Immune Regulation and Protective Immunity during Helminth and Protozoan Infections

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CHRISTIAN JOSEPH LECHNER
aus Stuttgart

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Dekan: Prof. Dr. Wolfgang Rosenstiel

1.Berichterstatter: Prof. Dr. P. Soboslay

2.Berichterstatter: Prof. Dr. H.-G. Rammensee

Table of Content

Zusammenfassung 1
Summary 4
Published Publications 7
Introduction 9
The Immune System 10
T helper cells 12
Cytokines and Chemokines 15
Pathogenesis and clearance: the impact of immune reactions in parasitic diseases 17
Immune modulation strategies of parasites and changes in immune reactivity following anti-parasite treatment 19
Polyparasitism: Immune reactivity towards parasite antigens and allergen in individuals 20
AIM of this study 22
Results and Discussion 24
Immune reactivity during severe and mild malaria tropica in infants 24
Impact of microfilariacidal treatment on the immune reactivity in Onchocerciasis 28
Altered immune reactivity in Alveolar Echinococcosis 30

Polyparasitism: Immune reactivity towards parasite antigens and allergens in individuals from different age groups 33

Conclusions 36

References 38

Publications 53

Curriculum Vitae 170

Acknowledgements 172

Zusammenfassung

Immunantworten beeinflussen Krankheitsverläufe und haben großen Einfluss auf die Entstehung von Pathologien. Sie können zur Beseitigung, aber auch zum chronischem Verlauf einer Infektion führen. Cytokine und Chemokine nehmen bei der Regulierung von Immunantworten eine zentrale Rolle ein. Ziel dieser Arbeit war die Aufklärung von Immunantworten und Immunregulation bei verschiedenen Helminthen- und Protozoen-Infektionen, sowie eine mögliche Rolle von Immunpathologien bei parasitären Erkrankungen.

Unterschiedliche Immunantworten konnten bei Patienten mit Alveolärer Echinokokkose (AE) beobachtet werden. Patienten mit stabilem und insbesondere fortschreitendem Krankheitsverlauf wiesen erhöhte Serum-Konzentrationen des proinflammatorischen IL-17B auf, während die Konzentrationen des löslichen Rezeptors IL-17RB in allen Patientengruppen stark erhöht war. Im Gegensatz hierzu wiesen alle AE Patientengruppen deutlich reduzierte Konzentrationen des proinflammatorischen Th17-Cytokins IL-17F auf. Dies deckte sich mit der Beobachtung, dass Periphere Mononukleäre Blutzellen von AE Patienten deutlich weniger IL-17F nach Stimulation mit *Echinococcus multilocularis* Antigenen produzierten. Diese Ergebnisse verdeutlichen eine selektive Unterdrückung von proinflammatorischen Immunantworten gegen Parasitenantigene. Die unterschiedlichen Sekretionsprofile in verschiedenen Patientengruppen legen einen möglichen Einsatz von IL-17 Cytokinen zur Beurteilung des Krankheitsverlaufs der Alveolären Echinokokkose nahe.

Chronische Parasitenerkrankungen können zu zellulärer Anergie führen. Eine solche Anergie kann in der humanen

Onchozerkose durch medikamentöse Intervention überwunden werden. Dies führt zu erhöhter Immunreaktivität, möglicherweise aber auch zu überschießenden Immunreaktionen und Immunpathologien. In Teilen dieser Arbeit wurde die Immunreaktivität bei Onchozerkose Patienten nach Ivermectin Behandlung untersucht. Das Absterben der Mikrofilarien (Mf) ging mit erhöhten Serum-Konzentrationen von Mediatoren der zellulären Immunität einher, wohingegen die Konzentration von Mediatoren der humoralen Immunität nahezu unverändert blieb. Diese Mediatoren der zellulären Immunität werden auch mit Auslösung von Immunpathologien in Zusammenhang gebracht. Die Ergebnisse dieser Arbeit unterstreichen die Reaktivierung der Immunreaktivität nach anti-parasitärer Behandlung und weisen auf mögliche Mechanismen bei der Entstehung von Immunpathologien hin.

Pathologien bei Krankheiten können entweder durch den Erreger oder durch das Immunsystem hervorgerufen werden. Gegenstand dieser Arbeit war unter anderem die Bestimmung von pro- und antiinflammatorischen Immunantworten bei leichter und schwerer Malaria Tropica bei Kindern. Verschiedene Th1, Th2 und Th17 Cytokine und Chemokine waren bei Kindern mit schwerer Malaria erhöht. Mehrere dieser Mediatoren scheinen eine wichtige Rolle in der Entstehung von Pathologien zu spielen. Die Ergebnisse dieser Arbeit weisen somit auf eine wichtige Rolle von Immunantworten bei der Pathologie-Entstehung bei schwerer Malaria Tropica hin.

Die Entwicklung von Immunantworten gegen endemisch vorkommende Parasiten schreitet sukzessive im Lauf des Lebens voran. Teil dieser Arbeit war die Bestimmung von

parasitenspezifischen Immunprofilen, welche sich im Laufe des Lebens durch permanente Infektion, mögliche Ausheilung und Reinfektion entwickeln. Während sich für pro-inflammatorische Cytokine und Chemokine eine parasitenspezifische Induzierbarkeit beobachten ließ, stieg mit zunehmendem Alter die Produktion anti-inflammatorischer Cytokine nach Stimulation mit allen Parasitenantigenen an. Diese Ergebnisse spiegeln die Entwicklung von parasitenspezifischen Immunantworten wider, aber auch eine Anpassung von regulatorischer Immunreaktivität, um eigenes Gewebe und Organe von chronischen Entzündungen und Schäden zu schützen.

Summary

Immune responses determine disease courses and have a major impact on the generation of pathologies. They can lead from clearance of infection to chronic disease course. Cytokines and chemokines are key mediators of such responses. Aim of this work was to elucidate immune reactions and putative immune regulation in different helminth and protozoan parasitic infections, and the possible impact of immune responses on pathology in parasitic diseases.

Distinct immune response patterns were observed in patients with different course of Alveolar Echinococcosis (AE). Patients with stable and especially progressive disease course presented with elevated serum levels of pro-inflammatory IL-17B while serum levels of soluble receptor IL-17RB were highly elevated in all AE patients. In contrast to this, serum levels of pro-inflammatory Th17-type IL-17F were drastically reduced in all AE patients. This observation paralleled with reduced secretion of IL-17F by Peripheral Blood Mononuclear Cells (PBMC) from AE patients after stimulation with *Echinococcus multilocularis* antigens, compared to healthy controls. These results reveal a selective suppression of pro-inflammatory immune responses against common parasite antigens. The distinct secretion patterns observed in different disease courses suggest a possible use of IL-17 family members in staging of Alveolar Echinococcosis.

Chronic parasitic diseases can lead to cellular anergy. In onchocerciasis, such anergy can be overcome by medical intervention. This will lead to reinstallment of immune reactivity, but might also lead to hyperactivation and immune

disorders. Parts of this work investigated immune reactivity in onchocerciasis patients following ivermectin treatment. Clearance of microfilaria (Mf) paralleled with enhanced serum levels of mediators of cellular immunity, while mediators of humoral immunity remained unchanged. However, these upregulated mediators of cellular immunity are not only involved in inflammation, but have also been allocated roles in various immune-mediated diseases. The results of this work underline the reactivation of immune reactivity following anti-parasitic treatment and suggest mechanisms which might lead to the generation of subsequent immune disorders.

Pathology in infections can be caused by the pathogen itself, but also by the immune system. Part of the current work sought to determine pro- and anti-inflammatory immune responses during mild and severe malaria tropica in infants. The results of this work showed an enhanced production of pro-inflammatory Th1, Th2, Th17 and further immune response mediators in severe malaria. Several of these cytokines and chemokines also seem to play a role in driving pathology. The results point out an important role of immune responses in the generation of pathology in infant malaria tropica.

The development of immune responses against endemic parasites takes time to develop during lifetime. Part of this work determined in different age groups the evolution of parasite-specific immune patterns, which are generated throughout repeated infection, possible clearance and reinfection. While parasite-specific secretion patterns were observed for different pro-inflammatory mediators, the production of regulatory mediators in response to all parasite antigens enhanced with age. The results mirror the

generation of parasite-specific responses but also an adaptation of regulatory immune reactivity to protect from chronic inflammation, thus avoiding organ and tissue damage.

Published Publications

1. Ayimba E, Hegewald J, Ségbéna AY, Gantin RG, Lechner CJ, Agosssou A, Banla M, Soboslay PT. Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe Plasmodium falciparum malaria. Clin Exp Immunol. 2011 Nov;166(2):218-26.

Contribution of C. Lechner to this Publication: Performance of Experiments, Data Analysis and Data Interpretation, Review of the Manuscript.

2. Lechner CJ, Gantin RG, Seeger T, Sarnecka A, Portillo J, Schulz-Key H, Karabou PK, Helling-Giese G, Heuschkel C, Banla M, Soboslay PT. Chemokines and cytokines in patients with an occult Onchocerca volvulus infection. Microbes Infect. 2012 May;14(5):438-46. Epub 2011 Dec 13.

Contribution of C. Lechner for this Publication: Giving assistance for performing Experiments, Performance of Experiments, Data Analysis and Data Interpretation, Manuscript Drafting and Revision.

3. Lechner CJ, Grüner B, Huang X, Hoffmann WH, Kern P, Soboslay PT. Parasite-specific IL-17-type cytokine responses and soluble IL-17 receptor levels in Alveolar Echinococcosis patients. Clin Dev Immunol. 2012;2012:735342. Epub 2012 Aug 30.

Contribution of C. Lechner to this Publication: Study Design, Performance of Experiments, Data Analysis and Data Interpretation, Manuscript Drafting and Revision.

4. Lechner CJ, Komander K, Hegewald J, Huang X, Gantin RG, Soboslay PT, Agossou A, Banla M, Köhler C. Cytokine and chemokine responses to helminth and protozoan parasites and to fungus and mite allergens in neonates, children, adults, and the elderly. Immun Ageing. 2013 Jul 15;10(1):29.

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Introduction

Parasitic infections contribute significantly to morbidity and mortality worldwide. In 2012 Malaria alone accounted for over 200 million infections and 660 000 deaths (WHO World Malaria Report 2012). Schistosomiasis affects approximately 240 million people, causing 200 000 deaths per year (WHO Schistosomiasis Progress Report 2013) while amebiasis is responsible for 100 million cases annually, causing 100 000 deaths (Mortimer et al. 2010).

Parasitic diseases are ubiquitous but most abundant in tropical and subtropical areas, where poor education, poverty and underdeveloped health care systems provide optimal conditions for establishment and persistence of diseases. Highest prevalence of hookworm infections were found in countries with a low Human Development Index rating and low Gross Domestic Product Purchasing Power (da Silva et al. 2003). The impact of parasitism on human societies is manifold - not only causing morbidity and mortality but also hampering socio-economic development. *Schistosoma mansoni* infected farm workers with high egg burden demonstrated significantly lower oxygen intake during agricultural work, compared to infection-free workers and workers with low egg burden (Abdel-Rahman et al. 1990). River blindness caused by filarial nematode *Onchocerca volvulus* is confined to fast-flowing streams, which serve as breeding sites for the parasite's vector *Simulium* spp., leading to human abandonment of fertile river valleys (Jamison et al. 2006). Chronic infections with intestinal nematodes cause wasting, stunting and anemia, and negatively affect cognitive development in children (Stephenson et al. 2000, Guyatt et al. 2000). Sick children demand intensive care from their parents

which impairs their capacity to work and weakens economic stability of families due to out-of-pocket expenses for treatment. Furthermore, chronic parasitic infections can alter the host's immune system, facilitating co-infections. Helminth-infected women have a higher risk of mother-to-child-transmission of HIV than uninfected women (Gallagher et al. 2005). Chronic parasite infections also have negative effects on vaccination efficacy (Malhotra et al. 1999, van Riet et al. 2007, Borkow et al. 2008, Cooper et al. 2001).

It is worth noting that many of these aforementioned parasitic diseases such as filariasis and intestinal nematode infections were recently grouped as Neglected Tropical Diseases (NTD). These diseases have received little research attention and funding in the past, but, following the establishment of the United Nations Millennium Development Goals, are starting to move into the limelight of research.

The Immune System

The human immune system has multifaceted duties. It protects the body against harmful pathogens and their products, while also controlling its own cells to prevent unregulated cell growth. At the same time, it has to tolerate non-pathogenic non-self components. Failure to do so can lead to auto-immune diseases, infections, cancer and allergies. The principle components of the immune system can be grouped in three pillars. The first pillar consists of physical and physiological barriers like skin, epithelia and secreted microbicidal fluids, like gastric acids. The second and third pillar comprise the innate and the adaptive immune

system. The innate immune system includes plasma proteins, like C reactive protein (CRP), proteins of the complement system, the mannan-binding lectin, as well as cells of myeloid origin, like monocytes, granulocytes, macrophages, Natural Killer (NK) cells, mast cells and Dendritic Cells (DC). Antigen recognition receptors of these cells, termed Pattern Recognition Receptors (PRR), are germline encoded. Most prominent PRR are Toll-like Receptors (TLR), whose naming refers to the Toll Receptor pathway of innate immunity found in *Drosophila*, which shows high similarity to the mammalian TLR signaling pathway. TLR recognize common pathogen-derived components, Pathogen Associated Molecular Patterns (PAMP), like flagellin, lipopolysaccharide and double-stranded RNA.

Neutrophil granulocytes, DC and macrophages are main phagocytes of the innate immune system; NK cells detect reduced expression of Major Histocompatibility Complex (MHC) Class I on somatic cells, which is a hallmark of intracellular replicating pathogens to evade detection. This reduced expression of MHC I ("missing self") activates NK cells to lyse infected cells. Macrophages, B cells, eosinophil granulocytes and DC further process components of phagocytized pathogens, presenting the fragments as antigens via MHC Class II to CD4+ T cells, which belong to the third pillar, the adaptive immune system.

The adaptive immune system consists of T and B lymphocytes, whose pathogen recognition receptors (T and B cell receptor) are not germline encoded, but undergo a complex receptor gene rearrangement during cell development. Naive CD4+ T helper cells (Th0 cells) recognize their receptor-specific antigen, leading to activation and differentiation into T helper (Th) cell subsets. These Th type

immune reactions initiate distinct immune responses and play a major role in clearance of infection and in immunity, but also in immune disorders. CD8+ cytotoxic T cells detect intracellular pathogens via antigen presentation on MHC Class I on infected cells, leading to killing of infected cells, while B cells secrete antigen-specific antibodies. The binding of antibodies to a pathogen can lead to various effector functions: neutralization, for example by preventing the attachment of a virus to a host cell; opsonization, by facilitating pathogen recognition and phagocytosis; agglutination, in which the antibodies' two binding sides bind to different pathogens, leading to complex formation; activation of the complement system, which can attack the pathogen, and Antibody Dependant Cell Cytotoxicity (ADCC). In ADCC, pathogen-derived antigens on the pathogen surface or on infected cells are recognized and bound by antibodies. Macrophages, eosinophil granulocytes and NK cells can bind to these antibodies via membrane-bound Fc receptor and degranulate granzymes and perforines to destroy target cells or pathogens. ADCC is an important mechanism against pathogens which are too big to phagocytize, e.g. parasitic helminths.

While the innate immune system mediates prompt immune reactions and recognizes common pathogen structures, adaptive immune responses are highly pathogen-specialized, take a prolonged time to develop, and can mediate immunity against future infections.

T helper cells

It was only during the 1980's that the pivotal role of Th immune responses against pathogens was elucidated through

the works of Timothy Mossman and Robert Coffman (Mossman et al. 1986). The initially proposed Th1/Th2 model, which attributed cellular pro-inflammatory immune responses to a Th1 response and humoral regulatory responses to a Th2 immune response was revised after the discovery of further Th cell subsets. Up until now, this has led to the definition of various Th subpopulations, currently Th1, Th2, Th9, Th17, Th22, regulatory T (Treg) cells and T follicular helper (TfH) cells. Th subpopulations show a high degree of plasticity, as early stages of Th cells still can switch from one subset to another (Zhu et al. 2010).

Th cell subsets can be defined by distinct secreted cytokines and chemokines, by differential expression of surface receptors and by different Clusters of Differentiation (CD). Th1 cells produce interleukin (IL)-12, interferon (IFN)-Gamma, express the C-C chemokine receptor (CCR) 5 (Loetscher et al. 1998) and are positive for intracellular transcription factor T-bet. Th2 cells secrete IL-4, IL-5 and IL-13 and are positive for GATA-3 and CCR3. The recently defined Th17 cell population can be induced by stimulation with IL-6/IL-23 (Pappu et al. 2011) and is characterized by production of IL-17A, IL-17F and by the transcription factor ROR gamma. Both natural and inducible regulatory T cells are Foxp3 and CD25 positive and secrete IL-10 and Transforming Growth Factor (TGF)-beta. Th22 cells produce IL-22 and Tumor Necrosis Factor (TNF)-alpha (Eyerich et al. 2009), while Th9 cells express IL-9 and intracellular transcription factor PU.1 (Goswami et al. 2012). TfH cells are positive for the CXC chemokine receptor (CXCR) 5 and secrete IL-21 (Crotty 2011). However, distinction patterns for Th subsets cannot be adopted dogmatically, as recently ROR gamma+ GATA-3+ IL-17 producing Th2 Cells were discovered (Wang et al. 2010),

and IL-10 is not only secreted by regulatory T cells, but also by Th0, Th1 and Th2 cells (de Vries et al. 1995).

The Th cell subsets drive distinct immune reactions and initiate different immune responses. Th1 responses are cell-mediated immune responses. They lead to increased proliferation of cytotoxic CD8+ cells and further activate macrophages, which enhances the killing of phagocytized pathogens through upregulated production of reactive oxygen radicals and nitric oxides. Reactive oxygen and nitric oxide species can also be released to harm pathogens which are too big to ingest. A tight regulation of macrophage activation is vital since exacerbated activity not only harms pathogens but will also lead to tissue destruction. Various autoimmune disorders were initially linked with Th1 type responses (Romagni 1999, Firestein et al. 2003), but now seem to be rather Th17 associated (Cianci et al. 2012, van Hamburg et al. 2011, Pappu et al. 2011).

Th2 immune responses are humoral responses and characterized by enhanced antibody production, eosinophil granulocyte and B cell proliferation, mechanisms that support the development of ADCC. Th2 responses can also lead to asthma and allergic diseases, characterized by infiltration of eosinophils, enhanced IgE levels and elevated mucus production (Hams & Fallon 2012, Levine & Wenzel 2010). Again, such classifications are not stringent, as during asthma Th1 type IFN-gamma seem to contribute to aggravated immune reactions (Heaton et al. 2005) and neutrophilic inflammation observed might be partly Th17 driven (Bullens et al. 2006).

Early studies linked pro-inflammatory Th17-type responses with immune disorders, but recent works indicate that these responses also facilitate protection against fungal and

bacterial infections (Ishigame et al. 2009). Th17 cells promote the release of various pro-inflammatory cytokines, generation of microbicidal peptides and neutrophil recruitment (Iwakura et al. 2011). Elevated levels of Th17 cytokines were observed in rheumatoid arthritis and inflammatory bowel disease (Pappu et al. 2011).

Regulatory T cells are suppressors of pro-inflammatory immune responses. They secrete anti-inflammatory cytokines and limit inflammatory responses to avoid tissue damage. The recently described Th9 cells seem to mediate protection against parasitic helminths (Khan et al. 2003), while Th22 cells protect against bacterial infections (Basu et al. 2012).

Cytokines and Chemokines

Cytokines are small secreted molecules which orchestrate immune responses, drive proliferation and activation of cell populations, but which also lead to immune suppression to limit inflammation and to prevent tissue damage. Through their key role as coordinators of immune reactions, cytokines have a major impact on the course of diseases.

The cytokines IL-1 beta, IL-6 and TNF-alpha are inducers of inflammation and secreted by macrophages and dendritic cells following pathogen encounter. They activate endothelia, are pyrogenic and promote increased vascular permeability to enhance the influx of leukocytes, complement proteins and antibodies to inflamed areas.

Th1 immune responses are driven by IL-12 and IFN-Gamma production. IL-12 enhances proliferation of cytotoxic T cells (CTL) and activates NK cells (Mehrota et al. 1993, Lehman et

al. 2001). IFN-gamma increases production of reactive oxygen species in macrophages and blocks IL-4 mediated B-Cell proliferation as well as IgG and IgE production (Rabin et al. 1986, Coffmann & Carty 1986).

Th2 type responses are induced by IL-4, IL-5 and IL-13. IL-4, formerly known as B-cell Stimulation Factor (BSF)-1, promotes activation and proliferation of B cells (Dubois et al. 1987), and the production of IgG and IgE (Coffman et al. 1986, OHara et al. 1985, Defrance et al. 1988). IL-5 drives differentiation of eosinophil granulocytes (Jabara et al. 1988). IL-13 enhances antibody generation, antibody class switch to IgE (Punnonen et al. 1993) and is important for recruitment and survival of eosinophil granulocytes (Horie et al. 1997).

Th17 development is dependent on IL-23 secretion (Harrington et al. 2006) and is characterized by release of IL-17A and IL-17F, which subsequently trigger the release of further pro-inflammatory cytokines and chemokines such as IL-8, TNF-alpha and IL-1 beta (Pappu et al. 2011). Both IL-17A and IL-17F induce neutrophil recruitment and production of antimicrobial peptides (Iwakura et al. 2011).

TGF-beta released by regulatory T cells blocks NK cell activation and T cell proliferation (Wilson et al. 2011, Tiemessen et al. 2003). It also inhibits IFN-gamma induced nitric oxide production in macrophages (Vodovotz et al. 1993). IL-10 blocks LPS-induced secretion of IL-1 beta, IL-6 and TNF-alpha by macrophages and secretion of IL-1 beta, IL-8 and TNF-alpha by granulocytes (Fiorentino et al. 1991, Cassatella et al. 1993) as well as antigen-specific proliferation of T cells (de Waal et al. 1991).

Chemokines are chemotactic cytokines, which can attract distinct leukocyte populations towards sites of inflammation.

The updated nomenclature grouped chemokines according to the composition of two neighboring cysteins, i.e. into (X)C, CC, CXC und CX3C chemokines (Zlotnik & Yoshie 2000). While some receptors, like CXCR4, only bind one chemokine other receptors have been shown to be highly promiscuous, such as CCR3 (Proudfoot 2002). Chemokines can be expressed in a homeostatic manner, being constitutively secreted by tissues and organs to facilitate cell trafficking, like PARC (CCL18) and Eotaxin-2 (CCL24). They can also act inflammatory, being upregulated during infections, like MCP-1 (CCL3), MCP-2 (CCL8) and MCP-3 (CCL7), or both inflammatory and homeostatic, like TARC (CCL17) or Eotaxin-1 (CCL11) (Zlotnik & Yoshie 2012). Some chemokines are differentially expressed by distinct Th subsets. TARC, 6CKine (CCL21) and MDC (CCL22) are Th2 type chemokines (Lebre et al. 2005, Veestergaard et al. 2000). The Th1 chemokines MIG (CXCL9) and IP-10 (CXCL10) bind to CXCR3. While the role of cytokines has been extensively studied in various infection models, chemokines have received much less attention.

Pathogenesis and clearance: the impact of immune reactions in parasitic diseases

Parasites dwell in different body compartments, pursue different propagation strategies and therefore elicit distinct immune reactions. Infection with intracellular protozoan parasites classically leads to cellular Th1 immune responses. Chronic helminth infections provoke a "modified Th2" immune response, which is characterized by Th2 immune responses with enhanced regulatory cytokine production (Diaz & Allen 2007).

Various studies have outlined critical roles of cytokines and chemokines in the course of parasitic diseases. Clearance of *Echinococcus multilocularis* metacestode infection is dependent on initial pro-inflammatory Th1 type immune responses, mediated by IL-12 and IFN-gamma, whereas Th2 immune responses, induced by IL-4, IL-5 and IL-10, lead to a chronic course of disease (Vuitton & Gottstein 2010). In human visceral leishmaniasis, enhanced IL-10 production is associated with increased parasite replication and pathogenesis, partly by blocking the intracellular killing of amastigote stages in macrophages (Nylén et al. 2007). Mice treated with Th1 chemokine IP-10 presented with a reduced parasite proliferation after challenge with *Leishmania donovani*, which was accompanied by decreased numbers of IL-10 producing T cells (Gupta et al. 2011).

Furthermore, immune reactions can directly contribute to pathogenesis in parasitic infections. During schistosomiasis, generation of pathogenic periportal fibrosis is dependent on pro-inflammatory cytokines like TNF-alpha (Henri et al. 2002) while anti-inflammatory Th2 immune responses prevent tissue damage, leading to host survival (Herbert et al. 2004). Th17 mediated immune responses seem to be associated with dire outcome of bladder pathology in human schistosomiasis (Mbow et al. 2013). Cerebral malaria with fatal outcome correlated with enhanced levels of pro-inflammatory chemokines Platelet Factor 4 (CXCL4) and IP-10 (Wilson et al. 2011). Reduced risk of cerebral malaria pathology is associated with decreased levels of chemokines MIP-1 alpha (CCL3), MIP-1 beta (CCL4) and Rantes (CCL5) in an experimental model (Clark et al. 2011).

Increased knowledge about such immune-mediated pathogenesis mechanisms and their key regulators is of

immense importance; differential expression patterns of cytokines and chemokines during distinct disease courses may predestine them as prognostic markers or as therapeutic targets. Therefore, detailed knowledge about their expression in health and in disease will not only deepen our understanding of the complex parasite-host interplay, but may also lead to novel treatment approaches.

Immune modulation strategies of parasites and changes in immune reactivity following anti-parasite treatment

Successful, persistent infections require countermeasures to modulate the host’s immune system. Therefore, parasites have evolved various strategies to interfere with the host's immune responses. Excretory/secretory (E/S) products from *E. multilocularis* onchosphaera larvae cause impaired LPS-induced DC maturation and induce DC apoptosis (Nono et al. 2012). *Onchocerca volvulus* onchocystatin (Ov17) suppresses antigen-induced proliferation and maturation of Peripheral Blood Mononuclear Cells (PBMC) (Schönemeyer et al. 2001). *E. histolytica* derived Monocyte Locomotion Inhibitory Factor (MLIF) blocks migration of leukocytes and oxidative burst (Velazquez et al. 2011). Intracellular production of regulatory cytokine TGF-beta by nematode *Onchocerca volvulus* has been reported, though a possible secretion remains unclear (Korten et al. 2009). *P. falciparum* seems to interfere with normal B-cell activation and differentiation, presumably via *P. falciparum* erythrocyte membrane protein (PfEMP) 1 (Scholzen et al. 2013). Eggs of intravascular helminth *S. mansoni* secrete a chemokine binding protein, which blocks

in vitro IL-8-induced neutrophil recruitment and leads to impaired inflammation *in vivo* (Smith et al. 2005).

In onchocerciasis, *O. volvulus*-specific cellular immune reactivity is drastically reduced during patent phase of infection, while *O. volvulus* specific antibody levels were enhanced in patent individuals, compared to post-patent individuals (Soboslay et al. 1997, Ottesen et al. 1995). In contrast, post-patent patients presented with highly elevated cellular immune reactivity (Soboslay et al. 1997). Ivermectin treatment will lead to microfilaria clearance without killing adults worms, thus resulting in a transient state of post-patency, which may lead to re-installment of cellular immune reactivity. Reactivation of cellular reactivity has been observed before, as ivermectin treatment led to re-installment of cellular reactivity towards *O. volvulus* antigens in patients (Akkufo et al. 1996, Soboslay et al. 1992, Soboslay et al. 1994). However, such sudden reactivation after long-lasting cellular anergy might also lead to hyperactive and uncontrolled immune reactions, resulting in immune disorders.

Polyparasitism: Immune reactivity towards parasite antigens and allergen in individuals

In areas that are endemic for multiple parasites, immune responses to parasite infections develop over long periods of time. A classical example is the interplay between the immune system and protozoan parasite *P. falciparum*. Repeated episodes of infection, host survival and reinfection will lead to development of semi-immunity, which requires constant reinfection to be maintained. Pro-inflammatory

immune responses may lead to clearance of infection but can also cause extensive tissue damage. Given the fact that clearance and protective immunity are not feasible in most parasitic infections, the immune system slowly adapts towards more regulated pro-inflammatory immune responses to avoid chronic and destructive inflammation (Diaz & Allen 2007). This may lead over time to the evolvement of parasite specific immune profiles, mirrored by differential cytokine and chemokine expression. The generation of such a balanced immune reactivity towards parasitic infections is up to now poorly elucidated.

AIM of this study

Immune responses are important to protect the body against infectious agents. Cytokines and chemokines are pivotal coordinators of such reactions. Through differential expression, they can elicit distinct immune responses, thus determining disease outcome, ranging from rapid expulsion to chronic course of infection. Furthermore, they have a major influence on pathogenesis during infection. Aim of this work was to elucidate differential cytokine expression patterns in distinct disease courses of human Alveolar Echinococcosis and of infant Malaria Tropica. Such patterns associated with different clinical outcome could lead to the use of cytokines or chemokines as biomarkers.

Parasites have evolved various ways to interfere with the hosts' immune responses. Parasite-induced immune suppression can be overcome by medical intervention, leading to re-installment of immune reactivity. Part of this work was to elucidate in human onchocerciasis the cytokine- and chemokine mediated re-installment of immune reactivity following anti-parasite treatment, which leads to parasite clearance but might also contribute to immune disorders.

Tropical and subtropical areas are often endemic for various parasite species. In contrast to bacterial and viral infections, infections with most parasites will not lead to protective immunity. Development of immune reactivity towards these parasites will be shaped over prolonged periods of time, by constant infection, clearance and reinfection. With ongoing maturation, the immune system will aim to balance pro-inflammatory responses against the parasite and regulatory responses to avoid collateral tissue destruction. The development of such balanced immune reactions in parasite

endemic areas is up to now not very well understood. Therefore, part of this work focused on parasite-specific inducibility of regulatory and pro-inflammatory cytokines and chemokines in different age groups.

Results and Discussion

Immune responses determine disease outcome and have major influence on pathogenesis. An ideal immune response leads to immediate pathogen clearance without eliciting pathology. Pathogens on the other side try to establish a productive infection without provoking threatening immune responses. These conflicting aims have resulted in a dynamic interplay between the immune system and infectious agents. It has led on one hand to pathogen-specific immune reactions, culminating in highly antigen-specific immune responses, on the other hand to immune modulation and immune suppression by pathogens.

The pathogen-host interplay can lead to various scenarios: in some cases, infections are cleared at early stages, resulting in an asymptomatic, abortive course; other infections, however, trigger full-blown responses, which can lead to elimination of the pathogen but may also cause damage to the body itself. Moreover, pathogens can persist within the host, by inducing immune suppression, which results in chronic infection accompanied by an anergic state of immune reactivity.

Immune reactivity during severe and mild malaria tropica in infants

Malaria tropica is a major cause of infant death in subtropical and tropical countries. Critical complications in malaria include severe anemia and cerebral complications (Idro et al. 2005). Two schools of thought exist which try to explain pathology in malaria (Faille et al. 2009). Supporters of pathogen-mediated pathology argue that *P. falciparum*

infected erythrocytes adhere to the vascular endothelium in the central nervous system, which will lead to obstruction of blood vessels, lowered blood flow and decreased oxygen transport. Immune-pathology advocates propose that neurological defects are directly caused by pro-inflammatory mediators like IFN-Gamma and TNF-alpha, which are released in response to the massive parasite presence in the blood (Faille et al. 2009). The latter proposition supports the theory of the "cytokine storm", in which unbalanced immune reactions result in major tissue and organ damage.

The observed increased production of various pro-inflammatory mediators like Th1 type chemokine MIG, Th2 type cytokines IL-33, IL-31 and IL-13 as well as Th17 cell chemoattractant MIP-3 alpha during severe malaria and the reduced production of IL-13 and MIG following treatment promote the concept of immune-mediated pathology. Elevated levels of MIG have been already observed in neurological disorders (Teixeira et al. 2004) and might play a role in the generation of cerebral malaria. In Multiple Sclerosis (MS), cerebral spinal fluid of patients with active MS attack contained elevated levels of MIG, while demyelinated MS brain lesions presented with high numbers of T cells which were positive for MIG receptor CXCR3 (Sorensen et al. 1999).

The alarmin IL-33 is expressed in the vascular capillary of the brain (Chapuis et al. 2009). It promotes eosinophil recruitment, and eosinophilia has been observed in severe malaria, accompanied by enhanced secretion of eosinophil neurotoxic protein and eosinophilic cationic protein (Kurzhals et al. 1998), which both exert neurodegenerative effects (Fredens et al. 1982). Neural pathology by eosinophils has been shown in cases of parasite associated eosinophilic

meningitis, like neuroschistosomiasis and eosinophilic meningitis associated angiostrongyliasis (Greif-Teixeira et al. 2009, Intapan et al. 2007). Thus, eosinophil recruitment and eosinophil derived products could play a role in the pathogenesis of malaria tropica.
The observed increased levels of CXCL16 in children with severe malaria might also play a direct role in pathology. Borst and coworkers have recently shown that CXCL16 induces platelet activation and adhesion (Borst et al. 2012). The enhanced levels of CXCL16 might trigger the adhesion of platelets, which leads to further endothelia activation and to clogging of platelets and parasitized erythrocytes, resulting in cerebral pathology and further organ failure. Levels of CXCL16, too, were reduced after therapeutic intervention. While certain cytokine concentrations drastically decreased after treatment, levels of IL-31 and IL-33 remained high after treatment. This might reflect long-lasting release of these proinflammatory cytokines, in the case of the alarmin IL-33 by damaged endothelia and epithelia.

Immune regulation can prevent extensive immune reactivity. Regulatory cytokines like IL-10 and IL-27 might play an important role in limiting pathology in malaria, but paradoxically, maybe also in aggravating it.
The reduced levels of IL-27 in severe cases of infant malaria compared to mild malaria might mirror the blockade of immune suppression due to uncontrolled activation of the immune system by massive quantities of *P. falciparum* blood stages. IL-27 is known for is attenuator function, limiting Th1, Th2 and Th17 responses (Sturmhofer & Hunter 2008). IL-27R KO mice infected with *P. berghei* presented with enhanced Th1 responses (Villegas-Mendez et al. 2013) and severe liver pathology while parasites were still effectively

cleared (Findlay et al. 2010). These results indicate that IL-27 might have a protective role in malaria.

The role of another regulatory cytokine, IL-10, seems to differ from IL-27. Various studies reported increased levels of IL-10 in severe cases of malaria (Awandare et al. 2006, Prakash et al. 2006). In this present work, elevated IL-10 levels in mild and severe malaria cases did not differ from each other, but were highly elevated, compared to uninfected children. However, plasma concentrations of IL-10 significantly decreased after treatment. It remains to debate whether this enhanced production mirrors a regulatory approach which is too weak to initiate effective anti-inflammatory responses because of massive parasitemia or whether IL-10 production might also contribute to pathology in malaria. Several studies support the latter notion. Administration of anti-IL-10 led to survival in a normally lethal *Plasmodium yoelii* infection model (Kobayashi et al. 2000) and IL-10 KO mice challenged with *P. yoelii* presented with reduced parasitemia and survived, compared to WT mice (Couper et al. 2008). It seems that while IL-27 might has beneficial effects in malaria tropica pathology, by down regulating immune-pathology inducing effects, an IL-10 mediated attenuation of immune reactivity might favor parasite survival and propagation, leading to severe course of disease.

Taken together, the results of this study suggest cytokines like IL-27, MIG and CXCL16 as predictive markers for staging of infant malaria and also to monitor therapy success. Several results of this work support a role of cytokines in driving pathology during malaria tropica. However, the overall image suggests that both immune system and parasite contribute to pathogenesis in malaria.

Impact of microfilariacidal treatment on the immune reactivity in Onchocerciasis

Ivermectin (Mectizan®) has been for decades the weapon of choice for treatment of onchocerciasis. Originally used as veterinary drug, first studies thirty years ago led to the use of ivermectin in human onchocerciasis patients (Campbell et al. 1983).

Ivermectin will kill Microfilaria (Mf) of *Onchocerca volvulus*, but not adults worms. In onchocerciasis, patency, i.e. the presence of Mf, has been associated with impaired cellular immune reactivity (Soboslay et al. 1992). Clearance of microfilaria will lead in patients to a transient state of post-patency and to the reinstallment of cellular immune responses (Soboslay et al. 1992, Soboslay et al. 1994). Such rapid reactivation of cellular immunity can lead to enhanced killing of residual Mf and to attacking of adult worms, but can also induce side effects.

Approximately one third of all onchocerciasis patientes suffer from skin lesions, which include onchodermatitis, atrophy and depigmentation (Timmann et al. 2003). Sowda, a common onchocerciasis-associated pathology, is characterized by hyperactive immune responses, leading to lichenified lesions. Patients suffering from sowda are rarely Mf positive (Siddiqui & Al-Kawajah 1991). As repeated ivermectin treatment will cause massive killing of Microfiolaria without killing adult worms, leading to restinallment of cellular reacitivity, this reactivation may also predispose patients for subsequent skin pathologies.

Reduced Mf burden paralleled with activation of immune reactivity three days after medical intervention.

Production of pro-inflammatory chemokines, mainly monocyte, T cell and eosinophil chemoattractant chemokines MCP-1, MCP-4 and Eotaxin-2 were highly elevated three days after treatment. These chemokines mediate infiltration of monocytes, macrophages, T cells and eosoinophils into dermal tissues and the killing of microfilaria. MCP-4 induces respiratory bursts from eosinophils (Petering et al. 1998) which might be used to attack migrating microfilaria. Furthermore, the neutrophile chemoattractants MPIF-1 and IL-8 were also enhanced directly following therapeutical intervention. Neutrophil granulocytes are first responders in infections, being quickly and numberously produced and directed to sites of inflammation. The enhanced levels of eosinophil and neutrophil chemoattranctants parallel with the observed infiltration of neutrophils and eosinophils during onchocerciasis (Nfon et al. 2006). All of these observations depict increased cellular reactivity mediated by chemokines after anthelmintic treatment.

However, secretion of these aforementioned immune response mediators might also lead to increased pathology. MCP-4 and MCP-1 have been proposed as biomarker in asthma and psoriasis, respectively (Kalayci et al. 2004, Lembo et al. 2013). Elevated levels of Eotaxin-2 have been observed in asthmatic patients which associated with sputum eosinophilia (Coleman et al. 2012) and increased numbers of eosinophils were detected in onchocerciasis patients with skin lesions (Timmann et al. 2003).

In contrast to the observed enhanced cellular immunity, mediators of humoral immune reactvity either remained stable, like B-cell acivating factor BCA-1, or even declined, like IL-13. This observed reduction of humoral immune reactivity in transient post-patency paralleled with reduced levels of *O.*

volvulus antigen specific antibodies observed in post patent individuals, compared to patent individuals. It seems that clearance of residual microfilaria and readaptation of immune responses in onchocerciasis are associated with pro-inflammatory cellular immune responses, comprising adaptive and innate immunity, while humoral response reacivity is downregulated. Furthermore, cytokines and chemokines seem to contribute substantially to the generation of such responses in onchocerciasis, and might also contribute to the generation of skin lesions observed in these patients.

Altered immune reactivity in Alveolar Echinococcosis

Alveolar Echinococcosis is a possibly life-threatening zoonosis, in which progressive growth the larval stage of *E. multilocularis* can lead to organ failure and fatality. Previous studies in AE have outlined that different course of infections can be linked with distinct immune responses (Gottstein & Felleisen 1995). In chronic course of infection, initially present pro-inflammatory Th1 responses are slowly skewed towards Th2 and Treg responses, characterized by enhanced production of IL-4, IL-5, IL-13, TGF-beta and IL-10 (Vuitton & Gottstein 2010, Aumüller et al. 2004, Sturm et al. 1995, Jenne et al. 1997, Zang et al. 2008). Initial proinflammatory Th1 responses on the other hand lead to expulsion of the parasite (Vuitton & Gottstein 2010). The outer layer of the metacestode, termed laminated layer, protects the parasite from harmful immune reactions like reactive oxygen and nitric species, but still allows a cross-talk between the inner germinal layer and the host (Vuitton & Gottstein 2010). It

facilitates the release of parasite derived E/S products, which can modulate the host's immune responses, but can also lead to metastasis-like dissemination of the metacestode to further organs and tissues. As medication of AE only acts parasitistatic, and as resection is not always curative, many AE patients require life long medication. Surveillance of metacestode growth hereby relies on imaging techniques like Magnet Resonance Tomography (MRT) and Positron Electron Tomography (PET). AE specific biomarkers could offer a sensitive approach, as changes in immune reacitivity could be first detected by differential secretion of immune response mediators.

The characterization of Th17 responses in the past years has been oscillating between different concepts. Initially associated with strictly pro-inflammatory properties and linked with immune disorders, later studies elucidated protective properties of Th17 responses against bacterial and fungal infections (Ishigame et al. 2009). Recent studies reveal new insights on this multifaceted cell population. The Th17 cell population is under normal conditions a small population, mainly confined to the intestine (Huber et al. 2012). Under inflammatory conditions, cell proliferation and migration are upregulated, while chemotaxis to sites of inflammation is mediated via chemokine CCL20/LARC and its receptor CCR6, which is present on Th17 cells (Kim et al. 2009). Inflammation induced in mice by anti-CD3 treatment led to diarrhea and intestinal tissue destruction, which paralleled with increased Th17 cell numbers (Esplugues et al. 2011). However, this inflammatory event was only transient, and seemed to be attenuated by reprogrammed Th17 cells, which interestingly secreted reduced levels of TNF-alpha and IL-2, but elevated levels of IL-10 (Esplugues et al. 2011). It seems now that Th17

cells possess both pro-inflammatory and regulatory properties.

Th17 cells are increased under inflammatory events in the liver (Chang et al. 2012), an organ is mainly affected by chronic metacestode growth, and further IL-17 family members like IL-17B have been detected in the gastro-intestinal tract, which is also afftected in AE. IL-17B, too, induces pro-inflammatory cytokine secretion (Li et al. 2000, Shi et al. 2000). Still, little is known about possible roles in diseases.

The eleveated serum levels of IL-17B and soluble receptor component IL-17RB observed in stable and progressive AE cases might signify rather innate immune responses, as IL-17B is expressed by cells of the intestine and stomach, and is a chemoattractant for neutrophil granulocytes (Shi et al. 2000), which represent one of the first cell populations involved in inflammation. IL-17B seems to drive early immune responses against various pathogens, and activation of neutrophils is a hallmark of early pro-inflammatory immune reactions. The role of solube IL-17RB is still open to discussion. On one hand its secretion might be modulated by *E. multilocularis* metacestode, to act as decoy receptor for IL-17B, on the other hand it may enhance the effect of IL-17B as previous reports suggest (Jung et al. 1999). The fact that progressive AE cases presented with highest levels of IL-17B and sIL-17RB seems to support the concept of parasite-induced immune modulation.

The observed reduced serum levels of IL-17F in AE patients paralleled with reduced IL-17F production from patients' PBMC after stimulation with *E. multilocularis* vesicle antigen, an E/S product which is present in late stages of metacestode development. This reduced production points out a possible

immune modulation towards common antigens of *E. multilocularis* during chronic course of AE. It seems to be highly cytokine specific, as unaltered production and plasma levels of Th17 member IL-17A were observed. Ishigame et al. have outlined distinct role of IL-17A and IL-17F in protection and in immune disorders (Ishigame et al. 2009). It appears that protection is rather IL-17F associated, which would be in accordance to its observed suppression in AE patients.

The distinct antigen-induced production levels of IL-17B and IL-17F in different stages of disease sheds a new light on the role of L-17 family members in AE and could predispose these cytokines as possible markers for disease staging. The observed modulation of IL-17F levels suggests an role of pro-inflammatory Th17 responses in AE, and further studies should elucidate putative effects of the yet poorly charazterized Th17 responses in Alveolar Echinococcosis.

Polyparasitism: Immune reactivity towards parasite antigens and allergens in individuals from different age groups

Infections with multiple parasites (co-infections) are disproportionally high in subtropical and tropical countries, where low education levels, poor hygienic conditions and disfunctional health care systems often facilitate the spread of infections (Supali et al. 2010).

Immune responses against these parasitic infections in these areas evolve over time and area shaped by iterative states of infection, possible clearance, and reinfection. The current

work outlined the gradual development of parasite-specific immune reactivity in different age groups.

The observed parasite-specific immune profiles in different age groups depict a dynamic evolution of immune responses in an infectious environment. While Th2 mediator IL-33 could be induced in neontates by *Ascaris* stimulation, suggesting priming events at early stages, further Th2 cytokines like IL-19 were only inducible in adults and elderly, with highly specific antigen stimulation patterns. The alarmin IL-33 is released by damaged epithelia and known to mediate immunity in experimental helminth infections (Hung et al. 2013). IL-19 induces pro-inflammatory cytokines and chemokines like IL-8, IL-1 beta and IL-6, but also attracts granulocytes (Liao et al 2004, Hsing et al. 2008). The release of IL-19 by PBMC in response to *Entamoeba* and *Ascaris* antigen might mirror its role in attracting neutrophils to attack migrating larvae of Ascaris and trophozoites of *Entamoeba*. The pro-inflammatory chemokines Eotaxin-2/CCL24 and MCP-4/CCL13, too, showed parasite-specific immune profiles, both being highly inducible in adults by *Ascaris* and *O. volvulus* antigen stimulation, while stimulation with *Entamoeba* antigen led to reduced levels. The increased inducibility of eosinophil chemoattractant Eotaxin-2/CCL24 following *O. volvulus* stimulation parallels with the observed infiltration of Eosinophils during onchocerciasis (Nfon et al. 2006, Berger et al. 2002).

Regulatory IL-27 was unspecifically inducible in all age groups. However, overall production levels of IL-27 increased with age. The pro-inflammatory monocyte and neutrophil chemokine MIP-1 delta was broadly and highly inducible in children and infants, while in adults and elderly, its inducibility was confined to stimulation with *Entamoeba*

antigen only. Neither of the measured chemokines elicited allergen induced immune responses, while in children, IL-33 was inducible by mite and fungus antigen, IL-19 was inducible by mite allergen in adults and elderly and IL-27 preferably expressed by children in response to mite allergen stimulation.

In contrast to many bacterial and viral infections, protective immunity against parasites is rather exception than rule. Therefore, chronic courses of disease often prevail. These chronic infections will lead to chronic inflammation, which would be detrimental to the body itself.
The broadly elevated production levels of IL-27 in adults and elderly could mirror an adaptation towards more regulatory immune reaction. Moreover, the work provides evidence that the immune system creates parasite-specific immune responses over prolonged periods of time; such pro-inflammatory immune reactivity will not clear or protect against parasitic infections, but rather lead to a balanced immune response. Previous works have already postulated the generation of 'modified Th2 response profiles' (Diaz & Allen 2007), which are characterized by pro-inflammatory responses being limited by regulatory mechanisms. The results of this work support this concept.

Conclusions

The present work investigated immune reactivity in different human parasitic infections. Cytokines and chemokines are pivotal elements in orchestrating immune reactions and provide good means to evaluate distinct types of immune reactions.

In AE, cytokine and chemokine responses revealed putative immune suppression patterns, which were elicited either directly by suppressed responsiveness to parasite antigens or by induction of putative decoy receptors. Production of certain pro-inflammatory cytokines (IL-17B) was enhanced, which seem to be too weak to induce successful clearance of infection and may rather mirror weak chronic inflammatory events during infection. The observed distinct parasite-specific inducibility of IL-17 family members in different patient groups might render these cytokines useful as predictive markers in AE; such biomarkers are sensitive and their determination can be carried out easily; however, further factors associated with distinct disease outcome should be investigated to ensure a reliable prediction about patient's course in AE.

In onchocerciasis, suppression of immune reactivity is observed in patency, leading to cellular anergy. The results of this work shows that transient interruption of patency results in reactivation of cellular immune responses, while humoral responses declined or remained stable. However, mediators of these cellular immune responses are also involved in generation of immune disorders, which might explain skin pathologies observed in onchocerciasis patients.

Pathology induced by the immune system itself plays a major role during many diseases. Severe cases of infant malaria tropica presented with increased production of Th1, Th2 and Th17 inducing cytokines and chemokines; immune response mediators with possible pathology-inducing effects like CXCL16 and IL-33 were upregulated during acute infection. Regulatory cytokines seem to play different roles in malaria tropica: while IL-27 levels were diminished in severe cases, IL-10 concentrations were highly enhanced in malaria tropica. This might mirror different roles of these regulatory mediators in preventing immune pathology on one side and in limiting parasite proliferation on the other.

In areas which are endemic for multiple parasites, the generation of immune responses to these parasitic diseases will develop after repeated infection, leading to balanced immune reactions. The immune system hereby aims at effective clearance while avoiding excessive pathology. The results of this work depict the generation of both parasite-specific pro-inflammatory and broadly-induced regulatory immune responses against endemic parasites.

In summary, the present work discloses possibly protective and pathology-inducing dynamics of immune reactivity in distinct parasitic infections; chronic helminth infections which are accompanied by both upregulated and suppressed cytokine responses and by cellular anergy, which can be overcome by treatment; acute protozoan infections in which cytokine and chemokine secretion might contribute to or protect against pathology; and the gradual development of immune reactivity against multiple parasites, resulting in both parasite-specific immune profiles and in enhanced regulatory immune responses.

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Publications

Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe *Plasmodium falciparum* malaria

Ayimba E[1,3], Hegewald J[3,6], Ségbéna AY[1], Gantin RG[5], Lechner CJ[3,6], Agosssou A[3,4], Banla M[2,3], Soboslay PT[3,6]

*1Centre National de Transfusion Sanguine,
Section Immunologie et Hématologie,
2Centre Hospitalier Universitaire Campus de Lomé,
Université de Lomé, Lomé, Togo,
3Institut National d'Hygiène – Onchocerciasis Reference Laboratory,
4Centre Hospitalier Régional,
Service Pédiatrie, Sokodé, Togo, Université de Lomé,
5Faculté Mixte de Médecine et Pharmacie, Service de Psychologie
Pathologique, Clinique et Medicale, Lomé, Togo,
6Institute for Tropical Medicine, University of Tübingen, Germany*

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ABSTRACT

Cytokine and chemokine levels were studied in infants (<5 years) with uncomplicated (MM) and severe malaria tropica (SM), and in *Plasmodium falciparum* infection-free controls (NEG). Cytokine plasma levels of interleukin (IL)-10, IL-13, IL-31 and IL-33 were strongly elevated in MM and SM compared to NEG ($P < 0.0001$). Inversely, plasma concentrations of IL-27 were highest in NEG infants, lower in MM cases and lowest in those with SM ($P < 0.0001$, NEG compared to MM and SM). The levels of the chemokines macrophage inflammatory protein (MIP3)-α/C–C ligand 20 (CCL20), monokine induced by gamma interferon (MIG)/CXCL9 and CXCL16 were enhanced in those with MM and SM ($P <$ 0·0001 compared to NEG), and MIP3-α/CCL20 and MIG/CXCL9 were correlated positively with parasite density, while that of IL-27 were correlated negatively. The levels of 6Ckine/CCL21 were similar in NEG,MM and SM. At 48–60 h post-anti-malaria treatment, the plasma concentrations of IL-10, IL-13, MIG/CXCL9, CXCL16 and MIP3-α/CCL20 were clearly diminished compared to before treatment, while IL-17F, IL-27, IL-31 and IL-33 remained unchanged. In summary, elevated levels of proinflammatory and regulatory cytokines and chemokines were generated in infants during and after acute malaria tropica. The proinflammatory type cytokines IL-31 and IL-33 were enhanced strongly while regulatory IL-27 was diminished in those with severe malaria. Similarly, MIP3-α/CCL20 and CXCL16, which may promote leukocyte migration into brain parenchyma, displayed increased levels, while CCL21, which mediates immune surveillance in central nervous system tissues, remained unchanged. The observed cytokine and chemokine production profiles and their dynamics

may prove useful in evaluating either the progression or the regression of malarial disease.

INTRODUCTION

Cytokines and chemokines can act as central contributors to severe and life-threatening illness; in particular their excess production, also described as the cytokine syndrome [1], may contribute decisively to pathogenesis and the severity of malarial disease. Parallels exist between falciparum malaria and other severe illnesses such as sepsis and influenza, where inflammatory cytokines as well as chemokines are important mediators of pathogenesis [1,2]. Chemokines bridge innate and adaptive immunity [3], regulate chemotactic recruitment of inflammatory cells, leukocyte activation, angiogenesis and haematopoiesis, and in addition may also regulate host immune responses decisively during intracellular as well as intestinal protozoan parasite infections [4–8]. Recent studies have shown that the profile of chemokine expression and their serumlevels varied with disease severity in children with acute *Plasmodium falciparum* malaria; notably, the beta chemokines macrophage inflammatory protein (MIP)-1α/CCL3 and MIP-1β/CCL4 were elevated while regulated upon activation normal T cell expressed and secreted (RANTES)/C–C ligand 5 (CCL5) appeared to be suppressed [9].
Resolution of *P. falciparum* infection requires proinflammatory immune responses that facilitate parasite clearance, while failure to regulate this inflammation leads to immune mediated pathology, but the sequelae of disease aggravation or its resolution still require further study for a better understanding of

pathogenesis as well as the prevention of malaria disease. The early production of proinflammatory T helper type 1 (Th1) cytokines, including tumour necrosis factor (TNF), interleukin (IL)-12 and possibly interferon (IFN)-γ may limit the progression from uncomplicated malaria to severe and life-threatening complications, but TNF can cause pathology if produced excessively [10–12].

Several studies support the idea that Th1 responses are important for clearance of *P. falciparum* malaria, and enhanced serum levels of IL-6 and IL-10 were observed in patients with severe *P. falciparum* malaria [13]. In young African children who presented with either mild or severe *P. falciparum* malaria, the acute-phase plasma IL-12 and IFN alpha (IFN-α) levels, as well as the whole-blood production capacity of IL-12, were lower in children with severe rather than mild malaria, and IL-12 levels were correlated inversely with parasitemia [14]. Further, TNF-α and IL-10 levels were significantly higher in those with severe malaria, being correlated positively with parasitemia, and children with severe anemia had the highest levels of TNF in serum [13]. The cytokine and chemokine imbalance measured in serum were suggested as useful markers for progression of cerebral malaria with fatal outcome; patients who died from malaria tropica had higher amounts of IL-6, IL-10 and TNF-α levels than those who survived; moreover, cerebral malaria (CM) was related to an inflammatory cascade characterized by dysregulation in the production of IP-10, IL-8, MIP-1β, platelet-derived growth factor (PDGF)-1, IL-1Rα, Fas-L, soluble TNF-receptor 1 (sTNF-R1) and sTNF-R2 [15].

This work addressed the levels of circulating proinflammatory and regulatory cytokines and chemokines in infants with acute *P. falciparum* infection, and our observations disclose clear differences

associated with progression and regression of malaria tropica.

MATERIALS AND METHODS

Study population

This work was conducted at the Centre Hospitalier Regional (CHR) in Sokodé in the Central Region of Togo. The study was approved by the Comite de Bioethique pour la Recherche en Sante (CBRS) in Togo, and by the Ethikkommission at University Clinics of Tübingen, Germany. Informed written consent was obtained from all parents for the participation of their children in this study. Infants of less than 5 years of age were recruited, and classification of malaria was performed according to previously published criteria [14], with severe malaria (SM) characterized by parasitemia of higher than 250 000 parasites/ml and/or the presence of severe anemia with haemoglobin concentrations of lower than 5 g/dl. Matched uncomplicated malaria (MM) patients were defined by parasitemia of lower than 250 000 parasites/ml and haemoglobin concentrations equal to or higher than 5 g/dl and the absence of any signs or symptoms of severe malaria [13]. *P. falciparum* exposed infants negative for parasites in thick blood film, and negative in rapid detection test kits for *P. falciparum* (Paracheck-Pf, Orchid, Biomedical Systems, Goa, India; OptiMAL-IT; Biorad, Marnes la Coquette, France), were defined as participants with previous malaria episode(s) and the actual absence of illness due to malaria within the last 2 weeks. Blood samples were obtained prior to treatment with anti-malarials and/or anti-pyretics, and immediately following primary diagnosis all *P. falciparum*-positive infants received anti-malarial and

appropriate supportive therapy as required and recommended by the Guidelines for Malaria Treatment indicated by the Ministry of Health in Togo. Infants with MM were treated with Coartem and Artemeter or Artesunate, and for SM, quinine perfusion or injectable Artemeter were applied as recommended. All hospitalized uncomplicated as well as severe malaria cases were followed until discharge from the hospital paediatric ward.

Chemokine and cytokine enzyme-linked immunosorbent assays (ELISAs)

Quantitative enzyme-linked immunosorbent assay (ELISA) was performed with commercially available assays to determine plasma levels of the cytokines IL-10, IL-13, IL-17F, IL-27, IL-31 and IL-33, as well as of the chemokines MIP3-α/CCL20, monokine induced by gamma interferon (MIG)/CXCL19, 6Ckine/CCL21 and CXCL16 (Duo-Set; R&D Minneapolis, MN, USA). Sample concentrations of each cytokine and chemokine were quantified from standard curves generated with recombinant chemokines/cytokines, and the lower limit for their detection was 50 pg/ml.

Statistical data analyses

For data analyses the statistical package jmp version 5.0.1·2 was used. For the cytokine and chemokine analyses, differences between groups were determined after logarithmic transformation to stabilize the variance of data [log (pg/ml + 1)]. The level of significance was adjusted according to Bonferroni–Holm ($\alpha = 0{\cdot}0025$). Paired data from patients were evaluated by *t*-test and unpaired data of patient groups were compared using Wilcoxon’s rank sum test.

RESULTS

Infant patient groups

A total of 392 infants 0.2–4.8 years of age were included in this investigation and Table 1 shows the characteristics of the infant patient groups; the endemic control group (NEG) were infants in whom *P. falciparum* was not detectable by means of thick blood smear and rapid antigen detection kits. The infant group with severe malaria (SM: > 250 000 parasites/ml; < 5 g/dl haemoglobulin) was significantly younger and had higher leukocyte counts than NEGs and uncomplicated malaria cases (MM: < 250 000 parasites/ ml; < 5 g/dl), and in both malaria patient groups haemoglobin levels were significantly lower compared to the levels in NEG infants ($P < 0.0001$).

Table 1. Characteristics of patient groups. Demographic data, leukocyte counts, haemoglobin concentrations and parasite densities in infant patient groups and controls. Infants with severe malaria (SM) were characterized by parasite densities > 250 000 parasites/ml blood (mean;min/max) and/or haemoglobin concentrations < 5 g/dl (mean; min/max). Uncomplicated malaria (MM) patients had < 250 000 parasites/ml and haemoglobin concentrations < 5 g/dl and no signs or symptoms of severe malaria. *Plasmodium falciparum*-exposed infants negative for parasites in thick blood film, and negative in rapid detection test kits for *P. falciparum* were defined as participants (NEG) with previous malaria episode(s) and the actual absence of illness due to malaria within the last 2 weeks. Significant differences (**P* = 0.0002; ***P* < 0.0001) between NEG and uncomplicated malaria (MM) or severe malaria (SM) patients are indicated. HB: haemoglobin.

Group	n	Male/ Female	Age (Years) (Min/Max)	Mean HB (g/dl) (Min/Max)	Parasite Density (Pf/µl blood)	Leukocytes (n/µl) (Min/Max)
NEG	81	33/48	2.7 (0.2/4.8)	11.61 (8.2/17.3)	0	6597 (3500/28000)
MM	184	99/85	2.1* (0.2/4.5)	8.56** (5/15.4)	44987 (50/250000)	10059** (1002/210000)
SM	127	70/57	1.8** (0.1/4.5)	4.22** (1.4/10.8)	165187 (50/2 100 000)	18020** (1100/121000)

Cytokine levels in infants with uncomplicated and severe malaria

Plasma levels of IL-10, IL-13, IL-17F, IL-27, IL-31 and IL-33 were quantified by specific ELISA in NEG, MM and SM infants (Fig. 1). In those negative for *P. falciparum* (NEG) the mean plasma IL-10 concentration was 120 pg/ml; with *P. falciparum* parasite presence it enhanced to 1030 pg/ml in MM and 1600 pg/ml in SM patients, significantly higher (for both $P < 0.0001$) when compared to NEG. The mean plasma concentrations of IL-13 were 230 pg/ml in MM and 380 pg/ml in SM. The mean levels of IL-17F were 2070 pg/ml, 3150 pg/ml and 2950 pg/ml in NEG, MM and SM infants, with differences ($P = 0.007$) between NEG and MM or SM groups, respectively. Plasma levels of IL-27 ranged between 1370 and 48 540 pg/ml, with mean concentrations greatly exceeding those of IL-10, IL-17F, IL-31 and IL-33 and, in contrast to the aforementioned measured cytokines, IL-27 concentrations were highest in NEG infants (23 320 pg/ml), lower in cases with uncomplicated malaria (MM: 15 530 pg/ml) and lowest in those children with severe malaria (SM: 10 850 pg/ml) ($P < 0.0001$, NEG compared to MM and SM). Mean levels of IL-31 and IL-33 in infants with MM were above those of the NEG group, and clearly higher ($P < 0.0001$) in SM infants compared to NEG. The concentrations of IL-31 were 1580 pg/ml in NEG, 2740 pg/ml in MM and 5940 pg/ml in SM. In all infant groups, IL-33 levels were considerably lower than those for IL-31, with IL-33 plasma concentrations at 90 pg/ml in parasite-free controls (NEG) which rose to 200 pg/ml in MM, reaching 310 pg/ml in SM cases (SM *versus* NEG; $P < 0.0001$).

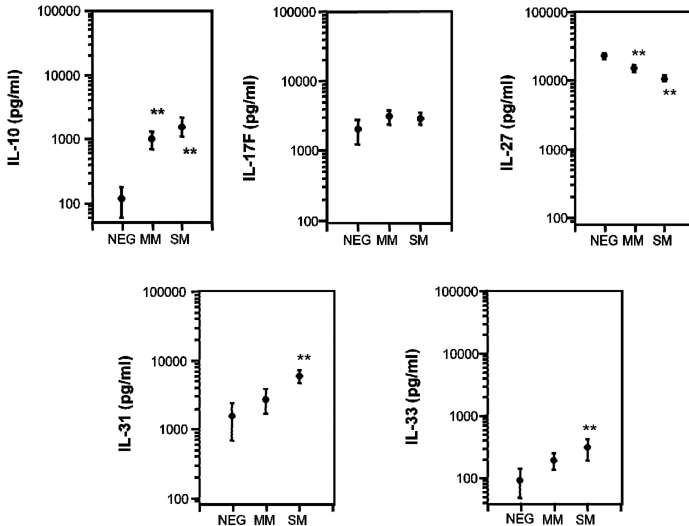

Fig. 1. Plasma concentrations of cytokines interleukin (IL)-10, IL-17F, IL-27, IL-31 and IL-33 were quantified in *Plasmodium falciparum*-infected infants (< 5 years) and in non-infected endemic controls (< 5 years). Cytokine concentrations are shown as means in pg/ml with the 95% lower and upper confidence interval. Infants with severe malaria (SM) were characterized by parasitaemias of higher than 250 000 parasites/ml and/or haemoglobin concentrations of less than 5 g/dl. Uncomplicated malaria (MM) patients were defined by parasitaemias of lower than 250 000 parasites/ml and haemoglobin concentrations equal or higher than 5 g/dl and the absence of any signs or symptoms of severe malaria. *P. falciparum*-exposed infants negative for parasites in thick blood film, and negative in rapid detection test kits for *P. falciparum* were defined as participants with previous malaria episode(s) and the actual absence of illness due to malaria within the last 2 weeks. Significant differences (** $P < 0{\cdot}0001$) between infection-free controls (NEG) and MM or SM patients are indicated. **$P < 0{\cdot}0001$ compared to NEG.

Chemokine levels in infants with mild and severe malaria
Plasma levels of MIP3-α/CCL20, MIG/CXCL9, the lymphoid and homeostatic chemokine 6Ckine/CCL21 and the inflammation-associated chemokine CXCL16 were quantified in NEG, MM and SM infants (Fig. 2). Concentrations of CCL20, CXCL16 and CXCL19 were enhanced in those with *P. falciparum*, while CCL21 remained at around 320 ± 5 pg/ml in NEG, MM and SM infants. The mean levels of CCL20 were 90 pg/ml in NEG infants, and were significantly higher ($P < 0.001$) in MM (550 pg/ml) and SM (900 pg/ml), with no difference between the MM and SM groups. For MIG/CXCL9, the concentrations were 720 pg/ml in NEG, and clearly enhanced ($P < 0.0001$) in MM (2180 pg/ml) and SM (3170 pg/ml) infants with no differences between the *P. falciparum*-infected groups.
Plasma concentrations of CXCL16 in NEG patients were 2930 pg/ml (mean) and the levels were enhanced in those with *P. falciparum*, to 5160 pg/ml in MM and 8840 pg/ml in SM cases. CXCL9 and CXCL16 levels were clearly higher ($P < 0.0001$) in SM than in NEG, and CXCL9 levels in SM were higher than those of MM patients ($P < 0.0001$).

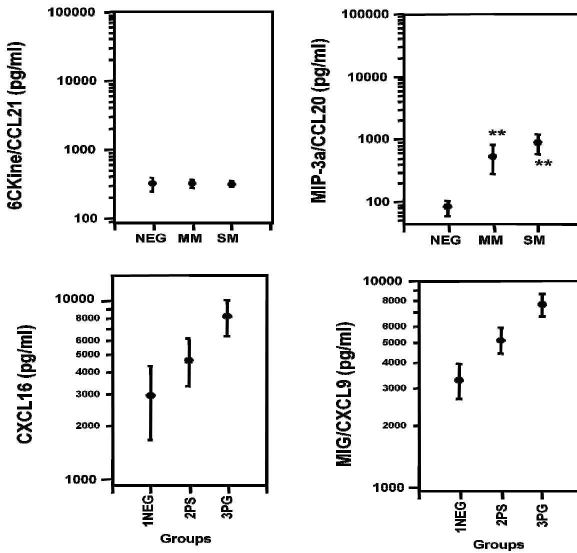

Fig. 2. Plasma concentrations of chemokines 6Ckine/CCL21, macrophage inflammatory protein (MIP)3-α/C–C ligand 20 (CCL20), CXCL16 and MIG/CXCL9 were quantified in *Plasmodium falciparum*-infected infants (< 5 years) and in non-infected endemic controls (< 5 years). Chemokine concentrations are shown as means in pg/ml with the 95% lower and upper confidence interval. Infants with severe malaria (SM) were characterized by parasitemias of higher than 250 000 parasites/ml and/or haemoglobin concentrations of less than 5 g/dl. Uncomplicated malaria (MM) patients were defined by parasitemias of lower than 250 000 parasites/ml and haemoglobin concentrations equal or higher than 5 g/dl and the absence of any signs or symptoms of severe malaria. *P. falciparum* exposed infants negative for parasites in thick blood film, and negative in rapid detection test kits for *P. falciparum* were defined as participants with previous malaria episode(s) and the actual absence of illness due to malaria within the last 2 weeks. Significant differences (** $P < 0.0001$) between infection-free controls (NEG) and MM or SM patients are indicated.

Cytokine and chemokine changes post-anti-malarial treatment

At 48–60 h post-anti-malarial treatment (Fig. 3), significantly diminished cytokine concentrations were detected for IL-10, IL-13 and the chemokines MIG/CXCL9, CXCL16 and MIP-3α/CCL20 (not shown). The mean levels of IL-17F, IL-27, IL-31 and IL-33 did not change at 48–60 h post-anti malaria treatment and with reduced parasitemia.

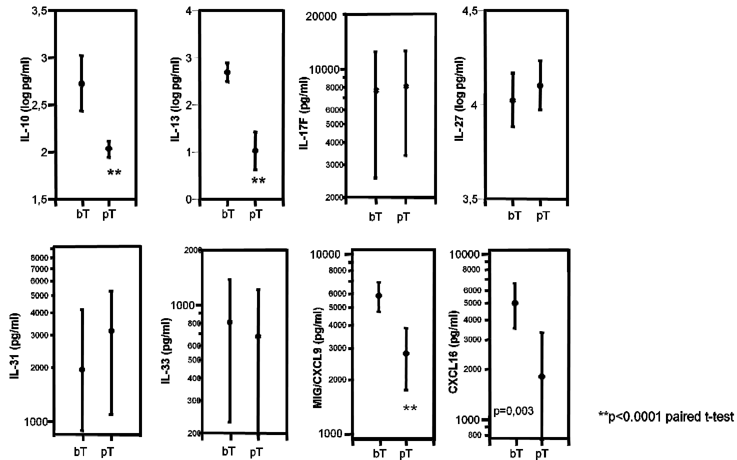

Fig. 3. Plasma concentrations of cytokines interleukin (IL)-10, IL-13, IL-17F, IL-27, IL-31, IL-33 and chemokines monokine induced by gamma interferon (MIG)/C–X–C ligand 9 (CXCL9) and CXCL16 were quantified in *Plasmodium falciparum*-infected infants (<5 years) before treatment (bT) with anti-malarials and/or anti-pyretics and at 48–60 h post-treatment (pT). Chemokine concentrations are shown as means in pg/ml with the 95% lower and upper confidence interval. Significant differences (** $P < 0.0001$, paired observations) between bT and pT are indicated.

Cytokine and chemokine correlations
In *P. falciparum*-infected infants, the levels of MIP3-α/ CCL20 ($r2$ = 0.28; P = 0.0002) and MIG/CXCL9 ($r2$ =0.33, P = 0.0005) were correlated positively with parasite density, while IL-27 displayed a weak negative correlation ($r2$ = -0.17; P = 0.01).

DISCUSSION

Naturally acquired protective immunity against malaria requires subclass-specific antibody responses [16–18], and the secretion of cytokines, chemokines and further immune mediators is essential for the regulation both of cellular effector mechanisms against *P. falciparum* blood-stage parasites and of organ-specific inflammation and pathogenesis [19,20]. In MM and SM infants substantial cytokine and chemokine levels were detected, which disclosed both innate and memory immune responsiveness. The first parasite encounter and sensitization to *P. falciparum* antigens may already occur prenatally and continue in infants shortly after birth [21]. *P. falciparum* infection during pregnancy is a major health problem in our study area [22,23], and prenatal and early life contact with plasmodial antigens has to be considered as a regularity. In infants, antibody responses and pronounced parasite-specific IL-10 production were found to be associated with faster *P. falciparum* parasite clearance [24], and the higher longevity of regulatory T cell (Treg)-type IL-10 compared to Th1-type IFN-γ responses [25] suggested that prenatal and early postnatal sensitization with *P. falciparum* antigens has occurred [26,27]. It is noteworthy that parasite-specific IL-10 responses were observed frequently and of high magnitude in umbilical cord blood cells from newborns of infected mothers [21–

23,28]. In the present work, plasma IL-10 levels were not correlated with parasite densities or the infants' age, and this further supported early life *P. falciparum*-specific immune sensitization and IL-10 induction. The role of IL-10 in malaria pathogenesis is controversial. High IL-10 levels were associated with cerebral malaria [13], with high parasite density and severe disease in children [29,30], while lower plasma concentrations of IL-10 occurred in those with severe malarial anemia [13,30]. IL-10 will modulate Th1-type responses to *Plasmodium* antigens by depressing proinflammatory TNF and also production of IFN-g by dampening the release of IL-12p70 [14]; however, in response to *P. falciparum* infection, cytokine profiles and their relative balance, not single pro- and anti-inflammatory T helper and T regulatory cytokines, may mediate protective immunity and disease severity [31].

With regard to the regulatory type IL-10, the Th2-type anti-inflammatory cytokine IL-13 disclosed similar levels and dynamics; it was enhanced in MM and SM infants and declined rapidly with parasite clearance following treatment. In 1–4-year-old children with acute uncomplicated *P. falciparum* malaria, increased IL-13 levels were found [32], which decreased up to day 2 post-treatment. IL-13 provides protection from LPS-induced lethal endotoxaemia similar to but independent from IL-10, and IL-13 can be considered as an immune modulator which might be beneficial in the treatment of septic shock [33]. As revealed recently, IL-13 mediated phagocytosis of *P. falciparum*-parasitized erythrocytes by alternative activated monocytes [34], and resistance to severe malaria through altered IL-13 production may be associated with a single nucleotide polymorphism in the IL-13 promoter [35].

As a cytokine with dual regulatory capacity, IL-27 will first initiate Th1-type IFN-γ responses and promote IL-10

synthesis by regulatory T cells, then attenuate inflammatory Th2 and Th17 cells [36] and depress proinflammatory cytokines and chemokines [37]. IL-27R-deficient mice infected with *Toxoplasma gondii*, *Trypanosoma cruzi* or *Leishmania donovani* first controlled parasite replication, but then developed lethal proinflammatory cytokine responses and succumbed to infection [38], and such mice infected with the intestinal helminth *Trichuris muris* developed an increased production of Th2-associated cytokines and were able to clear intestinal worms very early [39]. IL-27R-deficient mice were susceptible to *P. berghei* infection and developed Th1-mediated immune responses which, despite efficient parasite clearance, led to severe liver pathology [40]. The regulatory function of IL-27 via the induction of IL-10 and suppression of IL-17 secretion may help to prevented early manifestations of malarial disease, but IL-27 alone may not suffice to prevent chronic infection and severe malaria.

The capacity of IL-27 in suppressing Th17-type responses may be critical for pathology prevention; IL-17F levels were similarly high in MM, SM and NEG infants, and the unchanged IL-17F levels post-parasite clearance suggested that IL-17F may not be implicated in malaria progression or regression. Enhanced levels of Th17-associated cytokines have been detected in psoriasis, arthritis, asthma and bacterial and fungal infections [41], and Th17 cells might breach the blood–brain barrier and infiltrate the central nervous system (CNS) parenchyma [42], thereby inducing the production of other proinflammatory cytokines and chemokines which will attract effector cells and provoke tissue inflammation. However, IL-17 was not found to be associated with development of cerebral malaria in *P. berghei* infected mice [43], and in Ghanaian children

cerebral malaria mortality was not associated with IL-17 [15].
While IL-17F levels were similar in NEG, MM and SM infants, the cytokine IL-31, which has comparable effects to IL-17 [44], was highest in SM patients. IL-17 and IL-31 both have additive effects on secretion of cytokines and chemokines [44,45], and IL-31, a member of the gp130/IL-6 cytokine family [45], may recruit polymorphonuclear cells, monocytes and T cells to an inflammatory site *in vivo* [46]. IL-31 will induce the genes of inflammatory chemokines MIP-1β, MIP-3α, MIP-3β [47,48] and proinflammatory cytokines IL-6, IL-8, IL-16 and IL-32 [44,45]. IL-31-receptor-deficiency in mice injected with *Schistosoma mansoni* eggs resulted in severe pulmonary inflammation, enlarged granuloma and significantly more IL-4, IL-5 and IL-13 than in wild-type mice [48]. In allergic asthma patients, serum levels of IL-31 were elevated above controls [49], a further suggestion that the IL-31/IL-31R signalling pathway will regulate type 2 inflammations [48]. Another key player promoting Th2 type responses, the cytokine IL-33, is considered a mediator of pathology with allergies and septic shock [50–52]; IL-33 was suggested to function as an alarmin [53], to alert after endothelial or epithelial cell damage during trauma, stress or infection [53]. IL-33 levels were enhanced in infants with MM and SM, clearly above NEG, correlated positively with parasite densities, and diminished strongly following parasite clearance. Sequestration of *P. falciparum*-infected erythrocytes or the release of merozoites may have amplified IL-33 production by endothelial cells, and additional cytokines augmented by IL-33 are IL-5, IL-13, TNF and IL-3 [54]. Furthermore, IL-33 will promote splenomegaly, blood eosinophilia and epithelial hyperplasia, massive mucus production in lungs and pulmonary inflammation [55]. To what extent the enhanced production of IL-31 and IL-33

may contribute to pathogenesis of acute *P. falciparum* infection to cerebral inflammation and vascular obstructions should be investigated further.

For the development of cerebral malaria, an important role has been attributed to cytokines and chemokines [56,57]. With severe *P. falciparum* infection an increased production of MCP-1/CCL2, MIP-1α and MIP-1β, and also IL-8/CXCL8, has been observed [9,13], and the mortality risk with cerebral malaria (CM) was associated independently with the serum concentration of IP-10/CXCL10 [15]. The chemokines IP-10/CXCL10 and MIG/CXCL9, together with their common receptor CXCR3, are required for the development of murine CM [58]. MIG/CXCL9 and its receptor are expressed predominantly in Th1 cells, and MIG/CXCL9 is considered to be a predictive marker for antigen specific IFN-g-secreting peripheral blood mononuclear cells (PBMCs) in volunteers immunized with irradiated *P. falciparum* sporozoites [59]. In the present work, MIG/CXCL9 levels were highest in SM infants and lessened rapidly with parasite clearance after anti-malarial therapy, suggesting that MIG/CXCL9 may be an indicator for parasite multiplication or diminution, and possibly also for the sequestration of *P. falciparum*-infected erythrocytes (Pf-IRBC) in blood vessels of the CNS. MIP-3α/CCL20 will stimulate the migration, homing and maturation of leukocytes, and CCL20 together with CXCL1, CXCL2, IL-6 and IL-8 increased more than 100-fold in blood–brain barrier endothelial cells during Pf-IRBC contact, which suggests its participation in cellular defense during Pf-IRBC sequestration [60]. Astrocytes which line parenchymal blood vessels will respond in a pathogen-specific way to infection and release MIP-3α/CCL20 and CXCL16 [61]; both chemokines will promote Th1-type responses by enhancing IFN-γ and TNF-α release, and CXCL16 may attract neutrophil

granulocytes across the blood–brain barrier into the cerebrospinal fluid [62,63]. Both CCL20 and CXCL16 were elevated substantially in SM and MM infants; CCL20 correlated positively with parasite densities, and therefore CCL20 and CXCL16 should be investigated further as to what extent they contribute to the manifestation of CM. The chemokines 6Ckine/CCL21 and CCL19 are both involved in T lymphocyte migration into CNS tissues during immune surveillance and inflammation [64–66], and expression of their common receptor CCR4 is required for protective immune responses during acute *T. gondii* infection [67,68]. The abrogation of CCL21 function in mice with *L. major* infection resulted in failure to clear parasites from infected skin [68]. In the present work, 6Ckine/CCL21 plasma levels were similar in NEG, MM and SM infants, suggesting that with malaria progression or regression 6Ckine/CCL21, which may promote immune surveillance against intracellular parasite in CNS tissues, has not been activated or remained suppressed.

In summary, proinflammatory and regulatory cytokine and chemokines were generated in infants during progression and regression of acute malaria tropica. Proinflammatory type cytokines IL-31 and IL-33 were strongly enhanced, while regulatory IL-27 was lowered with severe malaria. Similarly, the chemokines CCL20 and CXCL16 which promote leukocyte migration into brain parenchyma increased while CCL21, which mediates immune surveillance in CNS tissues, remained unchanged. These cytokine and chemokine production profiles and their dynamics could be considered for evaluating the progression or regression of malarial disease.

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DISCLOSURE

The authors declare that no conflict of interest exists.

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Chemokines and cytokines in patients with an occult *Onchocerca volvulus* infection.

Lechner CJ[a,b], Gantin RG[b], Seeger T[a], Sarnecka A[a], Portillo J[a], Schulz-Key H[a,b], Karabou PK[c], Helling-Giese G[a,b], Heuschkel C[a,b], Banla M[d,b], Soboslay PT [a,b]

a Institute for Tropical Medicine, University of Tübingen, Germany
b Onchocerciasis Research Laboratories - Institute National d'Hygiene, Sokode, Togo
c National Onchocerciasis Control Programme, Kara, Togo
d Universite de Lome,
Centre Hospitalier Universitaire Campus de Lome, Togo

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ABSTRACT

Repeated ivermectin treatment will clear microfilaria (Mf) of *Onchocerca volvulus* from skin and eyes of onchocerciasis patients while adult filaria remains alive and reproductive, and such occult *O. volvulus* infection may persist for years. To investigate the effect of residual adult filaria on the immune response profile, chemokines and cytokines were quantified 1) in onchocerciasis patients who developed an occult *O. volvulus* infection (Mf-negative) due to repeated ivermectin treatments, 2) patients who became Mf-negative without ivermectin treatments due to missing re-infection, and 3) endemic and non-endemic *O. volvulus* Mf-negative controls. With occult *O. volvulus* infection, serum levels of pro-inflammatory chemokines MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, MPIF-1/CCL23 and CXCL8/IL-8 enhanced and approached higher concentrations as determined in infection-free controls, whilst regulatory and Th2-type cytokines and chemokines MCP-4/CCL13, MIP-1δ/CCL15, TARC/CCL17 and IL-13 lessened. Levels of Eotaxin-2/CCL24, MCP-3/CCL7 and BCA-1/CXCL13 remained unchanged. At 3 days post-initial ivermectin treatment, MCP-1/CCL2, MCP-4/CCL13, MPIF-1/CCL23 and Eotaxin-2/CCL24 were strongly enhanced, suggesting that monocytes and eosinophil granulocytes have mediated Mf clearance.
In summary, with occult and expiring *O. volvulus* infections the serum levels of inflammatory chemokines enhanced over time while regulatory and Th2-type-promoting cytokines and chemokines lessened; these changes may reflect a decreasing effector cell activation against Mf of *O. volvulus*, and in parallel, an enhancing inflammatory immune responsiveness.

INTRODUCTION

The elimination of onchocerciasis in Africa is considered achievable; in large areas of the African Programme for Onchocerciasis Control (APOC) the disease is no longer seen as a public health problem, but still, *Onchocerca volvulus* infection remains an obstacle to human health in several countries of the APOC, notably in Central Africa [1-5]. The likelihood for eradication of *O. volvulus* is currently limited as the only available and on large scale applicable control option is repeated ivermectin treatment. Ivermectin will not kill or sterilize adult filaria, the drug will clear microfilaria (Mf) of *O. volvulus* efficiently from skin and eyes [6,7], and even after repeated ivermectin treatments for several years, female adult *O. volvulus* remain alive and reproductive and *O. volvulus* infection may persist without detectable dermal Mf [8-10]. Such “occult onchocerciasis” may associate with a particular immune response profile and even predispose for distinct skin and lymphatic pathologies as seen with localized onchocerciasis in “sowda” or with lymphatic filariasis in “elephantiasis” patients in whom only rare or no Mf will be detected in skin or peripheral blood [11-14]. Following repeated ivermectin supported clearance of Mf, cellular proliferative responses will gradually re-activate in onchocerciasis patients, but treatment alone may not suffice to facilitate protective and adequately balanced Th1 and Th2 immune responses against *O. volvulus* infection [15,16]. Cytokines and chemokines may act as central contributors to the acquisition of immunity, and in particular, their excess or unbalanced production may contribute to pathogenesis and severity of disease. While cytokine response profiles were studied in onchocerciasis, lymphatic filariasis and schistosomiasis patients extensively [17-19], chemokines have received

less attention even though being recognized as central contributors to clearance of microfilaria and granuloma formation in filariasis and in schistosomiasis as well [20-24]. Previous investigations have shown that shortly after initial ivermectin treatment the serum chemokine levels of RANTES/CCL5, MDC/CCL22, CTACK/CCL27 and TARC/CCL17 enhanced [20-22], while those which activate inflammatory monocyte responses (MIP-1α/ CCL3, MIP-1β/CCL4) rose gradually during the first year post-initial treatment (p.i.t.). The initially enhanced chemokine levels may associate with massive destruction of Mf by effector cells, and the later rise of inflammatory chemokines with a re-activation of Th1-type immune responses. In the present work, chemokine and cytokine levels were studied in patients with an expiring *O. volvulus* infection, and how these mediators of inflammation and immune regulation changed while adult filarial parasites gradually exceeded their natural life span.

MATERIAL AND METHODS

2.1. Location of study

This study was conducted in Togo in West Africa, within the vector-controlled area of the Onchocerciasis Control Programme (OCP), where since 1977 (northern Togo) and 1985 (central Togo) transmission of infective third-stage larvae of *O. volvulus* has been reduced through control of the *Simulium* spp. black fly vectors [4]. Ivermectin treated onchocerciasis patients were from the Central and Kara Region, from the Villages Bougabou, Bounakou, Bouzalo, Kemeni, Mo, Sagbadai, Sirka, Tabalo and Tchapossi. All villages are covered by community directed annual ivermectin distribution of the National Onchocerciasis Control Programme

(PNLO/APOC), and situated in the special intervention zone (SIZ) where until December 2006 insecticides against black fly larvae were applied by the OCP [25].

2.2. Ivermectin treatment of patients

Onchocerciasis patients enrolled in a double-blind placebo controlled dose finding study of ivermectin for treatment of onchocerciasis [16,26]. The protocol of the study was reviewed and approved by the Ethics Commission of the Medical Board at University of Tübingen, the Advisory Council of the Ministry of Health in Togo and the Committee on Research Involving Human subjects of the World Health Organization. Authorization was granted by the Ministry of Health in Togo and reapproved every 4-5 years (no. 1999: 292//MS/CAB 2003431989; no. 261//MSP/DGSP/DRSP-RC). Patients were apparently healthy males and non-pregnant women with a body weight over 30 kg, without history of multiple allergies or drug intolerance, with skin biopsies positive for microfilaria of *O. volvulus* and with palpable nodules (onchocercomata). All onchocerciasis patients participated in regular surveys conducted by the National Onchocerciasis Control Programme (NOCP). At regular intervals thorough physical, parasitological and ophthalmological examinations were conducted and the density of *O. volvulus* microfilaria (Mf) was determined in skin biopsies (Mf/mg skin) taken from the right and left hip. At each time-point of examination all participants gave their informed consent, and for correct and complete understanding explanations were always given in the local language. The densities of microfilaria of *O. volvulus* (Mf/mg) in the skin were determined at the right and left scapula, iliac crest and calf by means of corneoscleral punch (Holth- or Walser-type). Patients were treated with ivermectin until month 18 with 100 mg/kg, 150 mg/kg or 200 mg/kg, or received placebo.

From month 18 onwards all patients received 150 mg/kg ivermectin and all patients were followed individually until 7 years post initiation of treatment (p.i.t.). Thereafter, ivermectin treatments (150 mg/kg) were applied annually by the National Programme for Onchocerciasis Control (PNLO/APOC) i.e. by community-directed ivermectin distribution.

2.3. Patients with postpatent O. volvulus infection and endemic and non-endemic controls

Onchocerciasis patients with postpatent *O. volvulus* infection and Mf-negative endemic controls (NEG) were from northern Togo, the Oti-Pendjari or Volta Blanche river basin, the core area of the former OCP (Phase III East, March 1977). They lived in the OCP sentinel villages Borgou (OCP village #138), Gale (#143), Koundjouare (#144), Mogou (#147), Panga (#140) and Warkambo (#142) where *Simulium* spp. vector control has been implemented since 1977. Patients became Mf-negative (postpatent) without receiving ivermectin treatment (n = 55, postpatent group). Individuals from northern Togo villages were followed by the OCP epidemiology unit (OCP/EPI) since 1977, skin biopsies were taken from the patients' left and right iliac crest, and the state of infection documented. The postpatent state of *O. volvulus* infection was attained when formerly Mf-positive patients remained Mf-negative on at least 2 follow up examinations (4-6 years) as determined by the OCP epidemiology survey teams (OCO/EPI). Endemic controls were residents in the OCP sentinel villages in central and northern Togo, and were never found positive for microfilariae of *O. volvulus* (n = 33, NEG) at all OCP/EPI surveys. Non-endemic controls were European travelers to the tropics (n = 60, CTRL).

2.4. Chemokine and cytokine ELISA

Cytokine and Chemokine concentrations were determined before the first ivermectin treatment (befT), three days (3d), three months (3mo), six months (6mo), 1 year (1y) post-initial treatment (p.i.t.), and 4 years (4y), 4 years +3 days (4y3d) and 7-8 years (7y) after repeated treatments. Quantitative enzyme-linked immunosorbent assays (ELISA) were used to determine chemokines and cytokines concentrations in serum (Duo-Set: R&D Minneapolis, (MN) USA). The concentrations of each chemokine and cytokine were calculated from standard curves generated with recombinant chemokines and cytokines; for all assays the detection limit was 50 pg/ml.

2.5. O. volvulus antigen-specific antibody ELISA

O. volvulus antigen-specific (OvAg-specific) IgG isotypes responses were determined by ELISA as previously described [16]. Isolation of *O. volvulus* and adult worm-derived antigen (OvAg) preparation was effected as described [27,28].

2.6. Statistical data analyses

For data analyses the statistic package JMP 5.0.1.2 was used. For cytokine and chemokine analyses, differences between groups were determined after logarithmic transformation to stabilize the variance of data [log (pg/ml + 1)]. The level of significance was adjusted according to Bonferroni-Holm. Paired data from patients were evaluated by t test, and unpaired data of patient groups were compared using the Wilcoxon rank sum test. The level of significance was adjusted according to Bonferroni-Holm; i.e. $\alpha = 0.0025$.

RESULTS

3.1. O. volvulus microfilaria (Mf) in patients

Repeated ivermectin treatments reduced the Mf density in patients (n = 138) from mean 89 Mf/mg at before-initial treatment to 3 Mf/mg skin at 48 months and 3 days (Mo48d3) post-initial treatment (p.i.t.) (Fig. 1). The initial Mf density lessened by 97% at Mo48d3, whilst 88% of the patients were then still Mf-positive. At 7 years p.i.t., all onchocerciasis patients were negative for Mf. In the *O. volvulus* postpatent group no Mf were detectable for at least 4 years, and NEG group individuals never presented with Mf in skin biopsies.

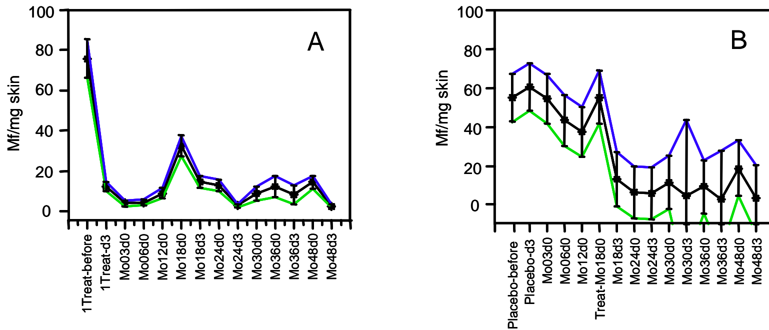

Fig. 1. Density of microfilariae (Mf) of *Onchocerca volvulus* in skin biopsies of onchocerciasis patients (Mf/mg skin) before and after treatments with ivermectin (Part A) or placebo (Part B). Patients were treated until month 18 with either 100 mg/kg, 150 mg/kg or 200 mg/kg ivermectin (n = 138), or received placebo (n = 48). From month 18 onwards all patients received 150 mg/kg ivermectin. Microfilarial densities were determined in patients at before treatment (1Treatbefore; ivermectin n =138; placebo n = 48), 3 days post-initial treatment (p.i.t.) (1Treat-d3; ivermectin n = 138; placebo n = 48), 3 months p.i.t. (Mo3d0; 122; 46), 6 months p.i.t. (Mo6d0; 110; 38), 12 months p.i.t. (Mo12d0; 108; 37), and, 18 months (Mo18d0; 126; 34), 18 months and 3 days (Mo18d3; 126; 33), 24 months (Mo24d0; 126; 35), 24 months and 3 days (Mo24d3; 124; 34), 30 months (Mo30d0; 121; 33), 36 months (Mo36d0; 111; 32), 36 months and 3 days (Mo36d3; 45; 13), 48 months (Mo48d0; 112; 31) and 48 months and 3 days (Mo48d3; 58; 20) after repeated treatments. Microfilarial densities are shown as means in Mf/mg skin with the 95% lower and upper confidence interval.

3.2. Monocyte chemoattractant proteins (MCP)

At before ivermectin treatment, lowest MCP-1 levels were detected in microfilaria (Mf)-positive onchocerciasis patients (Fig. 2). At 3 days post-initial treatment (p.i.t), MCP-1 significantly increased (before: 10 pg/ml; 3 days p.i.t.: 930 pg/ml; $p < 0.0001$). At 3 and 6 months p.i.t., MCP-1 concentration decreased to levels as observed before treatment, and then (MCP-1) gradually heightened until 7 years p.i.t. MCP-1 concentrations in patients at 7 years p.i.t. were similar to those of postpatent onchocerciasis patients and NEG controls.

Highest MCP-1 levels were measured in European CTRLs. The levels of MCP-3 (Fig. 2) ranged between 20 and 40 pg/ml, i.e. at the assay detection limit, and only 7 years p.i.t. MCP-3 elevated to 170 pg/ml. MCP-3 concentrations were similar in NEGs, CTRLs, postpatent and onchocerciasis patients.

MCP-4 concentrations in untreated onchocerciasis patients (Fig. 2) were at 550 pg/ml and those clearly higher than MCP- 1 and MCP-3 ($p < 0.0001$). At 3 days p.i.t., MCP-4 rose to 1200 pg/ml (min 310 pg/ml; max 3320 pg/ml; $p < 0.0001$), and thereafter, lessened at 3 months, 6 months and 1 year p.i.t. (170 pg/ml) to concentrations slightly lower as before the first ivermectin treatment and being similar as in postpatent patients and NEG and CTRLs. At 4 years p.i.t., MCP-4 was as high as 3 days p.i.t. (4 yrs p.i.t.: mean 920 pg/ml). At 7 years p.i.t., MCP-4 was at 620 pg/ml and above values measured during the first year p.i.t. MCP-4 did not attain the lower levels as in postpatent, NEG and CTRLs. The concentrations of MCP-1 and MCP-4 were clearly higher than those of MCP-3 (Fig. 2); only at 7 years p.i.t., the mean MCP-3 rose to 170 pg/ml. In patients who received placebo the MCP-1, MCP-3 and MCP-4 serum levels did not change until 12 months post-initial placebo.

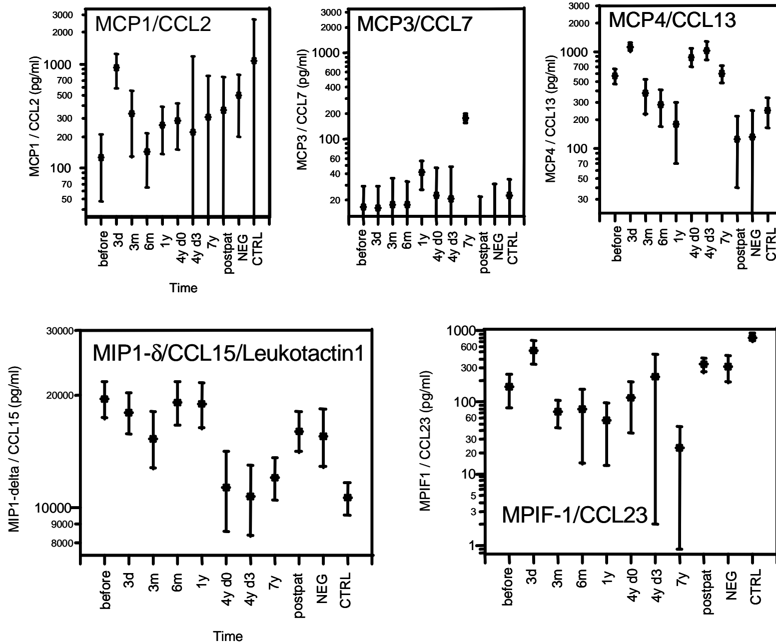

Fig. 2. Serum concentrations of monocyte chemotactic proteins MCP-1/CCL2, MCP-3/CCL7, MCP-4/CCL13, macrophage inflammatory protein MIP-1δ/CCL15 and myeloid progenitor inhibitory factor 1 (MPIF-1/CCL23) were quantified in onchocerciasis patients following repeated ivermectin treatment, in postpatent onchocerciasis patients (postpat), and in onchocerciasis-free endemic (NEG) and non-endemic European controls (CTRL). Chemokine concentrations are shown as means in pg/ml with the 95% lower and upper confidence interval. Patients were treated with ivermectin (100, 150 or 200 mg/kg ivermectin) annually and chemokine concentrations were determined before treatment (before), 3 days (3d), 3 months (3m), 6 months (6m) and 1 year (1y) post-initial treatment, and also, 4 years (4yd0), 4 years and 3 days (4yd3) and 7 years (7y) after repeated treatments. Postpatent onchocerciasis patients (postpat) originated from sentinel villages of the core area of the Onchocerciasis Control Programme (OCP) in northern Togo in whom *O. volvulus* infection had expired without ivermectin treatment. Negative (NEG) endemic controls were individuals from the Onchocerciasis Control Programme (OCP) in

northern Togo who were never found positive for *O. volvulus* infection. Non-endemic controls (CTRL) were *O. volvulus* infection-free European travellers returning from the tropics. Wilcoxon rank sum tests: $^{**}p < 0.0001$ compared with before treatment; $^{*}p < 0.002$ compared with before treatment.

3.3. Monocyte inflammatory proteins (MIP), myeloid progenitor inhibitory factor (MPIF) and tissue and activation regulated chemokine (TARC)

Before ivermectin treatment, MIP-1δ/CCL15 concentrations (Fig. 2) were at 19,860 pg/ml (mean) (min 9660 pg/ml; max 38,930 pg/ml). At 3 days p.i.t., MIP-1δ/CCL15 decreased, and remained such at 3 months p.i.t. At 6 months and 1 year p.i.t., concentration returned to values similar as before treatment. At 4 years p.i.t., MIP-1δ/CCL15 lessened further (mean 1 year p.i.t.: 18,770 pg/ml, mean 4 years p.i.t.: 10,980 pg/ml) and remained such at 7 years p.i.t. (mean 7 years p.i.t.: 12,100 pg/ml). Lowest MIP-1δ/CCL15 were measured in onchocerciasis patients at 4 years and 7 years p.i.t., and those were similar in CTRLs. In the postpatent and NEG groups, MIP-1δ/CCL15 levels were as in patients at 3, 6 and 12 months p.i.t.

MPIF-1/CCL23 was at 170 pg/ml (mean) in untreated patients (Fig. 2), it enhanced 3 days p.i.t. to 540 pg/ml (mean), thereafter diminished at 3 months p.i.t. (mean 80 pg/ml), was at 4 years p.i.t. at mean 120 pg/ml, and at 7 years p.i.t., MPIF- 1/CCL23 was below the assay detection limit (mean 10 pg/ ml). Higher MPIF-1/CCL23 levels were measured in CTRLs (mean 820 pg/ml), postpatent patients (mean 340 pg/ml) and in NEGs (mean 330 pg/ml), those concentrations were similar as detected in patients at 3 days p.i.t. (mean 500 pg/ml).

TARC/CCL17 concentrations were highest in Mf-positive patients (mean 1080 pg/ml) (Table 1), with postpatent *O. volvulus* infection levels of TARC lessened to 410 pg/ml, and in NEGs, TARC levels were at 520 pg/ml.

MIP-1α/CCL3 was highest in NEGs (mean 11,330 pg/ml) (Table 1), lower in postpatent patients (mean 2020 pg/ml) and lowest in *O. volvulus* Mf-positive patients (mean 410 pg/ml). As for MIP-1α/CCL3, the levels of

MIP-1β/CCL4 (Table 1) were highest in NEG controls (mean: 23,730 pg/ml), while in postpatent and patent onchocerciasis patients MIP-1β/CCL4 ranged similar (patent: mean 1370 pg/ml; postpatent: mean 820 pg/ml). In patients who received placebo the MIP-1δ/ CCL15, MPIF-1/CCL23, TARC/CCL17 and MIP-1α/CCL3 and of MIP-1β/CCL4 levels did not change until 12 months post-initial placebo.

Table 1. Serum concentrations of monocyte inflammatory proteins MIP-1α/CCL3 and MIP-1β/CCL4, and tissue and activation regulating chemokine TARC/CCL17 were quantified in onchocerciasis patients positive for microfilariae of *O. volvulus* (Patent), in postpatent onchocerciasis patients, and in onchocerciasis-free endemic controls (NEG). Chemokine concentrations are shown as means in pg/ml with the minimum and maximum values in each group. Postpatent onchocerciasis patients originated from sentinel villages of the core area of the Onchocerciasis Control Programme (OCP) in northern Togo, in these patients *O. volvulus* infection had expired without ivermectin treatment. Negative (NEG) endemic controls were individuals from the Onchocerciasis Control Programme (OCP) in northern Togo who were never found positive for *O. volvulus* infection (NEG).

Group	MIP-1α/CCL3 mean (pg/ml) (min/max)	MIP-1β/CCL4	TARC/CCL17
Patent (n=48)	410** (60/10990)	1370* (0/15190)	1080*,# (60/4660)
Postpatent (n=58)	2020** (60/83260)	820* (0/5400)	410 (0/390)
Negative (NEG) (n=54)	11330 (80/107640)	23730 (0/359390)	520 (0/1960)

3.4. Granulocytes and B cell activating chemokines

The chemokine Eotaxin-2/CCL24 was 900 pg/ml (mean) before treatment (min 60 pg/ml; max 3080 pg/ml) (Fig. 3), then enhanced at 3 days p.i.t. to 1290 pg/ml, followed by a strong decrease at 3 months p.i.t. (mean 650 pg/ml). At 4 years p.i.t., Eotaxin-2/CCL24 remained similar as before treatment and in the same range as in postpatent patients and NEGs. Highest concentrations of Eotaxin-2/CCL24 were found in CTRLs (mean 2010 pg/ml).

The B cell chemoattractant BCA-1/CXCL13 was at 120 pg/ml (mean) before treatment (Fig. 3), BCA-1 slightly enhanced after ivermectin treatments, and BCA-1 levels were higher in postpatent patients and NEGs and the differences never attained statistical significance. Highest levels of BCA-1/ CXCL13 were detected in CTRLs (mean 620 pg/ml).

The chemokine NAP-1/IL-8/CXCL8 concentrations were lowest (mean 170 pg/ml; min 0 pg/ml; max 3040 pg/ml) before ivermectin treatment. IL-8 enhanced at 3 days and at 3 months p.i.t., and reached at 6 months p.i.t. values of 2400 pg/ml clearly higher than before treatment ($p < 0.001$). At 1 year p.i.t., IL-8/CXCL8 concentrations (mean 3250 pg/ ml) were of similar magnitude as observed in NEGs (mean 320 pg/ml). At later time points post-initial treatment, IL-8/CXCL8 concentrations in the sera of onchocerciasis patients were not determined. The cytokine IL-13 (Fig. 3) was highest in untreated patients (mean 5230 pg/ml). After ivermectin treatment, at 3 days, 3 months, 6 months and 1 year p.i.t., IL- 13 concentrations in patient’s sera remained similar. At 4 years p.i.t., IL-13 levels declined, and at 7 years p.i.t. mean IL-13 concentrations were as in postpatent onchocerciasis patients (1820 pg/ml) and NEGs (1580 pg/ml). Lowest levels of IL-13 were measured in CTRLs. In patients who received placebo

the Eotaxin-2/CCL24, BCA-1/CXCL1, NAP-1/IL-8/CXCL8 and IL-13 levels did not change until 12 months post-initial placebo.

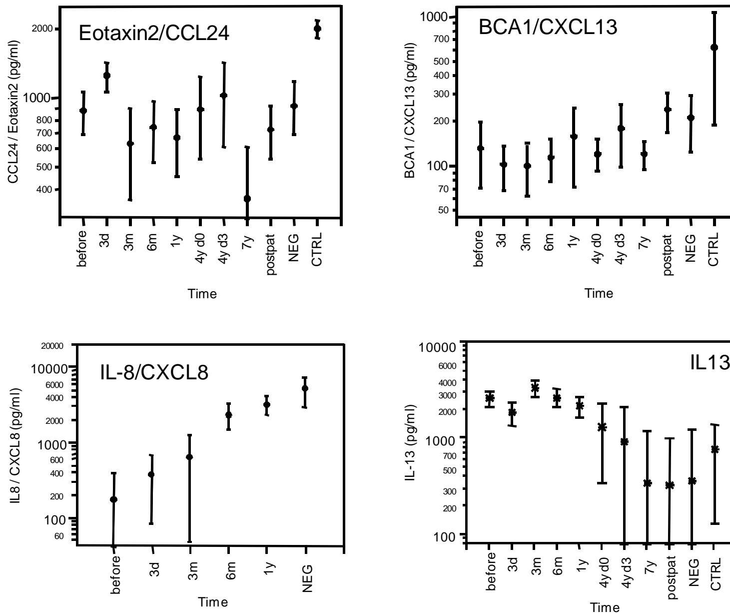

Fig. 3. Serum concentrations of Eotaxin-2/CCL24, B-cell chemoattractant BCA-1/CXCL13 and interleukin 13 (IL-13) were quantified in onchocerciasis patients following repeated ivermectin treatment, in postpatent onchocerciasis patients (postpat), in onchocerciasis-free endemic (NEG) and in non-endemic European controls (CTRL). Cytokine and chemokine concentrations are shown as means in pg/ml with the 95% lower and upper confidence interval. Patients were treated with ivermectin annually and chemokine concentrations were determined before treatment (before), 3 days (3d) post-initial treatment (p.i.t.), 3 months (3m) p.i.t., 6 months (6m) p.i.t., 1 year (1y) p.i.t., and 4 years (4yd0), 4 years and 3 days (4yd3) and 7 years (7y) after repeated treatments. Postpatent onchocerciasis patients (postpat) originated from the Onchocerciasis Control Programme (OCP) in northern Togo in whom O. volvulus

infection has expired without ivermectin treatment. Negative (NEG) endemic controls were individuals from the Onchocerciasis Control Programme (OCP) in northern Togo who were never found positive for O. volvulus infection. Non-endemic controls (CTRL) were O. volvulus infection-free European travellers returning from the tropics. Wilcoxon rank sum tests: **p < 0.0001 compared with before treatment; *p < 0.002 compared with before treatment.

3.5. Parasite-specific antibody responses with O. volvulus infection

O. volvulus antigen-specific antibody reactivity was strongest for IgG1 and IgG4 isotypes, highest in *O. volvulus* microfilaria-positive patients (mean IgG1 OD = 0.43; min 0.1; max 1.2) and clearly above ($p < 0.0001$) those OD values measured in postpatent and endemic NEGs (Fig. 4). The *O. volvulus*-specific IgG1 and IgG3 mean reactivity did not exceed OD values of 0.5 (Fig. 4), and as shown for the aforementioned isotypes IgG1 and IgG4, *O. volvulus* microfilaria-positive patients' IgG1 and IgG3 responses were above ($p < 0.0001$) those of patients with expired *O. volvulus* infection (postpatent) and NEG endemic controls.

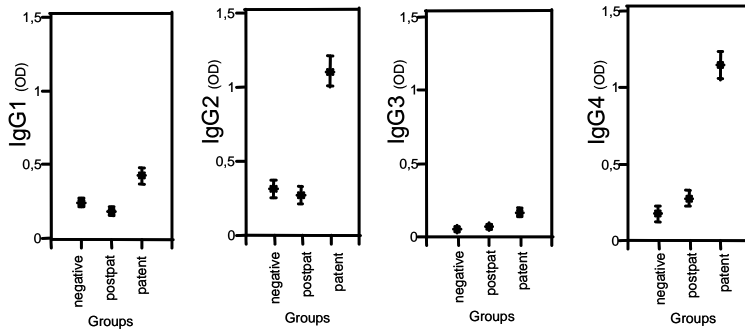

Fig. 4. *Onchocerca volvulus* antigen-specific IgG1, IgG2, IgG3 and IgG4 reactivity was determined in sera from *O. volvulus* microfilaria-positive onchocerciasis patients (patent, n = 14), in microfilariae-negative postpatent onchocerciasis patients (n = 51) originating from the Onchocerciasis Control Programme (OCP) core area in northern Togo in whom *O. volvulus* infection has expired without ivermectin treatment (postpatent), and in negative endemic controls (n = 49) originating from the Onchocerciasis Control Programme (OCP) core area in northern Togo in whom *O. volvulus* microfilariae were never found (negative). The mean Optical Densities (OD) with the 95% lower and upper confidence interval are shown. Wilcoxon rank sum tests: **$p < 0.0001$ compared with negative and postpatent groups.

DISCUSSION

Complete elimination of *O. volvulus* microfilaria (Mf) from the skin of onchocerciasis patients will require repeated ivermectin treatments. Such patients will harbour vivid adult *O. volvulus* without having Mf in their skin, i.e. an occult *O. volvulus* infection. In repeatedly with ivermectin treated patients, parasite-specific cellular immune responsiveness and Th1- and Th2-type cytokine production re-activated [13,15,29], and as observed in this study, with an expiring *O. volvulus* infection, serum levels of several pro-inflammatory chemokines gradually enhanced and approached the higher concentrations of Mf-negative controls. In contrast, regulatory chemokines and cytokines lessened and approached low infection-free control levels.

Each intervention with ivermectin will trigger immediate and acute phase immune responses, during which granulocytes and macrophages may act as the main effector cell population against filarial parasites [30,31]. Eosinophil granulocytes activated by e.g. IL-5 and Eotaxin may adhere and degranulate around larvae, thus creating sites of inflammation [32,33]. Shortly p.i.t. with ivermectin, Eotaxin-2/CCL24 and MCP-4/CCL13 enhanced and this may have promoted migration of eosinophils and macrophages into the patients' skin and triggered cellular killing of Mf. Both Eotaxin-2 and MCP-4 were elevated during allergic tissue reactions in human skin [34], and as previously observed, the gene expression of Eotaxin enhanced in dermal tissues of onchocerciasis patients after topical application of the microfilaricide diethylcarbamazine (DEC) [35]. Furthermore, severe inflammatory responses may occur in onchocerciasis patients after oral treatment with DEC [36]. At 3 days p.i.t., the chemokines MCP-1/CCL2, MCP-4/CCL13, MPIF-1/CCL23 and Eotaxin-2/CCL24

strongly enhanced, and such risen levels were not observed the following ivermectin treatments. Probably the remaining low numbers of dermal *O. volvulus* Mf did not activate eosinophils and macrophages similarly strong. Accumulation of macrophages, their activation and granuloma formation are central elements of the host immune defense against larvae and adult filaria [24,32]. MCP-1/CCL2 is required for the recruitment of macrophages during the acute phase of the anti-filarial immune response [24], and MCP-1 is pivotal for cellular recruitment into various tissues and inflammation [37]. MCP/CCL2 increased transiently at 3 days p.i.t., returned to the pre-treatment levels at month 6, and then gradually rose, however, without attaining the higher levels of NEGs and CTRLs. Similarly, the neutrophil activating protein 1 (NAP-1/IL-8/CXCL8) enhanced until 1 year post-initial treatment to levels as in NEGs. The steadily over time increasing MCP-1/CCL2 and NAP-1/IL-8/CXCL8 may have supported the recruitment of effector cells around microfilariae and adult filarial worms, thus preventing larval dissemination into dermal tissues. While indispensable for the recruitment of effector cells, an exceeding MCP-1/CCL2 production may contribute to disease manifestation and inflammation including asthma [38], experimental allergic encephalomyelitis (EAE) [39], pulmonary fibrosis [40], atherosclerosis [41] and schistosomiasis [42]. The increasing chemokine levels of MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, MPIF-1/CCL23 and NAP-1/IL-8/CXCL8 in *O. volvulus* postpatent and infection-free individuals may predispose them for inflammatory disorders; this was observed in school-age children in whom prevalence of allergen skin test reactivity and allergic symptoms increased after 15-17 years of repeated treatment with ivermectin [43].

The Th2 cytokines IL-4 and IL-13 will stimulate resident macrophages to differentiate into alternatively activated macrophages (aaMϕ) which have the capacity to down regulate inflammatory pathology associated with chronic filariasis [44,45]. The Th2-cytokine IL-13, which activates B-cells and enhances their IgG4 production, and also the levels of MIP-1δ/CCL15 and TARC/CCL17 [22] gradually decreased post ivermectin and these changes may be considered as indirect indicators for lessened numbers and decreased differentiation of macrophages activated alternatively [46]. Furthermore, the *O. volvulus*-specific IgG4 responses were clearly lower in postpatent and NEGs than with patent *O. volvulus* infection. IgG4 is a negative immune regulator for inflammatory responses [47], it will favour the generation of cellular hypo-responsiveness, and support asymptomatic and persisting helminth infections. Similarly, the MIP-1δ/CCL15 which promotes the maturation of eosinophils and the expression of eosinophil peroxidase (EPO) and major basic protein (MBP) [48] lessened after repeated ivermectin treatments to concentrations as observed in European CTRLs. The decreasing levels of IL-13, MIP-1δ/CCL15 and IgG4 with an expiring *O. volvulus* infection signify the dampening of a dominant Th2-immune responsiveness towards a mixed and balanced T helper cell profile as observed with postpatent infection and in controls.

The levels of MPIF-1/CCL23 and MCP-4/CCL13 first enhanced at 3 days p.i.t., both then gradually lessened during the first year p.i.t., and both did not attain to values as in postpatent patients and NEGs. MPIF-1/CCL23 is most closely related to MIP-1β/CCL4, both interact with receptor CCR1, MPIF-1/CCL23 production is induced by IL-4 and IL-13, and MPIF-1/CCL23-expressing cells were found at higher frequency in the epidermis of atopic dermatitis patients. MCP-4 was

suggested as a biomarker of asthma [49]. In onchocerciasis patients, the pre-treatment MCP-4/CCL13 levels exceeded those measured in patients with acute asthma exacerbation [49], and furthermore, MCP-4 rose above 1000 pg/m at 3 days p.i.t. and remained above 500 pg/ml at 4 and 7 years p.i.t. being much higher than in postpatent and controls. MCP-4/CCL13 is a chemoattractant for monocytes, eosinophils and basophils, and will also activate IFN-γ and IL-4 responses [50], and thus, MCP-4 will support and balance both Th1- and Th2-type cell responses. The higher MCP-4 levels in patients at 4 and 7 years p.i.t., as compared to postpatent cases and controls, may signify ongoing effector cell activation against the remaining adult and larval stages of *O. volvulus*, while low MCP-4 with postpatency may indicate that filarial infection has indeed expired. With postpatency, the diminished Th2-type chemoattractant TARC/CCL17 indicated that less Th2-type cells were attracted to and activated in dermal tissues [51], and this may contribute to lessened inflammatory skin reactions in onchocerciasis patients, i.e. papular rash and onchodermatitis.
In summary, with an expiring O. volvulus infection several chemokines and cytokines progressed to higher levels (IL-8, MCP-1, MIP-1α, MIP-1β, MPIF-1) or declined (IL-13, MCP-4, MIP-1d, TARC) to concentrations as measured in NEGs and CTRLs. Post-initial ivermectin treatment, the transiently risen chemokines Eotaxin-2, MCP-1, MCP-4, MPIF-1 may have attracted and activated effector cells which cleared Mf from the skin of patients. The gradually increasing levels of proinflammatory chemokines MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, MPIF-1/CCL23 and NAP-1/IL-8/CXCL8 promote monocyte and granulocyte effector function, but such enhanced cellular activation

may also predispose for allergic and inflammatory hyperresponsiveness.

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Parasite-Specific IL-17-Type Cytokine Responses and Soluble IL-17 Receptor Levels in Alveolar Echinococcosis Patients

Christian J. Lechner[1], Beate Grüner[2], Xiangsheng Huang[1], Wolfgang H. Hoffmann[1], Peter Kern[2], and Peter T. Soboslay[1]

[1] Institute for Tropical Medicine, University of Tübingen Clinics, 72074 Tübingen, Germany
[2] Division of Infectious Diseases and Clinical Immunology, Comprehensive Infectious Disease Center, University of Ulm Hospitals, 89081 Ulm, Germany

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ABSTRACT

Alveolar Echinococcosis (AE) caused by the cestode *Echinococcus multilocularis*, is a severe helminth infection of man, where unrestricted parasite growth will ultimately result in organ failure and fatality. The tissue-infiltrative growth of the larval metacestode and the limited efficacy of available drugs complicate successful intervention in AE; patients often need lifelong medication, and if possible, surgical resection of affected tissues and organs. Resistance to AE has been reported, but the determinants which confer protection are not known. In this study, we analyzed in patients at distinct stages of Alveolar Echinococcosis, that is cured, stable and progressive AE, as well as in infection-free controls, the cellular production and plasma levels of pro-inflammatory cytokines IL-17A, IL-17B, IL-17F and their soluble receptors IL-17RA (sIL-17RA) and IL-17RB (sIL-17RB). Significantly elevated levels of IL-17B and sIL-17RB were observed, whilst IL-17F and IL-17RA were reduced in patients with AE. Similarly, the cellular production of IL-17F and sIL-L7RA in response to *E. multilocularis* antigens was low in AE patients, while levels of sIL-17RB were highly enhanced. These observations suggest immune-modulating properties of *E. multilocularis* on IL-17 cytokine-mediated pro-inflammatory immune responses; this may facilitate the tissue infiltrative growth of the parasite and its persistence in the human host.

INTRODUCTION

Alveolar Echinococcosis (AE) of man can develop following the ingestion of eggs of *Echinococcus multilocularis.* Egg-hatched larvae will migrate into various host tissues, mainly liver, and their proliferative and tissue-infiltrative growth as metacestode larvae will cause damage and ultimately organ failure. Dissemination of cells of metacestode larvae may initiate metastasis-like parasite growth in secondary organs such as lungs and CNS which impairs surgical resection [1]. Since current chemotherapy with imidazoles is only parasitostatic, AE cases with inoperable parasite lesions require life-long medication [2].
In some cases of human AE, a spontaneous healing of the disease was observed [1, 3]. Such abortive cases are characterized by calcified parasite lesions suggesting the generation of immune responses which are able to limit parasite growth in humans [4]. Previous studies have shown that Th1- and Th2-type immune responses might be important for clearance of the infection and are associated with the chronic and progressive course of disease [4]; however, knowledge about the crucial determinants which limit parasite growth and disease progression remains scarce. IL-5 is the predominant cytokine expressed by PBMCs in AE patients [5], and Th2-type IL-3, IL-5, and IL-10 were enhanced in severely ill AE patients [6–8] while *E. multilocularis* antigen-induced IFN-γ and spontaneous IL-12 production were decreased [9, 10]. Most important, IL-12 and IFN-γ inhibited larval growth and metacestode dissemination in *E. multilocularis*-infected mice [11, 12], while the application of IFN-γ stopped disease progression in an AE patient [13].
Th1- and Th2-type immune responses in AE have extensively been studied, but pro-inflammatory and

regulatory chemokines as well as Th17-type cytokines have received less attention. Immune responses against metacestode larvae of *E. multilocularis* will create persistent sites of inflammation and the formation of peri-parasite granulomas. The chemokines CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES were highly elevated in AE patients [14], while mononuclear cells isolated from peri-parasite host granulomas secreted high amount of IL-10 and low amounts of IFN-γ disclosing an immune regulation which will counteract inflammatory responses in AE patients [15].

The role of IL-17 cytokines and Th17-type immune responses in AE disease is yet unexplored. The six family members identified (IL17A-F) exert mostly proinflammatory activities [16]. IL17A and IL17F, mediators of the recently described proinflammatory Th17-type immune responses, have been associated with inflammatory disorders like rheumatoid arthritis and inflammatory bowel disease [17, 18] but also with protection against extracellular bacteria and fungi [19, 20]. We analyzed levels of proinflammatory IL-17 members (IL-17A, IL-17B and IL-17F) as well as their soluble common receptors (IL-17RA and IL-17RB) in clinically staged AE patients, that is, cured, stable, and progressive AE, and in infection-free controls. The altered concentrations of IL-17B, Th17-type cytokine IL-17F, and their soluble receptors at distinct stages of AE disease suggest that these pro-inflammatory cytokines may contribute to the clinical outcome of *E. multilocularis* infection.

MATERIALS AND METHODS

2.1. Study Groups

The patient cohort consisted of 93 patients (58 females/35 males) diagnosed with Alveolar Echinococcosis at University of Ulm Clinics/Germany. The AE patients' mean age was 57 years, ranging from 17 to 83 years. Blood samples from 12 AE-free individuals from the Blood Transfusion Centre at University Clinics Tübingen served as controls. The UKT Tübingen and University of Ulm Clinics are situated in the federal state of Baden-Wurttemberg of Germany, a region endemic for *E. multilocularis* infections. In the AE patient groups, 23 cases were diagnosed with cured, 64 with stable, and 6 with progressive AE. The classification of AE patients in different clinical stages of AE was accomplished according to the World Health Organization- (WHO-) PNM (P = parasitic mass in the liver, N = involvement of neighboring organs, and *M* = metastasis) system previously published by Kern and coworkers [21]. Curative resection, stable disease, progressive disease, or presence of an apparently dead, fully calcified lesion was established by magnetic resonance imaging on the basis of lesion size and morphology at the respective follow-up intervals. This classification has been used for follow-up studies of AE patients [22, 23]. Written consent was obtained from all participating patients, and this study was approved by the Ethics Review Board at University of Ulm Clinics (Ethik-Kommissions Antrag number 71/2004).

2.2. In Vitro Culture of Echinococcus Multilocularis Metacestodes

E. multilocularis metacestodes were cultivated at 37℃, 5% CO_2 and saturated humidity as previously described [14]. For the generation of single-cell lines, *in vitro*

maintained *E. multilocularis* metacestode tissue blocks were cut into small pieces and cultured in RPMI 1640 supplemented with 5% FCS and 1% antibiotic-antimycotic solution (PAA, Cölbe, Germany) in cell tissue culture flasks at 37%, 5% CO_2, and saturated humidity. After 3 days cell culture flasks were washed with RPMI supplemented with antibiotics (as above) to obtain flask surface-adherent *E. multilocularis* derived cells. Adherent *E. multilocularis* single-cells (EmZ) were grown as above and flask cultures were split when cell overgrowth was observed. Cells were harvested, centrifuged, and stored at –80◦C for further use.

2.3. Antigen Preparation

The preparation of *E. multilocularis* metacestode and *Ascaris lumbricoides* antigens was performed as described previously [14]. Briefly, *E. multilocularis* metacestode tissues or adult *A. lumbricoides* were homogenized using a Ten Broek tissue grinder and subsequently ultrasonified (30% intensity, pulse 1 second for 8 minutes). The *Echinococcus* metacestode or *Ascaris* adult worm suspensions were then centrifuged at 4℃, sterile filtered (0.22 µm) and kept at –20℃. For *E. multilocularis* vesicle antigen preparation, entire *E. multilocularis* vesicles were collected, separated from *in vitro* culture medium, and vesicles were ruptured by sonication pulses (30% intensity, pulse 1 s for 1 min). Such disrupted vesicles were then homogenized, that is, grinded with a Ten-Broek tissue grinder on ice until a homogenous liquid extract was produced, then sonicated again (30% intensity, pulse 1 s for 8min) and thereafter the vesicle homogenate was centrifuged at 5000 g for 30 min at 4℃. The supern atant was sterile filtrated (0.22 µm) and stored at –70℃. For single-cell *E. multilocularis* antigen preparation, *in vitro* grown adherent *E.multilocularis* single-cells were

detached from the culture flask surface, and were collected and separated from *in vitro* culture medium by centrifugation (1.500 g for 5 minutes). The cell pellet was homogenized, that is, grinded with a Ten-Broek tissue grinder on ice until a homogenous liquid extract was produced, then sonicated (30% intensity, pulse 1 s for 8min) and thereafter the cell homogenate was centrifuged at 5000 g for 30 min at 4℃. The supern atant was sterile filtrated (0.22 μm) and stored at −70◦C. Protein concentrations were determined by bicinchoninic acid (BCA) protein determination (Pierce, Rockford, IL, USA). *Entamoeba histolytica* antigen (EhAg) was a kind gift of B. Walderich (Institute for Tropical Medicine, Tübingen, Germany).

2.4. Isolation of Peripheral Blood Mononuclear Cells (PBMC)

PBMC and plasma from AE patients and control individuals were isolated by means of Ficoll density gradient centrifugation as described previously [24]. PBMC were adjusted to a concentration of 2×10^6 cells per mL and dispersed into 48 well tissue culture plates with 0.5mL per well in RPMI 1640 supplemented with 5% FCS and 1% antibiotic-antimycotic solution. PBMC were stimulated for 24 and 48 hours with either 5 μL *E. multilocularis* metacestode antigen (Em, stock concentration 60 μg/mL), *E.multilocularis* single-cell antigen (EmZ, stock concentration 60 μg/mL), *E. multilocularis* vesicle antigen (EmV, stock concentration 60 μg/mL), *Ascaris* antigen (Asc, stock concentration 3.7 μg/mL), or *E. histolytica* antigen (Eh, stock concentration 100 μg/mL) or left unstimulated (base) at 37℃, saturated humidity, and 5% CO_2. Cells and cell culture supernatant were harvested, separated by centrifugation, and stored at −80℃.

2.5. Determination of Cytokine and Chemokine Concentrations

Cell culture supernatants and plasma were stored at –80℃ before use. Cytokine and chemokine concentrations were determined by sandwich enzyme-linked immunosorbent assay (ELISA) kits for IL-17A, IL-17B, IL-17F, IL-17RA, and IL-17RB (R&D Systems, MN, USA). The assays were performed according to the manufacturers' guidelines. Conversion of optical densities (OD) to final concentrations (pg/mL) was calculated by using cytokine-specific standard curves.

2.6. Data Analysis and Statistics

The statistical package JMP 9.0 (SAS Institute, Heidelberg, Germany) was used for statistical analyses. Significant differences of cytokine and chemokine concentrations were determined by analysis of variance (ANOVA) and Tukey's test. Due to multiple comparisons the level of significance was adjusted by the Bonferroni-Holm method.

RESULTS

3.1. Plasma Levels of Proinflammatory IL-17 Family Members and Soluble Receptor Components in AE Patients and Infection-Free Controls

Plasma concentrations of proinflammatory IL-17 family members IL-17A, IL-17B, IL-17F and their common soluble receptor subunits, sIL-17RA and sIL-17RB, were quantified in AE patients with different states of disease and in infection-free controls.

The levels of IL-17B were lowest in healthy controls and were significantly increased in all AE patient groups ($P < 0.01$ and $P < 0.001$) (Figure 1(a)). Within the AE patient group, lowest concentrations of IL-17B were detected in cured cases of AE, while highest concentrations were observed in progressive cases. Soluble IL-17RB levels were lowest in non-infected controls and highly elevated in all AE patient groups, while IL-17RB did not differ between patient groups (Figure 1(b)). Similar plasma concentrations of IL-17A were observed within AE patient groups and infection-free controls (Figure 2(a)).

In contrast, the concentrations of IL-17F, and its soluble receptor IL-17RA were the highest in infection-free controls. While plasma levels of IL-17F were significantly reduced in stable and progressive cases of AE ($P < 0.05$), significantly decreased levels of soluble IL-17RA concentrations were detected in all AE patient groups ($P < 0.01$ and $P < 0.001$) (Figures 2(b) and 2(c)).

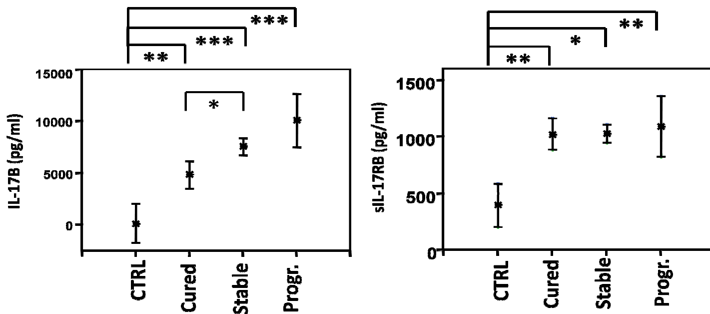

Figure 1: Plasma concentrations of interleukin (IL)-17B (part a) and soluble receptor IL-17RB (part b) in Alveolar Echinococcosis (AE) patients and in infection-free controls (CTRL). The patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) Alveolar Echinococcosis. The plasma concentrations are shown as the mean values in pg/mL with the 95% upper and lower confidence interval. The level of significance was adjusted by the Bonferroni-Holm method. Significant differences between the groups are indicated (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

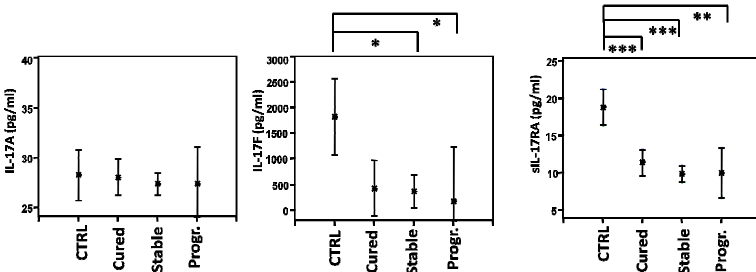

Figure 2: Plasma concentrations of interleukin (IL)-17A (part a) and IL-17F (part b) and of soluble IL-17RA (part c) in Alveolar Echinococcosis (AE) patients and infection-free controls (CTRL). Patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) Alveolar Echinococcosis. The plasma concentrations are shown as the mean values in pg/ml with the 95% upper and lower confidence interval. The level of significance was adjusted by the Bonferroni-Holm method. Significant differences between the groups are indicated (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

3.2. Echinococcus Multilocularis Antigen-(EmAg-) Induced Cellular Production of Soluble IL-17RA from AE Patients and Controls

The production of soluble IL-17RA, sIL-17RB, and IL-17F by peripheral blood mononuclear cells (PBMC) was investigated in AE patients and controls. Stimulation of PBMC with *Echinococcus multilocularis* vesicle (EmV) antigen for 24 hours did not result in cellular production differences of soluble IL-17RA between AE patients and infection-free control groups (Figure 3(a)). In addition, no differences within the AE patient groups could be observed. After 48 hours of stimulation a decreased cellular production of sIL-17RA by PBMC from all AE patient groups was observed, with production levels in healthy controls and stable AE cases being significantly different ($P < 0.01$) (Figure 3(b)). Production of cytokines and soluble receptors levels in response to *Ascaris lumbricoides* (AscAg) or to *Entamoeba histolytica* (EhAg) antigens did not differ between AE patient groups and controls.

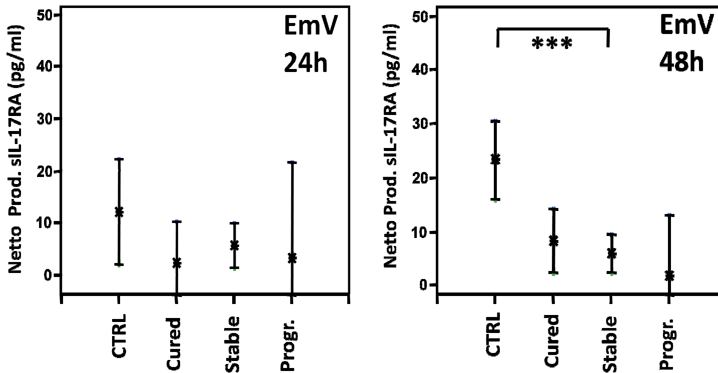

Figure 3: *Echinococcus multilocularis* antigen induced cellular production of soluble interleukin-17 receptor A (sIL-17RA) by peripheral blood mononuclear cells (PBMCs) from Alveolar Echinococcosis (AE) patients and infection-free controls (CTRL). Patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) Alveolar Echinococcosis. PBMCs from patients and controls were stimulated with *E. multilocularis* vesicle extract (EmV) for 24 (part a) and 48 hours (part b) or left without stimulation. Cytokine concentrations in cell culture supernatant were quantified by specific ELISA. The EmAg-induced cytokine production (Netto Prod.) was calculated by subtracting the cytokine production in not stimulated PBMC cultures from EmV-stimulated cytokine production. The cytokine production is shown as mean values in pg/mL with the 95% upper and lower confidence interval. The level of significance was adjusted by the Bonferroni-Holm method. The significant differences between groups are indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3.3. EmAg-Induced Cellular Production of Soluble IL-17RB from AE Patients and Controls

PBMC from progressive cases of AE produced high amounts of sIL-17RB following 24 and 48 hour stimulation with *Echinococcus multilocularis* vesicle (EmV) and single-cell (EmZ) extract (Figures 4(a) and 4(b)). The production difference between PBMC from progressive cases and the other groups was more prominent after 24 hours of stimulation than after 48 hours. The cellular production levels of sIL-17RB from infection-free controls and cured and stable cases did not differ in response to EmV or EmZ stimulation. Vesicle components, that is, parts of the laminated and germinal layer, but also hydatid fluid, which constitutes the largest volume of vesicles, may have conferred the observed effects on sIL-17RB production. The single-cell line extract (EmZ) may contain pro-inflammatory components from the inner germinal layer of the metacestode.

Figure 4A

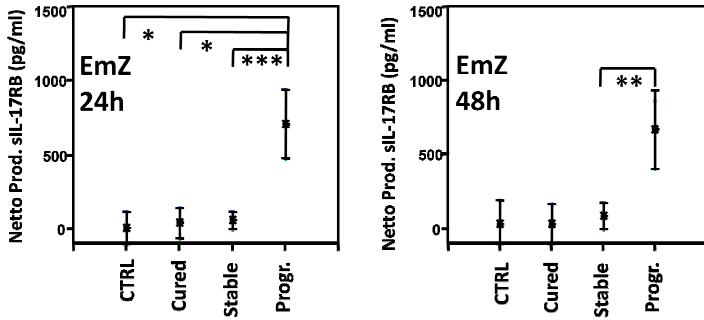

Figure 4B

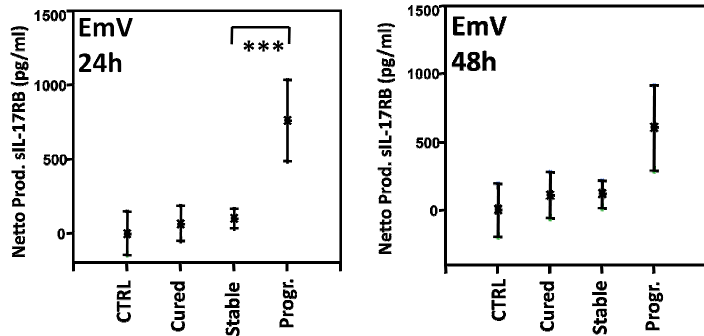

Figure 4: *Echinococcus multilocularis* antigen induced cellular production of soluble interleukin-17 receptor B (sIL-17RB) by PBMC from Alveolar Echinococcosis (AE) patients and infection-free controls (CTRL). Patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) Alveolar Echinococcosis. PBMCs from patients and controls were stimulated with *Echinococcus multilocularis* vesicle extract (EmV) (a) and single-cell extract (EmZ) (b) for 24 and 48 hours or left unstimulated. Cytokine concentrations in cell culture supernatant were determined by specific ELISA. The EmAg-induced cytokine production (Netto Prod.) was calculated by subtracting the cytokine production in not stimulated PBMC (Baseline) cultures from EmV-stimulated cytokine production (Brutto production). The cytokine production is shown as mean values in pg/mL with the 95% upper and lower confidence interval. The level of significance was adjusted by the Bonferroni-Holm method. The significant differences between groups are indicated (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

3.4. EmAg-Induced Cellular Production of IL-17F in AE Patients and Controls

Cellular production of IL-17F in response to *Echinococcus multilocularis* vesicle extract (EmV) for 24 and 48 hours was the highest in the control group (Figure 5). Compared to healthy controls, PBMC from all AE patient groups produced significant lower amounts of IL-17F in response to antigen stimulation ($P < 0.05$ and $P < 0.001$) (Figure 5). Stimulation of PBMC with EmV resulted in a similar IL-17F production in the three clinical AE patient groups (Figure 5). The IL-17F production by PBMC in response to *E. multilocularis* single-cell extract showed no difference between the studied groups (data not shown).

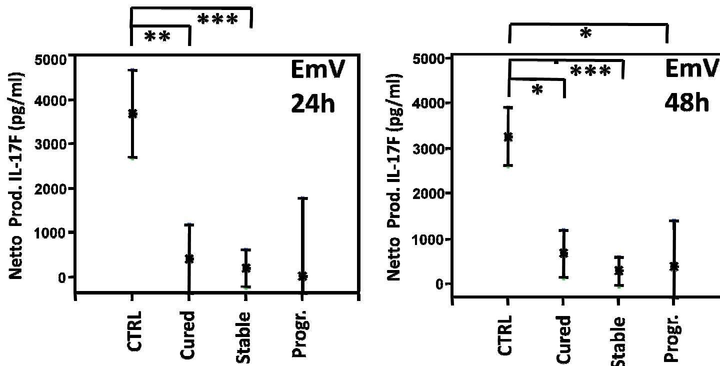

Figure 5: *Echinococcus multilocularis* antigen- (EmAg-) induced cellular production of interleukin-17F (IL-17F) by PBMC from Alveolar Echinococcosis (AE) patients and infection-free controls (CTRL). Patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) Alveolar Echinococcosis. PBMC from patients and controls were stimulated with *E. multilocularis* vesicle extract (EmV) for 24 (part a) and 48 hours (part b) or left unstimulated. Cytokine concentrations in cell culture supernatant were determined by specific ELISA. The EmAg-induced cytokine production (Netto Prod.) was calculated by subtracting the cytokine production in not stimulated PBMC cultures (baseline) from EmV-stimulated cytokine production (Brutto production). The cytokine production is shown as mean values in pg/mL with the 95% upper and lower confidence interval. The level of significance was adjusted by the Bonferroni-Holm method. The significant differences between groups are indicated (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

DISCUSSION

Spontaneously healed Alveolar Echinococcosis has been observed [3], and previous works indicate that Th2-type immune responses in AE are associated with progressive AE while proinflammatory Th1-type cytokines are important in protection and disease regression. Therefore, we focused on pro-inflammatory cytokines of the IL-17 family yet uncharacterized in AE and searched whether these immune mediators of inflammation and their receptors associated with progression or regression of AE. In the present study, pro-inflammatory IL-17 cytokine family members and their common receptors disclosed divergent cellular production profiles and plasma levels in AE patient groups and controls; Th17-type IL-17A levels were similar in patients with progressive, stable, and healed *E. multilocularis* metacestode lesions, IL-17B enhanced in AE patients, whilst the Th17-type IL-17F production was highest in controls and depressed in all AE patient groups. Such diverse and opposing cytokine profiles revealed distinct dynamics for each member of the IL-17 family during progression or regression of AE.
While Th17 immune responses may confer protection against infections with bacteria and fungi [25, 26], they may also initiate inflammatory responses which promote immune disorders like inflammatory bowel disease and rheumatoid arthritis [17, 18]. The IL-17A and IL-17F cytokines are best characterized, highly homologous, and were initially allocated similar characteristics. Recent findings, however, disclosed that IL-17F is dispensable for immune disorders like collagen-induced arthritis and experimental autoimmune encephalitis, while effective protection against *Staphylococcus aureus* and *Citrobacter rodentium* infections was dependent on the presence of both IL-17A and IL-17F activity [26]. IL-17F

is rather associated with protection while IL-17A seems to contribute equally to both protection and inflammatory disorders [26, 27].

In AE patients, irrespective of their stage of infection, IL-17F levels were depressed, and such cellular unresponsiveness and depressed cytokine production to *E. multilocularis* metacestode antigens have previously been observed [5, 6]. Furthermore, depressed IL-17F plasma and production levels persisted irrespective whether AE was cured, stable or progressive, suggesting continuing immune responses against residual parasite products. IL-17A and IL-17F are potent inducers of chemotaxis and inflammation, and both can be induced in PBMC by TGF-β, IL-6, and IL-21 secreted from antigen presenting cells (APC) [28, 29]. Both trigger proinflammatory responses by the release of neutrophil activating chemokine CXCL8/IL-8 [16] and of proinflammatory cytokines like TNF-α, IL-6 and IL-1β [30]. Suppression of Th17 immune responses has been demonstrated in infection models, where *Fasciola hepatica*-infected mice had a decreased production of IL-17 [31] and *Schistosoma mansoni*-infected mice with an elevated IL-17 production presented with a reduced adult worm burden [32]. The IL-17 receptor family consists of five dimer-forming subunits (IL-17RA to IL-17RE). IL-17A and IL-17F share the same receptor subunit IL-17RA [27] and IL-17RA is expressed ubiquitously by all cell types [30]. Plasma concentrations as well as the *E. multilocularis* antigen-induced cellular release of soluble IL-17RA were low in patients irrespective of their stage of AE and this paralleled the low plasma levels and the non-inducible cellular release of IL-17A by PBMC from AE patients (data not shown). The observed lessened EmAg-specific IL-17F and sIL-17RA levels in AE patients indicate a parasite-induced unresponsiveness occurring with active *E. multilocularis* infection and such cellular

anergy may facilitate survival of the parasite in its host. Parasite antigen-specific cellular anergy was similarly observed in filariasis or schistosomiasis patients, where patent infection, that is, with circulating microfilariae in filariasis or egg excretion in schistosomiasis patient, associated with cellular hyporeactivity to parasite-specific antigens; often the patients' cellular responses were lower than observed in endemic controls [33, 34].

Up to date, little is known about the biological properties of IL-17B. Its expression has been found in the monocyte-derived cell line THP-1, chondrocytes, and neurons [35–37], and IL-17B mRNA was detected in cells of the gastrointestinal tract, including stomach, pancreas, and small intestine [35]. IL-17B signals through binding to a homodimeric IL- 17RB complex and induces upon binding the release of proinflammatory TNF-α, IL-1β, and IL-6, CXCL8/IL-8 [36], and the migration of neutrophils to the peritoneal cavity in rats [38]. Protective properties of IL-17B in disease have not yet been reported. In AE patients, plasma concentrations of IL- 17B and its soluble receptor IL-17RB were strongly elevated and highest in those with progressive AE. The persistent exposure to growing *E. multilocularis* metacestodes may have triggered the release of IL-17B by cells of the gastrointestinal tract, leading to the recruitment of neutrophil granulocytes into peri-parasite lesions. While the effects of IL-17B are similar to those mediated by TNF-α, IL-17A, and IL-1β, its potency is limited [39]. The IL-17B-induced infiltration of neutrophils into the peritoneal cavity in rats required much higher concentrations compared to TNF-α and it was still considerably less effective than the cell migration induced by IL-17A [38]. The elevated IL-17B production in AE patients disclosed a proinflammatory response triggered by *E. multilocularis* antigens, but potentially not strong enough to limit the progressive parasite growth.

The decreasing concentration of IL-17B with an cured AE should be further evaluated as a prognostic marker in AE.
IL-17RB serves as receptor subunit for IL-17B and IL-17E [40], commonly expressed by cells of the intestine, but also by liver, pancreas, lung, and kidneys as well as on Th2 and Th9 cells [27, 30, 40, 41]. Plasma concentrations of soluble IL-17RB were highly elevated in AE with no significant differences between the patient groups, while PBMC from progressive AE cases produced high amounts of soluble IL-17RB in response to *E. multilocularis* antigens. While vesicle extracts will contain large amount of vesicle fluid, the single-cell extract will most likely be derived from the inner germinal layer of the metacestode, and therefore, the heightened proinflammatory IL-17 responses in patients with progressive AE may primarily be induced by vesicle fluid components and germinal cells and engaging the IL- 17RB activation pathway. Membrane bound and soluble IL-17RB are inducible in human antigen-presenting cells (APC) upon stimulation with Th2-type cytokines IL-4, IL- 10, IL-13 and TGF-*β* [42], and these cytokines are associated with progressive AE [5, 6, 8, 43, 44]. PBMC from AE patients produced elevated levels of Th2 cytokines IL-4, IL- 5, and IL-10 upon stimulation with crude *E. multilocularis* antigen [5]. Thus, the cellular production of sIL-17RB in AE patients in response to *E. multilocularis* antigen could result from an EmAg-induced production of Th2 cytokines, which subsequently triggered the release of sIL-17RB. The higher concentrations of sIL-17RB in patients may be a direct consequence of this Th2 polarization associated with chronic AE. The biological functions and importance of soluble IL-17 receptors in AE remain tentative; the soluble IL-17RB could act as decoy receptor for IL-17B. Previous studies have shown that the administration of

the soluble IL-4 receptor inhibited IL-4-mediated immune responses [45], while soluble IL-6 receptor amplified the effects of IL- 6 [46]. High amounts of circulating IL-17RB, as observed with progressive AE, could counteract the effects of IL-17B, thus silencing IL-17B-mediated pro-inflammatory responses in patients. Similar to IL-17F, the high amounts of soluble IL-17RB observed in plasma of patients with cured AE might indicate long-lasting effects of residual or inactive parasite lesions, whilst low levels of circulating soluble IL- 17RA together with low IL-17F production may reduce Th17 cytokine-mediated inflammation.

CONCLUSION

In summary, the present work discloses a modulation of proinflammatory IL-17 family members and Th17-type immune responses in AE; the persistently altered production of IL-17 cytokine family members and of their soluble receptors highlights the capacity of the *E. multilocularis* metacestode to exert long-lasting immune modulating effects, and further studies should address the protective and preventive potential of IL-17 cytokines during *E. multilocularis* infection.

CONFLICT OF INTERESTS

The authors have declared that they have no conflict of interest.

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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

Christian J Lechner[1], Karl Komander[1], Jana Hegewald[1], Xiangsheng Huang[1], Richard G Gantin[1,2], Peter T Soboslay[1,2], Abram Agossou[3], Meba Banla[4] and Carsten Köhler[1]

1Institute for Tropical Medicine, Eberhard Karls Universität Tübingen, Wilhelmstrasse 27, Tübingen 72074, Germany.
2Onchocerciasis Reference Laboratory, Sokodé, Togo.
3Centre Hospitalier Régional, Service Pédiatrie, Sokodé, Togo.
4Centre Hospitalier Universitaire Campus de Lomé, Université de Lomé, Lomé, Togo

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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.

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 effecter cells in parasite-invaded tissues and cells. Monocyte chemoattractive proteins such as MCP-4 recruit effecter 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 effecter 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. 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.

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

Cellular chemokine release to helminth and protozoan 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).

Table 2. The cellular production of interleukin (IL)-19, IL-27, IL-33, MCP-4/CC13, 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; 1x10^8 schizonts/ml) or not stimulated (Base), for 48 hours at 37%, 5% CO_2 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).

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)

Cellular chemokine release to helminth and protozoan 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, 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).

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

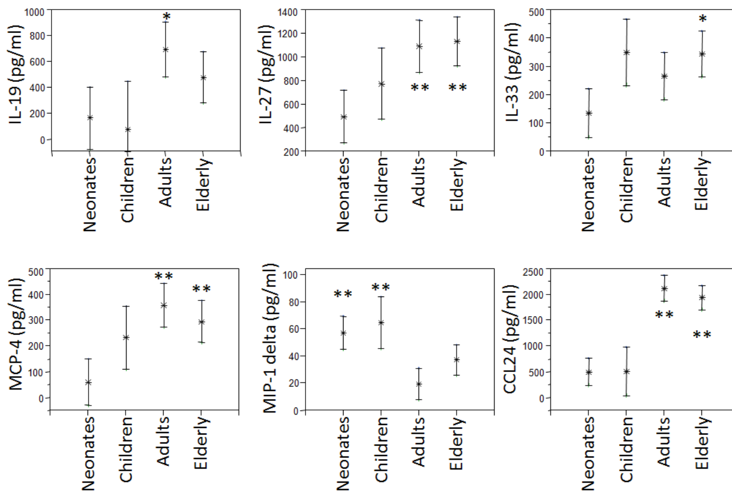

Figure 1B *O. volvulus* antigen-induced cellular production of IL-19, IL-27, IL-33, MCP-4, MIP-1delta and Eotaxin-2 from different age groups

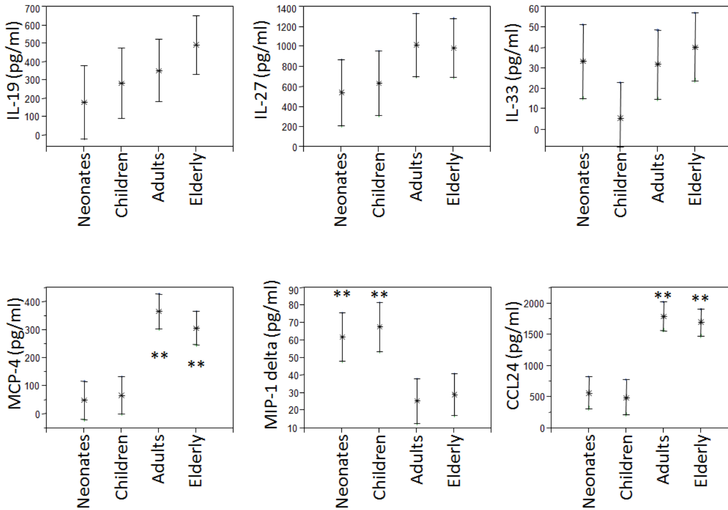

Figure 1. Cellular production of interleukin (IL)-19, IL-27, IL-33 MCP-4/CCL13, MIP-1delta/CCL15 and Eotaxin-2/CCL24 in response to *A. lumbricoides* (Asc) and *O. volvulus* (Ov) antigen by umbilical cord blood cells (UCBC) and peripheral blood mononuclear cells (PBMC) from different age groups is shown.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

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.

Table 3, 4. 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) (Table 3) or *D. farinae* (20 µg/ml) and D. pteronyssinus (20 ug/ml) (Table 4) for 48 hours at 37%, 5% CO_2 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.

Table 3

	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.

Table 4

	IL-19	IL-27	IL-33	MCP-4	CCL-24	MIP-1 delta
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

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 effecter cells may then adhere and attack tissue-infiltrating and migrating 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.
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 proinflammatory 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.

MATERIALS 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 Tchaoudjo). Adults were from the village of Bouzalo, near the city of Sokodé. Written consent was obtained from the childrens' 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 stuff during prenatal consultations at the CHR. Pregnant women received anti-parasite treatment in line with the national health guidelines of Togo both during prenatal consultations (PC) and after partition. All pregnant women received anti-malaria 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 1×10^6 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 1×106/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^2 at 37℃ and saturated humidity in the presence or abse nce (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. 1×10^8 schizonts/ml), *Dermatophagoides pteronyssinus* (Dp, final conc. 20 µg/ml), *Dermatophagoides farinae* (Df, final conc. 20 ug/ml), *Aspergillus fumigatus* (Af, final conc. 20 µg/ml), *Candida albicans* (Ca, final conc. 20 µg/ml), for 48 hours at 37℃elsius, 5% CO_2 and saturated humidity. Cells and cell culture supernatant were then harvested and stored at -20 Celsius 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℃. 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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Curriculum Vitae

Date of Birth: 22. January 1982
Nationality: German

Educational career:

1988-1992: Grundschule Kaltental
1992-2001: Fanny-Leicht-Gymnasium Stuttgart-Vaihingen

Civil Service:

2001/2002: Arbeiter-Samariter-Bund Stuttgart

Academic career:

Oct. 2002 – Sep. 2004	Studies in Biology Undergraduate Studies at the University of Hohenheim
Oct. 2004 – Feb. 2009	Studies in Biology Graduate Studies at the Eberhard-Karls-University, Tübingen Major subject: Cell Biology/Immunology Minor subjects: Parasitology and Virology

Feb. 2007 – June 2007	Exchange Semester at the University of Cape Town, South Africa.
Apr. 2008 – Feb. 2009	Diploma Thesis at the University Clinics of Tübingen, Medical Clinic II, Department of Hematology, Oncology and Immunology. Subject: "Untersuchung immunmodulatorischer Effekte der Tyrosinkinase- Inhibitoren Imatinib, Nilotinib und Dasatinib auf Dendritische Zellen."
Apr. 2010 - Nov. 2013	PhD at the University Clinics of Tübingen, Institute for Tropical Medicine.
Nov.-Dec. 2010 Feb.-Apr. 2011 Nov.-Dec. 2011 March - May 2012 Feb.- March 2013	Research stay in Togo/West Africa.

Honors:

Young Researcher Award in the category 'Diagnosis in Humans' at the XXIV. World Congress of Hydatidology in Urumqi, China.

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