

# Human Memory CD4<sup>+</sup> T Cell Immune Responses against *Giardia lamblia*

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The intestinal protozoan parasite *Giardia lamblia* may cause severe prolonged diarrheal disease or pass unnoticed as an asymptomatic infection. T cells seem to play an important role in the immune response to *Giardia* infection, and memory responses may last years. Recently, T<sub>H</sub>17 responses have been found in three animal studies of *Giardia* infection. The aim of this study was to characterize the human CD4<sup>+</sup> T cell responses to *Giardia*. Peripheral blood mononuclear cells (PBMCs) were obtained from 21 returning travelers with recent or ongoing giardiasis and 12 low-risk healthy controls and stimulated *in vitro* with *Giardia lamblia* proteins. Production of tumor necrosis factor alpha (TNF- $\alpha$ ), gamma interferon, interleukin-17A (IL-17A), IL-10, and IL-4 was measured in CD4<sup>+</sup> effector memory (EM) T cells after 24 h by flow cytometry. After 6 days of culture, activation and proliferation were measured by flow cytometry, while an array of inflammatory cytokine levels in supernatants were measured with multiplex assays. We found the number of IL-17A-producing CD4<sup>+</sup> EM T cells, as well as that of cells simultaneously producing both IL-17A and TNF- $\alpha$ , to be significantly elevated in the *Giardia*-exposed individuals after 24 h of antigen stimulation. In supernatants of PBMCs stimulated with *Giardia* antigens for 6 days, we found inflammation-associated cytokines, including IL-17A, as well as CD4<sup>+</sup> T cell activation and proliferation, to be significantly elevated in the *Giardia*-exposed individuals. We conclude that symptomatic *Giardia* infection in humans induces a CD4<sup>+</sup> EM T cell response of which IL-17A production seems to be an important component.

*Giardia lamblia* (synonyms, *G. duodenalis* and *G. intestinalis*) is a gastrointestinal protozoan parasite that can infect several different hosts, including humans (1, 2). Of the eight recognized genotypes of *G. lamblia*, assemblages A and B can infect humans (1, 3). *Giardia* infection of humans can produce an acute symptomatic disease with symptoms such as diarrhea with abdominal discomfort leading to weight loss and, at times, malabsorption syndrome (4, 5) or be asymptomatic (6). The infection may also become chronic, defined as giardiasis lasting >2 months (7).

Eradication of and protection against *Giardia* are likely to be dependent on both B cell-mediated antibody production and T cell-mediated immune responses (8, 9). Humans with immunodeficiencies in the form of common variable immunodeficiency (CVID) and impaired IgA function have an increased risk of developing chronic *Giardia* infections (10, 11). People living in areas where giardiasis is endemic, who are likely to have experienced numerous encounters with *Giardia*, are less prone to infection or reinfection, indicating that acquired immunity exists (12, 13).

Most of our knowledge regarding the cellular immune responses to *Giardia* is based on murine models. CD4<sup>+</sup> T cells have been shown to be necessary for immediate responses in mice, as the absence of these cells can lead to poor control of *Giardia*. Decreased or nonexistent CD4<sup>+</sup> T cells can give rise to chronic infection, indicating that these cells are crucial for the murine defense against both *G. lamblia* and *G. muris* (9, 14). Secretion of the cytokine tumor necrosis factor alpha (TNF- $\alpha$ ) in mice during *G. lamblia* infection has been shown to be important for determining the parasite load and the duration of an infection (15).

In mice, clearance of *G. lamblia* has been shown to be dependent on  $\alpha\beta$  T cells but not on specific polarizations into T<sub>H</sub>1 or T<sub>H</sub>2 subsets (9). Gamma interferon (IFN- $\gamma$ ), which is a crucial component of T<sub>H</sub>1 responses, has, however, been shown to be important for clearance of *G. muris* (16). A broad range of cyto-

kines probably secreted by CD4<sup>+</sup> T cells were found in the spleens and mesenteric lymph nodes of mice after *G. lamblia* infection (17) and could indicate that a range of T<sub>H</sub> responses may contribute to protection against *Giardia*.

Recently, *G. muris*-infected mice have been investigated for cytokine transcription patterns during infection. In one study, upregulation of peroxisome proliferator-activated receptor alpha, followed by upregulation of interleukin-17A (IL-17A), was found in the early phase of infection (18). IL-17A was also found to be upregulated in another mouse study, where IL-17A and its receptor were important for defense against and eradication of the parasite and needed for the transport of IgA into the lumen of the intestines (19). Likewise, IL-17A upregulation, in addition to increased FoxP3 mRNA levels, was found in proliferating CD4<sup>+</sup> T cells from calves infected with *G. lamblia* (20). On the basis of these three findings, IL-17A may be linked to protection and perhaps memory responses to *Giardia*.

*Giardia*-specific immune responses have also been investigated in humans. In one study, human intestinal and blood CD4<sup>+</sup> T cells were stimulated with *Giardia* trophozoites. IFN- $\gamma$  was se-

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creted, and cells were found to be proliferating in response to trophozoites, suggesting that *Giardia*-specific proliferation of CD4<sup>+</sup> T cells exists in humans as well (21). CD4<sup>+</sup> T cell memory immune responses in peripheral blood mononuclear cells (PBMCs) from humans were investigated 5 years after a large outbreak of *Giardia* occurred (22). *Giardia*-specific CD4<sup>+</sup> T cell responses were found to be present by the upregulation of surface activation markers (CD25/CD26 and CD45RO/HLA-DR) and higher proliferation rates of T cells in *Giardia*-exposed persons compared to those in controls.

The relative importance of the cytokines IL-17A, IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-10 in the T cell response to *Giardia* infection in humans has not been determined. The present study investigated *Giardia*-specific memory CD4 T cell immune responses in humans with regard to these cytokines by comparing PBMCs from individuals with recent or ongoing *Giardia* infections with those from healthy controls presumed to be unexposed.

## MATERIALS AND METHODS

***Giardia*-exposed individuals and low-risk controls.** Twenty-one adults with recent or ongoing symptomatic giardiasis were recruited by direct or indirect contact to serve as the *Giardia*-exposed group. None of these participants had been previously diagnosed with giardiasis. The infection had been acquired by traveling in areas where giardiasis is endemic, and the infections were laboratory confirmed by routine microscopy. The duration of *Giardia* infection was defined as the time from symptom onset to the date of successful treatment with antibiotics (i.e., verified by a negative stool sample). The time since giardiasis was defined as the time from successful treatment to the time of sample collection. If study participants had ongoing giardiasis at the time of sample collection, they received metronidazole as a first-line treatment or albendazole in combination with metronidazole as a second-line treatment.

An age- and sex-matched group of individuals with a low risk of ever having had giardiasis was recruited as controls. A low-risk control was defined as someone who had never traveled to an area where giardiasis is highly endemic (a low- or middle-income country), who had no known previous giardiasis or family members with giardiasis, and who had not drunk contaminated water in Bergen, Norway, during the 2004 *Giardia* outbreak.

To ascertain the *Giardia* infection status of all of the participants at the time of sample collection, a stool sample was analyzed by *Giardia* 18S small-subunit (SSU) PCR assay according to Verweij et al. (23). The type of *Giardia* assemblage responsible for infection was examined by genotyping of the triosephosphate isomerase (TPI) gene (24) with minor modifications. All of the study subjects had previously received the BCG vaccine against tuberculosis. None of the participants in this study had known immunosuppression, ongoing treatment with immunosuppressive medication, or autoimmune diseases.

***Giardia* antigens and negative and positive controls.** *Giardia* assemblage A (WB-C6, ATCC 50803) and B (GS/M, ATCC 50581) trophozoites were grown in TYDK medium (Diamond's TY-S-33 medium supplemented with bile as described by Keister [25]) at 37°C. The trophozoites were collected by three washing steps with cold, sterile, phosphate-buffered saline (PBS), followed by pelleting by centrifugation at 2,500 rpm at 4°C for 5 min. The pellet was resuspended in PBS, snap-frozen and thawed twice in liquid nitrogen, and then sonicated (three times for 30 s each at 50 W). Another centrifugation at 13,000 rpm at 4°C for 15 min was done to remove membrane and cell debris. The protein concentrations in the supernatants containing *Giardia* soluble proteins from the assemblage A (SSA) and assemblage B (SSB) fractions were measured with the Direct Detect system (EMD Millipore Corporation, Billerica, MA, USA); diluted in X-vivo 15 serum-free culture medium supplemented with L-glutamine, gentamicin, and phenol red (Lonza, Basel, Switzerland); and stored at -20°C. On the basis of pilot experiments with different SSA and SSB

dilutions, a final concentration of 10  $\mu$ g/ml was used for antigen stimulation of PBMCs.

X-vivo 15 medium without any additives was used as a negative control. Purified protein derivative (PPD; final concentration, 10  $\mu$ g/ml) from *Mycobacterium tuberculosis* (Statens Serum Institut, Copenhagen, Denmark) served as a protein antigen control. For the cytokine assay only, phorbol 12-myristate 13-acetate (PMA; final concentration, 20 ng/ml) and ionomycin calcium salt (IC; final concentration, 500 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) were used in combination as a positive mitogenic control (added for the last 6 h of the stimulation period). Lipopolysaccharide (LPS; final concentration, 1  $\mu$ g/ml) from *Salmonella enterica* serovar Typhimurium (Sigma-Aldrich) was used as a positive control in the cytokine assay. Staphylococcal enterotoxin B (SEB; final concentration, 1  $\mu$ g/ml) from *Staphylococcus aureus* (Sigma-Aldrich) was used as a positive control in the proliferation and surface activation marker assay.

**PBMC culture.** Venous blood was harvested and placed in BD Vacutainer CPT tubes with Na<sup>+</sup>/heparin (Becton Dickinson, Franklin Lakes, NJ, USA), and PBMCs were isolated by density gradient separation. The PBMCs were washed twice in PBS and dissolved in X-vivo medium.

For intracellular cytokine assays, PBMCs (10<sup>6</sup>/well) were cultured with antigens for 24 h and for the last 6 h in the presence of brefeldin A (10  $\mu$ g/ml) (Sigma-Aldrich). To measure proliferation and upregulation of surface activation markers, PBMCs were cultured for 144 h (2  $\times$  10<sup>5</sup>/well) with CellTrace Violet (10  $\mu$ l/ml) (Life Technologies, Carlsbad, CA, USA). Cells were cultured in duplicate or triplicate in 96-well V-bottom plates (Sarstedt, Nümbrecht, Germany) at 37°C in 5% CO<sub>2</sub> for both assays with 200  $\mu$ l of X-vivo medium with or without stimulation antigens.

**Flow cytometric cytokine assay.** After 24 h of stimulation, PBMC cultures from each participant were pooled, mixed, and washed two times with PBS by centrifugation. Dead cells were stained with the LIVE/DEAD Fixable Near-IR Dead Cell Stain kit (Life Technologies) as recommended by the manufacturer. The cells were washed twice with PBS and placed on ice before incubation with 10% normal human serum in PBS at 4°C for 15 min. The cells were washed twice with cold PBS and resuspended in the residual volume in the wells.

The cells were then stained for surface markers with fluorochrome-conjugated antibodies at 4°C in the dark for 30 min to allow gating of the following specific T cell populations: CD3-AF700, CD8a-BV711, CD45RA-BV510 (BioLegend, San Diego, CA, USA), CD4-peridinin chlorophyll protein-Cy5.5, CD14-allophycocyanin (APC)-H7, and CD197-phycoerythrin (PE)-CF594 (Becton Dickinson).

Intracellular cytokine staining was done after fixation and permeabilization with the BD Cytofix/Cytoperm Fixation/Permeabilization solution kit (Becton Dickinson) according to the manufacturer's instructions. The cells were stained at 4°C for 30 min in the dark. The intracellular fluorochrome-conjugated antibodies used were TNF- $\alpha$ -BV421, IL-4-APC, IL-17A-BV605 (BioLegend), IFN- $\gamma$ -fluorescein isothiocyanate (FITC), and IL-10-PE (Becton Dickinson).

Fluorescence minus one (FMO) controls were prepared for all of the intracellular cytokine antibodies. After staining, the cells were washed two times with Perm/Wash, followed by two PBS washing steps, before analysis on a flow cytometer on the same day. A typical cell acquisition consisted of 4  $\times$  10<sup>5</sup> (range, 0.12  $\times$  10<sup>6</sup> to 1  $\times$  10<sup>6</sup>) lymphocytes.

**Flow cytometric proliferation and activation marker assay.** After 144 h of culture, 100  $\mu$ l of supernatant from each participant was collected from the wells of the 96-V-well plate containing medium, SSA, and SSB. Supernatants from 19 *Giardia*-exposed individuals and 9 low-risk controls were stored at -70°C for later multiplex cytokine analysis.

The cells were washed out of the stimulation medium two times with PBS, and duplicate or triplicate cultures of cells from each participant were pooled and mixed. Viability staining, serum incubation, and washing steps before surface staining were done according to the flow cytometric cytokine assay protocol.

To separate T cells from the whole cell population, the same surface

TABLE 1 Characteristics of study participants by group including subgroups of *Giardia*-exposed individuals

Characteristic	<i>Giardia</i> -exposed group				Low-risk controls	P value <sup>a</sup>
	All	Subgroup with giardiasis:				
		Currently	For <8 wk	For >8 wk		
No. of individuals/group	21	5	11	5	12	NA <sup>f</sup>
No. (%) of females	11 (52.4)	2 (40.0)	8 (72.7)	1 (20.0)	8 (66.7)	0.486 <sup>b</sup>
Mean age, yr (SD)	38.7 (17.1)	31.0 (14.5)	46.9 (16.9)	28.4 (12.2)	43.0 (17.8)	0.389 <sup>c</sup>
Mean no. of wk infected (range)	12.5 (1–61)	20.2 (7–61)	3.36 (1–7)	25.0 (11–57)	NA	NA
Mean no. of wk since giardiasis (range)	33.9 (0–112)	NA	57.4 (1–112)	16.0 (5–32)	NA	NA
No. (%) with <i>Giardia</i> assemblage A	6 (35.3) <sup>d</sup>	2 (40.0)	4 (36.4)	0 (0) <sup>e</sup>	NA	NA

<sup>a</sup> P values are for comparisons of all *Giardia*-exposed groups with low-risk controls.

<sup>b</sup> Fisher's exact test was used for comparisons of categorical variables.

<sup>c</sup> The Mann-Whitney U test was used for comparisons of continuous variables between two groups.

<sup>d</sup> The *Giardia* assemblage responsible for infection could not be determined for 4 of the participants by TPI sequencing, and the percentage represents the ratio for 17 individuals. All of the assemblages were detected in the current-giardiasis group.

<sup>e</sup> The *Giardia* assemblage of two participants in this subgroup could not be characterized; the other three had assemblage B.

<sup>f</sup> NA, not applicable.

antibodies as used in the cytokine assay were used, except for the CD197 and CD45RA antibodies. To investigate activation and memory cell markers, CD25-APC, HLA-DR-FITC (Becton Dickinson), CD26-PE, and CD45RO-BV605 (BioLegend) were included. FMO controls for activation and memory cell markers were run in each assay.

The cells were stained for 30 min at 4°C in the dark, washed twice, and then fixed with 1% paraformaldehyde in PBS for 30 min on ice. Flow cytometric analysis was done on the same day. A typical cell acquisition consisted of  $6 \times 10^4$  (range,  $3 \times 10^4$  to  $10 \times 10^4$ ) lymphocytes.

**Supernatant cytokine assays.** Levels of IL-1 $\beta$ , IL-2 receptor (IL-2R), IL-4, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-22, IFN- $\gamma$ , TNF- $\alpha$ , granulocyte-macrophage colony-stimulation factor (GM-CSF), macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ ), MIP-1 $\beta$ , and CD40L were measured in culture supernatants after 144 h of stimulation. These cytokines were analyzed by two multiplex assays (Bio-Plex Pro human Th17 cytokine 10-plex assay and Bio-Plex Pro human cytokine group I 6-plex assay; Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. LPS-stimulated PBMCs were included as positive controls. Concentrations (pg/ml) in the detectable range were used for analysis. For molecules with values >50% below the lower limit of detection (IL-4 and IL-22), further analysis was not done.

Background responses in unstimulated cultures were subtracted from those in *Giardia*-stimulated cultures for each participant in this study as described in reference 26.

**Flow cytometric analysis.** Analysis and gating of flow cytometric data were done for CD4<sup>+</sup> T cells and the effector memory (EM) CD197<sup>+</sup> CD45RA<sup>+</sup> subpopulation to investigate surface activation, proliferation, and cytokine production, respectively (see Fig. S1 in the supplemental material). To determine the fluorescence properties of cells, a BD LSR Fortessa cell analyzer (Becton Dickinson) was used. To acquire data from cell samples in a flow cytometry standard (FCS) file format, BD FACSDiVa (v. 8; Becton Dickinson) was used. Gating and further flow cytometric analysis of the data were performed in FlowJo (v. X10; Tree Star, San Carlos, CA, USA). Polyfunctional EM CD4<sup>+</sup> T cells producing more than one cytokine at the same time were also analyzed with a Boolean gating tool. To further investigate these polyfunctional cells, SPICE (v. 5.0; M. Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA) was utilized. Background responses were always subtracted before statistical analysis.

**Statistical analysis.** The *Giardia*-specific immune responses of the *Giardia*-exposed group and the low-risk healthy controls were compared. To assess the statistical significance of comparisons between the groups and immune responses, the Mann-Whitney U nonparametric test, linear regression with Pearson's correlation coefficient test, and Fisher's exact

test were used. For subgroup comparisons within the *Giardia*-exposed individuals and the low-risk group, comparative analysis with the Kruskal-Wallis test was used and significant results were followed by pairwise analyses with the Mann-Whitney U nonparametric test. Individuals with giardiasis lasting >8 weeks (chronic) were also compared to individuals with giardiasis lasting <8 weeks to investigate whether there were response differences between *Giardia* infections lasting <8 weeks and those lasting >8 weeks.

The statistics software used was IBM SPSS (v. 21; IBM Corp., Armonk, NY). Cytokine combinations displayed in pie charts were compared between the two groups by performing a partial permutation test in SPICE as described by Roederer et al. (26).

P values of <0.05 were considered statistically significant.

**Ethics.** This study was approved by the Regional Committee for Medical Research Ethics of Western Norway (2013/1285/REK vest) and performed in compliance with the Declaration of Helsinki. Participation in this study was voluntary.

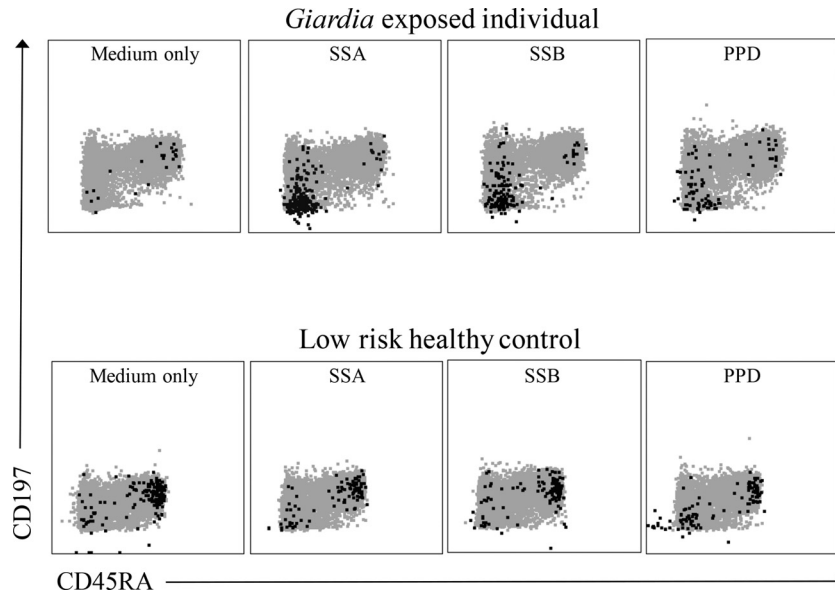
## RESULTS

**Characteristics of the study groups.** Twenty-one *Giardia*-exposed individuals and 12 healthy controls were included in this study. Five individuals in the *Giardia*-exposed group were unexpectedly still *Giardia* positive. They were analyzed together with the participants with recent *Giardia* infections but also as a separate group to compare their responses to the recently *Giardia*-exposed individuals and low-risk controls. The *Giardia*-exposed individuals with recent infections were further subgrouped according to the duration of infection (<8 or >8 weeks). Characteristics of the study population are displayed in Table 1.

**Intracellular cytokine expression.** Antigen-activated cytokine-producing cells were found to be concentrated in the CD4<sup>+</sup> CD197<sup>+</sup> CD45RA<sup>+</sup> EM T cell population (Fig. 1). These responses reflect a recall response and were therefore the main investigational focus of the cytokine assay. By including only this T cell subpopulation, unspecific responses seen in the CD197<sup>+</sup> CD45RA<sup>+</sup> population could be avoided.

Analysis of the cytokines TNF- $\alpha$ , IFN- $\gamma$ , IL-17A, IL-4, and IL-10 secreted by EM T cells after 24 h showed that the cytokines IL-4 and IL-10 were weakly expressed by all of the cells analyzed when they were stimulated with the SSA and SSB antigens. These two cytokines were therefore not included in the SPICE analysis. SSA responses were generally stronger, with relatively less unспе-





**FIG 1** IL-17A-producing CD4<sup>+</sup> T cells in a CD197<sup>-</sup> CD45RA<sup>-</sup> plot. Flow cytometric plots showing IL-17A-producing cells as black dots in a gray CD4<sup>+</sup> T cell population. The IL-17A-producing cells are concentrated in the CD197<sup>-</sup> CD45RA<sup>-</sup> EM T cell population for the PPD-positive control and in SSA and SSB for the *Giardia*-exposed individual.

cific responses in the low-risk controls than SSB responses, irrespective of the genotype of the recent or ongoing infection. For simplicity, mainly the SSA responses are presented in the further analyses. Because of the small number of cells available from one control person, only 11 low-risk controls were included in the cytokine analysis.

**Figure 2** shows the T cell cytokine response profiles for IL-17A, TNF- $\alpha$  and IFN- $\gamma$  to SSA, including polyfunctional cells, in the two different groups of participants. The relative percentages are expressed in bar graphs, and the proportions of the responses are expressed in pie charts. In the partial permutation analysis, where the distribution of all cytokine-expressing CD4<sup>+</sup> EM T cell populations was analyzed, the differences between the *Giardia*-exposed group and the controls did not reach statistical significance. However, the total number of IL-17A-positive CD4<sup>+</sup> EM T cells was significantly higher in the *Giardia*-exposed group than that in low-risk controls for both SSA (0.31 [0.41] versus 0.06 [0.11];  $P = 0.015$ ) and SSB (0.22 [0.30] versus 0.09 [0.21];  $P = 0.025$ ). For the responses of the different groups, see Table S2 in the supplemental material. Cells stimulated with SSA showed higher levels of CD4<sup>+</sup> EM T cells coproducing IL-17A and TNF- $\alpha$  in the *Giardia*-exposed group than in the low-risk control group.

The IL-17A responses were not dependent upon the assemblage with which the patient had been infected. A significant positive correlation between the SSA- and SBB-induced IL-17A responses was found ( $R^2 = 0.88$  and  $P < 0.001$ ) (data not shown).

Interestingly, the PPD internal protein control also showed higher values of cells expressing only IL-17A in the exposed group than in low-risk controls (0.07 [0.14] versus 0 [0];  $P = 0.009$ ). However, the total IL-17A expression difference did not reach significance for PPD (0.50 [0.56] versus 0.40 [0.51];  $P = 0.60$ ). No correlation between the upregulated IL-17A responses to PPD and the IL-17A responses to SSA and SSB was found (data not shown). IL-17A levels were not different between the groups for other internal positive controls (LPS and PMA-IC). CD4<sup>+</sup> T cells not producing TNF- $\alpha$ , IL-17A, or IFN- $\gamma$

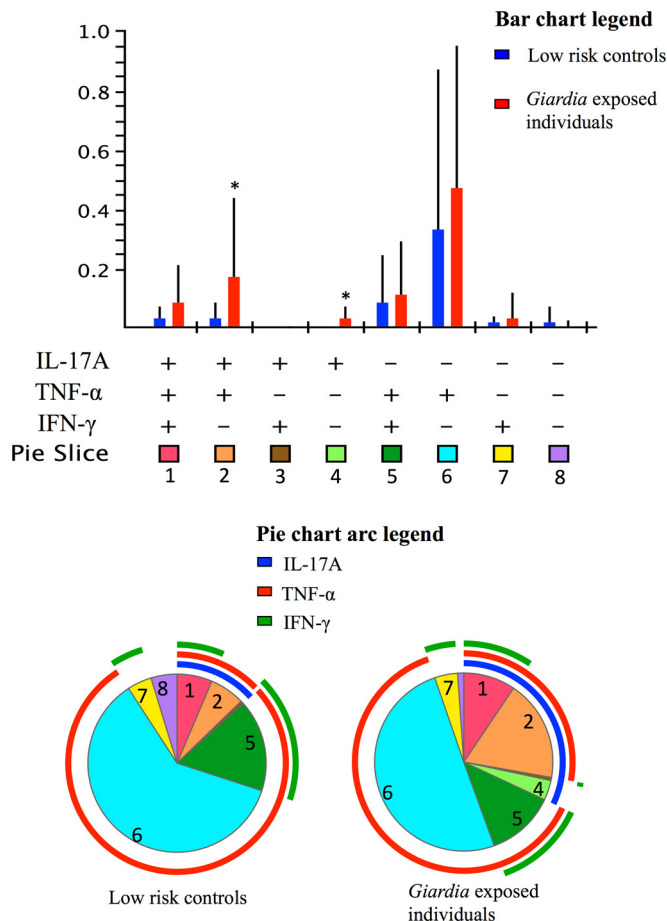
and hence producing IL-10 and/or IL-4 were not significantly different between the two groups.

#### Extracellular surface marker expression and proliferation.

To assess the magnitude of CD4<sup>+</sup> T cell activation and proliferation after 144 h of antigen stimulation, the cell markers CD25/CD26 and HLA-DR/CD45RO, proliferation, and a combination of HLA-DR/CD45RO and proliferation were investigated. The markers CD25/CD26 alone, and not in combination with HLA-DR/CD45RO, were only slightly upregulated in *Giardia*-exposed individuals and thus not included in the analysis. The mean percentages of activation and proliferation of CD4<sup>+</sup> T cells from *Giardia*-exposed individuals and low-risk controls are presented in **Fig. 3**. Because of the low cell numbers obtained from some of the *Giardia*-exposed individuals, only 18 individuals were included in this analysis and 4 of them had giardiasis at the time of sample collection.

SSA-activated CD45RO<sup>+</sup>/HLA-DR<sup>+</sup> CD4<sup>+</sup> T cell levels were higher in *Giardia*-exposed individuals than in low-risk controls (3.05 [6.62] versus 0.07 [1.21];  $P = 0.007$ ). Further, combining the four surface markers CD25/CD26 and CD45RO/HLA-DR and comparing the levels between the *Giardia*-exposed individuals and low-risk controls, we found significantly higher responses in SSA-stimulated cells in the exposed group (0.29 [2.42] versus 0.01 [0.24];  $P = 0.016$ ). To further investigate antigen-specific responses, the two surface markers CD45RO<sup>+</sup>HLA-DR<sup>+</sup> were combined with proliferating CD4<sup>+</sup> T cells. This analysis revealed significantly elevated responses in SSA-stimulated cells (2.50 [6.60] versus 0.03 [1.20];  $P = 0.006$ ).

**Comparison of cellular responses of low-risk controls and individuals with recent giardiasis with those of individuals with acute giardiasis.** We wanted to investigate how the cellular responses of individuals with current giardiasis would differ from those of low-risk controls and individuals with recent giardiasis. For the cytokine response, surface marker activation, and proliferation data and  $P$  values, see Tables S1 and S2 in the supplemental material.



**FIG 2** Polyfunctional EM CD4<sup>+</sup> T cell cytokine responses to SSA. The bar graph represents the relative mean frequencies of CD197<sup>-</sup> CD45RA<sup>-</sup> CD4<sup>+</sup> T cells producing different combinations of the cytokines IL-17A, TNF- $\alpha$ , and IFN- $\gamma$  or not producing any of these cytokines (IL-4<sup>+</sup> and/or IL-10<sup>+</sup> cells). \*,  $P < 0.05$ . The pie charts show data from low-risk controls and *Giardia*-exposed individuals. The pie slices represent the different cytokine combinations in the bar graph. The arcs surrounding the pie charts represent the total proportions of IL-17A-, TNF- $\alpha$ -, and IFN- $\gamma$ -producing cells.

The number of IL-17A/TNF- $\alpha$ - and IL-17A-positive EM CD4<sup>+</sup> cells was significantly higher in the current-giardiasis group than in low-risk controls and also significantly higher in individuals with current giardiasis than in recently *Giardia*-exposed individuals. Additionally, the IL-17A/TNF- $\alpha$ -positive EM CD4<sup>+</sup> T cell population was larger in recently *Giardia*-exposed individuals than that in low-risk controls. In SSB-stimulated cells, there were no differences in TNF- $\alpha$ , IFN- $\gamma$ , IL-10, or IL-4, but the number of IL-17A-producing cells was significantly higher in the group with current giardiasis than in low-risk controls.

With regard to surface activation marker and proliferation analyses, no significant differences were found between the recently *Giardia*-exposed group and the current-giardiasis group.

**Comparison of individuals with recent giardiasis lasting <8 or >8 weeks.** When stimulated with SSA, the percentage of polyfunctional EM CD4 T cells responding with the production of both IL-17A and TNF- $\alpha$  was found to be significantly higher in the subgroup with giardiasis for <8 weeks ( $n = 11$ ) (0.11 [0.10]) than in the subgroup with giardiasis for >8 weeks ( $n = 5$ ) (0.03 [0.02];

$P = 0.02$ ). The percentage of IL-17A TNF- $\alpha$  cells was also significantly higher in the individuals with giardiasis for <8 weeks than in the low-risk controls ( $n = 11$ ) (0.04 [0.06];  $P = 0.03$ ). No other T cell subsets in this subgroup comparison reached significance.

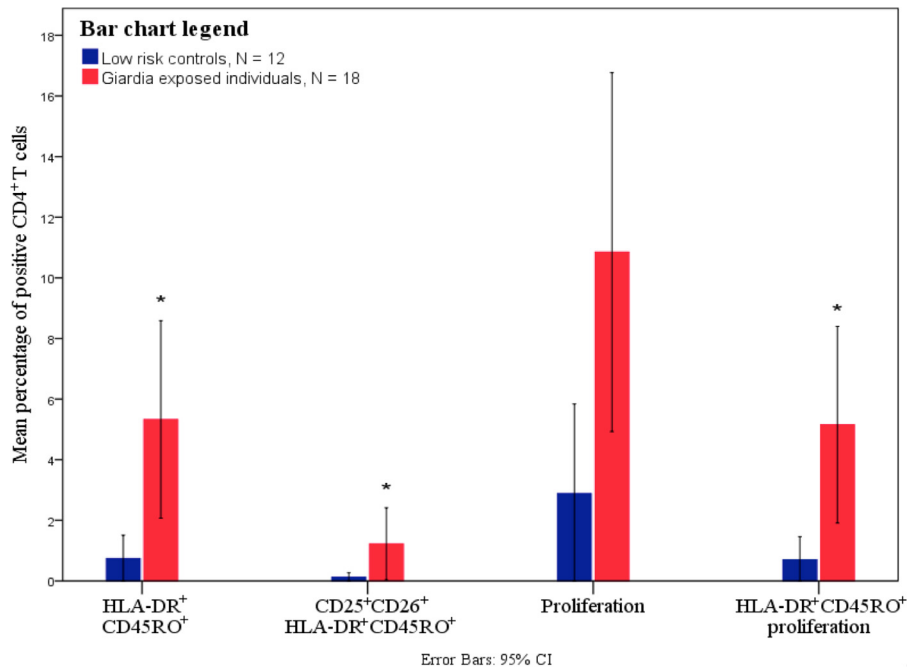
**Cytokines in supernatants.** A multiplex assay was done to investigate cytokine and chemokine production after 144 h of antigen stimulation. IL-1 $\beta$ , IL-6, IL-10, IL-17A, IFN- $\gamma$ , CD40L, TNF- $\alpha$ , IL-13, GM-CSF, and MIP-1 $\alpha$  levels were significantly higher in SSA-stimulated PBMCs from *Giardia*-exposed individuals than in those from low-risk controls (Table 2). Levels of IL-17A were also found to be increased in SSB-stimulated cells, and their correlation with the responses to SSA and SSB was therefore tested. IL-17A responses were correlated ( $R^2 = 0.82$ ), and the data were statistically significant ( $P < 0.001$ ).

## DISCUSSION

In this study, we found that CD4<sup>+</sup> T cell surface activation marker and proliferation responses to *Giardia* lysates were higher in *Giardia*-exposed individuals than in low-risk controls. IL-17A responses were found to be significantly higher in *Giardia*-exposed individuals than in low-risk healthy controls. Increased IL-17A levels were found both in the flow cytometric assay of EM CD4<sup>+</sup> T cells and in a cytokine analysis of the supernatants of *Giardia* lysate-stimulated PBMCs. Levels of cells expressing both IL-17A and TNF- $\alpha$  were higher in *Giardia*-exposed individuals than in low-risk controls, in those with current giardiasis than in recent giardiasis, and in those with recent giardiasis with a duration of <8 weeks than in those with longer-standing infections. These results indicate the presence of *Giardia* antigen-responsive polyfunctional CD4<sup>+</sup> EM T cells in *Giardia*-exposed individuals. Higher levels of CD4<sup>+</sup> EM T cells producing only IL-17A were also found in individuals with current giardiasis infections than in individuals with recent giardiasis infections or in low-risk controls. In the supernatant analysis, a broad range of inflammation-associated cytokines were found to be upregulated in the *Giardia*-exposed group (IL-17A, MIP-1 $\alpha$ , IL-1 $\beta$ , CD40L, TNF- $\alpha$ , IFN- $\gamma$ , IL-6, IL-10, IL-13, and GM-CSF).

Although T cells are important in the clearance of a *Giardia* infection, neither a specific T<sub>H</sub>1 nor a specific T<sub>H</sub>2 polarized response seems to be necessary for protection against *Giardia* infection in mice (9). In our study, both T<sub>H</sub>1-associated (IFN- $\gamma$ ) and T<sub>H</sub>2-associated (IL-13) cytokines were found to be upregulated in stimulated PBMC culture supernatants (Table 2), indicating redundant mechanisms or that another T cell polarization is more important. Increased IL-17A cytokine levels and IL-17A-positive T cell levels were found in both SSA and SSB assays. Because of these IL-17A responses, T<sub>H</sub>17 can be speculated to be a CD4<sup>+</sup> T cell polarization important in *Giardia* immune responses in humans. Individuals with current giardiasis infections had higher numbers of cells producing IL-17A than did recently *Giardia*-infected individuals. This difference could mean that individuals with current infections are in the process of becoming immune, while the IL-17A responses of recently *Giardia*-infected individuals are disappearing.

The cytokine IL-17A plays a role in proinflammatory responses for the recruitment or activation of different cell types belonging to the innate immune system such as neutrophils and macrophages (27). Further, IL-17A has been suggested to play an important role in protection after infection or vaccination, as deficiency in or reduced production of this cytokine can limit full protection



**FIG 3** Activation markers and proliferation of CD4<sup>+</sup> T cells stimulated with SSA. Average percentages of CD4<sup>+</sup> T cells showing proliferation and/or expression of surface activation markers are represented on the y axis, and the various markers and proliferation measures are presented on the x axis. \*,  $P < 0.05$ . CI, confidence interval.

(28). It has been reported that IL-17A is expressed mainly in CD4<sup>+</sup> CD45RO<sup>+</sup> memory T cells (29), and the main aim of our flow cytometric cytokine analysis was evaluation of responses in the EM T cell (CD197<sup>-</sup> CD45RA<sup>-</sup>) population. The EM CD4<sup>+</sup> T cell population was shown to be a significant source of IL-17A production in previously *Giardia*-exposed individuals, in contrast to controls. Recently, IL-17A was also shown to be associated with current *Giardia* infection in animal studies (18–20). In the first study, IL-17A was suggested to affect mice's capability to clear *Giardia* infection (18), and mice lacking the IL-17A receptor in their intestines have higher numbers of *Giardia* cysts in feces than do control mice. The present study supports the idea that IL-17A

may be important for rapid clearance of *Giardia* by humans as well, by the observation that individuals with a shorter disease duration had polyfunctional responsive EM T cells including an IL-17A response. Because of this finding, strong IL-17A responses may contribute to a shorter duration of *Giardia* infection in humans than in individuals with generally weaker IL-17A responses, who could have problems eliminating *Giardia*. However, the role of IL-17A and how it affects the course of a *Giardia* infection need to be investigated further.

In the second mouse study (19), IL-17A upregulation was essential for host protection against *Giardia*, and mice deficient in this cytokine and its receptor had difficulty eradicating the infection after 1 week. Further, a deficiency in IL-17A led to a defect in IgA transport into the lumen and thus affected the mouse's ability to clear *Giardia*.

In a study where calves infected with *Giardia* were investigated, upregulation of IL-17A was found to originate from CD4<sup>+</sup> T cells (20), providing evidence that these cells are important in protecting cattle against *Giardia* infection. Whether IL-17A has a protective role in human *Giardia* infection has to be investigated further. IL-17A has been shown in other studies to be important for both protection from and eradication of pathogens in association with the induction and stimulation of pathological inflammation during infection (30, 31). Many of the inflammation-related cytokines were significantly elevated in PBMC supernatants in the *Giardia*-exposed group (Table 2). The two cytokines GM-CSF and TNF- $\alpha$  have been shown to be mediators of neutrophil recruitment and activation and also cell survival and could originate from T<sub>H</sub>17 cells (31). The levels of the cytokine IL-1 $\beta$  were also increased in the supernatant analysis, and this cytokine has been suggested to be important for the development and maturation of IL-17A-producing cells (31).

**TABLE 2** Analysis of cytokines in supernatant of SSA-stimulated PBMCs cultured for 144 h

Cytokine	Median concn, pg/ml (SD)		P value
	Low-risk controls (n = 9)	<i>Giardia</i> -exposed group (n = 19)	
IL-1 $\beta$	0 (2.5)	4.72 (5.26)	0.007
IL-6	0 (0.0)	265 (554)	0.002
IL-10	0 (5.9)	7.59 (17.8)	0.02
IL-17A	0 (0.0)	11.5 (87.3)	<0.001
IFN- $\gamma$	0 (1,525)	566 (3,931)	0.02
sCD40L	0 (0.0)	6.99 (27.0)	0.007
TNF- $\alpha$	28.9 (258)	502 (594)	0.006
IL-9	0 (0.0)	0 (1.7)	NS <sup>a</sup>
IL-13	0 (0.0)	16.4 (86.8)	0.013
GM-CSF	0 (5.4)	11.2 (70.4)	0.011
MIP-1 $\alpha$	0 (0.0)	110 (327)	0.002
MIP-1 $\beta$	0 (0.0)	0 (986)	NS

<sup>a</sup> NS, not significant.

Increased levels of IL-17A were found in *Giardia*-exposed individuals in our flow cytometric cytokine assay, where the BCG vaccine antigen PPD was used as a positive control. IL-17A responses have previously been detected in CD4<sup>+</sup> T cells from healthy BCG-vaccinated individuals after 12 h of PPD stimulation (32). Our finding of increased levels of PPD-induced CD4<sup>+</sup> T cells producing only IL-17A in *Giardia*-exposed individuals could be coincidental or due to polarization of the PPD responses by the ongoing/recent giardiasis in this group. The *Giardia*-exposed group might also have boosted their PPD responses by recent travel in areas with an increased risk of reexposure to environmental mycobacteria. An alternative hypothesis is that individuals developing symptomatic giardiasis have a tendency toward T<sub>H</sub>17 polarized responses.

When we compared the responses of *Giardia* antigen-stimulated T cells in *Giardia*-exposed individuals and low-risk controls, some of the cells producing IL-17A were also producing TNF- $\alpha$ . Background responses found in low-risk healthy controls tended to be unspecific and with less polyfunctional EM cells than in individuals with recent or ongoing *Giardia* infections. Polyfunctional T cells can be linked to better and persistent protection from infection (28) and can be a sign of a good memory response. Polyfunctional T cells could therefore prove to be an important marker of protection from and clearance of *Giardia*.

Another cytokine responsible for anti-inflammatory responses, IL-10, was also found to be increased in SSA-stimulated cells. The higher levels of this cytokine could mean that SSA may also elicit a regulatory response in addition to inflammatory responses. Regulatory responses to *Giardia* have also been found in calves with current infections (20), where FoxP3 was significantly elevated. Regulatory responses may play an important role in giardiasis, and this may account for the negative association between *Giardia* infection and acute diarrhea seen in children in recent studies in countries where giardiasis is endemic (33, 34).

Increased levels of T cell markers specific for *Giardia*-exposed individuals have been shown before (22). Also in the present study, CD45RO/HLA-DR CD4 T cell responses were shown to be significantly elevated in SSA- and SSB-stimulated cells. Proliferation of CD4<sup>+</sup>  $\alpha$ B T cells in response to *Giardia* trophozoites has been found in a study in which calves were investigated (20). These findings are well in line with the results of the present study and provide evidence that CD4<sup>+</sup> T cells play a key role in the recall immune response to *Giardia* infections in calves and humans.

However, the EM CD4<sup>+</sup> T cell responses of *Giardia*-exposed individuals measured in this study are subtle, with small populations of cells responding. Subtraction of the unstimulated responses from the stimulated responses resulted in a number of negative values. A conservative analysis approach was taken in which an equal positive value was adjusted to zero for every negative value adjusted to zero (26).

Other cytokines more closely related to B-cell-mediated antibody production were also found to be elevated in our study. These cytokines include CD40L, IL-6, and MIP-1 $\alpha$ , and their importance for B cells and Ig promotion is described in references 28 and 35. IL-6 seems to play a crucial role in *G. lamblia* eradication in mice, as mice deficient in this cytokine could not control the acute phase of infection (36).

The intracellular *Giardia* protein mixtures used in the present study might not be the best for initiating good relevant anti-*Giardia* immune responses. The strongest immune re-

sponses to *Giardia* in humans are probably directed toward the variant-specific surface proteins (VSPs). Only 1 specific type, out of around 250, is expressed at any time point (37). Using semiconserved peptides from VSPs or recombinant *Giardia* trophozoites expressing all of the available VSPs could possibly have elicited stronger responses in *Giardia*-exposed individuals but could also have caused much larger variability, depending on which VSPs were expressed during *Giardia* infection. Responses to the *Giardia* antigens were also found in some low-risk controls. The true *Giardia* exposure status of the low-risk controls is hard to ascertain. Only individuals who had not traveled to regions where giardiasis is endemic were recruited, but still it is estimated that 2 to 5% of the population in an industrialized country has had giardiasis or has a current infection (6).

In the flow cytometric assay, some of the selected positive-control antigens were not capable of eliciting good IL-4 and IL-10 responses. In the multiplex assay, where proper controls were used, the IL-4 levels were below the detection limit and could not be analyzed. It is therefore likely that the number of cells producing IL-4 was low. It has been stated that IL-4 is expressed at low levels and consequently can be difficult to measure (29). It remains unclear whether this is a methodological problem or whether T<sub>H</sub>2 polarizations are not important for protection against *Giardia*. Better IL-4- and IL-10-eliciting positive controls could have been included in the flow cytometric assay to better interpret these results.

We observed much variability in the response measurements. The time since *Giardia* infection varied from 0 to >2 years, and the age span of the participants and our sample size were relatively small. Also, previous *Giardia* exposure(s), host-specific factors, and concomitant infections/microbiota account for some of the variability.

**Concluding remarks.** In this study, we found increased numbers of antigen-specific IL-17A-producing CD4<sup>+</sup> EM T cells after 24 h and in PBMC supernatants after 6 days of *Giardia* SSA and SSB stimulation. Interestingly, levels of responding polyfunctional EM CD4<sup>+</sup> T cells simultaneously producing IL-17A and TNF- $\alpha$  were elevated in the subgroup analysis of *Giardia*-exposed individuals with current infections and those infected for <8 weeks. Our findings indicate that T<sub>H</sub>17 responses are important during acute symptomatic *Giardia* infections in humans.

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C. S. Saghaug, K. Hanevik, and N. Langeland planned and designed the study. C. S. Saghaug carried out the experiments. S. Sørnes, S. Svård, D. Peirasmaki, and K. Hanevik contributed with reagents, methods, materials, and/or analysis programs. D. Peirasmaki and S. Svård produced the *Giardia* antigens. C. S. Saghaug, K. Hanevik, and N. Langeland analyzed the data. C. S. Saghaug, S. Sørnes, D. Peirasmaki, S. Svård, K. Hanevik, and N. Langeland wrote the paper.

K. Hanevik has served on an advisory board for Lupin Pharmaceuticals, Inc., Baltimore, MD. The other authors have no conflicts of interest to report.



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