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# Cytokine and chemokine responses to helminth and protozoan parasites and to fungus and mite allergens in neonates, children, adults, and the elderly

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## Abstract

**Background:** In rural sub-Saharan Africa, endemic populations are often infected concurrently with several intestinal and intravascular helminth and protozoan parasites. A specific, balanced and, to an extent, protective immunity will develop over time in response to repeated parasite encounters, with immune responses initially being poorly adapted and non-protective. The cellular production of pro-inflammatory and regulatory cytokines and chemokines in response to helminth, protozoan antigens and ubiquitous allergens were studied in neonates, children, adults and the elderly.

**Results:** In children schistosomiasis prevailed (33%) while hookworm and *Entamoeba histolytica/E. dispar* was found in up to half of adults and the elderly. *Mansonella perstans* filariasis was only present in adults (24%) and the elderly (25%). Two or more parasite infections were diagnosed in 41% of children, while such polyparasitism was present in 34% and 38% of adults and the elderly. Cytokine and chemokine production was distinctively inducible by parasite antigens; pro-inflammatory Th2-type cytokine IL-19 was activated by *Entamoeba* and *Ascaris* antigens, being low in neonates and children while IL-19 production enhanced "stepwise" in adults and elderly. In contrast, highest production of MIP-1delta/CCL15 was present in neonates and children and inducible by *Entamoeba*-specific antigens only. Adults and the elderly had enhanced regulatory IL-27 cytokine responses, with Th2-type chemokines (MCP-4/CCL13, Eotaxin-2/CCL24) and cytokines (IL-33) being notably inducible by helminth- and *Entamoeba*-specific antigens and fungus-derived allergens. The lower cellular responsiveness in neonates and children highlighted the development of a parasite-specific cellular response profile in response to repeated episodes of exposure and re-infection.

**Conclusions:** Following repeated exposure to parasites, and as a consequence of host inability to prevent or eliminate intestinal helminth or protozoa infections, a repertoire of immune responses will evolve with lessened pro-inflammatory and pronounced regulatory cytokines and chemokines; this is required for partial parasite control as well as for preventing inadequate and excessive host tissue and organ damage.

**Keywords:** Helminth, Protozoa, Parasite infection, Cytokine, Chemokine, Cellular response, Age groups

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## Background

Parasitic infections are common in countries with poor hygienic conditions, where a lack of sanitation and health care facilitates the transmission and the spread of helminths like *Ascaris lumbricoides*, *Schistosoma spp.*, hookworms, and protozoa like *Entamoeba histolytica/dispar* [1]. Approximately two billion people are infected with helminth parasites worldwide, and some intestinal parasites may affect up to 70% of an endemic population [2]. The dispersion of parasites often overlaps, and individuals living in such areas acquire multiple infections during their lifetime and are infected concurrently with several parasite species [1]. The encounter with parasite species elicits distinct and specific immune responses in their host; cytokines and chemokines are key players which regulate and polarize cellular reactivity and antibody responses to antigens and allergens. Helminth infections associate with an initial Th1 immune response during pre-patency, while during patency a Th2-type response prevails [3,4]. Generally with chronic helminth parasite infections Th2-type cytokine responses predominate [5], while Th1-type cytokine responses are important for protection against protozoa, e.g. amoebiasis [6] or *Plasmodium falciparum* malaria [7].

A specific, balanced and, to an extent, protective immunity develops over time in response to repeated parasite encounter, with immune responses initially being poorly adapted and non-protective [8,9]. Such incapability can result in parasite persistence and host tissue damage as a result of inappropriate inflammatory reactivity. With repeated episodes of infection, parasite clearance, and re-infection, immune responses to foreign antigens become increasingly specific and effective; however, the development of such immunity with increasing age is not well understood. Cytokines and chemokines may enhance, suppress, and regulate the expression of immunity to intravascular and intestinal parasites; moreover, they particularly promote chemotaxis and the activation of effector cells in parasite-invaded tissues and cells.

Monocyte chemoattractive proteins such as MCP-4 recruit effector cells [10] and inflammatory proteins such as MIP-1delta/CCL15 and Eotaxin2/CCL24 activate eosinophil granulocytes, monocytes, and lymphocytes and so contribute to inflammation [11-13]. Cytokines released as "alarmins" and mediators of inflammation, examples being IL-19 and IL-33, may enhance Th2 type immune reactions during infections with intestinal nematodes [14-17], while IL-19 promotes chemotaxis of neutrophil granulocytes and the production of IL-6 and TNF-alpha [18,19]. Regulatory cytokines such as IL-27 limit exacerbating Th17 and Th2 responses [20] and decrease immune pathology during malaria infection [21,22]. While with several cytokines and chemokines their role in parasitic infections has not yet been

investigated, others like Eotaxins were found to be important for effector cell recruitment during helminth infection.

In order to further clarify the extent to which these immune mediators become activated or suppressed during early life parasite exposure, and also with a view to later life chronic pathogen persistence, we studied the cellular production of pro-inflammatory and regulatory cytokines and chemokines in neonates, children, adults, and the elderly in response to helminth and protozoa infectious challenge and to ubiquitous allergen exposure. Distinctive production levels were observed between these groups, highlighting the development of a parasite-specific cellular responsiveness to repeated episodes of exposure and re-infection.

## Results

### Helminth and protozoa parasite infections in children, adults, and the elderly

The prevalence of parasite infections and parasite co-infections in children, adults, and the elderly is shown in Table 1. Free of parasite infection were 37% of the children, while 29% of adults and 21% of the elderly were negative for parasites in urine, blood, and stools. In children schistosomiasis prevailed (33%) while in adults and the elderly *Schistosoma mansoni* or *S. haematobium* were less than 10%. Hookworm infections were present in 22%, 26% and 34% of children, adults and the elderly, respectively, while *E. histolytica/dispar* was found in 22%, 37% and 55% of same. *M. perstans* filariasis was present only in adults (24%) and the elderly (25%). Multiple parasite infections were diagnosed in 41% of children, while such polyparasitism was present in 34% and 38% of adults and the elderly.

**Table 1 Prevalence of parasite infections and parasite co-infections in children, adults and elderly**

	Children (n=35)	Adults (n=39)	Elderly (n=42)
<i>Schistosoma haematobium/S. mansoni</i>	33%	8%	2%
Hookworm	22%	26%	34%
<i>Entamoeba histolytica/E. dispar</i>	22%	37%	55%
<i>Mansonella perstans</i>	0%	24%	25%
Infection-free	37%	29%	21%
Single parasite infection	22%	37%	41%
Multiple parasite infections	41%	34%	38%

Fresh stool samples were analyzed by microscopy for intestinal helminth eggs as well as protozoan cysts and trophozoites, and all stool samples were examined using the Kato-Katz technique. *Schistosoma haematobium* eggs were detected by filtration of urine samples and subsequent microscopic examination of filters, while microfilaria of *Mansonella perstans* were detected by microscopic analysis after gradient density centrifugation of whole blood samples as detailed in Materials and Methods.

### Cellular cytokine responses to helminth and protozoa parasite antigens in neonates, children, adults, and the elderly

Table 2 shows the antigen-inducible production levels of IL-19, IL-27, and IL-33 by umbilical cord blood cells (UCBC) and peripheral blood mononuclear cells (PBMC) (Data not shown for Asc and Ov). In neonates and children IL-19 did not change between not stimulated (Base) and antigen-stimulated UCBC and PBMC. In adults IL-19 production enhanced following *Ascaris* (Asc) and *Entamoeba* (Eh) antigens stimulation (for Eh  $p < 0.05$  compared to Base). In the elderly, Eh, *O. volvulus* (Ov) and Asc antigens stimulated IL-19 responses (for Eh  $p < 0.05$  compared to Base). The production levels of IL-27 were low in neonates and children, but IL-27 production enhanced overall in adults and the elderly, without significant differences between the groups. Spontaneous cellular release of IL-33 as well as amounts produced in response to antigens from

Eh, Ov and *Plasmodium* (Pf) remained low in all groups; only in response to Asc antigen were UCBC and PBMC from all four study groups found to secrete elevated amounts of IL-33 ( $p < 0.01$  for children and adults,  $p < 0.001$  for the elderly; compared to Base).

### Cellular chemokine release to helminth and protozoa parasite antigens in neonates, children, adults, and the elderly

Cellular production of MCP-4/CCL13, MIP-1delta/CCL15 and Eotaxin-2/CCL24 by UCBC and PBMC following stimulation with helminth- and protozoa-specific antigens is shown in Table 2 (Data not shown for Asc and Ov). Parasite antigen-induced MCP-4/CCL13 production by UCBC from neonates did not differ from spontaneous release. In children, baseline MCP-4/CCL13 production did not enhance when their PBMC were cocultured with Eh, Ov and Pf antigens. Similarly, in adults and the elderly,

**Table 2 Cytokines and Chemokines in response to parasite antigens**

Cytokine/chemokine	Study group	Base	Eh	Pf
IL-19	Neonates	137 (0/301)	240 (77/404)	271 (9/449)
	Children	204 (81/327)	382 (229/535)	189 (36/341)
	Adults	244 (114/375)	692 * (507/877)	370 (186/555)
	Elderly	257 (128/386)	726 * (544/909)	319 (137/502)
IL-27	Neonates	425 (307/542)	495 (378/612)	518 (393/642)
	Children	557 (429/686)	666 (513/820)	501 (340/661)
	Adults	719 (503/935)	1004 (699/1309)	886 (581/1191)
	Elderly	1050 (898/1202)	951 (738/1164)	932 (737/1128)
IL-33	Neonates	52 (17/86)	67 (31/104)	32 (0/71)
	Children	22 (0/110)	107 (2/212)	14 (0/123)
	Adults	29 (5/54)	51 (16/87)	52 (28/77)
	Elderly	45 (28/62)	76 (26/125)	62 (36/88)
MCP-4/ CCL13	Neonates	69 (40/99)	56 (26/86)	51 (19/82)
	Children	180 (120/240)	54 (0/126)	85 (40/130)
	Adults	129 (84/175)	14 (8/20)	135 (88/183)
	Elderly	124 (85/163)	12 (0/67)	133 (78/188)
Eotaxin-2/ CCL24	Neonates	537 (337/738)	540 (340/741)	419 (205/632)
	Children	358 (264/454)	319 (201/438)	360 (235/486)
	Adults	1398 (1223/1572)	544 ** (297/790)	1762 (1515/2008)
	Elderly	1430 (1262/1598)	340 *** (102/577)	1405 (1168/1643)
MIP-1delta/ CCL15	Neonates	63 (47/79)	68 (52/83)	64 (47/80)
	Children	46 (41/62)	56 (43/68)	41 (28/55)
	Adults	19 (12/29)	35 (24/46)	24 (13/34)
	Elderly	27 (16/37)	63 ** (49/78)	26 (12/41)

The cellular production of interleukin (IL)-19, IL-27, IL-33, MCP-4/CCL13, MIP-1delta/CCL15 and Eotaxin-2/CCL24 in response to parasite antigens by umbilical cord blood cells (UCBC) and peripheral blood mononuclear cells (PBMC) from different age groups is shown. UCBC from neonates (n=36) and PBMC were isolated from children (age 10-13 years; n=35), adults (18-45 years, n=39) and the elderly (50-80 years, n=42), and stimulated *E. histolytica* antigen (Eh; 10 µg/ml), *P. falciparum* antigen (Pf; 1x10<sup>8</sup> schizonts/ml) or not stimulated (Base), for 48 hours at 37%, 5% CO<sub>2</sub> and saturated humidity. Cytokine and chemokine concentrations in cell culture supernatant were determined by specific enzyme-linked immunosorbent assays (ELISA). The data are given as means with the lower and upper 95% confidence intervals (in brackets). (\*  $p < 0.05$ , \*\*  $p < 0.01$  \*\*\* $p < 0.001$ , compared to Base).

Eh and Pf antigens did not heighten MCP-4/CCL13 production, while Asc- and Ov-specific antigens strongly activated MCP-4/CCL13 by PBMC ( $p < 0.01$ , when compared to Base).

Production of the pro-inflammatory Eotaxin-2/CCL24 was low in neonates and children, irrespective of the antigens used for cell stimulation. In adults, *Ascaris* and also *Onchocerca* and *Plasmodium* antigen extracts enhanced Eotaxin-2/CCL24 release ( $p < 0.01$  for *Ascaris* when compared to Base), while stimulation with *Entamoeba* antigen significantly reduced Eotaxin-2/CCL24 levels ( $p < 0.01$  compared to Base). In the elderly, Eotaxin-2/CCL24 production enhanced in response to *Ascaris* ( $p < 0.05$ ), while depressed in response to *Entamoeba* antigen ( $p < 0.001$ ). Highest levels of MIP-1delta/CCL15 were produced by UCBC and PBMC from neonates and children, with no significant differences between the individual antigen stimulations. Overall MIP-1delta/CCL15 production was lower in adults (mean children 62 pg/ml, mean adults 25 pg/ml); only *Entamoeba* antigens slightly increased MIP-1delta/CCL15 production, with this *Entamoeba* antigen-specific activation being significant in PBMC from the elderly ( $p < 0.01$ ). All other parasite antigens elicited MIP-1delta/CCL15 production levels around baseline levels (Table 2).

#### **Helminth antigen-induced cellular production of cytokines (IL-19, IL-27, IL-33) and chemokines (MCP-4/CCL13, MIP-1delta/CCL15, Eotaxin-2/CCL24)**

The responses of UCBC and PBMC from neonates, children, adults, and the elderly to helminth-specific *A. lumbricoides* (Figure 1A) and *O. volvulus* antigens were evaluated (Figure 1B). Cellular response of IL-19, IL-27, IL-33 and MCP-4/CCL13 to *Ascaris* antigen was lowest in neonates, and enhanced in children, adults, and the elderly (Figure 1A). Highest production levels of IL-19, IL-27 and MCP-4/CCL13 were observed in adults and the elderly, without differences between the age-groups (Figure 1A). Eotaxin-2/CCL24 production was highly elevated in adults and the elderly as compared to neonates and children ( $p < 0.01$ ). In contrast, Asc antigen-induced production of MIP-1delta/CCL15 was clearly lower in adults than in neonates and children ( $p < 0.01$  compared to neonates). Broad confidence intervals for IL-19 and IL-33 were observed in neonates and children for all cytokines, while confidence intervals were smaller in adults (Figure 1A).

*O. volvulus* antigen-specific production of IL-19 while low in neonates was found to be enhanced “stepwise” in children, adults, and the elderly (Figure 1B). UCBC and PBMC from neonates and children produced lower amounts of IL-27 than did PBMC from adults and the elderly. Mean levels of IL-33 as produced by UCBC and PBMC from neonates, adults, and the elderly were similar; only IL-33 production was decreased in children in

response to Ov antigen. Production of MCP-4/CCL13 and Eotaxin-2/CCL24 was lowest in neonates and children, while PBMC from adults and the elderly produced significantly enhanced amounts of both chemokines ( $p < 0.01$ , compared to neonates and children). In contrast, the production levels of MIP-1delta/CCL15 were highest in neonates and children, and significantly lower in adults and the elderly ( $p < 0.01$ , compared to neonates and children) (Figure 1B).

#### **Fungus and mite allergen-induced cellular production of cytokines and chemokines in neonates, children, adults, and the elderly**

Tables 3 and 4 shows the cellular production of cytokines (IL-19, IL-27, IL-33) and chemokines (MCP-4/CCL13, Eotaxin-2/CCL24, MIP-1delta/CCL15) by neonatal UCBC and PBMC in response to *A. fumigatus*, *D. farinae* and *D. pteronyssinus* extracts.

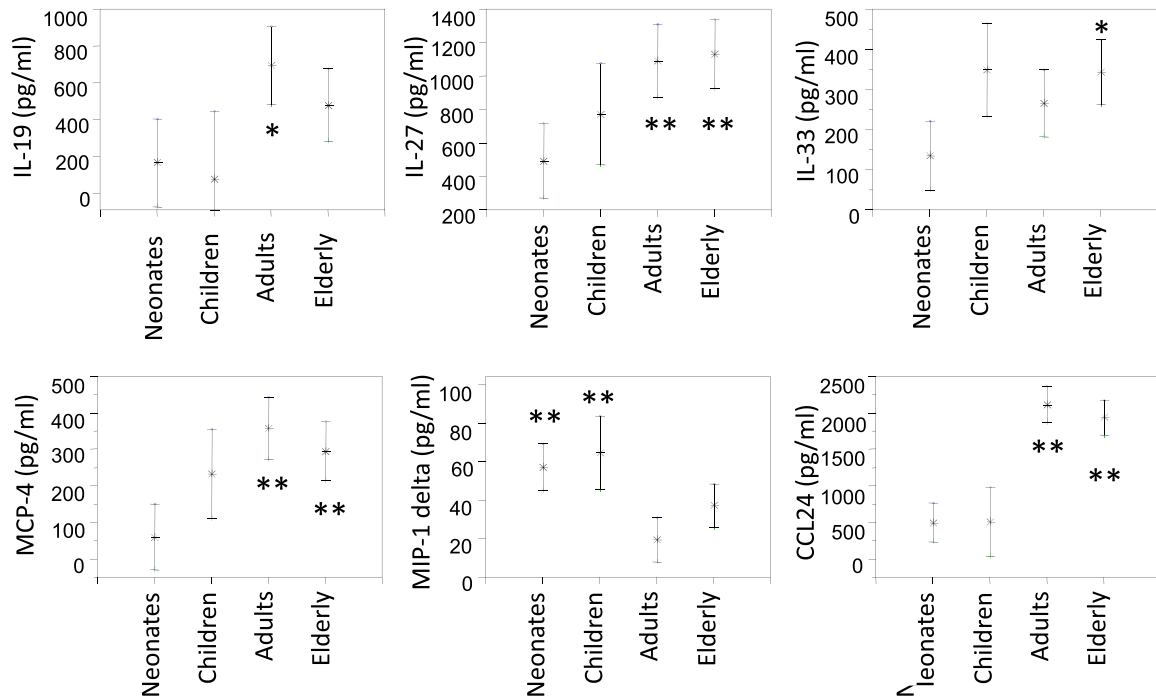
Allergen-stimulated production of IL-19 and IL-27 was lowest in UCBC from neonates, while IL-19 production levels were found to be enhanced in adults and the elderly (for mite allergens  $p < 0.01$ , compared to neonates). Cellular production of mite allergen-induced IL-27 was lowest in neonates but highly elevated in children. Both fungus (Af) and mite (Df) allergen induced cellular production of IL-33 were strongly elevated in children ( $p < 0.01$  and  $p < 0.001$ ), compared to neonates, adults, and the elderly). Allergen stimulation did not induce cellular MCP-4/CCL13, Eotaxin-2/CCL24 or MIP-1 delta/CCL15 production above baseline levels.

#### **Discussion**

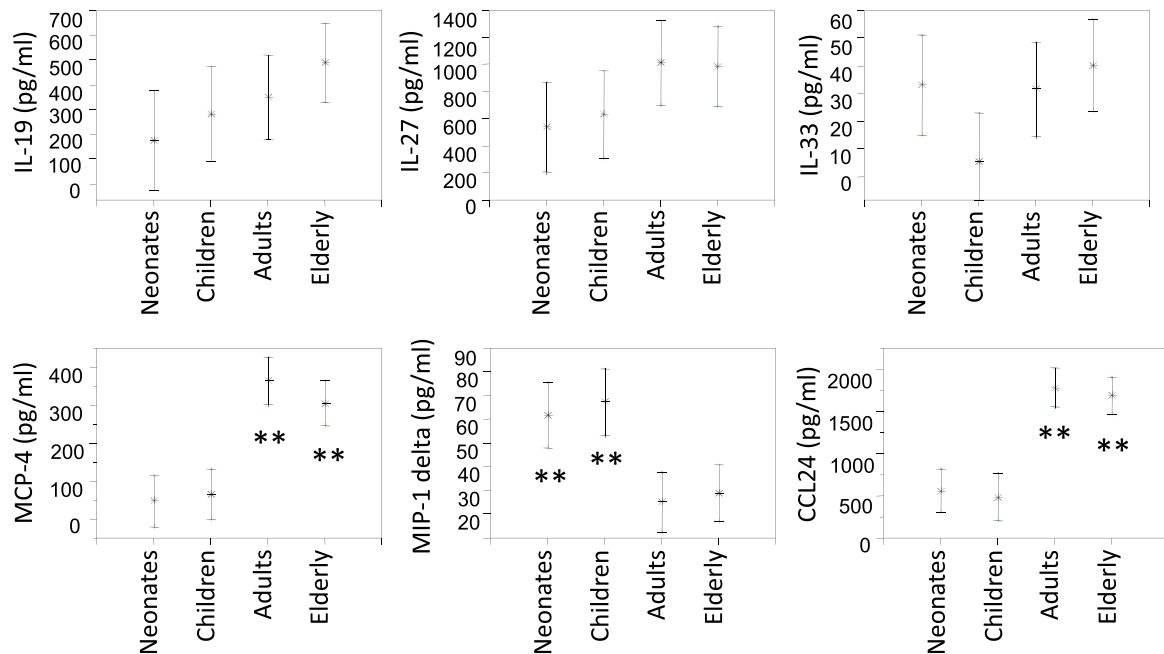
Immune memory and cellular effector responses against parasites may develop and take shape gradually with repeated exposure, pathogen persistence or their clearance, and also with re-infection. In the present work, cellular responsiveness in neonates and children was low while adults and the elderly had enhanced regulatory IL-27 cytokine responses, with Th2-type chemokines (MCP-4/CCL13, Eotaxin-2/CCL24) and cytokines (IL-19, IL-33) being inducible by parasite-specific antigens and allergens.

The pro-inflammatory Th2-type cytokine IL-19 while low in neonates and children was enhanced “stepwise” in adults and the elderly; IL-19 was predominantly activated by *Entamoeba* and *Ascaris* antigens and allergens also, signifying repeated encounter with protozoan and helminth parasites and environmental allergens – such responsiveness was not yet developed in neonates and children. High IL-19 levels were observed in patients with asthma and also in an allergen-inducible asthma animal model [18]. IL-19 was found to enhance IL-1beta, IL-6, and CXCL8/IL-8 release and to attract granulocytes [18,19]; such mobilized and activated effector cells may then adhere and attack tissue-infiltrating and migrating

**A** *Ascaris* antigen-induced cellular production of IL-19, IL-27, IL-33, MCP-4, MIP-1 delta and Eotaxin-2 from different age groups.



**B** *O. volvulus* antigen induced cellular production of IL 19, IL 27, IL-33, MCP-4, MIP-1 delta and Eotaxin-2 from different age groups.



**Figure 1** Cellular production of interleukin (IL)-19, IL-27, IL-33 MCP-4/CCL13, MIP-1delta/CCL15 and Eotaxin-2/CCL24 in response to *A. lumbricoides* (*Asc*) and *O. volvulus* (*Ov*) antigen by umbilical cord blood cells (UCBC) and peripheral blood mononuclear cells (PBMC) from different age groups is shown. **A.** *Ascaris* antigen-induced cellular production of IL-19, IL-27, IL-33, MCP-4, MIP-1delta and Eotaxin-2 from different age groups. **B.** *O. volvulus* antigen-induced cellular production of IL-19, IL-27, IL-33, MCP-4, MIP-1delta and Eotaxin-2 from different age groups.



**Table 3 Cytokines and chemokines in response to *Aspergillus fumigatus* allergen extract**

	IL-19	IL-27	IL-33	MCP-4	CCL-24	MIP-1 delta
Neonates	49 (0/603)	311 (0/1120)	62 (0/148)	0 (0/162)	283 (0/1198)	42 (0/87)
Children	ND	1096 (957/1236)	97** (80/113)	ND	ND	ND
Adults	280 (151/411)	823 (589/1056)	34 (7/60)	116 (72/162)	1728 (1474/1982)	29 (17/41)
Elderly	297 (172/424)	1136 (911/1363)	56 (32/81)	119 (75/163)	1558 (1315/1802)	31 (19/43)

\*\* p<0.01 in comparison to adults for IL-33.

Cellular production of interleukin (IL)-19, IL-27, IL-33 MCP-4/CCL13, MIP-1delta/CCL15 and Eotaxin-2/CCL24 in response to allergen extracts by umbilical cord blood cells from neonates (UCBC) or peripheral blood mononuclear cells (PBMC) from children (age 10-13 years), adults (18-45 years) and the elderly (50-80 years). UCBC or PBMC were stimulated with extracts from *A. fumigatus* (final concentration 20 µg/ml) (Part A) or *D. farinae* (20 µg/ml) and *D. pteronyssinus* (20 µg/ml) (Part B) for 48 hours at 37%, 5% CO<sub>2</sub> and saturated humidity. Cytokine and chemokine concentrations in cell culture supernatant were determined by enzyme linked immunosorbent assay (ELISA). The means of the brut production and the lower and upper 95% confidence intervals (in brackets) are shown. ND Not determined.

helminth larvae of *Ascaris* and hookworm. Furthermore, antigens of *Ascaris suis* and *E. histolytica* elicited strong chemotaxis and production of superoxide anions in neutrophil granulocytes [23,24]. IL-19 is a member of the IL-10 family; secreted by monocytes, epithelial cells and B cells [25-27], it exerts regulatory effects and, in mice, protects against colonic inflammation [28] and induces Th2 responses [29]. Inducible cellular IL-19 production in adults and elderly could therefore mirror an adaptation to intestinal protozoan and metazoan parasite challenge and allergen exposure over time. Similarly to IL-19, low amounts of IL-33 were detected in neonates; only *Ascaris* antigen activated IL-33 in children, suggesting early life priming by intestinal helminths. As a member of the IL-1 family, IL-33 promotes the generation of Th2 immune responses by inducing secretion of IL-4, IL-5 and IL-13 by T cells [30]. High levels of IL-33 were detected in patients with asthma or allergic rhinitis [31,32]. The "Alarmin" IL-33 is released by injured epithelia and endothelia following hookworm infection so as to attract leukocytes to the site of inflammation [33,34]; moreover, IL-33 supports reduction and expulsion of the intestinal helminths *Heligmosomoides pylorus*, *Trichuris muris* or *Nippostrongylus brasiliensis* from infected mice [17,35,36]. A recent study [14] has disclosed the importance of IL-33 during murine hookworm infection: in IL-33 gene knockout mice infected with *N. brasiliensis*, cellular production of the Th2-type cytokine IL-13 was lessened and eosinophil recruitment reduced, and accompanied by a delayed worm expulsion in these animals. In the present work, allergens of mite and fungus activated IL-33 in children, suggesting that such increase in IL-33 reflects an initial

responsiveness which, in later life and after repeated parasite exposure, is attenuated by regulatory cytokines like IL-27.

IL-27 production was low in neonates and children but gradually enhanced in adults and the elderly, with no difference between baseline and the antigen stimulation (Table 2). Mite allergen-induced production levels of IL-27 were highest in children, whereas in neonates, adults and the elderly, allergen stimulation did not induce IL-27 production above baseline levels (Tables 3 and 4). A member of the IL-12 family, IL-27 has been found to act as initiator and attenuator of immune responses [37,38], blocking both Th2- and Th17-type cytokines [37,39]. IL-27 exerts regulatory functions, mostly by inducing and regulating IL-10 and IL-17 [37,40]. Severe malaria tropica in children and non-healing *Leishmania major* infection in mice were accompanied by depressed levels of IL-27, despite high IL-10 [40,41]. IL-27R-deficient mice were able to control *Toxoplasma gondii* infection initially, but later succumbed due to inflammatory immune responses [42]; these mice will develop severe lung inflammation, elevated IgE levels, and eosinophilia [43]. Still, these regulatory properties of IL-27 cannot be adopted universally, as disruption of the IL-27 signaling pathway did not alter egg-induced immunopathology in an experimental schistosomiasis model [44]. While no differences in IL-27 production were observed following stimulation with parasite antigens between the age groups, enhanced IL-27 in adults and the elderly may reflect the stabilization of a regulatory cytokine network in response to repeated parasite encounter.

**Table 4 Cytokines and chemokines in response to *Dermatophagoides farinae/pteronysinus* allergen extracts**

	IL-19	IL-27	IL-33	MCP-4	CCL-24	MIP-1delta
Neonates	149 (39/260)	496 (330/662)	34 (14/55)	53 (28/79)	655 (448/862)	82(70/94)
Children	ND	1101*** (993/1208)	101*** (88/115)	ND	ND	ND
Adults	421 ** (328/514)	799 (640/958)	36 (17/56)	83 (59/107)	1249 (1059/1440)	26 (14/37)
Elderly	411 ** (321/501)	1077 (937/1217)	36 (20/51)	101 (78/124)	1377 (1194/1561)	25 (15/36)

For IL-19 and IL-27: \*\* p<0.01 in comparison to neonates, \*\*\* p<0.001 in comparison to neonates; for IL-33: \*\*\* p<0.001 in comparison to all groups.

Cytokines and chemokines are key players in regulating and polarizing cellular reactivity and antibody responses to pathogens and allergens. Helminth antigens induced Eotaxin-2/CCL24 and MCP-4/CCL13 production in adults and the elderly but not in neonates and children, as similarly observed for IL-19 and IL-33. Eotaxin-2/CCL24 activates Th2-type cytokines and chemoattracts eosinophil and basophil granulocytes. Elevated Eotaxin-2/CCL24 levels have been found in experimental helminth infections [45] and in acutely infected *S. mansoni* patients [12,46], and high Eotaxin-2/CCL24 levels were associated with increased liver damage in *S. mansoni*-infected mice. Following treatment of onchocerciasis patients with ivermectin, Eotaxin-2/CCL24 and MCP-4/CCL13 enhanced suggesting that these chemokines facilitated clearance of *O. volvulus* microfilariae by monocytes and eosinophil granulocytes [47]. Interestingly, protozoan *Entamoeba*-specific antigens depressed both Eotaxin-2/CCL24 and MCP-4/CCL13 in adults and the elderly, but not in children and neonates; such depressed responsiveness might support control *E. histolytica* infection, as elevated levels of Eotaxins were observed in mice with persistent *E. histolytica* infection [48]. The chemokine MCP-4/CCL13 attracts granulocytes, monocytes, and T cells, and it has been proposed as a biomarker in asthma [49] being up-regulated during both Th1- and Th2-type hyper-responses [10]. In the present work, MCP-4/CCL13 and Eotaxin-2/CCL24 were not produced in neonates, but were inducible by helminth antigens in adults and the elderly – an observation pointing to the gradual expansion of the parasite-specific immune response repertoire.

In stark contrast to the above studied cytokines and chemokines, the highest production of MIP-1delta/CCL15 was found in neonates and children, whereas MIP-1delta/CCL15 was low in adults and the elderly (Table 2). Cellular MIP-1delta/CCL15 release in response was not inducible by allergens in any group above baseline levels (Tables 3 and 4). The pro-inflammatory chemokine MIP-1delta/CCL15 attracts neutrophil granulocytes, T cells and monocytes [11]. In the present study MIP-1delta/CCL15 was inducible by *Entamoeba*-specific antigens only. Exposure of human monocytes to live microfilaria of *Brugia malayi* enhanced CCL15 mRNA expression, which was also present in IL-4 induced alternative activated macrophages [50]. With an expiring *O. volvulus* infection, low levels of MIP-1delta/CCL15 were detected in patients [47], while the reduced MIP-1delta/CCL15 observed in adults and the elderly may represent an immune adaptation towards lessened inflammatory responses against *E. histolytica*. Similarly, cellular reactivity towards allergens was highest in neonates and significantly reduced in adults and the elderly.

## Conclusion

In summary, helminth and protozoan antigens distinctly activated in adults Th2-type cytokines, effector cell-attracting chemokines and regulatory components, notably IL-27, while such responsiveness was not yet present in neonates and children. Following repeated exposure to parasites and as a consequence of host inability to prevent or eliminate intestinal helminth or protozoa infections, a repertoire of immune responses evolves with lessened pro-inflammatory and pronounced regulatory cytokines and chemokines – this is required for partial parasite control and also to prevent inadequate and excessive host tissue and organ damage.

## Material and methods

### Population study

The study was conducted at the Centre Hospitalier Regional (CHR) in the Central Region of Togo and approved and authorized by the Togolese Ministry of Health (292/99/MS/CAB, 0407/2007/MMSCAB/DGS, MS/DGS/DRS/RC/No. 220 and No. 261) and by the Ethics Committee of the University Clinics of Tübingen, Germany (No. 188/2008/BO2). A total of 152 individuals were included in the study and grouped by age: neonates (n= 36), children (10-13 yrs, n=35), adults (18-45 yrs, n= 39) and elderly (50-80 yrs, n=42). The children were all attending primary schools in suburban areas of the town of Sokodé (Prefecture Tchoudjo). Adults were from the village of Bouzalo, near the city of Sokodé. Written consent was obtained from the children's parents prior to participation. Peripheral Blood Mononuclear Cells (PBMC) or else Umbilical Cord Blood Cells (UCBC) was collected from all study participants. Blood, stool and urine samples were collected from children and from adults. Umbilical Cord Blood was obtained from mothers giving birth in the Central Hospital of Sokodé. Written informed consent was obtained from all mothers after thoroughly explaining to them the procedures and risks of this study; to ensure understanding, explanations were given in the local language by the medical staff during prenatal consultations at the CHR. Pregnant women received antiparasite treatment in line with the national health guidelines of Togo both during prenatal consultations (PC) and after partition. All pregnant women received antimalaria prophylaxis as recommended by national health guidelines - receiving either chloroquine at 300 mg/week, which was taken until partition, or a single dose of sulfadoxine/pyrimethamine at the end of the second trimester of pregnancy as well as a further dose at the beginning of the third. In the 4th month of pregnancy, all women received antihelminth treatment (albendazole, single dose 400 mg) and, after partition, they were treated against intestinal protozoan parasites (metronidazole) in line with the national health guidelines.

### Isolation of peripheral blood mononuclear cells (PBMC) and umbilical cord blood cells (UCBC) and cell culture experiments

Isolation of PBMC was carried out as described earlier [51,52]. In brief, 5-9 ml of venous blood was collected and PBMC were then isolated using Ficoll Density Centrifugation at 340 g for 35 minutes. Cells were collected, washed twice in Roswell Park Memorial Institute (RPMI) media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (Sigma, St. Louis, MO, USA). Cells were counted and cultured at a concentration of  $1 \times 10^6$  cells per well, supplemented in RPMI with 5% heat inactivated Fetal Calf Serum (FCS, Biochrom, Berlin, Germany). Umbilical cord blood was obtained from the placentas of healthy, full-term infants, after the placentas were delivered and separated from same. Blood samples were diluted 1:2 with RPMI (Gibco; Eching, Germany) supplemented with 25 mM HEPES buffer, 100 U/ml penicillin and 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B (as above). Umbilical cord mononuclear blood cells (UCBC) were isolated by Ficoll-Paque density gradient centrifugation at 340 g for 35 min at room temperature. UCBC were collected, washed twice in RPMI (as above) at 1400 rpm for 15 min and adjusted to  $1 \times 10^6$ /ml in RPMI (as above) supplemented with 10% heat inactivated FCS (as above). Freshly isolated UCBC or PBMC were cultured in 48 well plates in 5% CO<sub>2</sub> at 37°C and saturated humidity in the presence or absence (baseline) of the following antigens/allergens: *Onchocerca volvulus* antigen (OvAg, final concentration in cell culture 20 µg/ml), *Ascaris lumbricoides* antigen (AscAg, final conc. 5 µg/ml), *Entamoeba histolytica* antigen (EhAg, final conc. 10 µg/ml), *Plasmodium falciparum* schizonts (PfAg, final conc.  $1 \times 10^8$  schizonts/ml), *Dermatophagoides pteronyssinus* (Dp, final conc. 20 µg/ml), *Dermatophagoides farinae* (Df, final conc. 20 µg/ml), *Aspergillus fumigatus* (Af, final conc. 20 µg/ml), *Candida albicans* (Ca, final conc. 20 µg/ml), for 48 hours at 37°C and saturated humidity. Cells and cell culture supernatant were then harvested and stored at -20°C for further use.

### Preparation of antigens and allergens

*E. histolytica* antigen (EhAg; trophozoites; strain HM-1 axenic culture) was a gift from Dr. Brigitte Walderich (formerly: Institute for Tropical Medicine, University Clinics of Tübingen, Germany). *A. lumbricoides* or *O. volvulus* adult worms were isolated as described previously [52], then washed in phosphate-buffered saline (PBS) before being transferred into a Ten-Broek tissue grinder and homogenized extensively on ice. The homogenate was then sonicated twice (30% intensity) for 3 min on ice and centrifuged at 16,000 g for 30 min at 4°C. The supernatants were collected and sterile-filtered

(0.22 µm), and the protein concentration was then determined by BCA protein assay (Pierce, Rockford, USA). *D. pteronyssinus* (Dp), *D. farinae* (Df), *A. fumigatus* (Af) extracts were all purchased from Allergopharma (Rheinbeck, Germany). Crude antigen extracts of *P. falciparum* schizonts were kindly gifted by Dr. A. Luty and Dr. K. Brustoski (formerly: Institute for Tropical Medicine, University of Tübingen, Germany).

### Parasitological analysis

Analysis for helminth and protozoan infections was carried out as previously described [51,52]. Briefly, fresh stool samples were mixed with saline, dispersed on 2 microscope slides and analyzed for intestinal helminth eggs as well as protozoan cysts and trophozoites. All stool samples were examined using the Kato-Katz technique for quantification of helminth eggs per gram of stool (helm-TEST; Labmaster). *Schistosoma haematobium* eggs were detected by filtration of 10 ml urine (polycarbonate membrane, pore size 12 µm; Whatman). Microfilaria stages of *Mansonella perstans* were detected by microscopic analysis after Ficoll density centrifugation. Malaria Rapid Test (OptiMal™, TCS Biosciences, Birmingham UK) was used to determine infection with *P. falciparum*. Children showing signs of malaria (positive thick blood smears for *Plasmodium spp.* and fever or Malaria Rapid Test-positive) and diarrhea were excluded from the study. None of the children presented with *E. histolytica* trophozoites ingested with red blood cells in stool samples, bloody stools or clinical signs of invasive amoebiasis.

### Cytokine and chemokine determination by enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were tested for IL-19, IL-27, IL-33, Eotaxin-2/CCL24, MCP-4/CCL13 and MIP-1 delta/CCL15 using ELISA Assay Kits (R&D Systems). Assays were performed according to guidelines supplied by the manufacturer. Conversion of optical densities (OD) to final concentrations (pg/ml) was calculated by cytokine specific standard curves. Assay detection limits were 30 pg/ml for IL-19, 150 pg/ml for IL-27, 20 pg/ml for IL-33, 8 pg/ml for MCP-4/CCL13, 30 pg/ml for Eotaxin-2/CCL24 and 15 pg/ml for MIP-1 delta/CCL15.

### Statistical data analysis

The statistical package JMP 9.0 (SAS Institute) was used to analyze significant differences between the studied groups. Significant differences in cytokine and chemokine concentrations between studied groups were determined by Analysis of Variance (ANOVA) and Tukey's Test. Due to multiple comparisons, the level of significance was adjusted by Bonferroni-Holm-method.



### Competing interests

The authors have no competing conflicts of interest to declare.

### Authors' contributions

CK, MB, JH, AA, CJL, KK, XH: study proposal, data collection, study design, statistical analyses. CK, CJL, PTS, RGG: study design and coordination, interpretation of results, writing of manuscript and revision. CK, MB, AA, CJL, PTS: manuscript drafting and revision. All authors read and approved the final manuscript.

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