

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

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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 and IL-33 was clearly depressed in all AE patients, while regulatory IL-27, anti-inflammatory SDF-1/CXCL12, and eosinophil granulocyte attracting Eotaxin-1, Eotaxin-2, and Eotaxin-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, Eotaxin-3, and CXCL12 cytokine and chemokine responses, which were similarly present in all AE patient groups, while regulatory IL-27 was 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

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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 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 [11]. Th2-type cytokines IL-4, IL-10 and Th1-type interferon- γ (IFN- γ), and the chemokine MDC/CCL22 were produced by peripheral blood mononuclear cells (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, Eotaxin-2, and Eotaxin-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 nonallergic 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 (AE) 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 southwest 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, 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 °C, saturated humidity, and 5 % CO₂). 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 1 s for 8 min) on ice. The vesicle homogenate was then centrifuged at 5,000g for 30 min at 4 °C, and the supernatant was collected after sterile filtration (0.22 µm) as EmVAg and stored at –80 °C. For EmAg preparation, solid metacystode 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 °C. The supernatant was sterile-filtered (0.22 µm) and stored at –80 °C as EmAg. The protein concentration of these *E. multilocularis* antigens was determined using the bicinchoninic acid protein determination kit (Pierce, 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 °C. PBMC were cultured and stimulated as described previously [31]. Briefly, PBMC were adjusted to 1×10^7 cells/ml using complete RPMI 1640 medium (10 % FCS and 1 % antibiotic and antimycotic solution) and plated 500 µl/well onto 48-well flat-bottomed tissue culture plates. Then, the PBMC were cultured for 48 h at 37 °C, saturated humidity, and 5 % CO₂ in the presence of 50 µl EmVAg (final concentration 60 µg/ml) or 50 µl EmAg (final concentration 60 µg/ml) or remained unstimulated as a control (baseline). PBMC were cultured as a control (baseline). Cell-culture supernatant was collected and separated by centrifugation, and stored in –80 °C.

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 (5 µg/ml); nonspecific binding capacity of wells was blocked by PBST-BSA (PBS with 0.05 % Tween 20, 5 % bovine serum albumin) and 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 (1:1,000, Invitrogen, CA, USA), and the optical densities (OD) 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 48 h of stimulation with EmAg or EmVAg. From every patient, serum and plasma samples were collected. Cytokines and chemokines secretion were 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. 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 in 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 is shown in Fig. 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

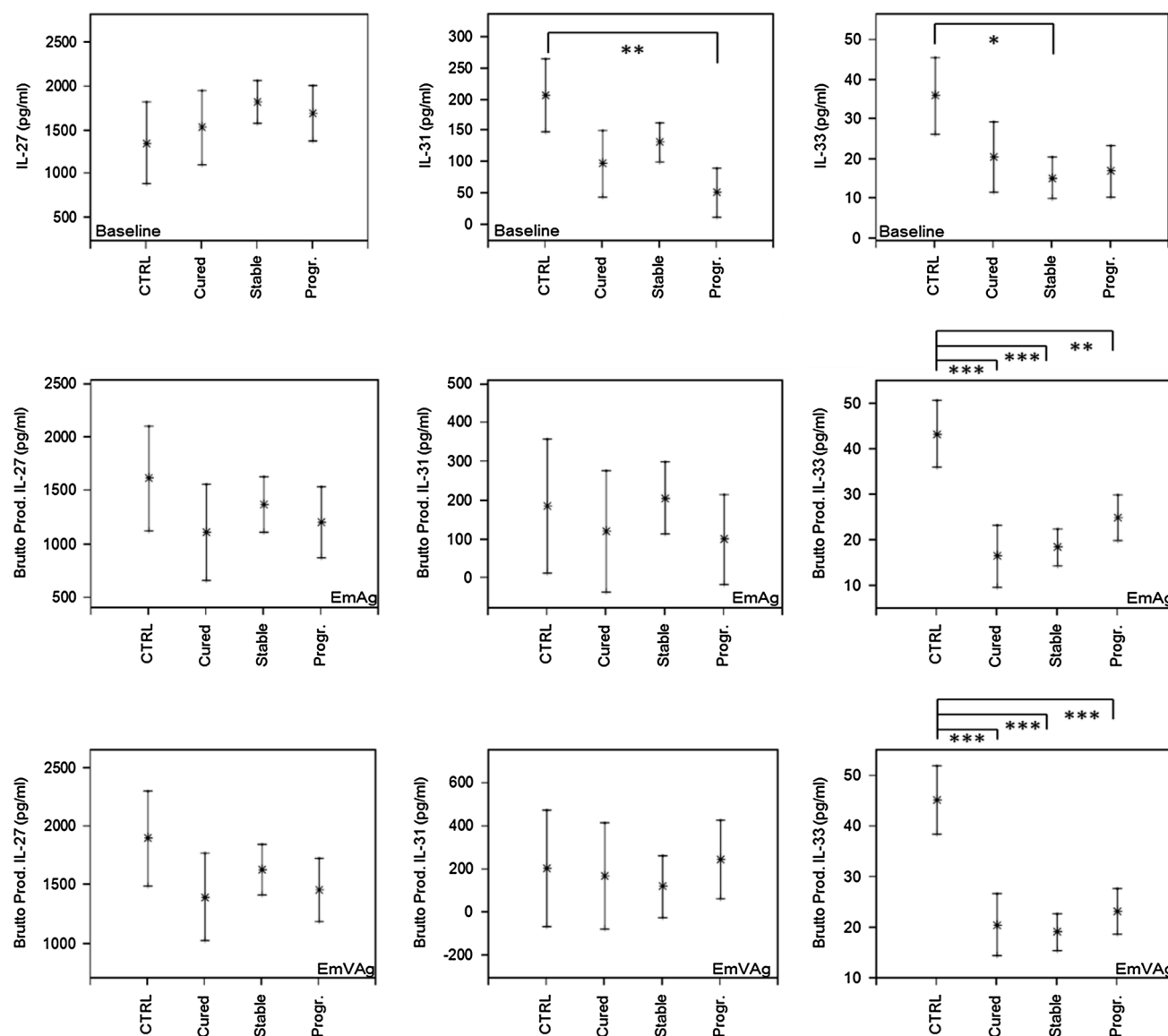


Fig. 1 Spontaneous and *E. multilocularis* antigen-induced cellular production of IL-27, IL-31, and IL-33 AE patients and infection-free controls ($n = 10$). AE patients were grouped according to their state of *E. multilocularis* infection, i.e., cured ($n = 12$), stable ($n = 36$), or progressive ($n = 21$). PBMC were cocultured for 48 h with either *E. multilocularis* vesicle antigen (EmVAg: 60 $\mu\text{g/ml}$) or *E. multilocularis* metacystode antigen (EmAg: 60 $\mu\text{g/ml}$). Cytokine concentrations

in cell-free culture supernatants were determined by cytokine-specific ELISA and are shown as baseline (spontaneous cytokine release without the addition of EmVAg or EmAg) and brutto production (cytokine release induced by antigen without the subtraction of the baseline spontaneous release). Production values between the groups were analyzed using Tukey's test, and significant differences are indicated (* $p < 0.05$; ** $p < 0.01$)

low in AE patients, and similar amounts were found in progressive, stable, and cured AE patients (data not shown).

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 is shown in Fig. 2. Spontaneous (baseline) release of CCL11, CCL24, CCL26, and CXCL-12 was highest in patients with progressive AE. Baseline levels of Eotaxin 1, Eotaxin-2 and Eotaxin-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,

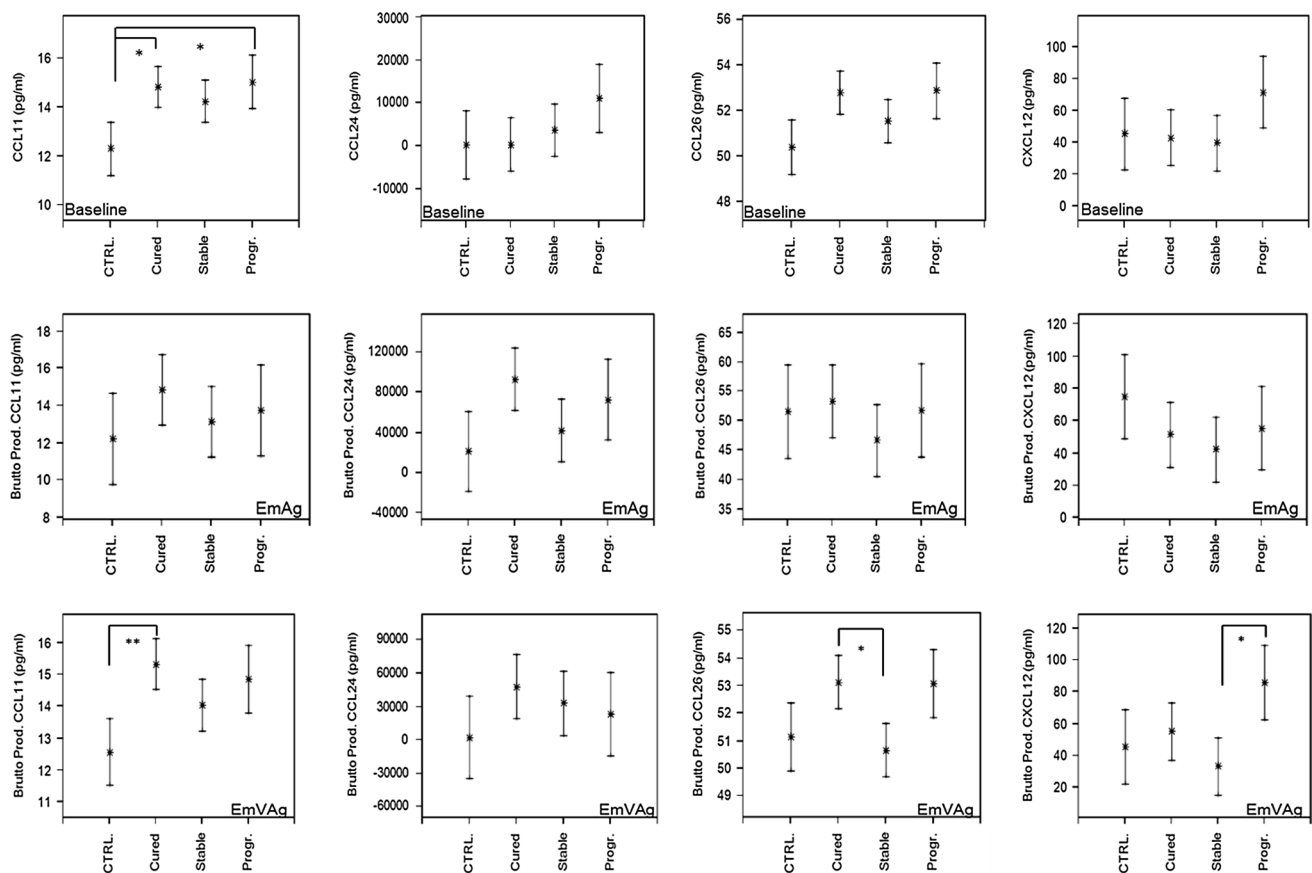


Fig. 2 Spontaneous and *E. multilocularis* antigen-induced cellular production of Eotaxin-1/CCL11, Eotaxin-2/CCL24, Eotaxin-3/CCL26, and SDF-1/CXCL12 AE patients and infection-free controls ($n = 6$). AE patients were grouped according to their state of *E. multilocularis* infection, i.e., cured ($n = 10$), stable ($n = 10$), or progressive ($n = 6$). PBMC were cocultured with either *E. multilocularis* vesicle antigen (EmVAg; 60 μ g/ml) or *E. multilocularis* entire metacystode antigen extract (EmAg; 60 μ g/ml) for 48 h. Cytokine

concentrations in cell-free culture supernatants were determined by cytokine-specific ELISA and are shown as baseline (spontaneous cytokine release without the addition of EmVAg or EmAg) and brutto production (cytokine release induced by antigen without the subtraction of the baseline spontaneous release). Production values between the groups were analyzed using Tukey's test, and significant differences are indicated (* $p < 0.05$)

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).

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

In AE patients and controls, antibody isotype responses to *E. multilocularis* metacystode (EmAg) and to *E. multilocularis* vesicle antigen extract (EmVAg) were quantified by ELISA; the results are shown in Fig. 3a–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 metacystode antigen extract, the *E. multilocularis* vesicle antigen appeared valuable for serological detection of echinococcosis and differentiation between the distinct states of AE.

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

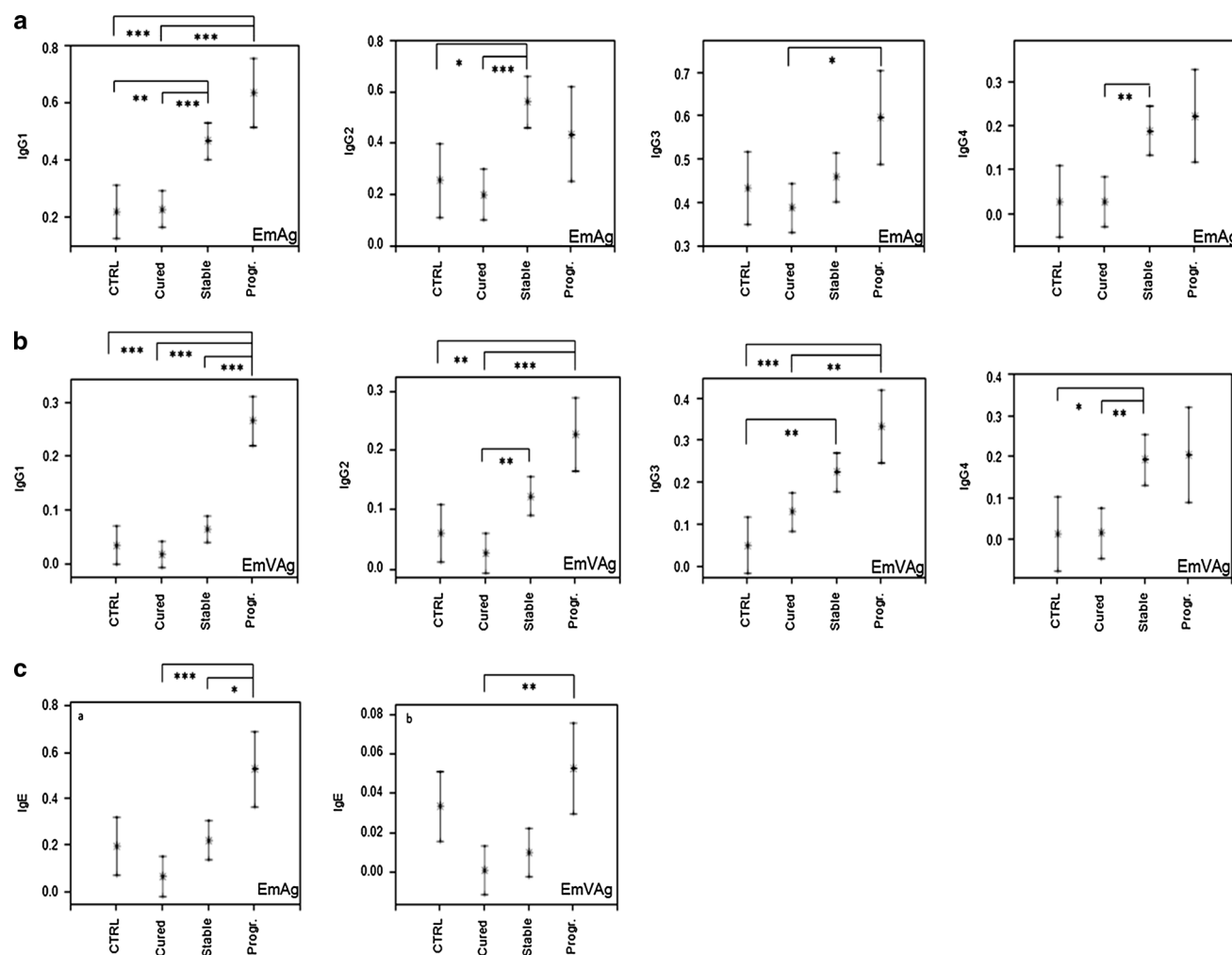


Fig. 3 Serum samples were collected from AE patients (cured, $n = 21$; stable, $n = 21$; progressive, $n = 6$) and infection-free controls ($n = 10$). Antibody IgG1, IgG2, IgG3, and IgG4 subclasses reactivity to *E. multilocularis* entire metacystode antigen extract (EmAg; **a**) and to *E. multilocularis* vesicle antigen (EmVAg; **b**) was determined by

ELISA. **c** IgE responses to EmAg and EmVAg. Statistical differences between patient groups and controls were analyzed using Tukey's test, and significant differences are indicated (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

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 metacystode lesions, reactivation of parasite growth, unresponsiveness to treatment, and metacystode lesions inaccessible for surgery.

To the best of our knowledge, the quantification of IL-27, IL-31, and IL-33 and the Eotaxins-1, Eotaxins-2 and Eotaxins-3 has not been investigated in AE yet. The observed response profiles with cured, stable, and progressive AE suggest 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, Eotaxin-2, and Eotaxin-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 is likely to be induced by viable and persistent *E. multilocularis* metacystodes in AE patients.

In contrast, spontaneous production of cytokines IL-31 and IL-33 was 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 such as *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 metacystodes of *E. multilocularis* release factors which impair the interaction between macrophages and T-cells in mitogen- and antigen-induced lymphoproliferative responses [40, 41]. Further, *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 MIP1 α 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-effector cell-mediated damage.

Both regulatory and pro-inflammatory cytokines and chemokines were generated by PBMC from AE patients after activation with *E. multilocularis* metacystode antigens. Previous studies have shown that Th1-type cellular response efficiently kill *E. multilocularis* metacystodes 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 effector responses are induced by vital metacystode tissues and still remain active in patients staged with cured AE. This indicates that not only viable and proliferating metacystodes 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 promoting 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* metacystode-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 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 it may help to improve the staging of AE patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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