *E. coli* (20). The Kato-Katz technique and the trichrome staining of wet mounts of concentrated stool were performed to identify *Ascaris lumbricoides*, *Entamoeba histolytica*, and *G. lamblia* ova in stool (34). Three lactose-fermenting colonies with morphology resembling that of *E. coli* (when present) were selected from MacConkey agar plates and speciated biochemically. Diarrheagenic *E. coli* was characterized by a single-multiplex technique, previously described (10), that detects the following pathogenic genes: heat-stable and heat-labile enterotoxins (*st* and *lt*) for enterotoxigenic *E. coli* (ETEC), intimin (*eaeA*) and bundle-forming pilus (*bfp*) for enteropathogenic *E. coli* (EPEC), Shiga toxins 1 and 2 (stx_1 and stx_2) and intimin (*eaeA*) for shiga toxin-producing *E. coli* (STEC), and invasive-associated loci (*ia1*) for enteroinvasive *E. coli* (EIEC).

Analysis of stool cytokines by ELISA. An aliquot from each fresh stool collected from children was frozen within 4 h after collection at −20°C. For this study, we analyzed cytokines from a subsample of children during the summer months of June, July, and August, since this period is when diarrheal *E. coli* is the most prevalent and when diarrheal rates reach their peak. As such, samples were extracted as described previously (29) and the supernatants were collected, frozen, and stored at −70°C. These supernatants then were assayed for the chemokines monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and IL-6, the Th1 cytokine gamma interferon (IFN-γ), the Th2 cytokines IL-4 and IL-5, and the T-regulatory cytokine (Treg) IL-10 by an enzyme-linked immunosorbent assay (ELISA) using paired ELISA-specific capture and biotinylated detecting antibody (Ab) (Pierce-Endogen, Rockford, IL, and R&D Systems, Minneapolis, MN). Peroxidases conjugated to streptavidin (Pierce-Endogen, Rockford, IL) were used to detect the capture Ab, and peroxidase activity was measured using 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate and read at a wavelength of 405 nm. Recombinant cytokines and chemokines were used to generate a standard curve, and levels of these cytokines and chemokines from the stool extracts were determined using the standard curve in 96-well plates according to the manufacturer's protocol. The unit for all cytokine assays was pg/ml normalized to mg/ml of protein per stool. The detection limit for these cytokine assays was 10 pg/ml.

Statistical analysis. The primary end points for this analysis were the durations of infections by ETEC, EPEC, and *G. lamblia* in children during the 3-month summer months of June, July, and August. These pathogens were selected for the analysis due to their importance as etiological agents in diarrhea among Mexican children (32). Infections by ETEC were defined as any lactose-positive bacteria isolated from stool that had the pathogenic genes for the heat-stable and heat-labile enterotoxins (*st* and *lt*), while infections by EPEC were defined by the absence of Shiga toxin genes and the presence of the genes for intimin (*eaeA*) for both typical EPEC (tEPEC) and atypical EPEC and the bundle-forming pilus (*bfp*) gene for tEPEC. An infection by *G. lamblia* was defined as a stool positive for the parasite. Separate analyses were not carried out for ETEC isolates that had only the *st* gene or the *lt* gene or for EPEC isolates with only the *eaeA* gene due to the small number of these pathotypes during the summer period. The beginning of an infection was defined as the midpoint in time between a stool negative for a pathogen and a subsequent positive stool. The end of the episode was defined as the midpoint between the last sequentially positive stool and the subsequent negative stool. Durations of pathogen infections were defined as encompassing the time between these two midpoints.

The means and standard deviations of infection duration first were calculated for each pathogen stratified by chemokine and cytokine values classified into three levels: nondetectable, less than the median of positives, and greater than or equal to the median of positives. These means were calculated separately for asymptomatic and symptomatic infections. The classification into levels was carried out because an important proportion of stool samples had no detectable levels of cytokines. These samples would have had to be eliminated from any analysis of durations if continuous variables for the chemokines and cytokines were used.

Weibull parametric regression survival time models then were fit to the durations of pathogen infections stratified by these chemokine and cytokine levels to test how differences in these levels were associated with durations. Children from both the vitamin A and placebo groups were included in these analyses. A variable indicating whether a child received the vitamin A supplement or a placebo was included in each model to control for supplementation. Statistical significance was set at a probability level of <0.05 and at <0.1 for interactions. The analysis was carried out using the STREG procedure from STATA (version 9.0) software (StataCorp, College Station, TX).

Sample size calculations. Sample sizes were calculated based on the assumption that the study population had a diarrheal disease rate of three episodes per child per year and that the vitamin A supplement would reduce this rate by approximately 20%. This effect approximated the reduction in diarrhea incidence reported by Barreto et al. in their vitamin A trial in Brazil (3). It was calculated that a sample size of 100 per group was required to detect a 20% reduction between the control and treatment group with 80 and 95% significance levels and an expected loss to follow-up of 20%. This calculation allowed for the repeated measurements of the outcome and a correlation between measurements at different time points of 0.7 (13).

The study was approved by the ethical review committees from the National Centre for the Health of Infants and Adolescences of Mexico (CENSIA) and the Harvard School of Public Health.

RESULTS

Number of enrolled children. Results from a subsample of 127 children monitored during the months of June, July, and August are included in this analysis. The remaining 73 children were not enrolled until the fall and were not included in the analysis. A total of 505 stool samples were collected from these 127 children, 262 samples from 70 children administered the placebo and 243 samples from 57 children administered the vitamin A supplement.

Clinical history of study children. A total of 149 pathogen isolates were identified in stools collected during the 3-month period of this analysis, representing 131 separate episodes of infection. ETEC infections were the most frequent (49), followed by EPEC infections (46). Additionally, 30 *Giardia* episodes were identified, while the remaining infections were due to Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli*

TABLE 1. Parametric hazard regression analyses of the association between innate and adaptive immune response cytokine levels and durations of asymptomatic EPEC infections[a]

Cytokine and response	No. of infections	Mean duration (+ SD, in days) of asymptomatic EPEC infections	Risk of asymptomatic EPEC infection ending	
			Hazards ratio (95% CI)	*P*
Innate immune				
IL-6				
ND	12	18.41 + 13.22	1.00	
<Median	3	14 + 30.12	15.7 (0.93–263.22)	0.05
>Median	3	21.5 + 5.07	0.90 (0.18–40.49)	0.90
TNF-α				
ND	10	26.7 + 220.15	1.00	
<Median	5	19.3 + 150.00	12.78 (10.94–84.13)	0.01
>Median	4	15.62 + 90.63	4.9 (0.77–310.25)	0.09
Adaptive immune				
IFN-γ (Th1)				
ND	14	19 + 120.45	1.00	
<Median	2	14.5 + 4.24	1.99 (0.42–90.31)	0.38
>Median	4	37.12 + 30.82	0.21 (0.06–00.72)	0.01
IL-4 (Th2)				
ND	13	25.30 + 21.15	1.00	
<Median	1	20.5	1.98 (0.20–19.63)	0.55
>Median	6	15.66 + 70.54	7.55 (1.56–36.01)	0.01
IL-5 (Th2)				
ND	12	26.75 + 21.79	1.00	
<Median	5	17.3 + 2.30	1.28 (0.33–40.96)	0.71
>Median	3	12 + 70.46	6.62 (1.41–30.95)	0.01
IL-10				
ND	10	16.7 + 11.05	1.00	
<Median	5	24.4 + 110.72	0.49 (0.16–10.46)	0.20
>Median	4	34.25 + 33.95	0.17 (0.04–0.64)	0.01

[a] Hazard rates were calculated after fitting the Weibull hazard model to the length of infection duration. Infection duration was defined as the time from the first positive stool until the first negative stool among sequentially collected stools. Reference groups are stool samples with no detectable levels of chemokine or cytokine. CI, confidence interval.

(EIEC), A. *lumbricoides*, and *E. histolytica*. Ninety-four percent of ETEC isolates (59/62) had only the *lt* heat-labile enterotoxin gene, while the remaining 3% had genes for both the heat-labile and *st* heat-stable enterotoxins. Seventy-eight percent of the EPEC isolates (43/59) were atypical, having only the *eaeA* intimin gene, while the remaining 22% had both the intimin and the bundle-form pilus gene (*bfp*). Eleven of 50 ETEC infections were associated with a diarrheal episode, while 23 of 47 EPEC infections were associated with an episode.

Associations between fecal cytokine responses and EPEC infection durations. Innate and adaptive cytokines were significantly associated with durations of EPEC, ETEC, and *G. lamblia* infections, but the directions of these associations were different for each pathogen and clinical outcome. Tables 1 and 2 present the hazard rates for durations of asymptomatic and symptomatic EPEC infections (both *eaeA* and *eaeA* plus *bfp* pathotypes) by cytokine levels. Asymptomatically infected children with intermediate fecal levels of IL-6 or intermediate and high fecal levels of TNF-α have increased risks of an episode ending, thus they have reduced durations of infection compared to that of children with no detectable levels. For the adaptive immune response, there is an increased risk of an asymptomatic infection ending among children with high levels of IL-4 and IL-5 in stool compared to that of children with no detectable levels. In contrast, children with high fecal levels of IFN-γ and IL-10 have reduced risks, thus they have increased infection durations compared to those of children with no detectable levels.

For symptomatic infections, children with intermediate fecal levels of IL-6 and IL-8 have an increased risk of an episode ending and reduced durations of infection compared to those of children with no detectable levels. For the adaptive immune response, an increased risk of an episode ending is found among children with intermediate levels of IL-5. In contrast, children with intermediate levels of IFN-γ have reduced risks, thus they have increased infection durations compared to those of children with no detectable levels. This contrasts with the increased risk and reduced duration found among symptomatically infected children who have high levels of IL-10.

Associations between fecal cytokines and ETEC infection durations. Tables 3 and 4 present the hazard rates for durations of asymptomatic and symptomatic ETEC infections (both *lt* and *st* plus *lt* pathotypes) by cytokine levels. Children with intermediate levels of TNF-α in stool have a reduced risk that an asymptomatic ETEC infection will end, thus they have longer infection durations compared to those of children with no detectable levels. Intermediate levels of IL-8 in children's stool, in contrast, are associated with an increased risk that an asymptomatic infection will end, leading to a reduced infection duration. Children with intermediate and high fecal levels of IL-4 have a greater risk of an asymptomatic ETEC episode

TABLE 2. Parametric hazard regression analyses of the association between innate and adaptive immune response cytokine levels and durations of symptomatic EPEC infections[a]

Cytokine and response	No. of infections	Mean duration (+ SD, in days) of symptomatic EPEC infections	Risk of symptomatic EPEC infection ending	
			Hazards ratio (95% CI)	*P*
Innate immune				
IL-6				
ND	8	18.68 + 8.48	1.00	
<Median	2	14 + 9.89	5.33 (0.89–31.85)	0.06
>Median	3	20 + 9.53	0.88 (0.23–31.85)	0.85
IL-8				
ND	9	23.44 + 20.28	1.00	
<Median	4	14.62 + 5.34	9.01 (1.83–44.35)	0.01
>Median	5	20.7 + 6.16	3.49 (0.90–13.54)	0.07
Adaptive immune				
IFN-γ (Th1)				
ND	8	19.06 + 7.97	1.00	
<Median	6	20.75 + 25.27	0.06 (0.008–0.45)	0.01
>Median	2	26.25 + 7.42	0.66 (0.18–2.36)	0.52
IL-5 (Th2)				
ND	11	18.18 + 7.89	1.00	
<Median	2	14.5 + 9.19	5.32 (1.20–23.53)	0.02
>Median	4	18.5 + 8.34	2.06 (0.61–6.9)	0.23
IL-10				
ND	10	22.25 + 19.57	1.00	
<Median	1	13.50	1.28 (0.18–9.27)	0.80
>Median	5	18.7 + 7.64	3.49 (0.95–12.87)	0.06

[a] Hazard rates were calculated after fitting the Weibull hazard model to the length of infection duration. Infection duration was defined as the time from the first positive stool until the first negative stool among sequentially collected stools. Reference groups are stool samples with no detectable levels of chemokine or cytokine. CI, confidence interval.

ending, thus they have reduced durations of infection compared to those of children with no detectable levels. In contrast, children with high fecal levels of IFN-γ and IL-10 have reduced risks, thus they have increased durations of asymptomatic ETEC infections compared to those of children with no detectable levels. For symptomatic ETEC infections, children with high fecal levels of IL-6 and TNF-α have greatly reduced risks that an infection will end, thus they have increased infection durations compared to those of children with no detectable levels. Children with intermediate and high levels of the adaptive IL-5 and IL-10 cytokines in stool also have reduced risks of an episode ending. In contrast, children with intermediate levels of IL-4 have an increased risk that a symptomatic ETEC infection will end, thus they have a reduced infection duration compared to that of children with no detectable levels.

Associations between fecal cytokines and *G. lamblia* infection durations. Only two *G. lamblia* infections were symptomatic, so separate analyses of asymptomatic and symptomatic durations were not carried out (Table 5). Children with intermediate fecal levels of the chemokine IL-8 had a significantly increased risk that *G. lamblia* infections would end, thus they had reduced infection durations compared to those of children with no detectable levels. In contrast, children with high levels of MCP-1 had a significantly decreased risk that a *G. lamblia* infection would end and thus significantly increased durations relative to those of children with no detectable concentrations. A significantly decreased risk of a *G. lamblia* infection ending was found among children with the highest fecal concentrations of IFN-γ and IL-4. Both intermediate and high levels of IL-5 also were associated with significantly decreased risks of *G. lamblia* infections ending, with intermediate levels being associated with the greatest reductions.

DISCUSSION

We have found significant differences in associations between levels of innate and adaptive immune response cytokines in stool and durations of asymptomatic and symptomatic infections by EPEC, ETEC, and *G. lamblia*. The most important differences were found between levels of the proinflammatory and regulatory cytokines and durations of asymptomatic and symptomatic DEP infections. These results suggest that specific cytokine responses play different roles in resolving specific pathogen infections among young children in a community setting.

The proinflammatory cytokines are strongly and positively associated with the resolution of EPEC infections in our study. The association between increased TNF-α and IL-6 levels and decreased durations of both asymptomatic and symptomatic EPEC infection is consistent with the role these cytokines play in eliminating infections by the related mouse pathogen *Citrobacter rodentium* (8, 15, 25, 42, 44). These proinflammatory cytokines, secreted by both enterocytes and local Peyer's patch lymphocytes, activate effector mechanisms at the epithelium (23). TNF-α, for example, may eliminate infections through its ability to activate macrophages and so increase their phagocytic activity. There still is no consensus on the importance of IL-8 in resolving infections, since the upregulation of IL-8 by the epithelium also can result in increased

TABLE 3. Parametric hazard regression analyses of the association between innate and adaptive immune response cytokine levels and durations of asymptomatic ETEC infections[a]

Cytokine and response	No. of infections	Mean duration (+ SD, in days) of asymptomatic ETEC infections	Risk of asymptomatic ETEC infection ending	
			Hazards ratio (95% CI)	*P*
Innate immune				
TNF-α				
ND	17	15.32 + 70.79	1.00	
<Median	6	27.58 + 12.76	0.29 (0.10–0.78)	0.01
>Median	7	21.28 + 11.66	0.45 (0.17–10.19)	0.11
IL-8				
ND	21	19.57 + 10.79	1.00	
<Median	6	14.75 + 50.13	5.90 (1.20–28.92)	0.03
>Median	10	19.9 + 100.65	1.89 (0.68–28.92)	0.21
Adaptive immune				
IFN-γ (Th1)				
ND	14	14.96 + 90.75	1.00	
<Median	10	25.65 + 90.85	0.34 (0.14–0.84)	0.02
>Median	9	18.83 + 90.50	0.61 (0.25–10.49)	0.28
IL-4 (Th2)				
ND	21	22.09 + 11.90	1.00	
<Median	7	17.78 + 40.91	2.85 (0.86–90.36)	0.08
>Median	8	13.81 + 70.00	3.50 (0.91–13.37)	0.06
IL-10				
ND	13	15.92 + 80.19	1.00	
<Median	12	23.41 + 12.23	0.42 (0.18–0.98)	0.04
>Median	7	19.07 + 10.57	0.65 (0.25–1.67)	0.37

[a] Hazard rates were calculated after fitting the Weibull hazard model to the length of infection duration. Infection duration was defined as the time from the first positive stool until the first negative stool among sequentially collected stools. Reference groups are stool samples with no detectable levels of chemokine or cytokine. CI, confidence interval.

inflammation and pathology following neutrophil infiltration (24, 28, 43, 44). This inconsistency may reflect the innate immune system's ability to provide a protective response but also produce negative consequences, including exacerbated tissue destruction, following the hyperactivation of this system (28). This possibility suggests that the proinflammatory cytokines are elevated as a result of inflammation independently of any role they play in infection resolution.

The results from our study suggest that the adaptive Th1, Th2, and Treg cytokine responses have contrasting roles in limiting EPEC infections. The findings that increased levels of IL-4 may play a role in resolving EPEC infections while increased levels of IFN-γ may increase infection durations is not consistent with the role of a Th1 response in resolving infections (17, 18, 45). However, Maaser et al. (33) have shown that B cells are of crucial importance for the eradication of *C. rodentium*, since mice lacking B cells could not clear the bacteria or decrease the bacterial burden during an extended period. This finding suggests that a strictly Th1 response is not adequate by itself in resolving infections.

A novel finding of the study is that increased IL-10 levels are associated with the prolongation and resolution of asymptomatic and symptomatic EPEC infections, respectively. IL-10 is an antiinflammatory cytokine produced by many cell types (4, 35, 38, 39). It acts principally to limit the damaging effects of the inflammatory response to pathogen infections largely through the inhibition of macrophage and dendritic cell function (9). However, high or dysregulated levels of IL-10 may result in chronic infection (14, 47). The association between high levels of IL-10 and the prolongation of asymptomatic EPEC infections in our study may reflect the negative effect of dysregulated IL-10 production on the clearance of EPEC infections.

For ETEC, the association of increased levels of TNF-α, IL-6, and IFN-γ with increased durations suggests that these components of the inflammatory responses are inadequate for resolving ETEC infections. In contrast, increased IL-8 levels appear to play an important role in infection resolution in our study. A number of studies have reported similar contrasting effects of ETEC infections on the expression of these cytokines. For example, experimental vaccines expressing ETEC fimbriae suppressed levels of TNF-α, IL-1, and IL-6 (22, 40), while *in vitro* and *in vivo* studies have reported that IL-8 levels are significantly increased following ETEC infections (16, 19). However, these studies do not indicate how the increased expression of these innate immune response cytokines relates to the resolution of ETEC infection. It is important to consider whether these cytokine levels could be elevated as a result of inflammation independently of any role they play in infection resolution.

The significant association between increased fecal IL-4 levels and decreased ETEC infection durations is consistent with the protective role of an upregulated Th2 cytokine response against ETEC infections (5, 6, 37). An anti-enterotoxin (anti-LT) intestinal IgA response upregulated by Th2 cytokines is important in resolving ETEC infection and so may be mediating this association (11). High levels of IL-10 in our study appear to be inadequate in resolving ETEC infections. This result is consistent with findings that LT-ETEC-infected individuals with IL-10 alleles associated with high IL-10 produc-

TABLE 4. Parametric hazard regression analyses of the association between innate and adaptive immune response cytokine levels and durations of symptomatic ETEC infections[a]

Cytokine and response	No. of infections	Mean duration (+ SD, in days) of symptomatic ETEC infections	Risk of symptomatic ETEC infection ending	
			Hazards ratio (95% CI)	*P*
Innate immune				
IL-6				
ND	2	17.25 + 6.01	1.00	
<Median	2	19.75 + 1.76	0.06 (0.002–2.08)	0.12
>Median	3	25 + 8.54	0.07 (0.005–1.01)	0.05
TNF-α				
ND	4	16.25 + 4.51	1.00	
<Median	1	16.00	0.14 (0.009–2.37)	0.17
>Median	3	26.66 + 6.02	0.01 (0.000–0.24)	0.00
Adaptive immune				
IL-4 (Th2)				
ND	4	19.75 + 5.45	1.00	
<Median	3	16.33 + 4.50	11.04 (1.39–88.26)	0.02
>Median	1	33.00	0.22 (0.026–2.00)	0.18
IL-5 (Th2)				
ND	6	17 + 4.01	1.00	
<Median				
>Median	2	29.5 + 4.94	0.12 (0.02–0.72)	0.02
IL-10				
ND	6	17 + 4.01	1.00	
<Median				
>Median	2	29.5 + 4.94	0.08 (0.009–0.80)	0.03

[a] Hazard rates were calculated after fitting the Weibull hazard model to the length of infection duration. Infection duration was defined as the time from the first positive stool until the first negative stool among sequentially collected stools. Reference groups are stool samples with no detectable levels of chemokine or cytokine. CI, confidence interval.

tion had a limited ability to rapidly clear infection (12). These findings suggest that the antiinflammatory effects of high levels of IL-10 inhibit the clearance of ETEC infections.

The increased durations of *G. lamblia* infections found among children with increased fecal IFN-γ, IL-4, and IL-5 levels in our study is not consistent with reports that these cytokines are elevated during the acute and elimination phases of infection (1). Overall, T-cell-dependent mechanisms are important in resolving acute *G. lamblia* infections (46). Intestinal IgA antibodies, which are upregulated by Th2 cytokines, are important in eradicating *G. muris* or *G. lamblia* infection (27). However, a strong Th1 response also is important in reducing *G. lamblia* intestinal trophozoites and fecal cyst counts (48, 49). The reduced durations of *G. lamblia* infections found among children with increased fecal IL-8 concentrations also is not consistent with reports that the infection of monolayers of human colon epithelial cells did not lead to the increased production of IL-8 or other chemokine or proinflammatory cytokine responses (23).

Overall, the clearest differences in associations between cytokine levels and pathogen infection durations in our study may relate to whether the pathogen induces a strong inflammatory response. Increased levels of the proinflammatory cytokines are associated with the more rapid resolution of infections by EPEC, which induces a strong inflammatory response, but reduced resolution of ETEC and *G. lamblia* infections, which do not. Interestingly, no similar contrasting associations are found for Th1 and Th2 cytokines for any of these three pathogens. Another important difference is the association between IL-10 levels and the resolution of EPEC asymptomatic and symptomatic infections. This finding may reflect the role IL-10 plays in counterbalancing the inflammatory response, which may be stronger in symptomatic than asymptomatic infections. As a result, increased levels of IL-10 may be more important in resolving symptomatic infections where the proinflammatory cytokines are overexpressed and causing tissue damage.

These associations may not represent the development of a protective immune response in the gut. The results for the diarrheal *E. coli* pathotypes may reflect the ability of these pathogens to actively downregulate a protective immune response as part of a strategy of immune response evasion. Virulence factors such as intimin, EspA, and lymphostatin produced by EPEC can modify the expression of a wide range of cytokines that effectively resolve infections (24, 26, 42, 44). As a result, it is not clear whether increases in cytokine expression during infection represent the development of a protective response that leads to the resolution of infection or the development of an inadequate response that can lead to prolonged infection. Our use of parametric hazard models to analyze infection durations is able to distinguish between these two outcomes and may provide insight into inconsistencies between different studies.

It is important to address a number of limitations of this study. First, biases may have been introduced into the analysis as a result of cytokine degradation. However, the treatment of all samples during collection, processing, storage, and analysis was identical, suggesting that such systematic bias was minimized. Additionally, the inclusion of children from the placebo and vitamin A groups in the analysis may have biased the

TABLE 5. Parametric hazard regression analyses of the association between innate and adaptive immune response cytokine levels and durations of *G. lambila* infections[a]

Cytokine and response	No. of infections	Mean duration (+ SD, in days) of *G. lamblia* infections	Risk of *G. lamblia* infection ending	
			Hazards ratio (95% CI)	*P*
Innate immune				
IL-8				
ND	10	26.6 + 24.66	1.00	
<Median	9	16.66 + 4.25	3.10 (1.01–9.45)	0.04
>Median	4	30.75 + 12.27	1.22 (0.37–4.07)	0.73
MCP-1				
ND	5	16.7 + 10.31	1.00	
<Median	10	16 + 2.99	1.37 (0.46–4.10)	0.56
>Median	9	30.88 + 25.02	0.26 (0.06–1.03)	0.05
Adaptive immune				
IFN-γ (Th1)				
ND	15	15.5 + 5.61	1.00	
<Median	5	19.9 + 14.82	0.39 (0.12–1.23)	0.11
>Median	5	44.8 + 25.33	0.06 (0.01–0.29)	0.00
IL-4 (Th2)				
ND	11	17.13 + 8.22	1.00	
<Median	8	21.56 + 13.16	0.51 (0.13–2.06)	0.35
>Median	8	28.31 + 26.11	0.23 (0.05–0.99)	0.05
IL-5 (Th2)				
ND	15	14.73 + 6.7	1.00	
<Median	4	38.75 + 26.47	0.14 (0.03–0.53)	0.00
>Median	5	29.1 + 21.32	0.24 (0.74–0.81)	0.02

[a] Hazard rates were calculated after fitting the Weibull hazard model to the length of infection duration. Infection duration was defined as the time from the first positive stool until the first negative stool among sequentially collected stools. Reference groups are stool samples with no detectable levels of chemokine or cytokine. CI, confidence interval.

results, given the effect vitamin A has on the immune response (29). This potential bias has been controlled effectively through the inclusion of a variable indicating whether the child was in the vitamin A or placebo group.

Overall, we have found that different fecal cytokine profiles are indeed associated with differences in pathogen infection duration. If these findings can be confirmed, they have important implications for the design of more effective vaccines that take into account those cytokines that are important in limiting infection and those that are inadequate. These results also can be used in the development of more cost-effective public health interventions. For example, they have important implications for the design of vitamin A supplementation programs, since the results suggest that communities that differ in indicators of pathogen prevalence and diarrheal disease burden should be targeted for interventions that differ in frequency, timing, and dosage of supplementation.

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