Immune Modulators with Parasite Infections

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von

Xiangsheng Huang
aus Shandong, China

Tübingen
2014

Tag der mündlichen Qualifikation: 06.06.2014

Dekan: Prof. Dr. Wolfgang Rosenstiel

1. Berichterstatter: Prof. Dr. Peter Soboslay

2. Berichterstatter: Prof. Dr. Nikolaus Blin

Table of Content

Zusammenfassung **1**

Summary **4**

Published Manuscripts **8**

Introduction **10**

Innate and adaptive immune responses 10

Cytokines and chemokines 13

The immune reactions in parasite diseases 16

AIM of this study 19

Results and Discussion **21**

The *IL2* and *IL2R alpha* polymorphism in Gabonese populations 21

Distinctive immune reactivity in Alveolar Echinococcosis 24

References **32**

Publications **51**

Curriculum Vitae **146**

Acknowledgements **148**

Zusammenfassung

Parasiten können ihrer Eliminierung entgehen und aus der Manipulation des wirtseigenen Immunsystems Nutzen für ihr eigenes Überleben ziehen. Sie sind in der Lage, regulatorische T-Zell (Treg)-Populationen zu induzieren, welche Ausmaß und Ausprägung von Effektor-T-Zell-Funktionen modulieren können. Dies kann zu ineffizienten Immunantworten bei Infektionen beitragen. Nukleotidveränderungen in der Promoter Region von Genen die Immunantworten regulieren, können nicht nur zu einer veränderten Genexpression führen, sondern dies kann sich auch in einer veränderten Empfänglichkeit für parasitäre Infektionen und in Veränderungen der immun regulatorisch wirkenden T-Zellen (Treg) widerspiegeln. In der vorliegenden Arbeit konnten zwei bekannte Einzelnukleotid-Polymorphismen (*Single Nucleotide Polymorphism, SNP*) (*rs2069762T/G* und *rs2067006T/G*) sowie eine bis dahin unbekannte CT Deletion (*#ss410961576*) an der Position -83/-84 der *IL2* Promoter Region bei allen 40 untersuchten, nicht miteinander verwandten gabunesischen Individuen identifiziert werden. Keines der untersuchten SNP Konstrukte wies im Vergleich zum Hauptallel (*rs2067006T/A*) eine veränderte Luciferase Aktivität *in vitro* auf, was möglicherweise mit der auf Tregs beschränkten Expression von FOXP3 zusammenhängt, welche zu einer leichte Veränderung der Effektor-T-Zell-Funktion führen könnte. Im Bezug auf *IL-2R alpha* (CD25) konnten zwei bereits bekannte Varianten (*rs12722617C/T* und *rs12722616C/T*) sowie eine bis dahin unbekannte C/T Variante (*#ss410961577*) an Position -409 der Promoter Region beschrieben

werden. Die SNP's *rs12722617C/T* und *rs12722616C/T* konnten bei allen untersuchten Individuen nachgewiesen werden und zeigten nach Stimulation keine Unterschiede zum Hauptallel. Die neu identifizierte Variante *#ss410961577* hingegen wies im Vergleich zum Hauptallel eine signifikant reduzierte Aktivität auf. Ein Vergleich mit der NCBI HapMap Datenbank ergab übereinstimmende Verteilungen der untersuchten SNP Varianten für *IL2* und *IL2R* alpha mit denen der Yoruba Population, welche eine ethnische Gruppe aus Subsahara Afrika darstellt. Die identifizierten regulatorischer SNP's könnten für weiterführende Untersuchungen zur Krankheitsanfälligkeit oder zur besseren Charakterisierung unterschiedlicher physiologischer Antworten verwendet werden.

Unterschiedliche Ausprägungen von wirtseigenen Immunantworten können zur Beseitigung einer Infektion beitragen, aber auch die Persistenz von Parasiten im Wirt und somit einen chronischen Infektionsverlauf fördern. In der vorliegenden Arbeit wurden Immunreaktivität und regulatorische Immunprozesse im Rahmen der Alveolären Echinokokkose (AE) untersucht. Klar unterscheidbare zelluläre und humorale Immunantwortprofile konnten hierbei bei Patienten mit unterschiedlichen Verlaufsformen der AE beobachtet werden. AE Patienten mit stabiler und progressiver Verlaufsform wiesen erhöhte Plasmakonzentrationen von IL-17B und seinem löslichen Rezeptor IL-17RB auf, während die Plasmakonzentrationen von IL-17F, IL-31, IL-33, Eotaxin-2/CCL24 sowie des regulatorischen IL-27 bei allen AE Patienten deutlich reduziert waren. Diese

Beobachtungen decken sich mit einer verminderten Produktion von IL-17F und IL-27 durch Periphere Mononukleäre Blutzellen (*Peripheral Blood Mononuclear Cells, PBMC*) von AE Patienten nach Stimulation mit *Echinococcus multilocularis* Metacestoden (Em) Antigenen. Im Gegensatz hierzu konnte nach Stimulation mit Em Antigenen eine erhöhte Produktion von IL-31 und CCL24 durch PBMC beobachten werden. Darüber hinaus war die Spontanproduktion von IL-31 und IL-33 durch PBMC bei allen AE Patienten reduziert, während regulatorisches IL-27, anti-inflammatorisches SDF-1/CXCL12 und Eosinophile Granulozyten aktivierendes Eotaxin-1, -2 und -3 (CCL11, CCL24, CCL26) mit progressivem Krankheitsverlauf zunahmen.

Antikörper Antworten gegen Em Antigen und *Echinococcus multilocularis* Vesikel (EmV) Antigen nahmen mit zunehmender Schwere der Erkrankung zu, wobei die höchste Reaktivität bei Patienten mit progressiver AE beobachtet werden konnte. Die geringste IgG Subklassen Reaktivität war bei Patienten mit ausgeheilter AE und bei Kontrollen nachweisbar. Weiterhin konnte, mit Ausnahme von IgG4, eine klar unterscheidbare Antikörper Reaktivität gegen EmV Antigen bei ausgeheilter, stabiler und progressiver AE beobachtet werden.

Die Resultate dieser Arbeit zeigen die Entwicklung eines ausgewogenen Reaktionsprofils von pro-inflammatorischen und regulatorischen Cytokinen und Chemokinen bei Patienten mit Alveolären Echinokokkose. Die Kenntnis dieser Reaktionsprofile hilft nicht nur einen schweren Krankeitsverlauf zu erkennen, sondern trägt

auch zur Identifizierung von regulatorischen Komponenten bei, die den Wirt gegen Schäden durch Effektorzellen des Immunsystems schützen können. Ein umfassendes Verständnis der Immunantworten bei einem progressiven oder ausheilenden Verlauf der Alveolären Echinokokkose, kann die Betreuung von AE Patienten verbessern und neue Interventionsmöglichkeiten im Sinne einer Immuntherapie eröffnen.

Summary

Parasites may escape their elimination and benefit for their survival in the host by manipulating the host immune response system. Parasites induce regulatory T cell (Tregs) populations that modulate the magnitude and expression of effector T cell functions thereby leading to an ineffective immune response during infections. Any nucleotide alteration in the promoter region may modify the gene expression, reflecting the level of susceptibility to a parasitic infection as well as Tregs expression. In this work, two known SNPs (*rs2069762T/G* and *rs2067006T/A*) and one novel *CT* deletion (*#ss410961576*) at the position -83/-84 were identified in the *IL2* promoter region in all the forty unrelated Gabonese individuals. None of the investigated SNP constructs showed a differential luciferase activity *in vitro* compared with the major allele (*rs2067006T/A*), which may relate to restricted FOXP3 expression in the Tregs leading to a slight alteration in T effector cell function. For the *IL2R alpha* (*CD25*), two previously described variants (*rs12722617C/T* and *rs12722616C/T*) and one novel C/T variant (*#ss410961577*) at position -409 were observed in the promoter region. The SNPs *rs12722617C/T* and *rs12722616C/T* were observed to be in absolute linkage in all individuals analyzed, and reveal no difference with the major allele after stimulation. The newly identified variant *#ss410961577* showed a significantly decreased activity when compared to the major allele. When compared to the NCBI HapMap database, all the frequencies of the reported SNP variants in *IL2* and *IL2R alpha* were found to be in accordance with the Yoruba

population which represents a Sub Saharan African group. The identified regulatory SNPs may be used for future prospective studies examining disease susceptibility or may better elucidate various physiological responses.

The diverse expression of the host immune responses may either help to clear infection or else allow for parasite persistence and chronic disease courses. In the present works, immune reactions and immune regulatory processes were studied during the course of Alveolar Echinococcosis (AE). Distinct humoral and cellular immune response profiles were observed in patients with different infection stages of AE. Elevated plasma levels of pro-inflammatory IL-17B and its soluble receptor IL-17RB were observed in stable and progressive AE patients. While plasma levels of pro-inflammatory IL-17F, IL-31, IL-33, Eotaxin-2/CCL24 and regulatory IL-27 were drastically depressed in all AE patients. These observations paralleled with reduced secretion of IL-17F and IL-27 by Peripheral Blood Mononuclear Cells (PBMC) from AE patients after stimulation with *Echinococcus multilocularis* antigens. In contrast, the expression of IL-31 and CCL24 by PBMC were enhanced after *E. multilocularis* antigen stimulation. In addition, the spontaneous release of IL-31 and IL-33 by PBMC was decreased in all AE patients, while regulatory IL-27, anti-inflammatory SDF-1/CXCL12 as well as eosinophil granulocyte attracting Eotaxin-1, -2 and -3 (CCL11, CCL24, CCL26) were enhanced with disease progression.

Antibody responses to *E. multilocularis* metacestode antigen (EmAg) and *E. multilocularis* vesicle antigen (EmVAg) were increased with disease progression, the

highest reactivity being observed in patients with progressive AE. Lowest IgG subclass responses were present in cured AE patients and controls. Furthermore, antibody reactivity against EmVAg distinctly separated between cured, stable or progressive AE, with the exception of IgG4.

These results reveal that equilibrated levels of pro-inflammatory and regulatory cytokines and chemokines have evolved to prevent severe disease manifestation and progression. At the same time, regulatory components may protect the host against immune-effecter cells mediated damage. Such distinctive response profiles could provide a better understanding of the immune response during disease progression or regression and improve the monitoring of AE patients.

Published Manuscripts

This thesis is based on the following publications:

1. Huang X, Kühne V, Kun JF, Soboslay PT, Lell B, Tp V. In-vitro characterization of novel and functional regulatory SNPs in the promoter region of *IL2* and *IL2R alpha* in a Gabonese population. BMC Med Genet. 2012 Dec 7;13:117.

Contribution of X. Huang to this Publication: Performance of Experiments, Data Analysis and Data Interpretation, Manuscript Drafting and Revision

2. 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.

Contribution of X. Huang to this Publication: Giving assistance for performing experiments and Data Analysis and Interpretation, Giving assistance for drafting Manuscript and Revision.

3. Huang X, Grüner B, Lechner CJ, Kern P, Soboslay PT. Distinctive cytokine, chemokine, and antibody responses in *Echinococcus multilocularis*-infected patients with cured, stable, or progressive disease. Med Microbiol Immunol. 2014 Feb 9.

Contribution of X. Huang to this Publication: Study design, Performance of Experiments, Data Analysis and Data Interpretation, Manuscript Drafting and Revision.

Further publications not included in this thesis:

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.
Contribution of X. Huang to this Publication: Giving assistance on Data Analysis and Data Interpretation, Giving assistance for drafting Manuscript and Revision.

5. Velavan TP, Bechlars S, Huang X, Kremsner PG, Kun JF. Novel regulatory SNPs in the promoter region of the TNFRSF18 gene in a Gabonese population. Braz J Med Biol Res. 2011 May; 44(5):418-20.
Contribution of X. Huang to this Publication: Giving assistance for performing experiments, Giving assistance for Revision.

Introduction

Innate and adaptive immune responses

Most pathogens which persist in their hosts can overcome the innate immune system, and an adaptive immune response is induced consequently for defense against them. The adaptive immune system consists of two major types of lymphocytes: B lymphocytes, which mature in the bone marrow and then differentiate into circulating plasma cells which secrete antibodies; and T lymphocytes, which mature in the thymus and may either kill pathogen-infected cells or help to eliminate extracellular pathogens. T cells become activated after having had contact with peptides on antigen-presenting cells, these peptides being presented as ligands by the major histocompatibility (MHC) complex.

Following maturation in the thymus, T lymphocytes will mainly develop into two classes: Cytotoxic T cells, which are CD8-positive, will detect and kill pathogen-infected cells when recognizing pathogen-specific peptide antigens presented on MHC class I molecule; The second T cell population are initially naïve CD4-positive T helper cells, which expand into effector T cells after their activation by pathogen-specific peptide antigens presented on MHC class II molecules. T helper cell may differentiate into Th1-, Th2- and Th17-type cells, as well as into regulatory T cells (Tregs) subsets which regulated immune responses.

Activated CD8-positive cytotoxic T cells will eliminate infection by inducing their target to undergo apoptosis,

and cytotoxic T cells may kill single infected cells in a strict antigen-restricted manner without creating widespread inflammation. The cytokine interferon-gamma (IFN-gamma), which is produced by CD8-postive cytotoxic T cells, will inhibit intracellular viral replication directly, and IFN-gamma is essential for the induction of MHC class I molecule expression and macrophage activation. In the MHC class I lessened mice, where CD8 T cell production is missing, the susceptibility to infection with the intracellular protozoan parasite *Trypanosoma cruzi* was increased (Munoz Fernandez et al. 1992).

T helper cell subsets are defined by the differing cytokine and chemokine secretion profiles, as well as by the differential expression of surface receptors. Both Th1- and Th2-T cell subsets are involved and predominate in acute and chronic infections with extracellular pathogens, respectively. During the initial pathogen encounter and antigen contact, the cytokines and chemokines produced by innate immune response drive the differentiation of Th1 and Th2 cells. When IFN-gamma is produced, the transcription factor STAT-1 (signal-transducing activator of transcription 1) can promote naïve T cells to differentiate into the type-1 T helper subset. Interleukin-4 (IL-4) will predominate upon the first antigen encounter, and is one of the most powerful trigger for the development of type-2 T helper cell, as well as the transcription factor STAT-4 and GATA-3. The development of Th17-type cells from naïve CD4 T cells will be promoted by IL-6 and transforming growth factor (TGF)-beta.

Th1-type cells can activate macrophages to control certain intracellular parasite infections by producing cytokines, such as IFN-gamma and TNF-alpha (Bogdan et al. 1990, Liew et al. 1990). Activated macrophages are tightly regulated by Th1 cells which help to minimize local tissue damage and energy consumption. Th1 cells can stimulate in humans the production of certain antibody subclasses, namely IgG1 and IgG3, which mainly act against bacterial pathogens and also by producing co-stimulatory signals which interact with naïve B lymphocytes. Furthermore, Th1 cells are traditionally associated with delayed-type hypersensitivity reactions (Glimcher & Murphy 2000, Coffman 2006).

T helper type-2 cells will also activate naïve B lymphocytes to produce certain antibody subclasses and will induce antibody subclass switching; this is related to the production of IgG2, IgG4 and IgE antibodies, those being involved in anti-helminth and anti-allergy type immune responses (Else & Finkelman 1998, Glimcher & Murphy 2000, Coffman 2006). Although initially in many infections mixed Th1- and Th2-type responses will evolve, such mixed type responses being mediated by “promiscuous” cytokines, but with long lasting parasite persistence and chronic infections a Th2- or a Th1-type immune response profile will predominate.

Th17 cells are often the first effector T cells subset which will develop following infection; Th17 cells can induce local epithelial and stromal cells to produce chemokines that recruit neutrophils to the site of infection. Several studies showed that IL-17 play an important role in promoting protection against bacteria and protozoan

parasite infections (Happel et al 2003, Kelly et al. 2005, Rudner et al. 2007). In addition, elevated levels of Th17 cytokines have been implicated in the induction of several autoimmune diseases (Bettelli et al. 2007).
Regulatory T cells (Treg) restrain inflammation and maintain tolerance through the secretion of inhibitory cytokines, mainly IL-10 and TGF-beta. Several studies have shown that Treg cells can also control effector T cell responses (Shevach et al. 2006). TGF-beta is a critical differentiation factor for the generation of Treg cells, but IL-6, an acute phase protein induced during inflammation, completely inhibits the generation of Treg cells induced by TGF-beta. Furthermore, IL-6 and TGF-beta together induce the differentiation of pathogenic Th17 cells from naive T cells. Thus, the generations of pathogenic Th17 cells by IL-6 that induce autoimmunity, and of regulatory T cells by TGF-beta that inhibit autoimmune tissue injury are selectively and dichotomously inducible (Bettelli et al. 2006).

Cytokines and chemokines

Cytokines are small immune modulating glycol proteins, which are secreted by a wide range of cells, including macrophages, B and T lymphocytes, as well as endothelial cells, fibroblasts, and stromal cells; cytokine modulate the proliferation and bioactivity of various cell types and many cytokines can be secreted by more than one cell type. The expression of cytokines can be regulated at the level of gene transcription and translation, at the protein synthesis levels and the cytokine synthesis

differs between cell types and their developmental stage and age.

The differentiation into Th1 cells will occur in the presence of IL-12 and IFN-gamma through toll-like receptor (TLR) signaling pathways. Also important in the early stage of Th1 differentiation is IL-2 (Liao et al. 2011); if an IL-12 production is inhibited, the secretion of IFN-gamma will lessen and the development of Th1 cells will dysfunction (Magram et al. 1996, O'Garra & Murphy 2009). IFN-gamma is the hallmark cytokine for Th1 cells and vital in directing Th1 cell differentiation, in parallel, IFN-gamma will suppress the production of Th2- and Th17-type cytokines.

The differentiation and evolution of Th2-type cells depends on the presence of IL-4 and the transcription factor GATA3 (Zhu et al. 2010). Th2-type cells will secrete IL-5, IL-6, and IL-13 (Paul & Zhu 2010), where IL-5 and IL-13 drive the differentiation, recruitment and survival of eosinophil granulocytes (Jabara et al. 1988, Horie et al. 1997), and the recently discovered Th2-type cytokine IL-33 can alter the Th2-type cell cytokine expression (Schmitz et al. 2005).

Th17-type cells were generated in the presence of IL-6, IL-1 beta and TGF-beta those often being synthesized by dendritic cells, and furthermore, IL-23 may expand and maintain Th17 cell populations (Bettelli et al. 2006, Dong 2006, Mangan et al. 2006). Th17 cells can produce IL-22, and together with IL-17, this population will induce an acute inflammatory response at the early stage of infection (Liang et al. 2006, Ma 2008). The IL-17 family contains six members, namely, IL-17A, IL-17B, IL-17C,

IL-17D, IL-17E and IL-17F. The cytokines IL-17A and IL-17F are highly homologous and bind to the same receptor, and both contribute similarly to the progress of inflammation and to the host defense against pathogens (Iwakura et al. 2008, Reynolds et al. 2010). The cytokine IL-17E appears to be involved in promoting Th2 cell immune responses playing a pivotal role in protection against parasitic worms (Fallon et al. 2006, Owyang et al. 2006).

Regulatory T cells (Treg) are associated with the cytokines TGF-beta, IL-10 and IL-2 (Vignali 2008). While the TGF-beta and IL-2 combination is essential for conversion of CD4+CD25- non-Treg cells into Treg cells, and thus enhancing the suppressive effects of Treg cells (Levings et al. 2001, Taylor et al. 2002, Horwitz et al. 2003). Furthermore, presence of other types of cells, in particular antigen-presenting cells (APCs), is critical for the generation of Tregs (Wan & Flavell 2006). Members of the TNF-nerve growth factor receptor family, notably TNFRSF18, is highly expressed on the surface of Treg cells, and studies have shown that TNFRSF18 signaling will modulate Tregs by regulating their generation and function (McHugh et al. 2002, Shimuzu et al. 2002). Furthermore, the suppressive activity of Treg cells can be abrogated by IL-6 when triggered by TLR expression on activated dendritic cells (DCs) (Pasare & Medzhitov 2003).

Chemokines are secreted proteins that attract cells bearing chemokine receptors, such as neutrophils and monocytes, out of the blood-stream and into the infected tissue. The chemokines are classified into two major (CXC and CC) and two minor (C and CXC) families based

on structural properties and their production in homeostatic and inflammatory conditions. Chemokines produced by antigen presenting cells or immune responses modulating cells, can regulate the migration of CD4-postivie lymphocyte subsets within the body (Sallusto & Baggiolini 2008). Studies have shown that T helper type 1 cells express CCR5 and CXCR3, while T helper type 2 cells express CCR4 and CCR8 (Zingoni et al. 1998, Sallusto & Lanzavecchia 2000). CCR6 is expressed both in Th17 cells and Treg cells (Singh et al. 2008, Kitamura et al. 2010). In addition, the CCL25-CCR9 axis is essential for T cell development in the thymus (Vicari et al. 1997). The CXCL12-CXCR4 axis plays pivotal roles during early B-cell development and is crucial for maintaining hematopoietic stem cells' function (Ara et al. 2003, Bonig et al. 2004, Tzeng et al. 2011). Furthermore, chemokines exhibit extraordinary specificity associated with certain human diseases, which suggested their possibility to be used as biomarkers (Zlotnik & Yoshie 2012).

The immune reactions in parasite diseases

Intracellular protozoan parasites usually stimulate typical Th1 immune responses, while chronic helminthes infections lead to characteristic Th2 immune responses (Díaz & Allen 2007). Several studies have described the importance of cytokines and chemokines in the prevention and elimination of parasite infection. With the tapeworm *Echinococcus spp.* infections, Th1-type cytokine IL-12 and IFN-gamma were identified to

successfully kill the larval stages of the parasite (metacestode) at the initial stages of development, whereas Th2 immune responses, induced by IL-4, IL-5 and IL-10, lead to a chronic course of disease (Vuitton & Gottstein 2010). A combined Th1 and Th2 cytokine profile appears crucial for prolonged metacestode growth and enhanced parasite survival. The Th2-related IgE synthesis and mast cell activation are responsible for anaphylactic reactions in cystic echinococcosis, but rare in alveolar echinococcosis (Vuitton 2003). Low IFN-gamma and high TNF-alpha are associated with severe hepatic fibrosis during *Schistosoma mansoni (S. mansoni)* infection (Henri et al. 2002, Booth et al. 2004). The increased expressions of IL-5, IL-10 and IL-13 are associated with the progression of schistosomiasis (de Jesus et al. 2004), while the T helper Type 2 cytokine IL-33 guides the immune response towards a beneficial type 2 response during *Trichuris muris* and *Nippostrongylus brasiliensis* infection (Humphreys et al. 2008, Neill et al. 2010).

Regulatory T cells are associated with parasite survival in helminthes infection, while controlling the infection-associated pathology in schistosomiasis (Hesse et al. 2004, Baumgart et al. 2006, D'Elia et al. 2009). In addition, Treg cells can lead to an improved parasite clearance in *Litomosoides sigmodontis* infected mice (Taylor et al. 2005). Together TGF-beta with IL-10 has been shown to suppress inflammatory responses in mice infected with *Heligmosomoides polygyrus* (*H. polygyrus*) (Setiawan et al. 2007, Ince et al. 2009). In contrast, Th17 cells mediate the development of immunopathology during *S. mansoni*

infection (Moustapha et al. 2013), and the inhibition of an IL-17 production by *H. polygyrus* infection may protect or reverse inappropriate inflammation in the Schistosoma-infected host (Eliott et al. 2004). Furthermore, IL-6 is essential in favoring the CD4+ T cells differentiate toward Th17 cells, and IL-6 can also decrease the Th2 type immune response and alter the Treg cells phenotype in mice, which will increase host susceptibility to *H. polygyrus* infection (Smith & Maizels 2014). A better knowledge about the differential expression patterns of cytokines and chemokines during the course of distinct parasitic infections may help to identify prognostic markers for disease progression or regression, and in addition, may help to identify therapeutic targets. Therefore, investigation on cytokine and chemokines expression profiles may lead to better understanding the parasite-host interplay and even evolve to new treatment approaches.

AIM of this study

1 Significant impact on the diversity among immune-related gene families is believed to be attributable to exposure and challenges with infectious pathogens and notably to repeated and persistent parasite infections. Variations in the promoter regions of genes can potentially modify the expression levels of immune response relevant genes by altering the function of the transcription pattern. Aim of this work was to identify SNPs in immune regulatory genes within populations that are naturally exposed to an array of parasites. The identified SNPs in the promoter regions of immune relevant genes may help to understand the relevance of sequence polymorphisms in populations exposed to poly-parasitic diseases and for backup studies examining disease susceptibility.

2 Cytokine and chemokine responses are pivotal to protect the body against foreign pathogens. Their expression may vary according to different parasite species, the duration of pathogen persistence, the parasite developmental stages and parasite tissue location within the infected host. Differential expression and cellular production of these immune mediators may find application to determine the stage of disease and outcome, such as cured, stable and progressive. The aim of this work was to clarify distinct profiles of cytokines, chemokines as well as antibody responses in certain clinically well defined stages of human Alveolar Echinococcosis. The distinctive responses profiles

together with the different clinical outcome could help to improve monitoring and staging of Alveolar Echinococcosis and may lead to the potential use of cytokines or chemokines as biomarkers, and it even open the avenue for their application as immune therapeutic molecules.

Results and Discussion

The *IL2* and *IL2R alpha* polymorphism in Gabonese populations

A significant impact on the diversity among immune gene families is believed to be attributable to invasion maneuvers performed by parasites. Therefore variations in the promoter regions of immune response genes can potentially amend the gene expression levels either by changing specificity of transcription binding sites or by altering the kinetics of transcription initiation (Velavan et al. 2011). In the present work, two known SNPs (*rs2069762T/G* and *rs2067006T/A*) and one novel *CT* deletion (*#ss410961576*) at the position −83/-84 were identified in *IL2* promoter region. None of the investigated SNPs altered the luciferase activity *in vitro* as compared to the major allele (*rs2067006T/A* was considered to be the major allele). In the major allele, when T allele was presented at the *rs2069762T/G* position, a potential binding site for the activation factor heterodimers of *TCF-11* (a transcription factor of the bzip type) and the *AP-1* protein MafG were exposed (Luna et al. 1995, Husberg et al. 2001, Shaulian & Karin 2002). Noteworthy, transcription factor *AP-1* plays a pivotal role in the regulation of IL-2 expression (Kang et al. 1992, Jain et al. 1992). Thus, the shifting from a T allele to a G will eradicate this potential binding site, which may reduce the binding affinity of the TCF-11/MafG complex and induce a relative decrease in IL-2 expression. The *rs2067006T/A* variant is positioned on the transcription site of *ETS-1*,

which belongs to *ETS* (E-twenty six) family. The *ETS-1* DNA-binding domain recognizes the core consensus DNA in target genes and acts either as a transcriptional activator or as repressor (Shaikhibrahim & Wernert 2012). Recent studies have demonstrated that *ETS-1* belongs to a large protein complex which may restrict the stable FOXP3 expression in Treg cells (Polansky et al. 2010). *FOXP3* acts as a master regulator for the natural Treg development and for their function (Hanel et al. 2011), while mature regulatory T cells expressing a non-functional fusion protein of FOXP3 lack any suppressor function (Lin et al. 2007). These results suggested that the studied Gabonese population may have a restricted FOXP3 expression leading to a indirect T effector cell function.

Furthermore, two known SNPs (*rs12722617C/T* and *rs12722616C/T*) and one novel C/T variant *(#ss410961577*) at the position −516 were identified when screened for the *IL2R alpha* (CD25) promoter region. Both the known SNP variants of the *IL2R alpha* (*rs12722617C/T* and *rs12722616C/T*) were observed in linkage in all studied individuals. No differential luciferase activity were found in the construct (*rs12722617C/T* + *rs12722616C/T*) when compared to the major allele. However, the novel variant (*#ss410961577C/T*) revealed a significantly increased activity in comparison to the construct and the major allele. This particular novel variant showed no relation with any putative transcription factor binding site, however, the variant rs12722616C/T which was in linkage with *rs12722617C/T* altered the transcription factor binding site *TBP* (TATA box binding

protein) and *C/EBPbeta* (CCAAT/enhancer binding protein beta). *TBP* (TATA binding protein) together with TATA associated factors (*TAFS*) make the transcription factor *TFIID* which binds the TATA box in combination with other transcription factors (Maldonado et al. 1990). All the above transcription factors along with the RNA polymerase II form a transcription initiation complex. Therefore, it is possible that the TATA box in the construct *rs12722616C/T* may contribute to the transcription initiation *in vitro* in Jurkat cells. The other transcription factor *C/EBP beta* is believed to modulate inflammatory processes. *C/EBP* together with Runt-related transcription factor (*RUNX*) might transactivate the leukocyte-specific-intercellular adhesion molecule 3 (*ICAM-3*) promoter (Estecha et al. 2012). Notabaly, *ICAM-3*, presented on the T cell surface induces the CD3-mediated up-regulation of IL-2R alpha (Hernandez-Caselles et al. 1993). A recent study has demonstrated in an experimental autoimmune encephalomyelitis model that C/EBP expression by dendritic cells (DC) influences the Th17 versus Treg differentiation but has little or no impact upon Th1 development (Tsai et al. 2011).

The identification and validation of such polymorphisms in immune regulatory genes may provide a basis for future studies on parasite susceptibility in a given population where T cell functions are compromised. The role of the described and novel variants of *IL2* and *IL2R alpha* still needs to be validated in terms of the specific role these play in different parasitic diseases.

Distinctive immune reactivity in Alveolar Echinococcosis

Metacestode larvae of the tapeworm *E. multilocularis* can cause Alveolar Echinococcosis (AE), a severe disease, which, if it remains untreated, can lead to organ failure and fatality. *E. multilocularis* infection, whether in rodents or humans, is accompanied by chronic inflammation (Gottstein & Hemphill 1997). As echinococcus metacestodes often live as long as their hosts, efficient immune-evasion mechanisms should developed, which may allow *Echinococcus spp*. larvae to develop to the largest pathogens to dwell in mammalian internal organs (Conchedda et al. 2004, Vuitton & Gottstein 2010). As current treatment and resection will not efficiently eliminate the infection, many AE patients require life-long medication and monitoring their metacestode growth by imaging techniques like Magnet Resonance Tomography (MRT) and Positron Electron Tomography (PET). Previous studies have shown that different course of infections can elicit distinct immune responses in AE patients (Gottstein & Felleisen 1995). Thus, AE-specific biomarkers could offer a sensitive approach for diagnosis and surveillance.

Immunoglobulins IgG1, IgG2, IgG4, and IgE were found to be highly reactive in cystic echinococcosis (CE) and in AE patients, which may indicate active *E. multilocularis* infestation and severe outcome of disease (Dreweck et al. 1997). Furthermore, predominant Th2-type cellular immune responses together with high levels of IgG4 and IgE were associated with progressive and severe AE

(Dreweck et al. 1997). Cellular effector responses appear crucial for the prevention of *E. multilocularis* metacestode growth and dissemination as well as for the protection of the host against parasite-induced inflammation and tissue damage. During *E. multilocularis* infection, CD4-positive T cell responses will progressively become depressed (Gottstein et al. 1994, Manfras et al. 2004), while regulatory T cells (Treg) were found to be strongly expanded in mouse *E. multilocularis* infection (Mejri et al. 2011).

Interventions that boost Th1 responses resulted in enhanced control of the larval *Echinococcus* (Vuitton & Gottstein 2010). In chronic infection, pro-inflammatory Th1-type responses will slowly lessen, while Th2-type and Treg responses are turned on by enhanced production of IL-4, IL-13, TGF-beta, IL-10 and MDC/CCL22 (Jenne et al. 1997, Aumüller et al. 2004, Vuitton & Gottstein 2010, Hübner et al. 2006), which may facilitate the tissue-infiltrative growth of the parasite and its persistence in the human host. The inflammation associated with *E. multilocularis* infection is typically granulomatous, which is a CD4-postivie T cell-dependent reaction and Th17 components were presented (Dai et al. 2004, Vuitton & Gottstein 2010). In addition, larval *Echinococcus spp.* infections probably induce strong regulatory responses, which control effector Th2-, Th1-, and possibly Th17-type responses.

Th17 responses play crucial roles in host against bacterial and fungal infections and in the development of inflammatory diseases by inducing the expression of genes encoding pro-inflammatory cytokines (TNF, IL-6),

chemokines (GRO-alpha/CXCL1, ENA-78/CXCL5) as well as antimicrobial peptides (Ishigame et al. 2009, Ahmed & Gaffen 2010, Kuchroo & Awasthi 2012). The tissue-infiltrative growth, its proliferation and the dissemination of the metacestode larval stage of *E. multilocularis* resembles cancer metastasis, and also, the liver is the mainly affected organ by chronic metacestode growth. In hepato-cellular carcinoma expanded Th17 cells were suggested to exert immunosuppressive functions (Greten et al. 2012), and similarities might be present in Alveolar Echinococcosis. Several studies showed that Th17 cells exert heterogenic functions in tumor microenvironments with different types and at different stages of cancer progression or regression (Peters et al. 2011, Ghoreschi et al. 2011). In addition, high levels of the chemokine SDF-1/CXCL12 in the tumor micro-environment may facilitate Th17 cell trafficking and migration into the tumor site (Zou & Restifo 2010).

Within the IL-17 cytokine family distinctive activities were observed for each member. The cytokine IL-17A showed tumor promoting activity by up-regulating the expression of pro-survival and pro-angiogenic genes, such as vascular endothelial growth factor (VEGF), IL-8 and IL-6 (Tartour et al. 1999, Numasaki et al. 2003, Wang et al. 2009); IL-17A also can indirectly stimulate anti-tumor activity by promoting type 1 immune responses (Martin-Orozco & Dong 2009, Kryczek et al. 2009). The IL-17B may induce pro-inflammatory cytokine secretion and was found highly expressed in the gastro-intestinal tract, where *E. multilocularis* metacestode are mostly present (Li et al. 2000, Shi et al. 2000). The IL-17F was shown to

play a protective role in colon tumor genesis (Tong et al. 2012). However, little is known about the cellular production, function and roles of the IL-17 cytokines in Alveolar Echinococcosis (AE).

Decreased IL-17F levels were observed in AE patients' serum as well as in cell culture supernatant of PBMC from AE patients' after stimulation with *E. multilocularis* vesicle antigen. These results suggest an appropriate immune activation against *E. multilocularis* antigens during the chronic course of AE. However, no differences were observed in the IL-17A levels of production in the same patients pointing towards a divergent immune regulation by the IL-17 type cytokines inducible by *E. multilocularis* antigens. Previously, IL-17A and IL-17F were shown to act in different way in protection against infection and in immune disorders as well (Ishigame et al. 2009). IL-17F has an earlier expression during the Th17 cell development than IL-17A (Lee et al. 2009), and IL-17A expression was more sensitive to the strength of T cell receptor signaling (Gomez-Rodriguez et al. 2009). It appears that IL-17F levels are associated with protection from the very beginning of infection, which is in accordance with the observed suppressed IL-17F levels in AE patients.

For IL-17B, it was detectable in several organs with high expression in chondrocytes and neurons (Li et al. 2000, Iwakura et al. 2011, Song & Qian 2013). Furthermore, IL-17B was suggested to play a potential pro-inflammatory role in disease processes (Yamaguchi et al. 2007). Elevated serum levels of IL-17B and of the soluble receptor component IL-17RB were observed in stable and

progressive AE cases and this suggested that IL-17B is involved in innate immune responses; it may enhance the migration of neutrophil granulocytes into the gastrointestinal tract or other epithelial structures, and lead to inflammatory immune reactions at targeted organs (Shi et al. 2000). IL-17B seems to drive early immune responses against various pathogens, and the activation of neutrophil granulocytes is the hallmark of early pro-inflammatory immune reactions. Furthermore, over-expression of IL-17RB was described to relate to non-recurrence after tamoxifen treatment in breast cancer (Ma et al. 2004), which may point out a potential involvement of IL-17B in tumor regression.

Eotaxin-1, Eotaxin-2, and Eotaxin-3 (CCL11, CCL24, and CCL26) are involved in the recruitment of eosinophil granulocytes, mast cells, and Th2 lymphocytes (Shin et al. 2003), and CCL11/Eotaxin-1 and CCL24/Eotaxin-2 were found to be pivotal in the immune response against *S. mansoni* infection (Sousa-Pereira et al. 2006). Similarly in the present work, CCL11/Eotaxin-1 and CCL24/Eotaxin-2 were enhanced in AE patients after activation with *E. multilocularis* metacestode antigens. With inflammation and immune activation, eotaxins attract eosinophil granulocytes to inflamed tissues and these results signify that such cellular effecter responses are induced by vital metacestode tissues, and still remain active in patients staged with cured AE. This indicates that not only viable and proliferating metacestodes of *E. multilocularis*, but residual parasite lesions will modulate cellular immune responses in AE patients. Similarly, elevated and CCL11/Eotaxin-1 and CCL24/Eotaxin-2 levels were

observed in eosinophil-mediated defense mechanism against helminthes (Del Pozo et al. 1999).

Furthermore, the spontaneous release of CXCL12 was highest in patients with progressive AE, indicating that CXCL12 participates in inflammatory processes with active AE. The CXC chemokine CXCL12 was found to be strongly expressed in lung and liver metastases (Müller et al. 2001), and CXCL12 will recruit leukocytes into infected tissues and promote inflammation (Berahovich et al. 2014). An up-regulated production of CXCL12 was also found in rheumatoid arthritis and asthma (Karin 2010, Negrete-Garcia et al. 2010).

The regulatory cytokines IL-27 can induce a strong proliferation of naïve human B cells, CD4+, and CD8+ T cells and will promote the development of Th1-type cells (Guzzo et al. 2010, Charlot-Rabiega et al. 2011, Schneider 2011). The cytokines IL-31 and IL-33 are pro-inflammatory Th2 type cytokines and are suggested to play a vital role in human T-cell-mediated skin diseases and allergic and non-allergic diseases (Ezzat et al. 2011, Dillon et al. 2004). The cytokine IL-33 will activate Th2 type helper cells, mast cells, and basophil granulocytes, thus supporting host defense against nematodes (Suzukawa et al. 2008, Yasuoka et al. 2011).

The spontaneous but not *E. multilocularis* antigen-driven release of IL-27 increased with disease severity in AE patients, suggesting the presence of immune regulatory mechanisms which may dampen inflammation induced by persistent *E. multilocularis*. The enhanced spontaneous release of IL-27 in AE patients with stable and progressive disease may indicate the emergence of a

regulatory component depressing pro-inflammatory cytokine and chemokine responses; such regulatory IL-27 production are likely to be induced by viable and persistent *E. multilocularis* metacestodes in AE patients.

Several studies have shown that metacestodes of *E. multilocularis* release factors which impair the interaction of macrophages and T-cells in mitogen- and antigen-induced lympho-proliferative responses (Rakha et al. 1991, Kizaki et al. 1993). By depressing Th1-type IFN-γ and inflammatory MIP-1 alpha/CCL3 while activating immune regulatory TARC/CCL17 and RANTES/CCL5 (Hübner et al. 2006, Kocherscheidt et al. 2008) *E. multilocularis* reduces inflammatory responses, prevents tissue destruction and organ damage, and facilitates parasite persistence within its host. Low levels of inflammation-associated IL-31 and IL-33 were observed in chronic AE supporting the notion that with parasite persistence, equilibrated levels of pro-inflammatory and regulatory cytokines and chemokines may have evolved to prevent disease progression and, at the same time, protect the host against immune-effecter cells mediated damage.

Distinctive antibody responses are known to correlate with the clinical state of helminthes infections, with IgG4 and IgE indicative of a Th2-type immune response and being prominent in chronic helminthes infections (Maizels et al. 1995; Mosmann & Sad 1996; Hussain et al. 1987). Progressively diminished *E. multilocularis* metacestode-specific IgG1, IgG3 and IgE responses were observed in AE patients during the regression from active to stable and cured disease, while IgG2 and IgG4 reactivity

remained similarly high in stable and progressive AE. Several studies have shown that increased IgE correlated with progressive AE (Ammann et al. 1996, Dreweck et al. 1997, Wellinghausen et al. 1999). Similarly, an enhanced IgE reactivity was observed in patients with progressive AE.

The antibody responses specific to *E. multilocularis* vesicle antigen (EmVAg) distinguished best between cured, stable or progressive AE; here the antibody subclasses reactivity, with the exception of IgG4, differentiated clearly between progressive and stable AE. Such distinctive immune response profiles with cured, stable and progressive AE suggests them as useful makers for disease monitoring and staging. Further, the combination with *E. multilocularis* vesicle-specific antibody responses should provide a better immune monitoring during progression and regression of AE.

References

Ahmed M, Gaffen SL. IL-17 in obesity and adipogenesis. Cytokine Growth Factor Rev. 2010 Dec;21(6):449-53.

Alcina A, Fedetz M, Ndagire D, Fernandez O, Leyva L, Guerrero M, Abad-Grau MM, Arnal C, Delgado C, Lucas M, et al. IL2RA/CD25 gene polymorphisms: uneven association with multiple sclerosis (MS) and type 1 diabetes (T1D). PLoS One 2009;4:e4137.

Ammann RW, Eckert J. Cestodes. Echinococcus. Gastroenterol Clin North Am 1996;25 (3):655-689

Antony PA, Paulos CM, Ahmadzadeh M, Akpinarli A, Palmer DC, Sato N, Kaiser A, Heinrichs C, Klebanoff CA, Tagaya Y, et al. Interleukin-2-Dependent Mechanisms of Tolerance and Immunity In vivo. The Journal of Immunology 2006;176:5255-5266

Ara T, Itoi M, Kawabata K, Egawa T, Tokoyoda K, Sugiyama T, Fujii N, Amagai T, Nagasawa. T. A role of CXC chemokine ligand 12/stromal cell-derived factor-1/pre-B cell growth stimulating factor and its receptor CXCR4 in fetal and adult T cell development in vivo. J Immunol. 2003 May 1;170(9):4649-55.

Attema JL, Reeves R, Murray V, Levichkin I, Temple MD, Tremethick DJ, Shannon MF. The human IL-2 gene promoter can assemble a positioned nucleosome that becomes remodeled upon T cell activation. J Immunol 2002:2466-2476.

Aumüller E, Schramm G, Gronow A, Brehm K, Gibbs BF, Doenhoff MJ, Haas H. *Echinococcus multilocularis* metacestode extract triggers human basophils to release interleukin-4. Parasite Immunol. 2004 Oct;26(10):387-95.

Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. CD4+CD25high regulatory cells in human peripheral blood. J Immunol. 2001 Aug 1;167(3):1245-53.

Baumgart M, Tompkins F, Leng J, Hesse M. Naturally occurring CD4+Foxp3+ regulatory T cells are an essential, IL-10-

independent part of the immunoregulatory network in Schistosoma mansoni egg-induced inflammation. J Immunol. 2006 May 1;176(9):5374-87.

Bayer AL, Yu A, Adeegbe D, Malek TR. Essential role for interleukin-2 for CD4(+)CD25(+) T regulatory cell development during the neonatal period. J Exp Med 2005;201:769-777.

Berahovich RD, Zabel BA, Lewén S, Walters MJ, Ebsworth K, Wang Y, Jaen JC, Schall TJ. Endothelial expression of CXCR7 and the regulation of systemic CXCL12 levels. Immunology. 2014 Jan;141(1):111-22.

Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006 May 11;441(7090):235-8.

Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol. 2007 Apr;8(4):345-50.

Bogdan C, Moll H, Solbach W, Röllinghoff M. Tumor necrosis factor-alpha in combination with interferon-gamma, but not with interleukin 4 activates murine macrophages for elimination of *Leishmania major* amastigotes. Eur J Immunol. 1990 May;20(5):1131-5.

Bonig H, Priestley GV, Nilsson LM, Jiang Y, Papayannopoulou T. PTX-sensitive signals in bone marrow homing of fetal and adult hematopoietic progenitor cells. Blood 2004 Oct 15;104(8):2299-306.

Booth M, Mwatha JK, Joseph S, Jones FM, Kadzo H, Ireri E, Kazibwe F, Kemijumbi J, Kariuki C, Kimani G, et al. Periportal fibrosis in human *Schistosoma mansoni* infection is associated with low IL-10, low IFN-gamma, high TNF-alpha, or low RANTES, depending on age and gender. J Immunol. 2004 Jan 15;172(2):1295-303

Bowes J, Ho P, Flynn E, Ali F, Marzo-Ortega H, Coates LC, Warren RB, McManus R, Ryan AW, Kane D, et al. Comprehensive assessment of rheumatoid arthritis

susceptibility loci in a large psoriatic arthritis cohort. Ann Rheum Dis 2012, 71(8):1350–1354.

Brand OJ, Lowe CE, Heward JM, Franklyn JA, Cooper JD, Todd JA, Gough SC. Association of the interleukin-2 receptor alpha (IL-2Ralpha)/CD25 gene region with Graves' disease using a multilocus test and tag SNPs. Clin Endocrinol (Oxf) 2007;66:508-512.

Brookes AJ. The essences of SNPs. Gene 1999;8:177-186

Charlot-Rabiega P, Bardel E, Dietrich C, Kastelein R, Devergne O. Signaling events involved in interleukin 27 (IL-27)-induced proliferation of human naive CD4+ T cells and B cells. J Biol Chem 2011;286 (31):27350-27362.

Coffman RL. Origins of the T(H)1-T(H)2 model: a personal perspective. Nat Immunol. 2006 Jun;7(6):539-41.

Collins FS, Guyer MS, Charkravarti A. Variations on a theme: cataloging human DNA sequence variation. Science1997;278:1580-1581.

Conchedda M, Gabriele E, Bortoletti G. Immunobiology of cystic echinococcosis. Parassitologia. 2004 Dec;46(4):375-80.

Dai WJ, Waldvogel A, Siles-Lucas M, Gottstein B. *Echinococcus multilocularis* proliferation in mice and respective parasite 14-3-3 gene expression is mainly controlled by an alphabeta CD4 T-cell-mediated immune response. Immunology. 2004 Jul;112(3):481-8.

de Jesus AR, Magalhães A, Miranda DG, Miranda RG, Araújo MI, de Jesus AA, Silva A, Santana LB, Pearce E, Carvalho EM. Association of type 2 cytokines with hepatic fibrosis in human *Schistosoma mansoni* infection. Infect Immun. 2004 Jun;72(6):3391-7.

del Pozo V, Arrieta I, Tunon T, Cortegano I, Gomez B, Cardaba B, Gallardo S, Rojo M, Renedo G, Palomino P, et al. Immunopathogenesis of human gastrointestinal infection by Anisakis simplex. J Allergy Clin Immunol 1999;104 (3 Pt 1):637-643.

D'Elia R, Behnke JM, Bradley JE, Else KJ. Regulatory T cells: a role in the control of helminth-driven intestinal pathology and worm survival. J Immunol. 2009 Feb 15;182(4):2340-8.

Díaz A, Allen JE. Mapping immune response profiles: the emerging scenario from helminth immunology. Eur J Immunol. 2007 Dec;37(12):3319-26.

Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. J Exp Med. 2001 Jun 4;193(11):1303-10.

Dillon SR, Sprecher C, Hammond A, Bilsborough J, Rosenfeld-Franklin M, Presnell SR, Haugen HS, Maurer M, Harder B, Johnston J, et al. Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. Nat Immunol 2004;5 (7):752-760.

Dong C. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. Nat Rev Immunol. 2006 Apr;6(4):329-33.

Dreweck CM, Luder CG, Soboslay PT, Kern P. Subclass-specific serological reactivity and IgG4-specific antigen recognition in human echinococcosis. Trop Med Int Health 1997;2 (8):779-787

Durand DB, Bush MR, Morgan JG, Weiss A, Crabtree GR. A 275 basepair fragment at the 5' end of the interleukin 2 gene enhances expression from a heterologous promoter in response to signals from the T cell antigen receptor. J.Exp.Med. 1987;165:395.

Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF Jr, Weinstock JV. *Heligmosomoides polygyrus* inhibits established colitis in IL-10-deficient mice. Eur J Immunol. 2004 Oct;34(10):2690-8.

Else KJ, Finkelman FD. Intestinal nematode parasites, cytokines and effector mechanisms. Int J Parasitol. 1998 Aug;28(8):1145-58.

Estecha A, Aguilera-Montilla N, Sánchez-Mateos P, Puig-Kröger A. RUNX3 Regulates Intercellular Adhesion Molecule 3 (ICAM-3) Expression during Macrophage Differentiation and Monocyte Extravasation. PLoS ONE 2012, 7(3): e33313.

Ezzat MH, Hasan ZE, Shaheen KY. Serum measurement of interleukin-31 (IL-31) in paediatric atopic dermatitis: elevated levels correlate with severity scoring. J Eur Acad Dermatol Venereol 2011;25 (3):334-339.

Fallon PG, Ballantyne SJ, Mangan NE, Barlow JL, Dasvarma A, Hewett DR, McIlgorm A, Jolin HE, McKenzie AN. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. J Exp Med. 2006 Apr 17;203(4):1105-16

Festen EA, Goyette P, Scott R, Annese V, Zhernakova A, Lian J, Lefèbvre C, Brant SR, Cho JH, Silverberg MS, et al. Genetic variants in the region harbouring IL2/IL21 associated with ulcerative colitis. Gut 2009, 58:799-804.

Fujita T, Shibuya H, Ohashi T, Yamanishi K, Taniguchi T. Regulation of human interleukin-2 gene: functional DNA sequences in the 5' flanking region for the gene expression in activated thymphocytes. Cell 1986;46:401

Ghoreschi K, Laurence A, Yang XP, Hirahara K, O'Shea JJ. T helper 17 cell heterogeneity and pathogenicity in autoimmune disease. Trends Immunol. 2011 Sep;32(9):395-401.

Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes Dev. 2000 Jul 15;14(14):1693-711.

Gomez-Rodriguez J, Sahu N, Handon R, Davidson TS, Anderson SM, Kirby MR, August A, Schwartzberg PL. Differential expression of interleukin-17A and -17F is coupled to T cell receptor signaling via inducible T cell kinase. Immunity. 2009 Oct 16;31(4):587-97.

Greten TF, Zhao F, Gamrekelashvili J, Korangy F. Human Th17 cells in patients with cancer: Friends or foe? Oncoimmunology. 2012 Nov 1;1(8):1438-1439.

Gottstein B, Felleisen R. Protective immune mechanisms against the metacestode of *Echinococcus multilocularis*. Parasitol Today. 1995 Sep;11(9):320-6.

Gottstein B, Hemphill A. Immunopathology of echinococcosis. Chem Immunol. 1997;66:177-208.

Gottstein B, Wunderlin E, Tanner I. *Echinococcus multilocularis*: parasite-specific humoral and cellular immune response subsets in mouse strains susceptible (AKR, C57B1/6J) or 'resistant' (C57B1/10) to secondary alveolar echinococcosis. Clin Exp Immunol 1994 May;96(2):245-52.

Guzzo C, Che Mat NF, Gee K. Interleukin-27 induces a STAT1/3- and NF-kappaB-dependent proinflammatory cytokine profile in human monocytes. J Biol Chem 2010;285 (32):24404-24411.

Happel KI, Zheng M, Young E, Quinton LJ, Lockhart E, Ramsay AJ, Shellito JE, Schurr JR, Bagby GJ, Nelson S, et al. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to Klebsiella pneumoniae infection. J Immunol. 2003 May 1;170(9):4432-6.

Hanel SA, Velavan TP, Kremsner PG, Kun JF. Novel and functional regulatory SNPs in the promoter region of FOXP3 gene in a Gabonese population. Immunogenetics 2011;63:409-415.

Henri S, Chevillard C, Mergani A, Paris P, Gaudart J, Camilla C, Dessein H, Montero F, Elwali NE, Saeed OK, et al. Cytokine regulation of periportal fibrosis in humans infected with *Schistosoma mansoni*: IFN-gamma is associated with protection against fibrosis and TNF-alpha with aggravation of disease. J Immunol. 2002 Jul 15;169(2):929-36.

Hernandez-Caselles T, Rubio G, Campanero MR, del Pozo MA, Muro M, Sanchez-Madrid F, Aparicio P. ICAM-3, the third LFA-1 counterreceptor, is a co-stimulatory molecule for both resting and activated T lymphocytes. Eur. J. Immunol. 1993, 23: 2799-2806.

Hesse M, Piccirillo CA, Belkaid Y, Prufer J, Mentink-Kane M, Leusink M, Cheever AW, Shevach EM, Wynn TA. The

pathogenesis of schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. J Immunol. 2004 Mar 1;172(5):3157-66.

Horie S, Okubo Y, Hossain M, Sato E, Nomura H, Koyama S, Suzuki J, Isobe M, Sekiguchi M. Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. Intern Med. 1997 Mar;36(3):179-85.

Horwitz DA, Zheng SG, Gray JD. The role of the combination of IL-2 and TGF-beta or IL-10 in the generation and function of CD4+ CD25+ and CD8+ regulatory T cell subsets. J Leukoc Biol. 2003 Oct;74(4):471-8.

Hubner MP, Manfras BJ, Margos MC, Eiffler D, Hoffmann WH, Schulz-Key H, Kern P, Soboslay PT. *Echinococcus multilocularis* metacestodes modulate cellular cytokine and chemokine release by peripheral blood mononuclear cells in alveolar echinococcosis patients. Clin Exp Immunol 2006;145 (2):243-251.

Humphreys NE, Xu D, Hepworth MR, Liew FY, Grencis RK. IL-33, a potent inducer of adaptive immunity to intestinal nematodes. J Immunol. 2008 Feb 15;180(4):2443-9.

Husberg C, Murphy P, Martin E, Kolsto AB. Two domains of the human bZIP transcription factor TCF11 are necessary for transactivation. J Biol Chem 2001; 276: 17641-17652.

Hussain R, Grogl M, Ottesen EA. IgG antibody subclasses in human filariasis. Differential subclass recognition of parasite antigens correlates with different clinical manifestations of infection. J Immunol 1987;139 (8):2794-2798

Ince MN, Elliott DE, Setiawan T, Metwali A, Blum A, Chen HL, Urban JF, Flavell RA, Weinstock JV. Role of T cell TGF-beta signaling in intestinal cytokine responses and helminthic immune modulation. Eur J Immunol. 2009 Jul;39(7):1870-8.

Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, Fujikado N, Tanahashi Y, Akitsu A, Kotaki H, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity. 2009 Jan 16;30(1):108-19.

Iwakura Y, Ishigame H, Saijo S, Nakae S. Functional specialization of interleukin-17 family members. Immunity. 2011 Feb 25;34(2):149-62.

Iwakura Y, Nakae S, Saijo S, Ishigame H. The roles of IL-17A in inflammatory immune responses and host defense against pathogens. Immunol Rev. 2008 Dec;226:57-79.

Jabara HH, Ackerman SJ, Vercelli D, Yokota T, Arai K, Abrams J, Dvorak AM, Lavigne MC, Banchereau J, De Vries J, et al. Induction of interleukin-4-dependent IgE synthesis and interleukin-5-dependent eosinophil differentiation by supernatants of a human helper T-cell clone. J Clin Immunol. 1988 Nov;8(6):437-46.

Jain J, Valge-Archer VE, Rao A: Analysis of the AP-1 sites in the IL-2 promoter. J Immunol 1992; 148: 1240-1250.

Jenne L, Kilwinski J, Scheffold W, Kern P. IL-5 expressed by CD4+ lymphocytes from *Echinococcus multilocularis*-infected patients. Clin Exp Immunol. 1997 Jul;109(1):90-7.

Kang SM, Beverly B, Tran AC, Brorson K, Schwartz RH, Lenardo MJ. Transactivation by AP-1 is a molecular target of T cell clonal anergy. Science 1992; 257: 1134-1138.

Karin N. The multiple faces of CXCL12 (SDF-1alpha) in the regulation of immunity during health and disease. J Leukoc Biol. 2010 Sep;88(3):463-73.

Kelly MN, Kolls JK, Happel K, Schwartzman JD, Schwarzenberger P, Combe C, Moretto M, Khan IA. Interleukin-17/interleukin-17 receptor-mediated signaling is important for generation of an optimal polymorphonuclear response against Toxoplasma gondii infection. Infect Immun. 2005 Jan;73(1):617-21.

Kitamura K, Farber JM, Kelsall BL. CCR6 marks regulatory T cells as a colon-tropic, IL-10-producing phenotype. J Immunol. 2010 Sep 15;185(6):3295-304.

Kizaki T, Ishige M, Bingyan W, Kumagai M, Day NK, Good RA, Onoe K. Interleukin-1-dependent mitogenic responses induced

by protoscoleces of *Echinococcus multilocularis* in murine lymphocytes. J Leukoc Biol 1993;53 (3):233-239

Klinker MW, Schiller JJ, Magnuson VL, Wang T, Basken J, Veth K, Pearce KI, Kinnunen L, Harjutsalo V, Wang X, et al. Single nucleotide polymorphisms in the IL2RA gene are associated with age at diagnosis in late-onset Finnish type 1 diabetes subjects. Immunogenetics 2010;62:101-107

Kocherscheidt L, Flakowski AK, Gruner B, Hamm DM, Dietz K, Kern P, Soboslay PT. *Echinococcus multilocularis*: inflammatory and regulatory chemokine responses in patients with progressive, stable and cured alveolar echinococcosis. Exp Parasitol 2008;119 (4):467-474.

Kruglyak L. The use of a genetic map of biallelic markers in linkage studies. Nat Genet 1997;17:21-24.

Kryczek I, Banerjee M, Cheng P, Vatan L, Szeliga W, Wei S, Huang E, Finlayson E, Simeone D, Welling TH, et al. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. Blood. 2009 Aug 6;114(6):1141-9.

Kuchroo VK, Awasthi A. Emerging new roles of Th17 cells. Eur J Immunol. 2012 Sep;42(9):2211-4.

Kuhner MK, Beeli P, Yamato J, Felsenstein J. Usefulness of single nucleotide polymorphism data for estimating population parameters. Genetics 2000;156:439-447.

Lee YK, Mukasa R, Hatton RD, Weaver CT. Developmental plasticity of Th17 and Treg cells. Curr Opin Immunol. 2009 Jun;21(3):274-80.

Levings MK, Sangregorio R, Roncarolo MG. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. J Exp Med. 2001 Jun 4;193(11):1295-302.

Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively

enhance expression of antimicrobial peptides. J Exp Med. 2006 Oct 2;203(10):2271-9.

Li H, Chen J, Huang A, Stinson J, Heldens S, Foster J, Dowd P, Gurney AL, Wood WI. Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family. Proc Natl Acad Sci U S A. 2000 Jan 18;97(2):773-8.

Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. Nat Immunol. 2011 Jun;12(6):551-9.

Liew FY, Li Y, Millott S. Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. J Immunol. 1990 Dec 15;145(12):4306-10.

Lin W, Haribhai D, Relland LM, Truong N, Carlson MR, Williams CB, Chatila TA. Regulatory T cell development in the absence of functional Foxp3. Nat Immunol 2007, 8:359–368.

Lowe CE, Cooper JD, Brusko T, Walker NM, Smyth DJ, Bailey R, Bourget K, Plagnol V, Field S, Atkinson M, et al. Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. Nat Genet 2007;39:1074-1082.

Luna L, Skammelsrud N, Johnsen O et al. Structural organization and mapping of the human TCF11 gene. Genomics 1995; 27: 237-244.

Ma HL, Liang S, Li J, Napierata L, Brown T, Benoit S, Senices M, Gill D, Dunussi-Joannopoulos K, Collins M, et al. IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. J Clin Invest. 2008 Feb;118(2):597-607.

Ma XJ, Wang Z, Ryan PD, Isakoff SJ, Barmettler A, Fuller A, Muir B, Mohapatra G, Salunga R, Tuggle JT, et al. A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer Cell. 2004 Jun;5(6):607-16.

Magram J, Connaughton SE, Warrier RR, Carvajal DM, Wu CY, Ferrante J, Stewart C, Sarmiento U, Faherty DA, et al. IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity. 1996 May;4(5):471-81.

Maizels RM. Parasite immunomodulation and polymorphisms of the immune system. J Biol 2009, 8:62.

Maizels RM, Sartono E, Kurniawan A, Partono F, Selkirk ME, Yazdanbakhsh M. T-cell activation and the balance of antibody isotypes in human *lymphatic filariasis*. Parasitol today 1995;11(2):50–56

Maldonado E, Ha I, Cortes P, Weis L, Reinberg D. Factors involved in specific transcription by mammalian RNA polymerase II: role of transcription factors IIA, IID, and IIB during formation of a transcriptioncompetent complex. Mol Cell Biol 1990, 10:6335-6347.

Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 2006 May 11;441(7090):231-4.

Manfras BJ, Reuter S, Wendland T, Boehm BO, Kern P. Impeded Th1 CD4 memory T cell generation in chronic-persisting liver infection with *Echinococcus multilocularis*. Int Immunol. 2004 Jan;16(1):43-50.

Márquez A, Orozco G, Martínez A, Palomino-Morales R, Fernández-Arquero M, Mendoza JL, Taxonera C, Díaz-Rubio M, Gómez-García M, Nieto A, et al. Novel association of the interleukin 2-interleukin 21 region with inflammatory bowel disease. Am J Gastroenterol 2009, 104:1968-1975.

Martin-Orozco N, Dong C. The IL-17/IL-23 axis of inflammation in cancer: friend or foe? Curr Opin Investig Drugs. 2009 Jun;10(6):543-9.

Matesanz F, Fedetz M, Collado-Romero M, Fernandez O, Guerrero M, Delgado C, Alcina A. Allelic expression and interleukin-2 polymorphisms in multiple sclerosis. J Neuroimmunol 2001; 119:101-105.

Mattuzzi S, Barbi S, Carletto A, Ravagnani B, Moore PS, Bambara LM, Scarpa A. Association of polymorphisms in the IL1B and IL2 genes with susceptibility and severity of systemic sclerosis. J Rheumatol 2007;34(5):997-1004

Mbow M, Larkin BM, Meurs L, Wammes LJ, de Jong SE, Labuda LA, Camara M, Smits HH, Polman K, Dieye TN, et al. T-helper 17 cells are associated with pathology in human schistosomiasis. J Infect Dis. 2013 Jan 1;207(1):186-95.

McHugh RS, Whitters MJ, Piccirillo CA, Young DA, Shevach EM, Collins M, Byrne MC CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. Immunity. 2002 Feb;16(2):311-23.

McKeigue PM. Mapping genes that underlie ethnic differences in disease risk: methods for detecting linkage in admixed population, by conditioning on parental admixture. Am J Hum Genet 1998; 63:241-251.

Mejri N, Müller N, Hemphill A, Gottstein B. Intraperitoneal *Echinococcus multilocularis* infection in mice modulates peritoneal CD4+ and CD8+ regulatory T cell development. Parasitol Int. 2011 Jan;60(1):45-53.

Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996;17 (3):138-146.

Muñoz-Fernández MA, Fernández MA, Fresno M. Activation of human macrophages for the killing of intracellular *Trypanosoma cruzi* by TNF-alpha and IFN-gamma through a nitric oxide-dependent mechanism. Immunol Lett. 1992 Jun;33(1):35-40.

Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001 Mar 1;410(6824):50-6.

Negrete-García MC, Velazquez JR, Popoca-Coyotl A, Montes-Vizuet AR, Juárez-Carvajal E, Teran LM. Chemokine (C-X-C motif) ligand 12/stromal cell-derived factor-1 is associated with leukocyte recruitment in asthma. Chest. 2010 Jul;138(1):100-6.

Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, Bucks C, Kane CM, Fallon PG, Pannell R, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. Nature. 2010 Apr 29;464(7293):1367-70.

Nickerson DA, Whitehurst C, Boysen C, Charmley P, Kaiser R, Hood L. Identification of clusters of biallelic polymorphic sequence-tagged sites (pSTSs) that generate highly informative and automatable markers for genetic linkage mapping. Genomics 1992;12:377-387.

Numasaki M, Fukushi J, Ono M, Narula SK, Zavodny PJ, Kudo T, Robbins PD, Tahara H, Lotze MT. Interleukin-17 promotes angiogenesis and tumor growth. Blood. 2003 Apr 1;101(7):2620-7.

O'Garra A, Murphy KM. From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce T(H)1 development. Nat Immunol. 2009 Sep;10(9):929-32.

Owyang AM, Zaph C, Wilson EH, Guild KJ, McClanahan T, Miller HR, Cua DJ, Goldschmidt M, Hunter CA, Kastelein RA, Artis D. Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. J Exp Med. 2006 Apr 17;203(4):843-9.

Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? Nat Rev Immunol. 2010 Apr;10(4):225-35.

Paranjape SM, Kamakaka RT, Kadonaga JT. Role of chromatin structure in the regulation of transcription by RNA polymerase II. Annu. Rev. Biochem. 1994;63:265.

Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science. 2003 Feb 14;299(5609):1033-6.

Peters A, Pitcher LA, Sullivan JM, Mitsdoerffer M, Acton SE, Franz B, Wucherpfennig K, Turley S, Carroll MC, Sobel RA, et al. Th17 cells induce ectopic lymphoid follicles in central nervous system tissue inflammation. Immunity. 2011 Dec 23;35(6):986-96.

Polansky JK, Schreiber L, Thelemann C, Ludwig L, Kruger M, Baumgrass R, Cording S, Floess S, Hamann A, Huehn J. Methylation matters: binding of Ets-1 to the demethylated Foxp3 gene contributes to the stabilization of Foxp3 expression in regulatory T cells. J Mol Med (Berl) 2010, 88:1029–1040.

Rakha NK, Dixon JB, Carter SD, Craig PS, Jenkins P, Folkard S. *Echinococcus multilocularis* antigens modify accessory cell function of macrophages. Immunology 1999;74 (4):652-656

Reya,T, Bassiri H, Biancaniello R, Carding SR. Thymic stomal-cell abnormalities and dysregulated T-cell development in IL-2-deficient mice. Dev.Immunol. 1998, 5:287.

Reynolds JM, Angkasekwinai P, Dong C. IL-17 family member cytokines: regulation and function in innate immunity. Cytokine Growth Factor Rev. 2010 Dec;21(6):413-23.

Rudner XL, Happel KI, Young EA, Shellito JE. Interleukin-23 (IL-23)-IL-17 cytokine axis in murine Pneumocystis carinii infection. Infect Immun. 2007 Jun;75(6):3055-61.

Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alphachains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151-1164.

Sallusto F, Baggiolini M. Chemokines and leukocyte traffic. Nat Immunol. 2008 Sep;9(9):949-52.

Sallusto F, Lanzavecchia A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. Immunol Rev. 2000 Oct;177:134-40.

Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005 Nov;23(5):479-90.

Schneider R, Yaneva T, Beauseigle D, El-Khoury L, Arbour N. IL-27 increases the proliferation and effector functions of

human naive CD8+ T lymphocytes and promotes their development into Tc1 cells. Eur J Immunol 2007;41 (1):47-59.

Schubert LA, Jeffery E, Zhang Y, Ramsdell F, Ziegler SF. Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. J Biol Chem 2001;276(40):37672-9.

Setiawan T, Metwali A, Blum AM, Ince MN, Urban JF Jr, Elliott DE, Weinstock JV. *Heligmosomoides polygyrus* promotes regulatory T-cell cytokine production in the murine normal distal intestine. Infect Immun. 2007 Sep;75(9):4655-63.

Shahram F, Nikoopour E, Rezaei N, Saeedfar K, Ziaei N, Davatchi F, Amirzargar A. Association of interleukin-2, interleukin-4 and transforming growth factor-beta gene polymorphisms with Behcet's disease. Clin Exp Rheumatol 2011;29:S28-S31.

Shaikhibrahim Z, Wernert N. ETS transcription factors and prostate cancer: the role of the family prototype ETS-1 (review). Int J Oncol 2012;40:1748-1754.

Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol 2002; 4: E131-136.

Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. Immunol Rev. 2006 Aug;212:60-73.

Shi Y, Ullrich SJ, Zhang J, Connolly K, Grzegorzewski KJ, Barber MC, Wang W, Wathen K, Hodge V, Fisher CL, et al. A novel cytokine receptor-ligand pair. Identification, molecular characterization, and in vivo immunomodulatory activity. J. Biol. Chem. 2000;275:19167–19176.

Shimizu J, Yamazaki S, Takahashi T, Ishida Y, Sakaguchi S. Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. Nat Immunol. 2002 Feb;3(2):135-42.

Shin HD, Kim LH, Park BL, Jung JH, Kim JY, Chung IY, Kim JS, Lee JH, Chung SH, Kim YH, et al. Association of Eotaxin gene

family with asthma and serum total IgE. Hum Mol Genet 2003;12(11):1279-1285

Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. J Immunol. 2008 Jan 1;180(1):214-21.

Smith KA, Maizels RM. IL-6 controls susceptibility to helminth infection by impeding Th2 responsiveness and altering the Treg phenotype in vivo. Eur J Immunol. 2014 Jan;44(1):150-61.

Song X, Qian Y. The activation and regulation of IL-17 receptor mediated signaling. Cytokine. 2013 May;62(2):175-82.

Sousa-Pereira SR, Teixeira AL, Silva LC, Souza AL, Antunes CM, Teixeira MM, Lambertucci JR. Serum and cerebral spinal fluid levels of chemokines and Th2 cytokines in *Schistosoma mansoni* myeloradiculopathy. Parasite Immunol 2006;28(9):473-478.

Stauber DJ, Debler EW, Horton PA, Smith KA, Wilson IA. Crystal structure of the IL-2 signaling complex: paradigm for a heterotrimeric cytokine receptor. PNAS 2006, 103:2788-2793

Suzukawa M, Koketsu R, Iikura M, Nakae S, Matsumoto K, Nagase H, Saito H, Matsushima K, Ohta K, Yamamoto K, et al. Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. Lab Invest 2000;88(11):1245-1253.

Tartour E, Fossiez F, Joyeux I, Galinha A, Gey A, Claret E, Sastre-Garau X, Couturier J, Mosseri V, Vives V, et al. Interleukin 17, a T-cell-derived cytokine, promotes tumorigenicity of human cervical tumors in nude mice. Cancer Res. 1999 Aug 1;59(15):3698-704.

Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, Maizels RM. Removal of regulatory T cell activity reverses hyporesponsiveness and leads to filarial parasite clearance in vivo. J Immunol. 2005 Apr 15;174(8):4924-33.

Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory

cells inhibits graft-versus-host disease lethality. Blood. 2002 May 15;99(10):3493-9.

Tong Z, Yang XO, Yan H, Liu W, Niu X, Shi Y, Fang W, Xiong B, Wan Y, Dong C. A protective role by interleukin-17F in colon tumorigenesis. PLoS One. 2012;7(4):e34959.

Tsai VW, Mohammad MG, Tolhurst O, Breit SN, Sawchenko PE, Brown DA. CCAAT/enhancer binding protein-delta expression by dendritic cells regulates CNS autoimmune inflammatory disease. J Neurosci 2011;31:17612-17621.

Tzeng YS, Li H, Kang YL, Chen WC, Cheng WC, Lai DM. Loss of CXCL12/SDF-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. Blood. 2011 Jan 13;117(2):429-39.

Velavan TP, Bechlars S, Huang X, Kremsner PG, Kun JF. Novel regulatory SNPs in the promoter region of the TNFRSF18 gene in a Gabonese population. Braz J Med Biol Res 2011;44:418-420.

Velavan TP, Bechlars S, Tomiuk J, Kremsner PG, Kun JF. Molecular characterization of regulatory polymorphisms in the promoter region of the STAT6 gene in a Gabonese population. Mem Inst Oswaldo Cruz 2011, 106:65–69.

Velavan TP, Ojurongbe O. Regulatory T cells and parasites. J Biomed Biotechnol 2011;201(1):520940.

Velavan TP, Buyukyazici B, Kremsner PG, Kun JF. Combined promoter haplotypes of the IL10R genes are associated with protection against severe malaria in Gabonese children. Immunogenetics 2012;64:87-95.

Vicari AP, Figueroa DJ, Hedrick JA, Foster JS, Singh KP, Menon S, Copeland NG, Gilbert DJ, Jenkins NA, Bacon KB, et al. TECK: a novel CC chemokine specifically expressed by thymic dendritic cells and potentially involved in T cell development. Immunity. 1997 Aug;7(2):291-301.

Vignali D. How many mechanisms do regulatory T cells need? Eur J Immunol. 2008 Apr;38(4):908-11.

Vuitton DA. The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? Acta Trop. 2003 Feb;85(2):119-32.

Vuitton DA, Gottstein B. *Echinococcus multilocularis* and its intermediate host: a model of parasite-host interplay. J Biomed Biotechnol. 2010:923193.

Wan YY, Flavell RA.. The roles for cytokines in the generation and maintenance of regulatory T cells. Immunol Rev. 2006 Aug;212:114-30

Wang J, Wicker LS, Santamaria P. IL-2 and its high-affinity receptor: Genetic control of immunoregulation and autoimmunity. Seminars in Immunology 2009;21:363-371.

Wang L, Yi T, Kortylewski M, Pardoll DM, Zeng D, Yu H. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. J Exp Med. 2009 Jul 6;206(7):1457-64.

Wellinghausen N, Jochle W, Reuter S, Flegel WA, Grunert A, Kern P. Zinc status in patients with alveolar echinococcosis is related to disease progression. Parasite Immunol 1999;21 (5):237-241

Wolffe AP. Centromeric chromatin: histone deviants. Curr. Biol. 1995;5:452.

Workman JL, Kingston RE. Alteration of nucleosome structure as a mechanism of transcriptional regulation. Annu. Rev. Biochem. 1998;67:545.

Yamanouchi J, Rainbow D, Serra P, Howlett S, Hunter K, Garner VE, Gonzalez-Munoz A, Clark J, Veijola R, Cubbon R, et al. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. Nature Genet. 2007;39(3): 329-337.

Yamaguchi Y, Fujio K, Shoda H, Okamoto A, Tsuno NH, Takahashi K, Yamamoto K. IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis. J Immunol. 2007 Nov 15;179(10):7128-36.

Yasuoka S, Kawanokuchi J, Parajuli B, Jin S, Doi Y, Noda M, Sonobe Y, Takeuchi H, Mizuno T, Suzumura A. Production and functions of IL-33 in the central nervous system. Brain Res 2011;1385:8-17.

Yeh J, Spicuglia S, Kumar S, Sanchez-Sevilla A, Ferrier P, Imbert J. Control of IL-2Ra gene expression: structural changes within the proximal enhancer/core promoter during T-cell development. Nucl. Aci. Res. 2002;30 (9): 1944-1951.

Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature 2007;445(7130):936-40.

Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). Annu Rev Immunol. 2010;28:445-89.

Zlotnik A, Yoshie O. The chemokine superfamily revisited. Immunity. 2012 May 25;36(5):705-16.

Zou T, Satake A, Corbo-Rodgers E, Schmidt AM, Farrar MA, Maltzman JS, Kambayashi T. Cutting edge: IL-2 signals determine the degree of TCR signaling necessary to support regulatory T cell proliferation in vivo. J Immunol 2012;189:28–32.

Zou W, Restifo NP. T(H)17 cells in tumour immunity and immunotherapy. Nat Rev Immunol. 2010 Apr;10(4):248-56.

Publications

In vitro characterization of novel and functional regulatory SNPs in the promoter region of *IL2* and *IL2R alpha* in a Gabonese population

Xiangsheng Huang[1†], Vera Kühne[1†], Jürgen F. J. Kun[1], Peter T Soboslay[1], Bertrand Lell[1,2], Velavan TP[1*]

[1] Institute of Tropical Medicine, University of Tübingen, Wilhelmstrasse 27, Tübingen 72074, Germany.
[2] Centre de Recherche Médicale de Lambaréné (CERMEL), Lambaréné P.B.118, Gabon.

[†] Equal contributors

BMC Med Genet. 2012 Dec 7;13:117

Abstract

Background: The selection pressure imposed by the parasite has a functional consequence on the immune genes, leading to altered immune function in which regulatory T cells (Tregs) induced by parasites during infectious challenges modulate or thwart the T effector cell mechanism.

Methods: We identified and investigated regulatory polymorphisms in the immune gene *IL2* and its receptor *IL2R alpha* (also known as *CD25*) in Gabonese individuals exposed to plentiful parasitic infections.

Results: We identified two reported variants each for *IL2* and its receptor *IL2R alpha gene loci.* Also identified were two novel variants, -83 /-84 *CT* deletions (*ss410961576)* for *IL2* and -409*C/T* (*ss410961577)* for *IL2R alpha*. We further validated all identified promoter variants for their allelic gene expression using transient transfection assays. Three promoter variants of the *IL2* locus revealed no significant expression of the reporter gene. The identified novel variant (*ss410961577C/T*) of the *IL2R alpha* revealed a significant higher expression of the reporter gene in comparison to the major allele ($P<0.05$). In addition, the *rs12722616C/T* variant of the *IL2R alpha* locus altered the transcription factor binding site *TBP* (TATA box binding protein) and *C/EBP beta* (CCAAT/enhancer binding protein beta) that are believed to regulate the Treg function.

Conclusions: The identification and validation of such regulatory polymorphisms in the immune genes may provide a basis for future studies on parasite susceptibility in a population where T cell functions are compromised.

Key words: *IL2, IL2R alpha, CD25*, Polymorphism, Transfection, Regulatory T cells

Background

Parasites increase their survival rate in the host by means of a complex interaction with the host immune system. However, understanding such interaction on the part of the host and parasite during infections still remains a fundamental issue. In such processes, it is believed that the host regulatory T (Tregs) cells play an essential role [1,2]. The parasite induces a regulatory T cell (Tregs) population that can modulate the magnitude of effector T cell functions, thereby leading to a subtle immune response during infections [3]. The regulatory T cell populations remain diverse; a few of them are induced during infections while the others are considered to be natural Tregs vitally implicated in averting autoimmunity [4]. Tregs are believed to influence host inflammatory and immune responses via mechanisms of cell-to-cell contact, inhibitory cytokines, and cytokine deprivation [3]. Pathogen driven selection operating on the host immune genes can impose a nucleotide variation in the primed sequence whereby substantial changes in gene expression is directed [2]. Human gene expression is a controled transcriptional process in the promoter region of a given gene and is regulated by *cis*-acting DNA sequence elements. Any nucleotide alteration in the promoter region is likely to alter the gene expression, reflecting the level of susceptibility to a parasitic infection as well as Treg expression [5]. A number of loci are known to be associated with Treg activity. Genes such as *IL10, IL13, STAT6, TNFRSF18, TLRs* and *FOXP3* have been established as key players in regulating Tregs [2,4-7]. One such gene of interest is the interleukin 2 (*IL2*) and

its receptor *IL2R* alpha (*CD25*) that are known to modulate the proliferation and differentiation of T cells and are essential for peripheral homeostasis of the $CD4^+CD25^+$ Tregs [8].

The human *IL2* is located on the q arm of the chromosome 4 (specifically 4q27) and its receptor *IL2R* alpha (*CD25*) maps to the p arm of the chromosome 10 (specifically 10p15.1). The human IL-2 is primarily produced by T cells in response to antigenic stimulation and is a major mediator of the immune response [9]. Studies have demonstrated that IL-2 is essential for the proliferation and maintenance of Tregs and can disrupt Treg homeostasis [10,11], whereas the IL-2R plays a significant role in Treg differentiation and proliferation [12]. Tregs have been shown to constitutively express IL-2R (CD25), allowing Tregs to respond to low levels of IL-2 produced by conventional CD4+ T cells [10,11]. The removal of IL-2 from activated T cells can lead to a deprivation of cells, which is indicated by studies on *IL2*-deficient mice [13]. The IL-2 receptor has three chains, α, β and γ, which constitute the high affinity *IL2* receptor. The *IL2R* alpha (*CD25*) is responsible for activating the *IL2* signaling complex and regulates the signal transduction [14,15]. The IL2-R alpha subunit forms the largest of the three IL-2/IL2-R interfaces. Association of α chain with the β and γ heterodimer creates a receptor with a much higher affinity for IL-2 than the β and γ chains receptor [16]. Antigen recognition by the T cell receptor induces the synthesis or activation of transcription factors such as *NFAT, AP-1*, and *NFκB*, which are located in the promoter region of the *IL2* gene and are essential for

activating its transcription [17]. Studies have pointed to the single nucleotide polymorphisms (SNPs) located within the upstream -10 kb of the *IL2* gene that includes the promoter region, and possibly even beyond, thereby contributing to *IL2* transcriptional properties *in vivo* [18]. The inhibition of *IL2R* alpha (*CD25*) during thymocyte differentiation is related to *IL2R* alpha promoter after response to pre-TCR signals and is essential for the specific response of mature T cells later on [19]. Additionally, studies have shown that SNPs within *IL2R* alpha are associated with both Grave's disease and Type1 diabetes [20,21]. Reports have indicated that polymorphisms in the genes encoding *IL2* are associated with ulcerative colitis, inflammatory bowel disorder, rheumatoid arthritis, and Behcet's disease [22-25], whereas the receptor of *IL2,* the *IL2R* alpha variants were associated with type I diabetes and multiple sclerosis [21,26,27].

In the current study, our goal is to identify regulatory single nucleotide polymorphisms (SNPs) in the promoter region of the *IL2* and its receptor *IL2R* alpha (*CD25*) gene loci, doing so in a Sub-Saharan African population exposed to a wide array of parasitic diseases. In this study, with a view to identifying regulatory SNPs in the promoter regions of the *IL2* and its receptor *IL2R* alpha (*CD25*), we sequenced the promoter region of such genes as were upstream of the transcriptional start site, using samples from 40 unrelated Gabonese individuals. The identified regulatory SNPs were further validated for their allelic gene expression, which may possibly be correlated with various physiological responses.

Methods

Genomic DNA Isolation

Forty DNA samples were collected from unrelated Gabonese individuals; informed written consent for participation in the study was obtained from all participants. Blood samples were collected from adult male patients with uncomplicated malaria at the Medical Research Unit of the Albert Schweitzer Hospital, Lambaréné, Gabon, between August and November 2004 [28]. All uncomplicated malaria individuals were male adults living in malaria endemic countries; usually such individuals will not show symptoms of malaria although carrying parasites in their blood. The study subjects represent another cohort where we investigated novel chemotherapy against malaria [29]. To avoid complications arising from unnoticed pregnancy, only males were chosen. The study was approved by the local ethics committee of the International Foundation of the Albert Schweitzer Hospital. Genomic DNA from whole blood was isolated using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany).

Sequencing and SNP identification

For purposes of sequencing analysis, gene and genomic sequences of the *IL2* (NM_000586) and *IL2R alpha* (CD25) (NM_000417) were obtained from the SNPper database [http://snpper.chip.org/]. PCR primers were designed to amplify the promoter region of the gene using the PRIMER3 Software [http:// www genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi]. The promoter regions of the human *IL2* and *IL2R* alpha (*CD25*)

genes were amplified by polymerase chain reaction (PCR). The primer pairs employed for amplifying promoter regions of the *IL2* were *IL2F*: 5'-TAAATAAGGCCATAGAATGG-3' and *IL2R*: 5'-GTTACATTAGCCCACACTTA -3'. The primer pairs employed to amplify promoter regions of *IL2R* alpha were *IL2RF*: 5'- GATCCACCCACCTTGGTCTA -3' and *IL2RR*: 5'-GGCAGCCAGGCACCATGATGAAC -3' (MWG Operon, Germany). In brief: PCR were carried out in 20 μl reaction volumes with 5ng of genomic DNA, 1x PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM of $MgCl_2$; Qiagen), 0.125mM of dNTPs, 0.5mM of each primer and 1 U Taq DNA polymerase (Qiagen, Hilden, Germany) on a PTC-200 Thermal cycler (MJ Research, USA). Thermal cycling parameters for the amplification of both *IL2 and IL2R* alpha were as follows: initial denaturation at 94 °C for 5min, followed by 40 cycles of 15 sec at 94 °C denaturation, 60 sec at 60 °C annealing temperature, 60 sec at 72 °C extension, followed by a final extension of 10 min at 72 °C. PCR products were cleaned up using Exo-SAP-IT (USB, Affymetrix, USA) and 1μl of the purified product was directly used as templates for sequencing, using the BigDye terminator v. 2.0 cycle sequencing kit (Applied Biosystems, USA) on an ABI *3130 XL* DNA sequencer, according to the manufacturer's instructions. Polymorphisms in the promoter regions were identified by assembling the sequences, the respective reference sequences being obtained from the SNP per database (http://snpper.chip.org) using Codon code Aligner 4.0 software (http://www.codoncode.com/), and were then

reconfirmed visually from their respective electropherograms. [stimmt diese Korrektur? Ja!!]

Cloning and construct preparation

The SNPs identified in the promoter regions had their polymorphism status reconfirmed by subsequent cloning procedures. Those genomic DNA sequences identified as having SNPs in the promoter regions of the *IL2* and *IL2R* alpha were amplified with infusion primers (flanks a 15bp homology to the linearized pGL3 vector) and were then cloned to a linearized pGL3 basic vector. In brief, PCR amplifications were carried out in 50 μl reaction volumes under the same program conditions as mentioned above. The amplified PCR products was analyzed by electrophoresis in 1.5% agarose gels, using a 100 bp DNA ladder molecular size marker (Invitrogen, Karlsruhe, Germany), and PCR -products were gel eluted and purified using a Nucleospin kit (Macherey-Nagel, Düren, Germany) before being cloned to the pGL3 basic vector using Infusion advantage PCR cloning kit (Clonetech, Mounatain view, CA). Plasmids were isolated using QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany). To ensure accuracy of the sequenced promoter regions, several independent plasmids containing inserts were sequenced in both directions using appropriate primer pairs. The plasmid exhibiting the confirmed polymorphism was transformed into one shot *E. coli* (Invitrogen, Karlsruhe, Germany). Two independent colonies were selected from these transformations and maxi prep was performed using Endofree plasmid maxi kit (Qiagen, Hilden, Germany).

Transient transfection assays

We tested the activities of the observed polymorphic promoters for both *IL2* and *IL2R* alpha, using Jurkat T cell lines. Basically, four independent transfection experiments for each construct in duplicates were performed with Jurkat T cells (DSMZ, Braunschweig, Germany). Jurkat T cells (0.8X10 ^{6} cells/µl) were grown in a RPMI 1640 (Sigma-Aldrich, Hamburg, Germany) supplemented medium containing 10% FBS, 2mM L-Glutamine, and 1% Streptamycin-pencillin substrate (Invitrogen, Karlsruhe, Germany). Jurkat cells (0.8X10 ^{6} cells/µl) were transfected with TransIT reagent (Mirus Bio, Madison, USA) as recommended by the manufacturer. In brief: 120µl of TransIT reagent was added to 3ml of RPMI 1640 serum free medium (Sigma-Aldrich, Hamburg Germany), this being then incubated for 20 min at room temperature. Each of the 24 well plates was then seeded with 0.5ml (0.8X10 ^{6} cells/µl) of Jurkat T cells, along with 500ng of plasmid DNA constructs and 20ng of Renilla, before being allowed to incubate for 20 minutes. After incubation, 52µl of TransIT+RPMI serum free medium mix were suspended across each well. The whole procedure was performed in 2X24 well plate formats. After 24 hours, one plate was induced with 20ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Hamburg, Germany) as well as 25µg/ml Concanavalin A (Sigma-Aldrich, Hamburg, Germany). After 24 hours, cells were harvested by centrifugation, then washed twice with phosphate-buffered saline and lysed in 100µl of 1x passive lysis buffer (Promega, Mannheim, Germany). After incubation

for 20 min at room temperature on a rocking platform, 10µl of the lysate was used to measure luciferase activity in the SIRIUS luminometer (Berthold detection system, Pforzheim, Germany). We employed the dual luciferase reporter assay system (Promega, Mannheim, Germany). For each experiment, a plasmid expressing constitutively Renilla luciferase in low amounts was used as a positive control [30], while a promoterless plasmid (pGL3 basic) was integrated as a negative control. Each construct was measured 8 times, then both stimulated and non stimulated with two different DNA preparations. Relative luciferase activity was calculated as luciferase firefly/luciferase Renilla multiplied by 1000.

Transcription factor binding search

An extensive search for transcription factor binding sites for the observed SNPs in the promoter region was performed using a TF-Search online tool (http://www.gene-regulation.com/pub/programs/alibaba2) that utilizes TRANSFAC 4.0.

Statistical analysis

Data were normalized and then analyzed by StatView (http://www.statview.com). The mean ratio (Luciferase/Renilla) across all measurements was considered for purposes of calculating relative luciferase activity. The luciferase activity of the two different *IL2* and *IL2R* alpha (*CD25*) promoter variants was comparatively analyzed by a t-test (before and after stimulation). In addition, each construct's activity was compared to the activity of the major allele (common alleles) in both an

induced and a non induced state. The statistically significant level was set as 0.05.

Results

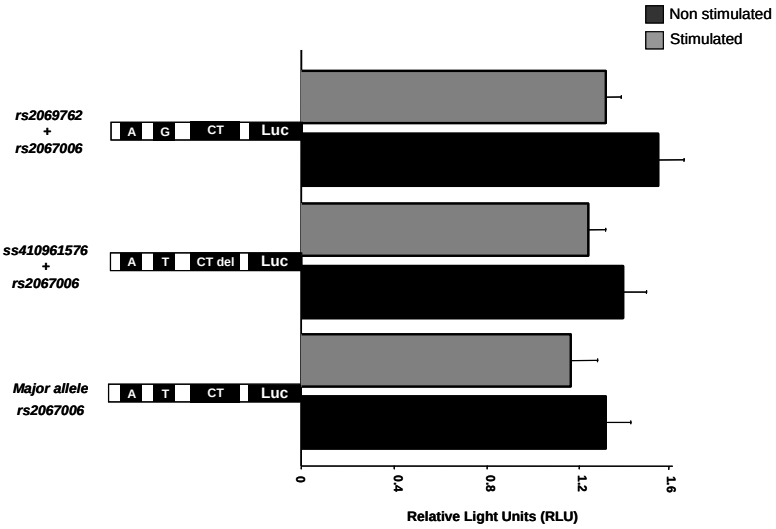
All forty subjects were sequenced for the entire promoter region of the *IL2* and *IL2R* alpha (*CD25*) and were then investigated for described and novel SNPs. For the *IL2* promoter region, two predescribed SNPs (*rs2069762T/G* and *rs2067006T/A*) were observed. In addition, we observed a novel *CT* deletion at the position -83/-84 in one individual. This novel SNP was submitted to the SNPper database and a corresponding ID obtained (*#ss410961576*). The corresponding references SNP ID (#rs); observed allele frequencies from the investigated *IL2* gene are summarized in Table 1. In order to characterize these variants in terms of function, a transient transfection assay were performed using Jurkat T cells. The activity of the three different *IL2* promoter variants analyzed by luciferase activities is compared in Fig. 1. The *p*-values of the two observed SNP constructs of the *IL2* variant remained insignificant in comparison to the major allele before and after stimulation ($P > 0.05$). When explicitly examined for a transcription factor binding site in the observed variants of *IL2*, the *rs2067006T/A* variant was found to be positioned on the transcription site *ETS-1*, which is a member of the *ETS* (*E-twenty six*) family of transcription factors that function as transcriptional activators or repressors in numerous genes believed to be involved in stem cell development, cell senescence, and death. The other two variants (*#ss410961576CTdel* and *rs2069762T/G)* do not alter any putative transcription factor binding site.

Table 1. Genetic variants identified in the promoter regions of the *IL2* gene locus

SNP (rs#)	Position	Polymorphism	Flanking sequences	Genotype	Analysed individuals	Allele	Frequency [a]	Frequencies (Hapmap YRI, CEU, CHB) [b]
ss410961576	-83,84	CT deletion	ATTTT [CT/-] GAGTT	CT/CT	39	CT	0.975	NA
				del/del	1	del	0.025	
rs2069762	-100	T >G	TTTTA [T/G] GACAA	TT	38	T	0.950	0.966, 0.768 , 0.733
				TG	2	G	0.050	0.034, 0.232, 0.267
rs2067006	-191	T>A	TGTTT [T/A] ATCAA	AT	0	T	0.000	0.000, 0.000, 0.000
				AA	40	A	1.000	1.000, 1.000, 1.000

a: Frequency corresponds for both allele and genotypes; b: YRI: Yoruba population representing Sub- Saharan African individual group, CEU: European, CHB: Han Chinese; NA: data not available

Figure 1: Comparison of the activity of the three identified *IL2* promoter variants analyzed by luciferase activities. The ratio of the relative light units (firefly/renilla) is given. *P* values are calculated by t-Test from four different experiments performed in duplicates with two different DNA preparations. Each construct was induced by PMA and ConA. The letters indicate the presence of mutations T/G, CT del and T/A.



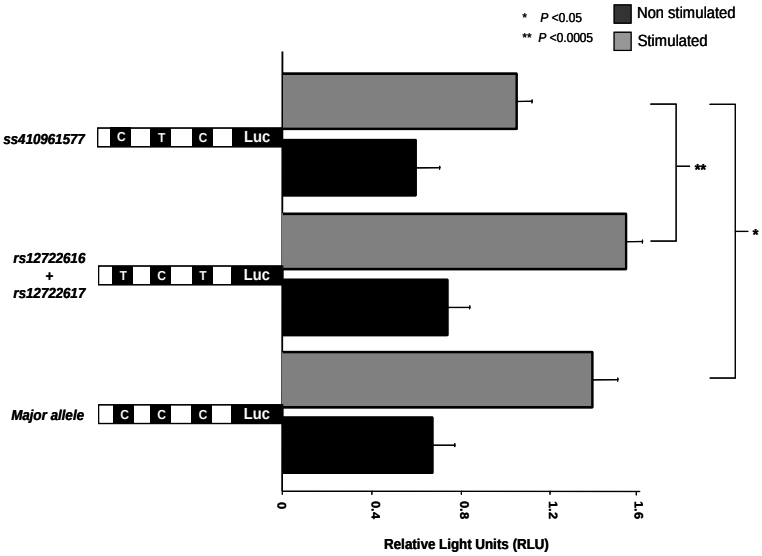
For the *IL2R* alpha (*CD25*), two described variants in the promoter region (*rs12722617C/T* and *rs12722616C/T*) were observed. In addition, we observed a novel *C/T* variant at the position -409 in two individuals. This novel SNP was submitted to the SNPper database and a corresponding ID was obtained (#*ss410961577*). The corresponding references SNP ID (#rs); observed allele frequencies from investigated *IL2R* alpha (CD25) are summarized in Table 2. The activity of the three different *IL2* promoter variants analyzed by luciferase activities is compared in Fig. 2. The variant *rs12722617C/T* and *rs12722616C/T* were observed to be in absolute linkage in all the individuals analyzed. The *p*-values of the SNP construct (*rs12722617C/T + rs12722616C/T)* of the *IL2R alpha* variant remained insignificant in comparison to the major allele before and after induction ($P > 0.05$), but remained significant when compared to the new variant in a stimulated state ($P < 0.0005$). The observed novel variant *#ss410961577C/T* showed significantly increased activity in comparison to the major allele ($P < 0.05$). When explicitly examined for a transcription factor binding site in the observed variants of *IL2R alpha (CD25)*, two variants (*rs12722617C/T and #ss410961577C/T)* were found not to alter any putative transcription factor binding site. However, the SNP variant (*rs12722616C/T*), which was in linkage disequilibrium (LD) with *rs12722617C/T,* is positioned at the transcription factor binding site *TBP* (TATA box binding protein) and *C/EBP* beta (CCAAT/enhancer binding protein beta).

Table 2. Genetic variants identified in the promoter regions of the *IL2R alpha (CD25)* gene locus

SNP (rs#)	Position	Polymorphism	Flanking sequences	Genotype	Analysed individuals	Allele	Frequency [a]	Frequencies (Hapmap YRI, CEU, CHB) [b]
rs12722617	-398	C>T	TTCGC [C/T] GCATC	CC	35	C	0.875	0.932, 1.000, NA
				CT	5	T	0.125	0.068, 0.000, NA
ss410961577	-409	C>T	GGATC [C/T] TTCAG	CC	38	C	0.950	NA
				CT	2	T	0.050	
rs12722616	-516	C>T	AACAC [C/T] TTATA	CC	35	C	0.875	0.924, 1.000, 1.000
				CT	5	T	0.125	0.076, 0.000, 0.000

a: Frequency corresponds for both allele and genotypes; b: YRI: Yoruba population representing Sub- Saharan African individual group, CEU: European, CHB: Han Chinese; NA: data not available

Figure 2: The activity of the three identified *IL2R alpha* promoter variants as compared by luciferase activities. The ratio of the relative light units (firefly/renilla) is given. *P* values are calculated by t-Test in four different experiments performed in duplicates with two different DNA preparations. Each construct was induced by PMA and ConA. The letters indicate the presence of polymorphic variants.



Discussion

The underlying idea of the current study is to understand how regulatory SNPs in populations that are naturally exposed to array of parasites contribute to immune outcome. A significant impact on diversity among immune gene families is believed to be attributable to invasion maneuvers performed by the parasites. Therefore, variations in the promoter regions of these immune system genes can potentially amend the gene expression levels, either by changing specificity of transcription binding sites or by altering the kinetics of transcription initiation [7]. In our current study, we identified two known SNPs (*rs2069762T/G* and *rs2067006T/A*) and one novel *CT* deletion (*#ss410961576*) at the position -83/-84 when screened for *IL2* promoter region. None of the investigated SNP constructs revealed differential luciferase activity compared to the major allele. In the current investigation, the *IL2* variant (*rs2067006T/A*) was considered to be the major allele, as all the individuals were found to harbor this particular variant. When compared to the NCBI HapMap database, the frequencies of the observed SNP variants (*rs2067006T/A* and *rs2069762T/G* of *IL2*) were in accordance with the Yoruba population of Nigeria, which represents a Sub Saharan African group. The *rs2067006T/A* variant is positioned on the transcription site *ETS-1*, a member of the *ETS* (*E-twenty six*) family of transcription factors. The *ETS-1* DNA-binding domain recognizes the core consensus DNA in target genes and acts either as transcriptional activators or repressors [31]. Recent studies have demonstrated that *ETS-1* belongs to a large

protein complex which binds to the Treg-specific demethylated region (TSDR) in the *FOXP3* locus, thereby restricting the stable *FOXP3* expression in the Tregs [32]. *FOXP3* is described as a master regulator of natural Tregs development and function [4], while mature regulatory T cells expressing a non-functional fusion protein of *FOXP3* lack any suppressor function [33]. Since all the investigated individuals inherited this variant, we believe that the studied population may have a restricted *FOXP3* expression leading to a subtle T effector cell function.

In our study we also identified two known SNPs (*rs12722617C/T* and *rs12722616C/T*) along with one novel C/T variant (*#ss410961577*) at the position -516 when screened for the *IL2R alpha* (*CD25*) promoter region of the *IL2R alpha*. Both predescribed SNP variants of the *IL2R alpha* (*rs12722617C/T* and *rs12722616C/T*) were observed in linkage in all studied individuals. We did not observe a differential luciferase activity compared to the major allele for the construct (*rs12722617C/T* + *rs12722616C/T*). However, the identified novel variant (*#ss410961577C/T*) revealed significant increased activity in comparison to the constructs with the major allele. This particular novel variant did not alter any putative transcription factor binding site. But the variant *rs12722616C/T,* which was in LD with *rs12722617C/T,* altered the transcription factor binding site *TBP* (TATA box binding protein) and *C/EBP* beta (CCAAT/enhancer binding protein beta). The *TATA* box is a type of promoter sequence that indicates the transcriptional start site whereby a genetic sequence can be read and decoded. It

is named after the conserved DNA sequence TATAAA. *TBP* (TATA binding protein) together with TATA associated factors (*TAFS*) make the transcription factor *TFIID* which binds the TATA box in combination with other transcription factors (*TFIIB, TFIIA, TFIIE, TFIIF, TFIIH*) [34]. All the above transcription factors along with RNA polymerase II enzyme form a transcription initiation complex. Therefore, it is possible that the *TATA* box in the construct *rs12722616C/T* contributes to the transcription initiation in vitro in the Jurkat cells. The other transcription factor *C/EBP* beta (CCAAT/enhancer binding protein beta) is believed to modulate inflammatory processes. A recent study has demonstrated in an experimental autoimmune encephalomyelitis model that *C/EBP* expression by dendritic cells (DC) influences Th17 versus Treg differentiation but has little or no impact upon Th1 development [35]. Examining the HapMap database, all the frequencies of reported variants of the *IL2R alpha* loci were found to be in accordance with the frequencies in the Yoruba population.

Human *IL2* and its receptor *IL2R* alpha are described as constituting a potent T cell growth factor and are mainly produced by activated CD4+ T cells, though also by naïve CD8+ T cells, dendritic cells, and thymic cells [36]. Human IL-2 is considered to be vital for the development of $CD4^{+}CD25^{+}$ regulatory T cells [37]. Also, studies have demonstrated that IL-2 potentially plays a role in the thymic development of Tregs [38,39]. Studies have shown that polymorphisms in the *IL2* are associated with many diseases, including auto immune diseases. Recent studies have demonstrated that the studied *IL2* promoter

variant (*rs2069762T/G*) is associated with *Helicobacter pylori* infection [40], multiple sclerosis [41], pathogenesis of childhood lymphoma [42], pathogenesis of new-onset diabetes after transplantation (NODAT) [43], measles-specific cellular immunity [44], a higher risk of acute rejection episodes during kidney transplantation [45] and in allergic disorders [46] . However, the role of the described and novel variants of *IL2* and *IL2R alpha* in this study needs to be validated in terms of the specific role these play in different parasitic diseases.

Conclusions

In summary, the regulatory SNPs identified in this current study will provide useful information for understanding the relevance of sequence polymorphisms in populations exposed to many parasitic diseases and may serve as a basis for backup studies examining disease susceptibility

Conflicting interests

The authors declare that they have no conflicting interests

Authors' contributions

BL designed the field study; VTP and JFJ Kun designed and supervised the experiments; XH and VK performed the experiments; PTS and VTP analyzed the data; BL and VTP contributed materials/analysis tools; XH and VTP wrote the paper. All authors read and approved the final manuscript.

Acknowledgements

We thank Andrea Weierich and Velia Grummes for technical assistance during the study period. The project was funded by a grant from the EU commission – TRANCHI (INCO-CT-2006-032436).

References

1. Belkaid Y, Rouse BT: **Natural regulatory T cells in infectious disease.** *Nat Immunol* 2005, **6:** 353-360.

2. Maizels RM: **Parasite immunomodulation and polymorphisms of the immune system.** *J Biol* 2009, **8:** 62.

3. Velavan TP, Ojurongbe O: **Regulatory T cells and parasites.** *J Biomed Biotechnol* 2011, **2011:** 520940.

4. Hanel SA, Velavan TP, Kremsner PG, Kun JF: **Novel and functional regulatory SNPs in the promoter region of FOXP3 gene in a Gabonese population.** *Immunogenetics* 2011, **63:** 409-415.

5. Velavan TP, Buyukyazici B, Kremsner PG, Kun JF: **Combined promoter haplotypes of the IL10R genes are associated with protection against severe malaria in Gabonese children.** *Immunogenetics* 2012, **64:** 87-95.

6. Velavan TP, Bechlars S, Huang X, Kremsner PG, Kun JF: **Novel regulatory SNPs in the promoter region of the TNFRSF18 gene in a Gabonese population.** *Braz J Med Biol Res* 2011, **44:** 418-420.

7. Velavan TP, Bechlars S, Tomiuk J, Kremsner PG, Kun JF: **Molecular characterization of regulatory polymorphisms in the promoter region of the STAT6 gene in a Gabonese population.** *Mem Inst Oswaldo Cruz* 2011, **106:** 65-69.

8. Bayer AL, Yu A, Adeegbe D, Malek TR: **Essential role for interleukin-2 for CD4(+)CD25(+) T regulatory cell development during the neonatal period.** *J Exp Med* 2005, **201:** 769-777.

9. Zou T, Satake A, Corbo-Rodgers E, Schmidt AM, Farrar MA, Maltzman JS *et al*.: **Cutting edge: IL-2 signals determine the degree of TCR signaling necessary to support regulatory T cell proliferation in vivo.** *J Immunol* 2012, **189:** 28-32.

10. Cheng G, Yu A, Malek TR: **T-cell tolerance and the multi-functional role of IL-2R signaling in T-regulatory cells.** *Immunol Rev* 2011, **241:** 63-76.

11. Setoguchi R, Hori S, Takahashi T, Sakaguchi S: **Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization.** *J Exp Med* 2005, **201:** 723-735.

12. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD *et al*.: **In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression.** *J Exp Med* 2009, **206:** 751-760.

13. Reya T, Bassiri H, Biancaniello R, Carding SR: **Thymic stromal-cell abnormalities and dysregulated T-cell development in IL-2-deficient mice.** *Dev Immunol* 1998, **5:** 287-302.

14. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH: **Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood.** *J Exp Med* 2001, **193:** 1285-1294.

15. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M: **Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.** *J Immunol* 1995, **155:** 1151-1164.

16. Grant AJ, Roessler E, Ju G, Tsudo M, Sugamura K, Waldmann TA: **The interleukin 2 receptor (IL-2R): the IL-2R alpha subunit alters the function of the IL-2R beta subunit to enhance IL-2 binding and signaling by mechanisms that do not require binding of IL-2 to IL-2R alpha subunit.** *Proc Natl Acad Sci U S A* 1992, **89:** 2165-2169.

17. Attema JL, Reeves R, Murray V, Levichkin I, Temple MD, Tremethick DJ *et al*.: **The human IL-2 gene promoter can assemble a positioned nucleosome that becomes remodeled upon T cell activation.** *J Immunol* 2002, **169:** 2466-2476.

18. Wang J, Wicker LS, Santamaria P: **IL-2 and its high-affinity receptor: genetic control of immunoregulation and autoimmunity.** *Semin Immunol* 2009, **21:** 363-371.

19. Yeh JH, Spicuglia S, Kumar S, Sanchez-Sevilla A, Ferrier P, Imbert J: **Control of IL-2Ralpha gene expression: structural changes within the proximal enhancer/core promoter during T-cell development.** *Nucleic Acids Res* 2002, **30:** 1944-1951.

20. Brand OJ, Lowe CE, Heward JM, Franklyn JA, Cooper JD, Todd JA *et al*.: **Association of the interleukin-2 receptor alpha (IL-2Ralpha)/CD25 gene region with Graves' disease using a multilocus test and tag SNPs.** *Clin Endocrinol (Oxf)* 2007, **66:** 508-512.

21. Lowe CE, Cooper JD, Brusko T, Walker NM, Smyth DJ, Bailey R *et al*.: **Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes.** *Nat Genet* 2007, **39:** 1074-1082.

22. Bowes J, Ho P, Flynn E, Ali F, Marzo-Ortega H, Coates LC *et al*.: **Comprehensive assessment of rheumatoid arthritis susceptibility loci in a large psoriatic arthritis cohort.** *Ann Rheum Dis* 2012.

23. Festen EA, Goyette P, Scott R, Annese V, Zhernakova A, Lian J *et al*.: **Genetic variants in the region harbouring IL2/IL21 associated with ulcerative colitis.** *Gut* 2009, **58:** 799-804.

24. Marquez A, Orozco G, Martinez A, Palomino-Morales R, Fernandez-Arquero M, Mendoza JL *et al*.: **Novel association of the interleukin 2-interleukin 21 region with inflammatory bowel disease.** *Am J Gastroenterol* 2009, **104:** 1968-1975.

25. Shahram F, Nikoopour E, Rezaei N, Saeedfar K, Ziaei N, Davatchi F *et al*.: **Association of interleukin-2, interleukin-4 and transforming growth factor-beta gene polymorphisms with Behcet's disease.** *Clin Exp Rheumatol* 2011, **29:** S28-S31.

26. Alcina A, Fedetz M, Ndagire D, Fernandez O, Leyva L, Guerrero M *et al*.: **IL2RA/CD25 gene polymorphisms: uneven association with multiple sclerosis (MS) and type 1 diabetes (T1D).** *PLoS One* 2009, **4:** e4137.

27. Klinker MW, Schiller JJ, Magnuson VL, Wang T, Basken J, Veth K *et al*.: **Single-nucleotide polymorphisms in the IL2RA gene are associated with age at diagnosis in late-onset Finnish type 1 diabetes subjects.** *Immunogenetics* 2010, **62:** 101-107.

28. Kreidenweiss A, Kremsner PG, Dietz K, Mordmuller B: **In vitro activity of ferroquine (SAR97193) is independent of chloroquine resistance in Plasmodium falciparum.** *Am J Trop Med Hyg* 2006, **75:** 1178-1181.

29. Supan C, Mombo-Ngoma G, Dal-Bianco MP, Ospina Salazar CL, Issifou S, Mazuir F *et al*.: **Pharmacokinetics of ferroquine, a novel 4-aminoquinoline, in asymptomatic carriers of Plasmodium falciparum infections.** *Antimicrob Agents Chemother* 2012, **56:** 3165-3173.

30. Juliger S, Bongartz M, Luty AJ, Kremsner PG, Kun JF: **Functional analysis of a promoter variant of the gene encoding the interferon-gamma receptor chain I.** *Immunogenetics* 2003, **54:** 675-680.

31. Shaikhibrahim Z, Wernert N: **ETS transcription factors and prostate cancer: the role of the family prototype ETS-1 (review).** *Int J Oncol* 2012, **40:** 1748-1754.

32. Polansky JK, Schreiber L, Thelemann C, Ludwig L, Kruger M, Baumgrass R *et al*.: **Methylation matters: binding of Ets-1 to the demethylated Foxp3 gene contributes to the stabilization of Foxp3 expression in regulatory T cells.** *J Mol Med (Berl)* 2010, **88:** 1029-1040.

33. Lin W, Haribhai D, Relland LM, Truong N, Carlson MR, Williams CB *et al*.: **Regulatory T cell development in the absence of functional Foxp3.** *Nat Immunol* 2007, **8:** 359-368.

34. Maldonado E, Ha I, Cortes P, Weis L, Reinberg D: **Factors involved in specific transcription by mammalian RNA polymerase II: role of transcription factors IIA, IID, and IIB**

during formation of a transcription-competent complex. *Mol Cell Biol* 1990, **10:** 6335-6347.

35. Tsai VW, Mohammad MG, Tolhurst O, Breit SN, Sawchenko PE, Brown DA: **CCAAT/enhancer binding protein-delta expression by dendritic cells regulates CNS autoimmune inflammatory disease.** *J Neurosci* 2011, **31:** 17612-17621.

36. Nelson BH: **IL-2, regulatory T cells, and tolerance.** *J Immunol* 2004, **172:** 3983-3988.

37. Nelson BH: **IL-2, regulatory T cells, and tolerance.** *J Immunol* 2004, **172:** 3983-3988.

38. Malek TR, Porter BO, Codias EK, Scibelli P, Yu A: **Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors.** *J Immunol* 2000, **164:** 2905-2914.

39. Malek TR, Yu A, Vincek V, Scibelli P, Kong L: **CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2.** *Immunity* 2002, **17:** 167-178.

40. Schmidt HM, Ha DM, Taylor EF, Kovach Z, Goh KL, Fock KM *et al*.: **Variation in human genetic polymorphisms, their association with Helicobacter pylori acquisition and gastric cancer in a multi-ethnic country.** *J Gastroenterol Hepatol* 2011, **26:** 1725-1732.

41. Fedetz M, Ndagire D, Fernandez O, Leyva L, Guerrero M, Arnal C *et al*.: **Multiple sclerosis association study with the TENR-IL2-IL21 region in a Spanish population.** *Tissue Antigens* 2009, **74:** 244-247.

42. Song N, Han S, Lee KM, Choi JY, Park SK, Jeon S *et al*.: **Genetic Variants in Interleukin-2 and Risk of Lymphoma among Children in Korea.** *Asian Pac J Cancer Prev* 2012, **13:** 621-623.

43. Kim YG, Ihm CG, Lee TW, Lee SH, Jeong KH, Moon JY *et al*.: **Association of genetic polymorphisms of interleukins**

with new-onset diabetes after transplantation in renal transplantation. *Transplantation* 2012, **93:** 900-907.

44. Haralambieva IH, Ovsyannikova IG, Kennedy RB, Vierkant RA, Pankratz VS, Jacobson RM *et al*.: **Associations between single nucleotide polymorphisms and haplotypes in cytokine and cytokine receptor genes and immunity to measles vaccination.** *Vaccine* 2011, **29:** 7883-7895.

45. Park SJ, Yoon YC, Kang SW, Kim TH, Kim YW, Joo H *et al*.: **Impact of IL2 and IL2RB genetic polymorphisms in kidney transplantation.** *Transplant Proc* 2011, **43:** 2383-2387.

46. Braegger CP, Spencer J, MacDonald TT: **Ontogenetic aspects of the intestinal immune system in man.** *Int J Clin Lab Res* 1992, **22:** 1-4.

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
2Division of Infectious Diseases and Clinical Immunology, Comprehensive Infectious Disease Center, University of Ulm Hospitals, 89081 Ulm, Germany

Clin Dev Immunol. 2012;2012:735342.

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.

1. 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.

2. 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°C, 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°C, sterile filtered (0.22 µm) and kept at −20°C. 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°C. The supernatant was sterile filtrated (0.22 µm) and stored at −70°C. 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°C. The supernatant 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°C, saturated humidity, and 5% CO_2. Cells and cell culture supernatant were harvested, separated by centrifugation, and stored at −80°C.

2.5. Determination of Cytokine and Chemokine Concentrations

Cell culture supernatants and plasma were stored at −80°C 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.

3. 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)).

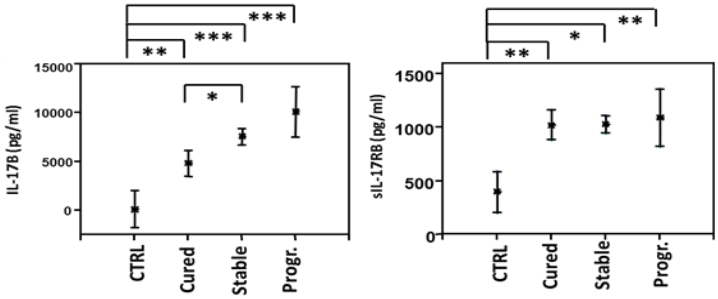


Figure 1: Plasma concentrations of interleukin (IL)-17B (part a) and soluble receptor IL-17RB (part b) in Alveolar Echinococcosis (AE) patients and in infection-free controls (CTRL). The patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) Alveolar 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).

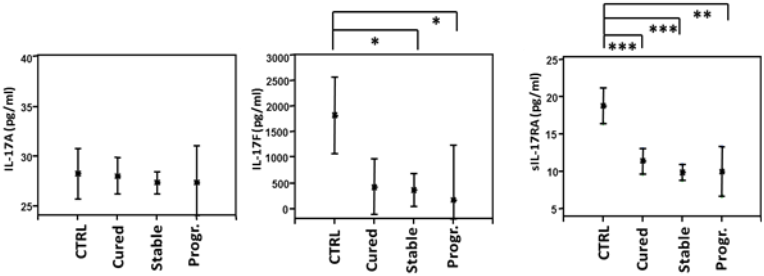


Figure 2: Plasma concentrations of interleukin (IL)-17A (part a) and IL-17F (part b) and of soluble IL-17RA (part c) in Alveolar Echinococcosis (AE) patients and infection-free controls (CTRL). Patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) 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.

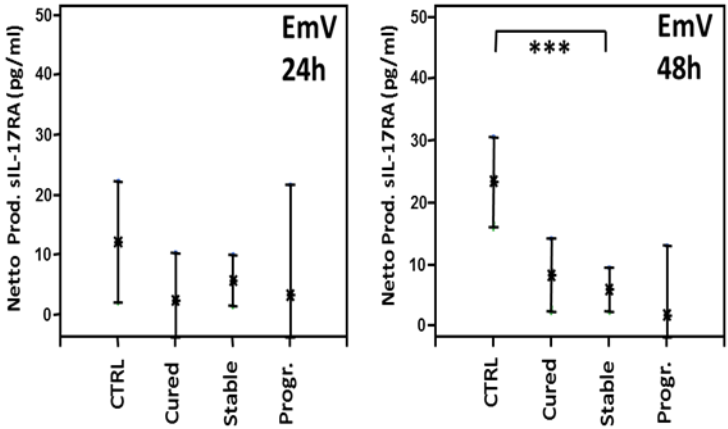


Figure 3: *Echinococcus multilocularis* antigen induced cellular production of soluble interleukin-17 receptor A (sIL-17RA) by peripheral blood mononuclear cells (PBMCs) from Alveolar Echinococcosis (AE) patients and infection-free controls (CTRL). Patients were grouped according to their state of infection, 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

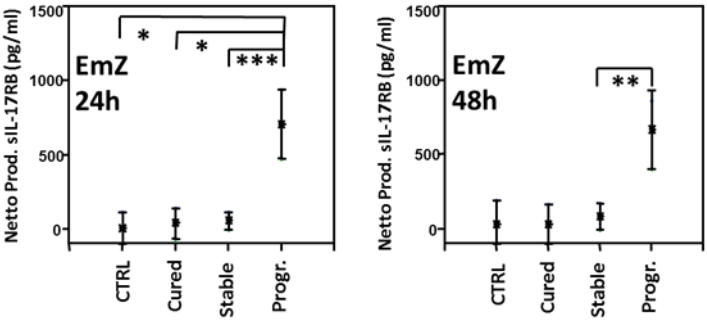


Figure 4B

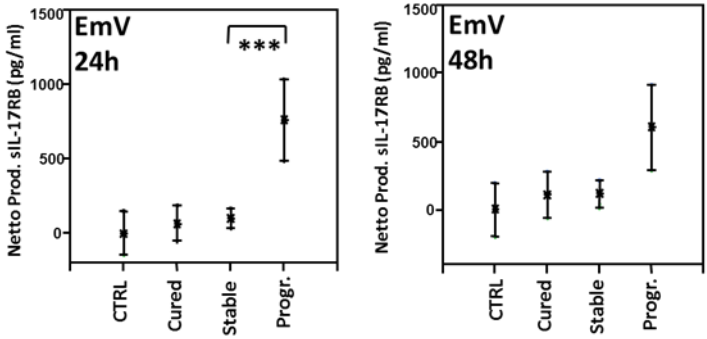


Figure 4: *Echinococcus multilocularis* antigen induced cellular production of soluble interleukin-17 receptor B (sIL-17RB) by PBMC from Alveolar Echinococcosis (AE) patients and infection-free controls (CTRL). Patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) Alveolar Echinococcosis. PBMCs from patients and controls were stimulated with *Echinococcus multilocularis* single-cell extract (EmZ) (a) and vesicle extract (EmV) (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).

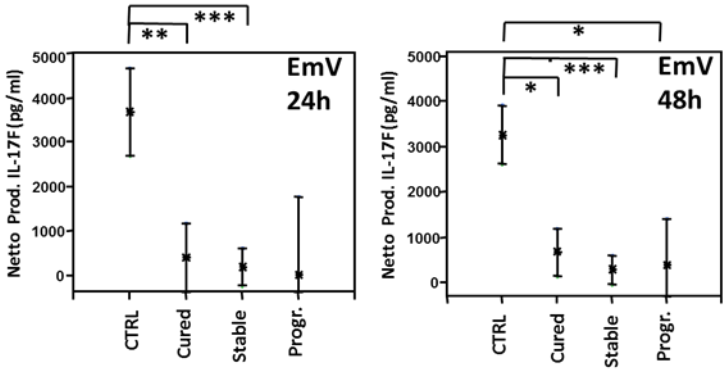


Figure 5: *Echinococcus multilocularis* antigen- (EmAg-) induced cellular production of interleukin-17F (IL-17F) by PBMC from Alveolar Echinococcosis (AE) patients and infection-free controls (CTRL). Patients were grouped according to their state of infection, that is, cured, stable, or progressive (Prog.) 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 ***$P < 0.001$).

4. 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.

5. 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.

Acknowlegment

This work was supported by the Baden-Württemberg Stiftung (Grant P-LS-AL2/06).

References

[1] M. Piarroux, R. Piarroux, R. Giorgi et al., “Clinical features and evolution of alveolar echinococcosis in France from 1982 to 2007: results of a survey in 387 patients,” *Journal of Hepatology*, vol. 55, no. 5, pp. 1025–1033, 2011.

[2] P. Kern, “Clinical features and treatment of alveolar echinococcosis,” *CurrentOpinion in InfectiousDiseases*, vol. 23, no. 5, pp. 505–512, 2010.

[3] B. Gottstein and R. Felleisen, “Protective immune mechanisms against the metacestode of *Echinococcus multilocularis*,” *Parasitology Today*, vol. 11, no. 9, pp. 320–326, 1995.

[4] D. A. Vuitton, “The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite?” *Acta Tropica*, vol. 85, no. 2, pp. 119–132, 2003.

[5] D. Sturm, J. Menzel, B. Gottstein, and P. Kern, “Interleukin-5 is the predominant cytokine produced by peripheral blood mononuclear cells in alveolar echinococcosis,” *Infection and Immunity*, vol. 63, no. 5, pp. 1688–1697, 1995.

[6] N. Wellinghausen, P. Gebert, and P. Kern, “Interleukin (IL)-4, IL-10 and IL-12 profile in serum of patients with alveolar echinococcosis,” *Acta Tropica*, vol. 73, no. 2, pp. 165–174, 1999.

[7] L. Jenne, J. Kilwinski, W. Scheffold, and P. Kern, “IL-5 expressed by CD4+ lymphocytes from *Echinococcus multilocularis*-infected patients,” *Clinical and Experimental Immunology*, vol. 109, no. 1, pp. 90–97, 1997.

[8] V. Godot, S. Harraga, M. Deschaseaux et al., “Increased basal production of interleukin-10 by peripheral blood mononuclear cells in human alveolar echinococcosis,” *European Cytokine Network*, vol. 8, no. 4, pp. 401–408, 1997.

[9] M. Schmid, H. Samonigg, H. St¨oger et al., “Use of interferon *γ* and mebendazole to stop the progression of alveolar hydatid disease: case report,” *Clinical Infectious Diseases*, vol. 20, no. 6, pp. 1543–1546, 1995.

[10] M. P. Hübner, B. J.Manfras, M. C.Margos et al., "*Echinococcus multilocularis* metacestodes modulate cellular cytokine and chemokine release by peripheral blood mononuclear cells in alveolar echinococcosis patients," *Clinical and Experimental Immunology*, vol. 145, no. 2, pp. 243–251, 2006.

[11] I. Emery, C. Leclerc, K. Sengphommachanh, D. A. Vuitton,and M. Liance, "In vivo treatment with recombinant IL-12 protects C57BL/6J mice against secondary alveolar echinococcosis," *Parasite Immunology*, vol. 20, no. 2, pp. 81–91, 1998.

[12] M. Liance, S. Ricard-Blum, I. Emery, R. Houin, and D. A. Vuitton, "*Echinococcus multilocularis* infection in mice: in vivo treatment with a low dose of IFN-*γ* decreases metacestode growth and liver fibrogenesis," *Parasite*, vol. 5, no. 3, pp. 231–237, 1998.

[13] L. Jenne, J. Kilwinski, P. Radloff, W. Flick, and P. Kern, "Clinical efficacy of and immunologic alterations caused by interferon *γ* therapy for alveolar echinococcosis," *Clinical Infectious Diseases*, vol. 26, no. 2, pp. 492–494, 1998.

[14] L. Kocherscheidt, A. K. Flakowski, B. Grüner et al., "*Echinococcus multilocularis*: inflammatory and regulatory chemokine responses in patients with progressive, stable and cured alveolar echinococcosis," *Experimental Parasitology*, vol. 119, no. 4, pp. 467–474, 2008.

[15] S. Harraga, V. Godot, S. Bresson-Hadni, G. Mantion, and D. A. Vuitton, "Profile of cytokine production within the periparasitic granuloma in human alveolar echinococcosis," *Acta Tropica*, vol. 85, no. 2, pp. 231–236, 2003.

[16] Y. Iwakura, H. Ishigame, S. Saijo, and S. Nakae, "Functional specialization of interleukin-17 family members," *Immunity*, vol. 34, no. 2, pp. 149–162, 2011.

[17] S. Y. Hwang, J. Y. Kim, K. W. Kim et al., "IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-kappaB- and PI3-kinase/Akt-dependent

pathways.," *Arthritis Research & Therapy*, vol. 6, no. 2, pp. R120–R128, 2004.

[18] J. Seiderer, I. Elben, J. Diegelmann et al., "Role of the novel Th17 cytokine IL-17F in inflammatory bowel disease (IBD): upregulated colonic IL-17F expression in active Crohn's disease and analysis of the IL17F p.His161Arg polymorphism in IBD," *Inflammatory Bowel Diseases*, vol. 14, no. 4, pp. 437–445, 2008.

[19] K. Noda, S. Kodama, S. Umemoto, N. Nomi, T. Hirano, and M. Suzuki, "Th17 cells contribute to nontypeable *Haemophilus influenzae*-specific protective immunity induced by nasal vaccination with P6 outer membrane protein and *α*-galactosylceramide," *Microbiology and Immunology*, vol. 55, no. 8, pp. 574–581, 2011.

[20] S. Hanna and A. Etzoni, "New host defense mechanisms against Candida species clarify the basis of clinical phenotypes," *Journal of Allergy and Clinical Immunology*, vol. 127, no. 6, pp. 1433–1437, 2011.

[21] P. Kern, H. Wen, N. Sato et al., "WHO classification of alveolar echinococcosis: principles and application," *Parasitology International*, vol. 55, pp. S283–S287, 2006.

[22] D. Tappe, M. Frosch, Y. Sako et al., "Close relationship between clinical regression and specific serology in the follow-up of patients with alveolar echinococcosis in different clinical stages," *American Journal of Tropical Medicine and Hygiene*, vol. 80, no. 5, pp. 792–797, 2009.

[23] D. Tappe, Y. Sako, S. Itoh et al., "Immunoglobulin G subclass responses to recombinant Em18 in the follow-up of patients with alveolar echinococcosis in different clinical stages," *Clinical and Vaccine Immunology*, vol. 17, no. 6, pp. 944–948, 2010.

[24] A. Eger, A. Kirch, B. Manfras, P. Kern, H. Schulz-Key, and P. T. Soboslay, "Pro-inflammatory (IL-1*β*, IL-18) cytokines and IL-8 chemokine release by PBMC in response to *Echinococcus multilocularis* metacestode vesicles," *Parasite Immunology*, vol. 25, no. 2, pp. 103–105, 2003.

[25] H. R. Conti, F. Shen, N. Nayyar et al., “Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis,” *Journal of Experimental Medicine*, vol. 206, no. 2, pp. 299–311, 2009.

[26] H. Ishigame, S. Kakuta, T. Nagai et al., “Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses,” *Immunity*, vol.30, no. 1, pp. 108–119, 2009.

[27] S. L. Gaffen, “Structure and signalling in the IL-17 receptor family,” *Nature Reviews Immunology*, vol. 9, no. 8, pp. 556–567, 2009.

[28] M.Veldhoen, R. J. Hocking, C. J. Atkins, R.M. Locksley, and B. Stockinger, “TGF*β* in the context of an inflammatory cytokine milieu supports *de novo* differentiation of IL-17-producing T cells,” *Immunity*, vol. 24, no. 2, pp. 179–189, 2006.

[29] L. Zhou, I. I. Ivanov, R. Spolski et al., “IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways,” *Nature Immunology*, vol. 8, no. 9, pp. 967–974, 2007.

[30] R. Pappu, V. Ramirez-Carrozzi, and A. Sambandam, “The interleukin-17 cytokine family: critical players in host defence and inflammatory diseases,” *Immunology*, vol. 134, no. 1, pp. 8–16, 2011.

[31] K. P. Walsh, M. T. Brady, C. M. Finlay, L. Boon, and K. H. G. Mills, “Infection with a helminth parasite attenuates autoimmunity through TGF-*β*-mediated suppression of Th17 and Th1 responses,” *Journal of Immunology*, vol. 183, no. 3, pp. 1577–1586, 2009.

[32] H. Tallima, M. Salah, F. R. Guirguis, and R. El Ridi, “Transforming growth factor-*β* and Th17 responses in resistance to primary murine schistosomiasis mansoni,” *Cytokine*, vol. 48, no. 3, pp. 239–245, 2009.

[33] S. Mahanty, H. E. Luke, V. Kumaraswami, P. R. Narayanan, V. Vijayshekaran, and T. B. Nutman, “Stage-specific induction of cytokines regulates the immune response

in lymphatic filariasis," *Experimental Parasitology*, vol. 84, no. 2, pp. 282– 290, 1996.

[34] I. R. C. Viana, A. Sher, O. S. Carvalho et al., "Interferon-*γ* production by peripheral blood mononuclear cells from residents of an area endemic for Schistosoma mansoni," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 88, no. 4, pp. 466–470, 1994.

[35] H. Li, J. Chen, A. Huang et al., "Cloning and characterization of IL-17B and IL-17C, two newmembers of the IL-17 cytokine family," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 2, pp. 773–778, 2000.

[36] T. Kokubu, D. R. Haudenschild, T. A.Moseley, L. Rose, and A. H. Reddi, "Immunolocalization of IL-17A, IL-17B, and their receptors in chondrocytes during fracture healing," *Journal of Histochemistry and Cytochemistry*, vol. 56, no. 2, pp. 89–95,2008.

[37] E. E. Moore, S. Presnell, U. Garrigues et al., "Expression of IL-17B in neurons and evaluation of its possible role in the chromosome 5q-linked form of Charcot-Marie-Tooth disease," *Neuromuscular Disorders*, vol. 12, no. 2, pp. 141–150, 2002.

[38] Y. Shi, S. J. Ullrich, J. Zhang et al., "A novel cytokine receptorligand pair: identification, molecular characterization, and *in vivo* immunomodutory activity," *Journal of Biological Chemistry*, vol. 275, no. 25, pp. 19167–19176, 2000.

[39] Y. Yagi, A. Andoh, O. Inatomi, T. Tsujikawa, and Y. Fujiyama, "Inflammatory responses induced by interleukin-17 family members in human colonic subepithelial myofibroblasts," *Journal of Gastroenterology*, vol. 42, no. 9, pp. 746–753, 2007.

[40] J. Lee, W. H. Ho, M. Maruoka et al., "IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1," *Journal of Biological Chemistry*, vol. 276, no. 2, pp. 1660–1664, 2001.

[41] H. S. Yu, P. Angkasekwinai, S. H. Chang, Y. Chung, and C. Dong, "Protease allergens induce the expression of IL-25 via

Erk and p38 MAPK pathway," *Journal of Korean Medical Science*, vol. 25, no. 6, pp. 829–834, 2010.

[42] A. Gratchev, J. Kzhyshkowska, K. Duperrier, J. Utikal, F. W. Velten, and S. Goerdt, "The receptor for interleukin- 17E is induced by Th2 cytokines in antigen-presenting cells," *Scandinavian Journal of Immunology*, vol. 60, no. 3, pp. 233–237, 2004.

[43] E. Aumüller, G. Schramm, A. Gronow et al., "*Echinococcus multilocularis* metacestode extract triggers human basophils to release interleukin-4," *Parasite Immunology*, vol. 26, no. 10, pp. 387–395, 2004.

[44] S. Zhang, S. Hüe, D. Sène et al., "Expression of major histocompatibility complex class I chain-related molecule A, NKG2D, and transforming growth factor-β in the liver of humans with alveolar echinococcosis: new actors in the tolerance to parasites?" *Journal of Infectious Diseases*, vol. 197, no. 9, pp. 1341–1349, 2008.

[45] T. Jung, K. Wagner, C. Neumann, and C. H. Hausser, "Enhancement of human IL-4 activity by soluble IL-4 receptors in vitro," *European Journal of Immunology*, vol. 29, no. 3, pp. 864–871, 1999.

[46] S. A. Jones, S. Horiuchi, N. Topley, N. Yamamoto, and G. M. Fuller, "The soluble interleukin 6 receptor: mechanisms of production and implications in disease," *FASEB Journal*, vol. 15, no. 1, pp. 43–58, 2001.

Distinctive cytokine, chemokine and antibody responses in *Echinococcus multilocularis*-infected patients with cured, stable or progressive disease

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

[1] Institute for Tropical Medicine, University of Tübingen, Wilhelmstraße 27, 72074 Tübingen, Germany, e-mail: peter.soboslay@uni-tuebingen.de

[2] Section of Infectious Diseases and Clinical Immunology, University of Ulm, Robert-Koch-Straße 8, 89081 Ulm, Germany

Med Microbiol Immunol. 2014 Feb 9

Abstract

Metacestode larvae of the tapeworm *Echinococcus multilocularis* can cause alveolar echinococcosis (AE), a severe parasitic disease in man, which, if it remains untreated, may cause organ failure and death. Spontaneous and parasite antigen-induced cellular responses were studied in patients with cured, stable and progressive AE to differentiate the response profiles between the distinct states of infection. Antibody reactivity was evaluated in AE patients with cured, stable, and progressive disease. The spontaneous cellular release of pro-inflammatory IL-31, IL-33 was clearly depressed in all AE patients, while regulatory IL-27, anti-inflammatory SDF-1/CXCL12 and eosinophil granulocyte attracting Eotaxin-1, -2 and -3 (CCL11, CCL24, CCL26) were enhanced with disease progression. Such distinctive response profiles could be applied for monitoring of AE disease progression or regression. *E. multilocularis* metacestode (Em) antigens (entire metacestode EmAg as well as EmVesicles) stimulated *in vitro* IL-31, IL-33, Eotaxin-1, -3 and CXCL12 cytokine and chemokine responses, which were similarly present in all AE patient groups, while regulatory IL-27 were suppressed and pro-inflammatory Eotaxin-2 was enhanced. *E. multilocularis* metacestode-specific IgG1, IgG3 and IgE responses progressively diminished with regression from active to stable and cured AE. IgG2 and IgG4 reactivity remained similarly high in stable and progressive cases, and lessened only with cured AE. Antibody reactivity against *E. multilocularis* vesicle antigen distinctively separated

between cured, stable or progressive AE, with the exception of IgG4.
In sum, the combined and longitudinal study of several cytokines and chemokines, together with the evaluation of *E. multilocularis* vesicle-specific antibody responses, should provide a better understanding of the immune response during progression and regression of AE, and may help to improve the staging of AE patients.

Keywords *Echinococcus multilocularis*; Cytokines; Chemokines; Antibody; Staging

Introduction

For the tapeworm *Echinococcus multilocularis*, the definitive hosts are mainly foxes and dogs, and rarely man also can become infected by accidentally ingesting *E. multilocularis* eggs [1]. In the human host, the egg-hatched cestode larvae (metacestode) will predominantly develop in the liver, may proliferate and cause tissue damage and ultimately organ failure. The metacestode can also spread to secondary organs by infiltrative growth or metastatic dissemination. Humans infected with *E. multilocularis* may remain asymptomatic without evident parasite growth for decades, and yet the infection may subsequently progress into a chronic or progressive state. Indeed, the number of established infections was found to be far lower than the estimated number of exposures [2,3] - only 1 out of 10 subjects serologically positive for *E. multilocularis* will grow metacestode larvae and develop AE [1]. Evidence points to a host-parasite interplay and modulation of the host immune responses as decisive determinants permitting progressive parasite growth and host tissue infiltration. Progression to severe AE may be very slow and the infection is often detected incidentally by imaging techniques [4], but late diagnosis of clinical manifestations without adequate treatment may result in case fatality. Thus, early detection of viable and proliferating metacestodes and appropriate intervention will lead to a better prognosis.

Detection of helminth-specific immunoglobulins is highly sensitive and specific using affinity-purified and recombinant antigens [5-7]. IgG1, IgG2, IgG4 and IgE were found to be highly reactive in cystic echinococcosis

(CE) and in AE patients, which may indicate active *E. multilocularis* infestation and severe outcome of disease [8]. The combined use of ELISA and Western Blot assays has been recommended for the differential diagnosis of human echinococcosis [9,10]. Furthermore, predominant Th2-type cellular immune responses together with high levels of IgG4 and IgE were associated with progressive and severe AE [8].

Cellular effector responses appear crucial for prevention of *E. multilocularis* metacestode growth and dissemination as well as for protection of the host against parasite-induced inflammation and tissue damage [11]. Th2-type cytokines IL-4, IL-10 and Th1-type interferon-gamma (IFN-γ) and the chemokine MDC/CCL22 were produced by PBMC from AE patient in response to EmAg [12-14]. Besides, immune-modulating properties of *E. multilocularis* on Th17 cytokine-mediated pro-inflammatory immune responses were observed in AE patients [15], which may facilitate the tissue-infiltrative growth of the parasite and its persistence in the human host. Eotaxin-1, -2 and -3 (CCL11, CCL24 and CCL26) are involved in the recruitment of eosinophils, mast cells and Th2 lymphocytes [16] and CCL11 and CCL24 were proved to be pivotal in the immune response against *Schistosoma mansoni* infection [17]. Regulatory cytokines IL-27 can induce a strong proliferation of naive human B cells, CD4+ and CD8+ T-cells and will promote the development of Th1 cells [18-20]. The pro-inflammatory IL-31 and IL-33 are Th2-type cytokines and are suggested to play a vital role in human T-cell mediated skin diseases and allergic and non-allergic diseases

[21,22]. IL-33 will activate Th2-type helper cells, mast cells and basophil granulocytes, thus supporting host defense against nematodes [23,24].

In the present study, we analyzed the parasite antigen-specific cytokine, chemokine and antibody responses in patients with cured, stable and progressive AE, and in infection-free controls. Dynamic profiles of the humoral and cellular immune responses were observed during disease progression and regression, and these observations may help to improve monitoring and staging of AE.

Materials and Methods

Study participants

All alveolar echinococcosis patients were admitted for consultations at the Echinococcosis Reference Center (University Hospitals of Ulm; 38 females/29 males, mean age was 55 years). The Echinococcosis Center in Ulm and University Hospitals of Tübingen are located in south west of the federal state of Baden-Württemberg in Germany, both towns being 70 km apart, in an endemic area for *E. multilocularis*. It was estimated that about 61 cases of AE will be detected annually in Germany [25]. The AE patients in the present study were classified into cured, stable and progressive according to WHO-PNM criteria which were established by the WHO Informal Working Group on Echinococcosis [26]. Diagnosis and classification of AE was performed using positive imaging, serology and histology according to the WHO-PNM system (P = parasitic mass in the liver, N = involvement of neighboring organs, and M = metastasis) [26], and most patients were re-examined regularly during follow-up. Echinococcosis patients were diagnosed and grouped as cured (n=10), stable (n=36) and progressive (n=21) AE. All healthy controls (n=10) were blood donors at University Hospitals of Tübingen; all controls were negative in *E. multilocularis* antigen-specific antibody ELISA responses which confirmed their infection-free state. All patients gave their written consent to participate in this study, and approval for the latter was obtained from the ethical board of the University Clinics of Ulm (Ethik-Kommision Antragsnummer 71/2004).

In vitro culture of *E. multilocularis* metacestode

The *E. multilocularis* metacestode was collected from an infected AE patient in Germany, and thus belonging to the *E.multilocularis* Europe strain. *E. multilocularis* metacestodes was cultured *in vitro* as previously described [27,28]. Briefly, metacestode tissues were removed aseptically from the peritoneal cavity of infected *Meriones unguiculatus* and cut into small tissue blocks. These tissues were cultivated using complete RPMI 1640 medium (5% FCS and 1% antibiotic and antimycotic solution) in an incubator (37 ℃, saturated humidity, and 5% CO_2). After several weeks, metacestodes start to proliferate and produce daughter vesicles. The culture medium was renewed weekly and viable *E. multilocularis* metacestode vesicles were collected for *E. multilocularis* vesicle antigen (EmVAg) preparation.

Preparation of *E. multilocularis* antigens

EmAg and EmVAg were prepared as previously described [29,30]. For EmVAg preparation, *E. multilocularis* vesicles were homogenized and sonicated (30% intensity, pulse 1s for 8 min) on ice. The vesicle homogenate was then centrifuged at 5000g for 30 min at 4 ℃, the supernatant was collected after sterile-filtration (0.22μm) as EmVAg and stored at -80 ℃. For EmAg preparation, solid metacestode tissues were ground on ice in a Ten-Broek tissue grinder until a homogenous liquid extract emerged; the liquid extract was then collected and centrifuged at 15,000g for 30 min at 4 ℃. The supernatant was sterile-filtered (0.22μm) and stored at -80℃ as EmAg. The protein concentration of these *E.*

multilocularis antigens was determined using the bicinchoninic acid protein determination kit (Pierece, Rockford, IL, USA).

Isolation and activation of peripheral blood mononuclear cells

Whole blood samples from AE patients were processed at the blood transfusion center at the University Hospitals of Ulm. Buffy-coated cells of healthy controls were processed at the blood transfusion center at the University Hospital of Tübingen. PBMC and plasma from AE patients and healthy controls were isolated by Ficoll-Paque (Pharmacia, Freiburg, Germany) density gradient centrifugation as described previously [31]. From every AE patient serum was collected and stored at -80℃. PBMC were cultured and stimulated as described previously [31]. Briefly, PBMC were adjusted to $1x10^7$cells/ml using complete RPMI 1640 medium (10% FCS and 1% antibiotic and antimycotic solution) and plated 500µl/well onto 48-well flat-bottomed tissue culture plates. Then, the PBMC were cultured for 48h at 37℃, saturated humidity and 5% CO_2 in the presence of 50µl EmVAg (final concentration 60ug/ml) or 50µl EmAg (final concentration 60ug/ml) or remained un-stimulated as a control (baseline). PBMC were cultured as a control (baseline). Cell culture supernatant was collected and separated by centrifugation, and stored in -80℃.

Evaluation of *E. multilocularis*-specific IgG subclass and IgE responses in AE patients

To evaluate parasite-specific serological reactivity, the levels of IgG subclasses (IgG1, IgG2, IgG3 and IgG4) and IgE in AE patients sera against EmAg or EmVAg were quantified by enzyme-linked immunosorbent assay (ELISA) as previously described [8,29]. Briefly, microtitre plates (Maxisorb, Nunc, Denmark) were coated with EmAg or EmVAg (5µg/ml); nonspecific binding capacity of wells was blocked by PBST-BSA (PBS with 0.05% Tween 20, 5% Bovine serum albumin), then washed with washing buffer before application of AE patient sera (1:8 diluted). Total IgE and IgG subclasses were detected with HRP-conjugated mouse anti human IgG subclasses or IgE antibodies (1:1000, Invitrogen, CA, USA) and the optical densities were then measured at 450 nm using a microplate autoreader (EL808, BioTek instrument Inc., VT, USA).

Determination of cytokine and chemokine production by PBMC and plasma

PBMC culture supernatants were collected for further analysis after 48h of stimulation with EmAg or EmVAg. From every patient serum and plasma samples were collected. Cytokines and chemokines secretion was quantified using sandwich ELISA kits for IL-27, IL-31, IL-33, CCL11, CCL24, CCL26 and CXCL12 (R&D Systems, Minneapolis, MN, USA). The measurement was performed according to the guidelines of the manufacturers. Optical densities (OD) were transformed into concentrations (pg/ml) according to the cytokine/chemokine-specific standard curve.

Data analysis and statistics

The statistical package JMP10 was used to compile statistical analyses. Significant differences of cytokines, chemokines levels and immunoglobulin responses were determined using Tukey's test (with Bonferroni correction).

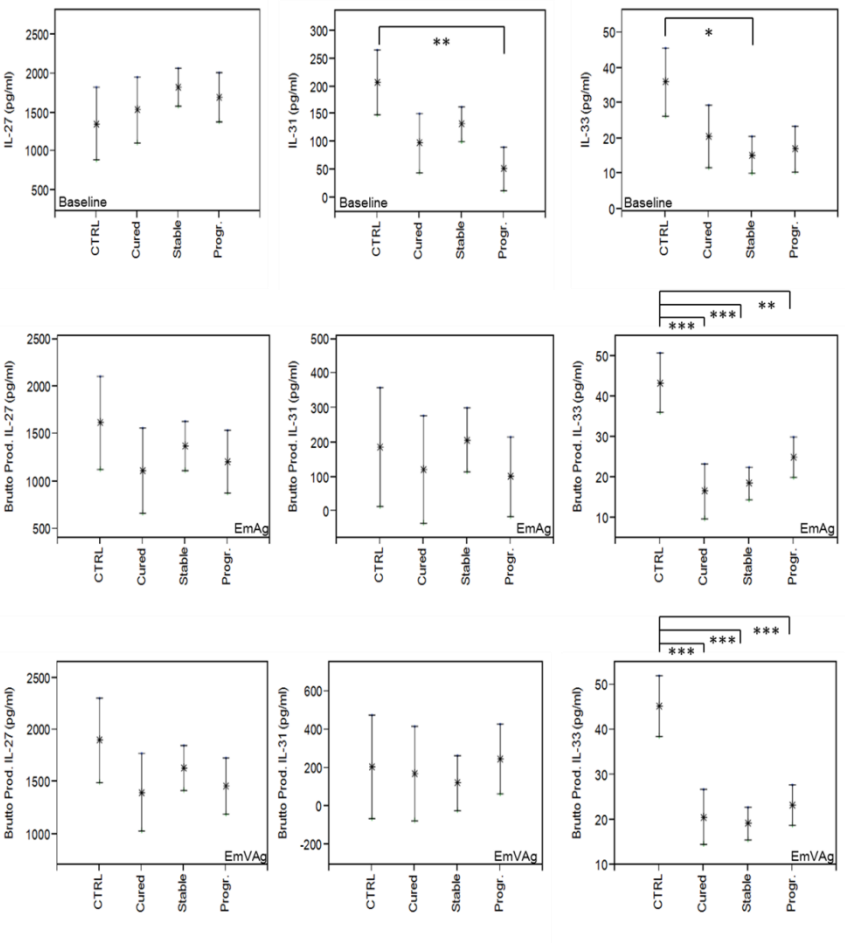
Results

E. multilocularis antigen-induced cytokine (IL-27, IL-31 and IL-33) production in AE patients and infection-free controls

The cellular production of regulatory IL-27, pro-inflammatory IL-31 and IL-33 by PBMC *in vitro* are shown in Figure 1. The spontaneous release of IL-31 and IL-33 was low in AE patients, while IL-27 levels were elevated. Different baseline levels of IL-27, IL-31 and IL-33 corresponded with the states of infection in AE patients; IL-31 and IL-33 were low in AE patients at all states of infection and highest in infection-free controls. In contrast, regulatory cytokine IL-27 was spontaneously released highest by PBMC in increasing amounts as AE progressed with severity. Such distinctive response profiles could be applied for monitoring progression or regression of AE disease. After PBMC were co-cultured with EmAg or EmVAg, a low production of IL-27 and IL-33 in AE patients was observed, but significantly higher amounts of IL-33 were observed in controls when compared with AE patients. The production of IL-31 in AE patients was inducible by EmAg and EmVAg above baseline, but IL-31 levels were similar in AE patients and controls. Cytokine IL-27 and IL-33 production was not inducible by EmAg or EmVAg. The plasma levels of IL-27, IL-31 and IL-33 were low in AE patients, and similar amounts were found in progressive, stable and cured AE patients (data not shown).

Fig.1

Spontaneous and *E. multilocularis* antigen-induced cellular production of IL-27, IL-31 and IL-33 alveolar echinococcosis (AE) patients and infection-free controls (n=10). AE patients were grouped according to their state of *E. multilocularis* infection, i.e. cured (n=12), stable (n=36) or progressive (n=21). Peripheral blood mononuclear cells (PBMC) were co-cultured for 48h with either *E. multilocularis* vesicle antigen (EmVAg: 60 μg/ml) or *E. multilocularis* metacestode antigen (EmAg: 60 μg/ml). Cytokine concentrations in cell-free culture supernatants were determined by cytokine-specific ELISA and are shown as baseline (spontaneous cytokine release without addition of EmVAg or EmAg) and brutto production (cytokine release induced by antigen without subtraction of the baseline spontaneous release). Production values between the groups were analyzed using Tukey's test and significant differences are indicated. (* $p < 0.05$; ** $p < 0.01$)

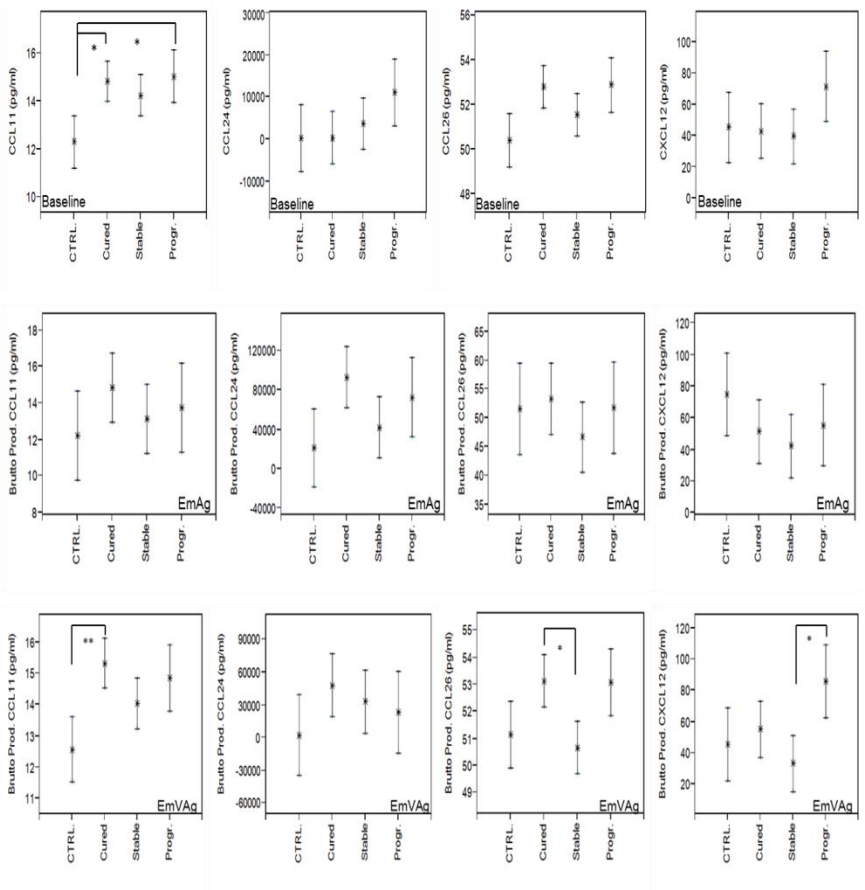

IL-27 (pg/ml)
IL-31 (pg/ml)
IL-33 (pg/ml)
Baseline
Brutto Prod. IL-27 (pg/ml)
Brutto Prod. IL-31 (pg/ml)
Brutto Prod. IL-33 (pg/ml)
EmAg
EmVAg
CTRL
Cured
Stable
Progr.
*
**
***

E. multilocularis antigen-induced chemokine (Eotaxin-1/CCL11, Eotaxin-2/CCL24, Eotaxin-3/CCL26 and SDF-1/CXCL12) production in AE patients and infection-free controls

The *in vitro* production by PBMC of CC-chemokines eotaxin-1, eotaxin-2 and eotaxin -3 (CCL11, CCL24 and CCL26) and of CXC-chemokine CXCL12 are shown in Figure 2. Spontaneous (baseline) release of CCL11, CCL24, CCL26 and CXCL-12 was highest in patients with progressive AE. Baseline levels of Eotaxin 1, -2 and -3 decreased as AE regressed from progressive to stable and cured infection. When PBMC from AE patients were stimulated with EmAg and EmVAg, production of CCL11 was enhanced in all AE patient groups to similar levels. *E. multilocularis* vesicle antigen (EmVAg) activated CCL26 production in cured and progressive AE patients, while EmVAg strongly stimulated CXCL12 release only in PBMC from progressive AE patients. The plasma levels of CCL11, CCL26 and CXCL12 did not differ between the study groups (data not shown).

Fig.2

Spontaneous and *E. multilocularis* antigen-induced cellular production of Eotaxin-1/CCL11, Eotaxin-2/CCL24, Eotaxin-3/CCL26 and SDF-1/CXCL12 alveolar echinococcosis (AE) patients and infection-free controls (n=6). AE patients were grouped according to their state of *E. multilocularis* infection, i.e. cured (n=10), stable (n=10) or progressive (n=6). Peripheral blood mononuclear cells (PBMC) were co-cultured with either *E. multilocularis* vesicle antigen (EmVAg: 60 ug/ml) or *E. multilocularis* entire metacestode antigen extract (EmAg: 60 ug/ml) for 48h. Cytokine concentrations in cell-free culture supernatants were determined by cytokine-specific ELISA and are shown as baseline (spontaneous cytokine release without addition of EmVAg or EmAg) and brutto production (cytokine release induced by antigen without subtraction of the baseline spontaneous release). Production values between the groups were analyzed using Tukey's test, and significant differences are indicated. (* $p < 0.05$)

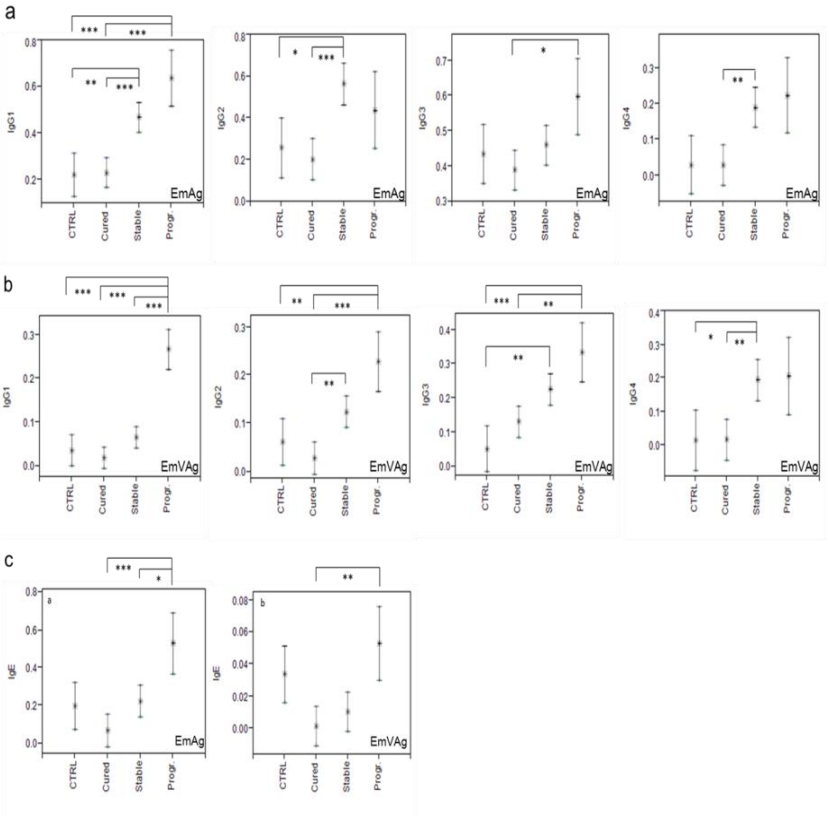

CCL11 (pg/ml)
CCL24 (pg/ml)
CCL26 (pg/ml)
CXCL12 (pg/ml)
Baseline
Brutto Prod. CCL11 (pg/ml)
Brutto Prod. CCL24 (pg/ml)
Brutto Prod. CCL26 (pg/ml)
Brutto Prod. CXCL12 (pg/ml)
EmAg
EmVAg
CTRL.
Cured
Stable
Progr.

E. multilocularis antigen-specific antibody responses in AE patients and infection-free controls

In AE patients and controls, antibody isotype responses to *E. multilocularis* metacestode (EmAg) and to *E. multilocularis* vesicle antigen extract (EmVAg) were quantified by ELISA; the results are shown in Figure 3 a, b and c. IgG1, IgG2, IgG3 and IgG4 responses to EmAg and EmVAg increased with disease progression, the highest reactivity being observed in patients with progressive AE. IgG2 and IgG4 responses were similarly high in patients with stable and progressive AE. Lowest IgG subclass responses were present in cured AE patients and controls. Parasite antigen-specific IgE reactivity was highest in patients with progressive AE, and IgE responses were at similar levels with cured and stable AE. Thus, apart from the entire EmAg metacestode antigen extract, the *E. multilocularis* vesicle antigen appeared valuable for serological detection of echinococcosis and differentiation between the distinct states of AE.

Fig.3

Serum samples were collected from alveolar echinococcosis (AE) patients (cured, n=21; stable, n=21; progressive, n=6) and infection-free controls (n=10). Antibody IgG1, IgG2, IgG3 and IgG4 subclasses reactivity to *E. multilocularis* entire metacestode antigen extract (EmAg; Panel a) and to *E. multilocularis* vesicle antigen (EmVAg; Panel b) was determined by ELISA. Panel c) shows IgE responses to EmAg and EmVAg. Statistical differences between patient groups and controls were analyzed using Tukey's test, and significant differences are indicated. (**p*<0.05; ** $p < 0.01$; *** $p < 0.001$)


a
IgG1
IgG2
IgG3
IgG4
EmAg
CTRL
Cured
Stable
Progr
b
IgG1
IgG2
IgG3
IgG4
EmVAg
CTRL
Cured
Stable
Progr
c
IgE
EmAg
IgE
EmVAg
CTRL
Cured
Stable
Progr

Discussion

Cytokines and chemokines link innate and adaptive immunity [32]; they can act as central contributors to severe and life-threatening illness and are also an important regulatory element against inflammatory diseases. During the course of AE, both regulatory and inflammatory cytokines and chemokines levels will change in AE patients, so cytokine profiling at distinct states of AE may help not only to better understand host-parasite interactions but to reveal whether the disease is progressive, stable or cured. In the present cohort three times as much stable, twice as much progressive, than cured AE patients were studied; such imbalance of patients groups is reflecting the course of AE where progressive disease may become stabilized by clinical intervention in most patients. However, the regression from stable disease to cure is difficult to achieve, often due to multiple metacestode lesions, re-activation of parasite growth, unresponsiveness to treatment and metacestode lesions inaccessible for surgery.

To our best knowledge, the quantification of IL-27, IL-31 and IL-33 and the Eotaxins -1, -2 and -3 has not been investigated in AE yet. The observed response profiles with cured, stable and progressive AE suggests them as useful makers for disease monitoring. Furthermore, the application of proliferating and tissue invading *E.multilocularis* vesicles as antigen to dissect between disease stages has the potential to refine AE serological diagnosis and patients monitoring. Here, our work presents new findings which show that by combined monitoring of cytokines (IL-27, IL-31 and IL-33) together

with eotaxin -1, -2, -3 chemokines and antibody subclass responses to proliferating vesicles of *E. multilocularis* may offer an approach for distinctive staging of AE.

Spontaneous but not antigen-driven release of IL-27 increased with disease severity in AE patients, suggesting the presence of immune regulatory mechanisms which may dampen inflammation induced by persistent *E. multilocularis*. The biphasic cytokine IL-27 first potentiates Th1-type responses and inflammatory signals generated during the early phase of infection; thereafter, with persistent pathogen exposure and chronic infection, IL-27 inhibits both pro-inflammatory Th2 and Th17 cytokine releases [33]. Enhanced spontaneous release of IL-27 in AE patients with stable and progressive disease thus indicates the emergence of a regulatory component depressing pro-inflammatory cytokine and chemokine responses; such regulatory IL-27 production are likely to be induced by viable and persistent *E. multilocularis* metacestodes in AE patients.

In contrast, spontaneous production of cytokines IL-31 and IL-33 were found to be lowest in AE patients with progressive disease. Preferentially produced by Th2-type helper cells, IL-31 is a potent inducer of pro-inflammatory effects in monocytes and macrophages [34,35], and the “alarmin” IL-33 is associated with cellular damage and Th2-type inflammatory disease pathogenesis [36]. High expression of IL-33 was observed in unbalanced Th1/Th2 immunity biased parasite infections, such as visceral leishmaniasis [37], toxoplasmosis [38] and severe malaria [39]. Multi-cellular parasites like *E. multilocularis* may escape elimination from their host by interfering with

immune defense mechanisms, while the secretion of immune modulators by *E. multilocularis* inhibits T cells and diverts the activation of monocytes and macrophages. Several studies have shown that metacestodes of *E.multilocularis* release factors which impair the interaction of macrophages and T-cells in mitogen- and antigen-induced lymphoproliferative responses [40,41]. Futher, *E. multilocularis* protoscoleces can modify the proliferation of murine T and B cells, and alter the cytokine secretion in macrophages/monocytes [42]. By depressing Th1-type IFN-γ and inflammatory MIP1α while activating immune regulatory TARC/CCL17 and RANTES [14,30], *E. multilocularis* reduces inflammatory responses, prevents tissue destruction and organ damage, and facilitates parasite persistence within its host. Observed low levels of inflammation-associated IL-31 and IL-33 with chronic AE support the notion that with parasite persistence, equilibrated levels of pro-inflammatory and regulatory cytokines and chemokines may have evolved to prevent disease progression and, at the same time, protect the host against immune-effecter cells mediated damage.

Both regulatory and pro-inflammatory cytokines and chemokines were generated by PBMC from AE patients after activation with *E. multilocularis* metacestode antigens. Previous studies have shown that Th1-type cellular response efficiently kill *E. multilocularis* metacestodes and control echinococcosis [11,43], whereas with disease progression Th2 responses prevail [44-46]. Prominent IL-5 expression by CD4+ T lymphocytes was induced in AE patients [47], activating

eosinophil granulocytes; in the present investigation, we observed enhanced spontaneous releases of eotaxins in all AE patient groups. With inflammation and immune activation, eotaxins attract eosinophil granulocytes to inflamed tissues; our findings signify that such cellular effecter responses are induced by vital metacestode tissues, and still remain active in patients staged with cured AE. This indicates that not only viable and proliferating metacestodes of *E. multilocularis*, but residual parasite lesions as well modulate cellular immune responses in AE patients. Similarly, elevated CCL11 and CCL24 levels were observed in human neurocysticercosis and human angiostrongyliasis [48,49], and eosinophil-mediated defense mechanism against helminthes was of paramount importance [50]; however, eotaxins and eosinophil accumulations were also suggested as having a disease-promoting role in atopic dermatitis and bronchial asthma [16,51]. The CXC chemokine CXCL12/SDF-1 was strongly expressed in lung and liver metastases [52], recruiting leukocytes into infected tissues and promote inflammation [53]. An up-regulated production of CXCL12 was found in rheumatoid arthritis, nephritis and asthma [54-56]. The spontaneous release of CXCL12 was highest in patients with progressive AE, indicating that CXCL12 participates in the inflammatory progress with active AE.

Distinctive antibody responses are known to correlate with the clinical state of helminthes infections, with IgG4 and IgE indicative of a Th2-type immune response and prominent in chronic helminthes infections [5,7,57]. In cystic echinococcosis, a prevalent IgG4 response was

associated with cystic growth and disease progression [58], while IgG1 and IgG4 subclasses were dominant for AE in man [59].

In the present investigation, *E. multilocularis* metacestode-specific IgG1, IgG3 and IgE responses progressively diminished with regression from active to stable and cured AE, while IgG2 and IgG4 reactivity remained similarly high in stable and progressive cases, and diminished only in cured AE. As previously shown, IgE responses decrease rapidly in cystic echinococcosis and in AE patients following surgery or successful chemotherapy [60,61]; it was similarly observed in this study that specific IgE levels against EmAg dramatically decreased in cured AE patients. Several studies have also shown that increased IgE correlated with progressive AE [8,62,63]; similarly, in this study, patients with progressive AE had the highest IgE reactivity. The EmVAg specific antibodies performed best in distinguishing between cured, stable or progressive AE; here antibody subclasses reactivity, with the exception of IgG4, differentiated clearly between progressive and stable AE.

In sum, combined and longitudinal study of several cytokine and chemokines, in combination with evaluation of *E. multilocularis* vesicle-specific antibody responses, should provide a better understanding of the immune response during progression and regression of AE, and it may help to improve the staging of AE patients.

Acknowledgments

This work was supported by the Baden-Württemberg Stiftung (Grant P-LS-AL2/06).

Xiangsheng Huang was supported by the Chinese Scholarship Council.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Gottstein B, Saucy F, Deplazes P, Reichen J, Demierre G, Busato A, Zuercher C, Pugin P (2001) Is high prevalence of Echinococcus multilocularis in wild and domestic animals associated with disease incidence in humans? Emerg Infect Dis 7 (3):408-412.
2. Bresson-Hadni S, Laplante JJ, Lenys D, Rohmer P, Gottstein B, Jacquier P, Mercet P, Meyer JP, Miguet JP, Vuitton DA (1994) Seroepidemiologic screening of Echinococcus multilocularis infection in a European area endemic for alveolar echinococcosis. Am J Trop Med Hyg 51 (6):837-846
3. Rausch RL, Wilson JF, Schantz PM, McMahon BJ (1987) Spontaneous death of Echinococcus multilocularis: cases diagnosed serologically (by Em2 ELISA) and clinical significance. Am J Trop Med Hyg 36 (3):576-585
4. Gottstein B, Hemphill A (1997) Immunopathology of echinococcosis. Chem Immunol 66:177-208
5. Maizels RM, Sartono E, Kurniawan A, Partono F, Selkirk ME, Yazdanbakhsh M (1995) T-cell activation and the balance of antibody isotypes in human lymphatic filariasis. Parasitol Today 11 (2):50-56.
6. Hagan P, Blumenthal UJ, Dunn D, Simpson AJ, Wilkins HA (1991) Human IgE, IgG4 and resistance to reinfection with Schistosoma haematobium. Nature 349 (6306):243-245.
7. Mosmann TR, Sad S (1996) The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 17 (3):138-146.
8. Dreweck CM, Luder CG, Soboslay PT, Kern P (1997) Subclass-specific serological reactivity and IgG4-specific antigen recognition in human echinococcosis. Trop Med Int Health 2 (8):779-787
9. Wen H, Craig PS, Ito A, Vuitton DA, Bresson-Hadni S, Allan JC, Rogan MT, Paollilo E, Shambesh M (1995) Immunoblot evaluation of IgG and IgG-subclass antibody responses for

immunodiagnosis of human alveolar echinococcosis. Ann Trop Med Parasitol 89 (5):485-495

10. Tappe D, Gruner B, Kern P, Frosch M (2008) Evaluation of a commercial Echinococcus Western Blot assay for serological follow-up of patients with alveolar echinococcosis. Clin Vaccine Immunol 15 (11):1633-1637.

11. Vuitton DA (2003) The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? Acta Trop 85 (2):119-132.

12. Sturm D, Menzel J, Gottstein B, Kern P (1995) Interleukin-5 is the predominant cytokine produced by peripheral blood mononuclear cells in alveolar echinococcosis. Infect Immun 63 (5):1688-1697

13. Godot V, Harraga S, Beurton I, Deschaseaux M, Sarciron E, Gottstein B, Vuitton DA (2000) Resistance/susceptibility to Echinococcus multilocularis infection and cytokine profile in humans. I. Comparison of patients with progressive and abortive lesions. Clin Exp Immunol 121 (3):484-490.

14. Hubner MP, Manfras BJ, Margos MC, Eiffler D, Hoffmann WH, Schulz-Key H, Kern P, Soboslay PT (2006) Echinococcus multilocularis metacestodes modulate cellular cytokine and chemokine release by peripheral blood mononuclear cells in alveolar echinococcosis patients. Clin Exp Immunol 145 (2):243-251.

15. Lechner CJ, Gruner B, Huang X, Hoffmann WH, Kern P, Soboslay PT (2012) Parasite-specific IL-17-type cytokine responses and soluble IL-17 receptor levels in Alveolar Echinococcosis patients. Clin Dev Immunol 2012:735342.

16. Shin HD, Kim LH, Park BL, Jung JH, Kim JY, Chung IY, Kim JS, Lee JH, Chung SH, Kim YH, Park HS, Choi JH, Lee YM, Park SW, Choi BW, Hong SJ, Park CS (2003) Association of Eotaxin gene family with asthma and serum total IgE. Hum Mol Genet 12 (11):1279-1285

17. Sousa-Pereira SR, Teixeira AL, Silva LC, Souza AL, Antunes CM, Teixeira MM, Lambertucci JR (2006) Serum and cerebral spinal fluid levels of chemokines and Th2

cytokines in Schistosoma mansoni myeloradiculopathy. Parasite Immunol 28 (9):473-478.

18. Charlot-Rabiega P, Bardel E, Dietrich C, Kastelein R, Devergne O (2011) Signaling events involved in interleukin 27 (IL-27)-induced proliferation of human naive CD4+ T cells and B cells. J Biol Chem 286 (31):27350-27362.

19. Schneider R, Yaneva T, Beauseigle D, El-Khoury L, Arbour N (2011) IL-27 increases the proliferation and effector functions of human naive CD8+ T lymphocytes and promotes their development into Tc1 cells. Eur J Immunol 41 (1):47-59.

20. Guzzo C, Che Mat NF, Gee K (2010) Interleukin-27 induces a STAT1/3- and NF-kappaB-dependent proinflammatory cytokine profile in human monocytes. J Biol Chem 285 (32):24404-24411.

21. Ezzat MH, Hasan ZE, Shaheen KY (2011) Serum measurement of interleukin-31 (IL-31) in paediatric atopic dermatitis: elevated levels correlate with severity scoring. J Eur Acad Dermatol Venereol 25 (3):334-339.

22. Dillon SR, Sprecher C, Hammond A, Bilsborough J, Rosenfeld-Franklin M, Presnell SR, Haugen HS, Maurer M, Harder B, Johnston J, Bort S, Mudri S, Kuijper JL, Bukowski T, Shea P, Dong DL, Dasovich M, Grant FJ, Lockwood L, Levin SD, LeCiel C, Waggie K, Day H, Topouzis S, Kramer J, Kuestner R, Chen Z, Foster D, Parrish-Novak J, Gross JA (2004) Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. Nat Immunol 5 (7):752-760.

23. Suzukawa M, Koketsu R, Iikura M, Nakae S, Matsumoto K, Nagase H, Saito H, Matsushima K, Ohta K, Yamamoto K, Yamaguchi M (2008) Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. Lab Invest 88 (11):1245-1253.

24. Yasuoka S, Kawanokuchi J, Parajuli B, Jin S, Doi Y, Noda M, Sonobe Y, Takeuchi H, Mizuno T, Suzumura A (2011) Production and functions of IL-33 in the central nervous system. Brain Res 1385:8-17.

25. Torgerson PR, Keller K, Magnotta M, Ragland N (2010) The global burden of alveolar echinococcosis. PLoS Negl Trop Dis 4 (6):e722.

26. Kern P, Wen H, Sato N, Vuitton DA, Gruener B, Shao Y, Delabrousse E, Kratzer W, Bresson-Hadni S (2006) WHO classification of alveolar echinococcosis: principles and application. Parasitol Int 55 Suppl:S283-287.

27. Dreweck CM, Soboslay PT, Schulz-Key H, Gottstein B, Kern P (1999) Cytokine and chemokine secretion by human peripheral blood cells in response to viable Echinococcus multilocularis metacestode vesicles. Parasite Immunol 21 (8):433-438.

28. Hemphill A, Stettler M, Walker M, Siles-Lucas M, Fink R, Gottstein B (2003) In vitro culture of Echinococcus multilocularis and Echinococcus vogeli metacestodes: studies on the host-parasite interface. Acta Trop 85 (2):145-155.

29. Soboslay PT, Luder CG, Hoffmann WH, Michaelis I, Helling G, Heuschkel C, Dreweck CM, Blanke CH, Pritze S, Banla M, et al. (1994) Ivermectin-facilitated immunity in onchocerciasis; activation of parasite-specific Th1-type responses with subclinical Onchocerca volvulus infection. Clin Exp Immunol 96 (2):238-244

30. Kocherscheidt L, Flakowski AK, Gruner B, Hamm DM, Dietz K, Kern P, Soboslay PT (2008) Echinococcus multilocularis: inflammatory and regulatory chemokine responses in patients with progressive, stable and cured alveolar echinococcosis. Exp Parasitol 119 (4):467-474.

31. Eger A, Kirch A, Manfras B, Kern P, Schulz-Key H, Soboslay PT (2003) Pro-inflammatory (IL-1beta, IL-18) cytokines and IL-8 chemokine release by PBMC in response to Echinococcus multilocularis metacestode vesicles. Parasite Immunol 25 (2):103-105.

32. Luster AD (2002) The role of chemokines in linking innate and adaptive immunity. Curr Opin Immunol 14 (1):129-135.

33. Anderson CF, Stumhofer JS, Hunter CA, Sacks D (2009) IL-27 regulates IL-10 and IL-17 from CD4+ cells in nonhealing Leishmania major infection. J Immunol 183 (7):4619-4627.

34. Cornelissen C, Luscher-Firzlaff J, Baron JM, Luscher B (2012) Signaling by IL-31 and functional consequences. Eur J Cell Biol 91 (6-7):552-566.

35. Kasraie S, Niebuhr M, Werfel T (2010) Interleukin (IL)-31 induces pro-inflammatory cytokines in human monocytes and macrophages following stimulation with staphylococcal exotoxins. Allergy 65 (6):712-721.

36. Nile CJ, Barksby E, Jitprasertwong P, Preshaw PM, Taylor JJ (2010) Expression and regulation of interleukin-33 in human monocytes. Immunology 130 (2):172-180.

37. Rostan O, Gangneux JP, Piquet-Pellorce C, Manuel C, McKenzie AN, Guiguen C, Samson M, Robert-Gangneux F (2013) The IL-33/ST2 axis is associated with human visceral leishmaniasis and suppresses Th1 responses in the livers of BALB/c mice infected with Leishmania donovani. MBio 4 (5):e00383-00313.

38. Jones LA, Roberts F, Nickdel MB, Brombacher F, McKenzie AN, Henriquez FL, Alexander J, Roberts CW (2010) IL-33 receptor (T1/ST2) signalling is necessary to prevent the development of encephalitis in mice infected with Toxoplasma gondii. Eur J Immunol 40 (2):426-436.

39. Ayimba E, Hegewald J, Segbena AY, Gantin RG, Lechner CJ, Agosssou A, Banla M, Soboslay PT (2011) Proinflammatory and regulatory cytokines and chemokines in infants with uncomplicated and severe Plasmodium falciparum malaria. Clin Exp Immunol 166 (2):218-226.

40. Rakha NK, Dixon JB, Carter SD, Craig PS, Jenkins P, Folkard S (1991) Echinococcus multilocularis antigens modify accessory cell function of macrophages. Immunology 74 (4):652-656

41. Kizaki T, Ishige M, Bingyan W, Kumagai M, Day NK, Good RA, Onoe K (1993) Interleukin-1-dependent mitogenic

responses induced by protoscoleces of Echinococcus multilocularis in murine lymphocytes. J Leukoc Biol 53 (3):233-239

42. Kizaki T, Kobayashi S, Ogasawara K, Day NK, Good RA, Onoe K (1991) Immune suppression induced by protoscoleces of Echinococcus multilocularis in mice. Evidence for the presence of CD8dull suppressor cells in spleens of mice intraperitoneally infected with E. multilocularis. J Immunol 147 (5):1659-1666

43. Gottstein B, Haag K, Walker M, Matsumoto J, Mejri N, Hemphill A (2006) Molecular survival strategies of Echinococcus multilocularis in the murine host. Parasitol Int 55 Suppl:S45-49.

44. Emery I, Liance M, Leclerc C (1997) Secondary Echinococcus multilocularis infection in A/J mice: delayed metacestode development is associated with Th1 cytokine production. Parasite Immunol 19 (11):493-503

45. Zhang W, You H, Li J, Zhang Z, Turson G, Aili H, Wang J, McManus DP (2003) Immunoglobulin profiles in a murine intermediate host model of resistance for Echinococcus granulosus infection. Parasite Immunol 25 (3):161-168.

46. Al-Qaoud KM, Abdel-Hafez SK (2005) Humoral and cytokine response during protection of mice against secondary hydatidosis caused by Echinococcus granulosus. Parasitol Res 98 (1):54-60.

47. Jenne L, Kilwinski J, Scheffold W, Kern P (1997) IL-5 expressed by CD4+ lymphocytes from Echinococcus multilocularis-infected patients. Clin Exp Immunol 109 (1):90-97

48. Evans CA, Garcia HH, Hartnell A, Gilman RH, Jose PJ, Martinez M, Remick DG, Williams TJ, Friedland JS (1998) Elevated concentrations of eotaxin and interleukin-5 in human neurocysticercosis. Infect Immun 66 (9):4522-4525

49. Intapan PM, Niwattayakul K, Sawanyawisuth K, Chotmongkol V, Maleewong W (2007) Cerebrospinal fluid eotaxin and eotaxin-2 levels in human eosinophilic

meningitis associated with angiostrongyliasis. Cytokine 39 (2):138-141.

50. del Pozo V, Arrieta I, Tunon T, Cortegano I, Gomez B, Cardaba B, Gallardo S, Rojo M, Renedo G, Palomino P, Tabar AI, Lahoz C (1999) Immunopathogenesis of human gastrointestinal infection by Anisakis simplex. J Allergy Clin Immunol 104 (3 Pt 1):637-643.

51. Petering H, Kluthe C, Dulkys Y, Kiehl P, Ponath PD, Kapp A, Elsner J (2001) Characterization of the CC chemokine receptor 3 on human keratinocytes. J Invest Dermatol 116 (4):549-555.

52. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A (2001) Involvement of chemokine receptors in breast cancer metastasis. Nature 410 (6824):50-56.

53. Berahovich RD, Zabel BA, Lewen S, Walters MJ, Ebsworth K, Wang Y, Jaen JC, Schall TJ (2014) Endothelial expression of CXCR7 and the regulation of systemic CXCL12 levels. Immunology 141 (1):111-122.

54. Balabanian K, Couderc J, Bouchet-Delbos L, Amara A, Berrebi D, Foussat A, Baleux F, Portier A, Durand-Gasselin I, Coffman RL, Galanaud P, Peuchmaur M, Emilie D (2003) Role of the chemokine stromal cell-derived factor 1 in autoantibody production and nephritis in murine lupus. J Immunol 170 (6):3392-3400

55. Karin N (2010) The multiple faces of CXCL12 (SDF-1alpha) in the regulation of immunity during health and disease. J Leukoc Biol 88 (3):463-473.

56. Negrete-Garcia MC, Velazquez JR, Popoca-Coyotl A, Montes-Vizuet AR, Juarez-Carvajal E, Teran LM (2010) Chemokine (C-X-C motif) ligand 12/stromal cell-derived factor-1 is associated with leukocyte recruitment in asthma. Chest 138 (1):100-106.

57. Hussain R, Grogl M, Ottesen EA (1987) IgG antibody subclasses in human filariasis. Differential subclass

recognition of parasite antigens correlates with different clinical manifestations of infection. J Immunol 139 (8):2794-2798

58. Daeki AO, Craig PS, Shambesh MK (2000) IgG-subclass antibody responses and the natural history of hepatic cystic echinococcosis in asymptomatic patients. Ann Trop Med Parasitol 94 (4):319-328

59. Wen H, Craig PS (1994) Immunoglobulin G subclass responses in human cystic and alveolar echinococcosis. Am J Trop Med Hyg 51 (6):741-748

60. Rigano R, Profumo E, Ioppolo S, Notargiacomo S, Ortona E, Teggi A, Siracusano A (1995) Immunological markers indicating the effectiveness of pharmacological treatment in human hydatid disease. Clin Exp Immunol 102 (2):281-285

61. Gottstein B, Eckert J, Woodtli W (1984) Determination of parasite-specific immunoglobulins using the ELISA in patients with echinococcosis treated with mebendazole. Z Parasitenkd 70 (3):385-389

62. Wellinghausen N, Jochle W, Reuter S, Flegel WA, Grunert A, Kern P (1999) Zinc status in patients with alveolar echinococcosis is related to disease progression. Parasite Immunol 21 (5):237-241

63. Ammann RW, Eckert J (1996) Cestodes. Echinococcus. Gastroenterol Clin North Am 25 (3):655-689

Curriculum Vitae

Xiangsheng Huang

Date/place of birth: July 18th, 1985 in Shandong, China
Nationality: Chinese

Education

2003.09- 2007.07	Bachelor, Study Veterinary Medicine at Qingdao Agriculture University, China Topic: “Effect of chitosan-Fe on the growth and immune functions of piglets” under the supervision of Prof. Lianqin Zhu
2007.09-2009.12	Master, Study Prevention Veterinary Medicine at Jilin University, China Topic: “Evaluate the immune-protection of subunit vaccine and DNA vaccine encoding *T. gondii* ADF gene against *Toxoplasma gondii* infection in BALB/c mice” under supervision of Prof. Guocai Zhang and Prof. Xichen Zhang

2010.01-2014.05	Ph.D. candidate at Institute for Tropical Medicine at University of Tübingen, Germany Dissertation title: “Immune modulators with parasite infections” under supervision of Prof. Dr. Peter Soboslay

Acknowledgements

I would like to thank Prof. Peter Soboslay for giving me the opportunity of performing and completing my PhD in his laboratory and for his support and advice throughout these past years.

I dedicate this thesis to the memory of Prof. Dr. Jürgen F. Kun, my former supervisor, who brought me into Germany. He gave me the chance to join his research group by the begining of 2010. His patience, guidance, support and enthusiasm helped a lot on my study.

I owe gratitude to Prof. Nicolaus Blin for his willingness to act as second supervisor of my dissertation. Thank you for your time.

I must express my gratitude to Dr. Velavan TP for his support on my work. Thank you for you unconditional help and friendship.

I thank Prof. Dr. Peter Kern and Dr. Beate Grüner from the University Clinics of Ulm for their support and cooperation in carrying out the Alveolar Echinococcosis study.

In particularly, I would like to thank Christian Lechner, Wolfgang Hoffmann, Clemens Unger, Richard Gantin and Albert Lalremruata for their invaluable support, both in work and apart from work.

I would like to extent my thanks to all colleagues and staff of ITM for your assistance and advice in scientific questions and many memorable moments.

I thank the Chinese Scholarship Council for funding of my study in Tübingen.

I'd like to thank my parents and parents-in-law. You care about me all the time, especially for being always by my side to support and encourage me.

我也要感谢我的父母和岳父母。这么多年来，你们一直在远方关心和牵挂着我并不断的给予我支持和鼓励。

With all my love I also would like to express my gratitude to my wife Qianqian Ma. Thanks for your unconditional support and trust throughout these past years. I dedicate this work to you.