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Echinococcus multilocularis metacestodes modulate cellular cytokine and chemokine release by peripheral blood mononuclear cells in alveolar echinococcosis patients

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Summary

Infection with the cestode *Echinococcus multilocularis* causes human alveolar echinococcosis (AE), a life-threatening disease affecting primarily the liver. Despite the severity of AE, clinical symptoms often develop only many years after infection, which suggests that *E. multilocularis* has developed mechanisms which depress anti-parasite immune response, thus favouring immune evasion. In this study we examined the production of cytokines, chemokines and the expression of CD molecules on peripheral blood mononuclear cells (PBMC) from AE patients and healthy controls in response to *E. multilocularis* metacestode culture supernatant, viable *E. multilocularis* vesicles and *E. multilocularis* vesicle fluid antigen *in vitro*. After 48 h of co-culture, *E. multilocularis* metacestode culture supernatant and *E. multilocularis* vesicles depressed the release of the proinflammatory cytokine interleukin (IL)-12 by PBMC. This effect was dose-dependent and a suppression of tumour necrosis factor (TNF)-α and IL-12 was observed even when PBMC were activated with lipopolysaccharide (LPS). Comparing proinflammatory cytokine release by AE patients and controls showed that the release of IL-12 and TNF-α was reduced in AE patients, which was accompanied by an increased number of $CD4^+$ $CD25^+$ cells and a reduced release of the Th2 type chemokine CCL17 (thymus and activation regulated chemokine, TARC), suggesting an anti-inflammatory response to *E. multilocularis* metacestode in AE patients. Instead the production of interferon (IFN)-γ and the expression of CD28 on $CD4^+$ T cells were increased in PBMC from AE patients when compared to controls. This was accompanied by a higher release of the Th2-type chemokine CCL22 (macrophage derived chemokine, MDC) supporting that *E. multilocularis* also generates proinflammatory immune responses. These results indicate that *E. multilocularis* antigens modulated both regulatory and inflammatory Th1 and Th2 cytokines and chemokines. Such a mixed profile might be required for limiting parasite growth but also for reducing periparasitic tissue and organ damage in the host.

Keywords: alveolar echinococcosis, cytokine and chemokine responses, *Echinococcus multilocularis*

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Introduction

In infected humans, proliferating metacestodes of *Echinococcus multilocularis* progressively infiltrate infested tissues and organs, mainly liver, and cause alveolar echinococcosis (AE). Infection is often undetected for many years of parasite persistence, and often found only incidentally by imaging diagnostic techniques [1]. Epidemiological and clinical data, e.g. the high prevalence of *E. multilocularis* in foxes in endemic areas but the very low incidence of AE in the human population, suggest that exposure to *E. multilocularis* does not progress to clinical disease in all cases because many subjects present abortive and spontaneously healed lesions after infection [2–5]. The progressive parasite growth in human host tissues appears not to cause fulminant and exacerbated inflammation and immediate organ damage, but often a

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'latent', non-apparent disease. This supports that *E. multilocularis* metacestodes have developed mechanisms which depress anti-parasite responses favouring immune evasion and, moreover, metacestodes may restrict or modulate inflammatory responses which could cause tissue damage and pathology to the human host. Cellular effector mechanisms are considered to be the key defence against metacestode growth and dissemination [6]. Peripheral blood mononuclear cells (PBMC) from AE patients generate substantial amounts of Th1 and Th2 cytokines and chemokines when activated with *E. multilocularis* metacestode antigens or viable *E. multilocularis* vesicles. While interleukin (IL)-5 was found to be the predominant cytokine produced by activated PBMC in AE patients [7], the levels of tumour necrosis factor (TNF)-α, IL-10, IL-12, IL-13 and sIL-2R in sera correlated with the actual state of clinical disease [8–11]. Therefore, regulatory and inflammatory immune mediators, notably cytokines and chemokines, may contribute to tissue and organ damage and disease progression in AE patients [12–14]. However, metacestodes and their secreted products will also depress proinflammatory cytokine and proliferative responses over time [12,15]. In the present study we analysed the anti-inflammatory properties of *E. multilocularis* metacestodes on the cellular production of proinflammatory cytokines and Th2-type chemokines in AE patients and healthy controls. Furthermore, the potential of *E. multilocularis* metacestodes to promote $CD4^+$ $CD25^+$ differentiation and the capacity of secretory products of *E. multilocularis* to modulate inflammatory cytokine generation, also after lipopolysaccharide (LPS)-induced activation, were examined. We found that proliferating *E. multilocularis* selectively stimulated Th2-type chemokine release, depressed proinflammatory cytokines, also in the presence of LPS, and viable vesicles of *E. multilocularis* promoted $CD4^+$ $CD25^+$ T cell differentiation in AE patients. While such parasite–host interplay may only limit metacestode growth and dissemination to some extent, it may favour parasite growth without generating inflammation and immediate organ damage to the host.

Materials and methods

Study participants

The study population consisted of a total of 28 AE patients admitted to the University Hospitals of Ulm and 55 healthy controls received from the University Hospitals of Tübingen. The AE patients and infection-free controls came from south-western Germany (Baden-Württemberg and Bayern). The Echinococcosis Centre and University Clinics of Ulm and the Clinics of Tübingen are situated in an endemic area for *E. multilocularis*, both towns being 70 km apart. The mean age of AE patients was 57·1 years, ranging from 18 to 79 years. Seventeen AE patients were female, 11 male. In the AE patient group were 10 cured patients, 12 patients had stable infections and six patients had progressive AE. The control group for fluorescein activated cell sorter (FACS) analysis and cytokine/chemokine determination consisted of 34 healthy controls (16 female, 18 male) with an mean age of 40·4 years (23–63 years). Diagnosis of AE was achieved by positive imaging, serology and histology, and most patients were re-examined regularly during follow-up. All patients gave written consent to participate in this study and approval for the investigation was obtained from the ethical board of the University Clinics of Ulm (Ethikkommission Antrag no. 71/2004). All patients except five were treated with benzimidazole (albendazole or mebendazole).

Isolation of peripheral blood mononuclear cells (PBMC)

Whole blood samples of donor blood from AE patients as well as controls were processed at the blood transfusion centre at University Clinics of Ulm. PBMC from AE patients and healthy endemic controls were isolated by Ficoll-Paque (Pharmacia, Freiburg, Germany) density gradient centrifugation of heparinized venous peripheral blood. Cell culture experiments were conducted as described previously [12]. Briefly, PBMC were adjusted to 1×10^7/ml in complete medium: RPMI-1640 containing 25 mM HEPES, 2 mM l-glutamine (Gibco, Eching, Germany), 10% heat-inactivated fetal calf serum (FCS) (Biochrom KG, Berlin, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin and 0·25 μg/ml amphotericin B (Sigma, St Louis, MO, USA). PBMC (1×10^7) were plated in duplicate in 48-well flat-bottomed tissue culture plates (Costar 3548, New York, USA) and co-cultured with either 60 μg/ml EmVF (*E. multilocularis* vesicle fluid antigen), 5 μg/ml *Escherichia coli* LPS (026:B6; Sigma), 5 μg/ml phytohaemagglutinin (PHA) (Sigma), EmMed (*E. multilocularis* metacestode culture supernatant) or 5–10 EmVes (viable *E. multilocularis* metacestode vesicles), both constituting 20% of the final cell culture volume and incubated for 24–96 h at 37°C, saturated humidity and 5% CO_2. Cells from AE patients were cultured in corresponding concentrations. To analyse the effect of EmMed and EmVes on LPS-induced cytokine release by PBMC, cells were adjusted to 2×10^6/ml in complete medium (as above) and preincubated for 2 h in the presence of EmMed and EmVes before stimulation with 100 ng/ml *E. coli* LPS (026:B6; Sigma).

In vitro culture of *E. multilocularis* metacestodes

In vitro culture of *E. multilocularis* metacestodes was carried out as described previously [10,14]. Briefly, metacestode tissues were removed aseptically from the peritoneal cavity of infected jirds (*Meriones unguiculatus*) and cut into tissue blocks. These were cultivated in complete medium (as above) at 37°C, saturated humidity and 5% CO_2. Several weeks later metacestodes started to proliferate and produce daughter vesicles. Twice a week the culture medium was

renewed and *E. multilocularis* metacestode culture supernatant (EmMed) and vesicles (EmVes) were collected and used immediately for *in vitro* stimulation of PBMC or stored below −20°C for further use.

Preparation of *E. multilocularis* vesicle fluid antigen (EmVF)

Cryopreserved *E. multilocularis* vesicles were homogenized and sonicated (30% intensity, pulse 1 s for 8 min) on ice. The vesicle homogenate was centrifuged at 5000 ***g*** for 30 min at 4°C. The supernatant was sterile-filtrated (0·22 μm). The protein concentration was determined by the bicinchoninic acid (BCA) protein determination kit (Pierce, Rockford, IL, USA).

Determination of cytokine and chemokine production by PBMC

Cell-free culture supernatants were collected after 24, 48, 72 and 96 h and stored below −20°C until further use. Cytokine and chemokine secretion by stimulated PBMC was quantified by sandwich enzyme-linked immunosorbent assay (ELISA) using monoclonal and polyclonal antibodies for TNF-α, IL-12 + p40, IFN-γ (Biosource, Ratingen, Germany), macrophage-derived chemokine (MDC) and thymus and activation regulated chemokine (TARC) (R&D Systems, Minneapolis, MN, USA) as recommended by the manufacturers and as described previously [12].

FACS analysis

Freshly isolated PBMC were cultured at 37°C, with saturated humidity and 5% CO_2 in a concentration of 1×10^7 cells/ml in complete medium (as above) in the presence of either 60 μg/ml EmVF, 5 μg/ml *E. coli* LPS (026:B6) or EmVes constituting 20% of the final cell culture volume. After 24 h vesicles were carefully removed and the remaining supernatant and cells were collected and centrifuged at 400 ***g***, 4°C for 5 min. Cells were stained for 30 min at a concentration of 4×10^5 cells/well in 96-well flat-bottomed plates (Costar, NY, USA). Staining was conducted with phycoerythrin (PE)-conjugated mouse IgG anti-human CD4 (BD PharMingen, San Diego, USA), PE-conjugated mouse IgG anti-human CD8 (BD PharMingen, San Diego, USA), fluorescein isothiocyanate (FITC)-conjugated mouse IgG anti-human CD25 (BD PharMingen, San Diego, CA, USA) and FITC-conjugated mouse IgG anti-human CD28 (BD PharMingen). All staining was compared against the relevant isotype controls (IgG1 for FITC and IgG2α for PE). Following two washing steps with FACSflow (400 g, 4°C, 5 min) cells were resuspended with phosphate-buffered saline (PBS) containing 0·2% formaldehyde and stored overnight at 4°C. For FACS analysis a FACSCalibur running CellQuest version 3·3 was used.

Statistical analysis

To analyse differences in concentrations of cytokines and chemokines between AE patients and controls, mean values over time (24, 48, 72 and 96 h) were determined. Significant differences between groups were determined after logarithmic transformation to stabilize the variance of data [log (pg/ml + 0·5)] for *t*-test. The level of significance was adjusted according to Bonferroni–Holm (25 tests; $\alpha = 0{\cdot}002$).

To illustrate the influence of EmMed and EmVes on spontaneous or LPS-induced cytokine release, cytokine levels were expressed as mean in pg/ml or ng/ml ± s.e.m. Dunnett's test was used for statistical analysis, and the variance of data was stabilized by logarithmic transformation [log (pg/ml + 0·5)]. The level of significance was adjusted according to Bonferroni–Holm (16 tests; $\alpha = 0{\cdot}0031$).

For FACS analysis mean values of gated cells are given as percentage ± s.e.m. and differences between AE patients and controls were evaluated by *t*-test. For this analysis data were stabilized by converting percentage values to arcsin values [arcsin (square root of values in percentage/100)].

Results

Cytokine and chemokine production by AE patients and controls

PBMC from healthy controls and AE patients were stimulated *in vitro* with *E. multilocularis* antigen EmMed, EmVes, EmVF or the mitogen PHA and cellular production of the Th1 cytokines IL-12, TNF-α and IFN-γ, as well as the Th2 chemokines MDC and TARC (Table 1), were quantified by ELISA. Results from AE patients with cured, stable or progressive disease were grouped, as they showed no significant differences in cytokine and chemokine release after stimulation with *E. multilocularis* antigens or PHA/*E. coli* LPS, except for TNF-α in the presence of PHA. Furthermore, in those AE patients from our study who were receiving benzimidazole treatment, cytokine and chemokine production did not differ from those AE patients not being treated.

IL-12

Most AE patients showed a diminished spontaneous release of IL-12 when compared with healthy controls. Addition of antigens from *E. multilocularis* enhanced this effect, i.e. significantly reduced IL-12 production by AE patients compared to healthy controls in presence of EmVes (EmVes $P = 0{\cdot}001$; EmMed $P = 0{\cdot}01$; EmVF $P = 0{\cdot}02$). Activation of PBMC with the mitogen PHA resulted in a similar IL-12 production by PBMC from AE patients and controls ($P = 0{\cdot}10$).

TNF-α

Stimulation with EmVes and PHA increased in AE patients and controls TNF-α production, while EmMed and EmVF

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Table 1. *In vitro* production of Th1 cytokines interleukin (IL)-12, tumour necrosis factor (TNF)-α and interferon (IFN)-γ as well as Th2 chemokines macrophage derived chemokine (MDC) and thymus and activation regulated chemokine (TARC) by peripheral blood mononuclear cells (PBMC) from alveolar echinococcosis patients (EP) and infection-free controls (BS) is shown in pg/ml as means and ranges. Spontaneous (medium) cytokine/chemokine production or in response to *Echinococcus multilocularis* metacestode culture supernatant (EmMed), vesicles (EmVes), vesicle fluid antigen (EmVF), photohaemagglutinin (PHA) and *Escherichia coli* lipopolysaccharide (LPS) (used only for TARC), respectively, are shown. The number of AE patients tested for cytokine and chemokine production as well as their number according to their state of infection (cured/stable/progressive) is indicated for each experiment. Concentrations of cytokines/chemokines released into the cell culture supernatant were quantified after 24, 48, 72 and 96 h by enzyme-linked immunosorbent assay (ELISA) and means over time are given. Concentration of MDC was analysed after 96 h. Differences in cytokine/chemokine production between alveolar echinococcosis patients and controls were evaluated as log values [log (pg/ml + 0·5)] by *t*-test and the level of significance adjusted by Bonferroni–Holm ($*P < 0{\cdot}05$, Bonferroni-Holm: 25 tests, $**\alpha = 0{\cdot}002$).

	EP (cured/stable/progressive)	Medium	EmMed	EmVes	EmVF	PHA/*E. coli* LPS
IL-12	BS ($n = 9$)	376 (0–2974)	385 (0–3013)*	510 (0–3915)**	382 ($n = 8$) (0–2394)*	365 ($n = 8$) (22–2179)
	AE ($n = 12$) (6/3/3)	28 (0–133)	14 (0–87)	10 (0–49)	24 (0–123)	105 (0–368)
TNF-α	BS ($n = 22$)	2958 (56–23039)*	3534 (94–16871)*	6396 ($n = 21$) (215–24129)**	2222 (117–14370)*	16453 ($n = 6$) (428–37182)
	AE ($n = 23$) (8/10/5)	648 (0–3110)	564 (0–3189)	1425 (0–10013)	1182 (0–8563)	6987 ($n = 12$) (875–25096)
IFN-γ	BS ($n = 19$)	27 (0–101)	29 (0–102)*	46 (0–169)	107 (0–881)*	3170 ($n = 4$) (250–10451)
	AE ($n = 17$) (6/7/4)	132 (0–467)	259 (0–1620)	252 (0–1487)	542 (0–2073)	380 ($n = 6$) (121–658)
MDC	BS ($n = 8$)	2011 (207–6322)*	2295 (48–8897)**	279 (0–1572)**	4089 (0–11855)*	3700 (0–9247)*
	AE ($n = 12$) (6/3/3)	4390 (1552–6622)	6700 (2717–11441)	3542 (0–11272)	8255 (1855–15713)	8494 (4841–11847)
TARC	BS ($n = 14$)	396 (35–3509)**	159 (32–425)*	34 (12–64)	316 (30–1187)	74 (16–198)
	AE ($n = 11$) (3/6/2)	32 (0–87)	71 (4–172)	34 (0–100)	123 (4–549)	119 (0–356)

had no effect on TNF-α release. Production of the proinflammatory cytokine was constantly lower in AE patients compared to controls, i.e. cells from AE patients spontaneously released less TNF-α ($P = 0{\cdot}003$) as well as after antigen stimulation with EmMed ($P = 0{\cdot}003$), EmVes ($P = 0{\cdot}0005$) and EmVF ($P = 0{\cdot}04$). AE patients with acute infections ($n = 3$) produced higher amounts of TNF-α after PHA stimulation (16123 pg/ml, range 8124–25096 pg/ml) compared to AE patients with abortive infection (3942 pg/ml, range 875–6510 pg/ml; $n = 6$; $P = 0{\cdot}03$).

IFN-γ

Spontaneous IFN-γ secretion did not differ significantly between AE patients and controls, but PBMC from AE patients tended to have a higher secretion of IFN-γ than controls. Stimulation with EmMed ($P = 0{\cdot}01$) and EmVF ($P = 0{\cdot}01$) enforced this effect clearly; however, PHA augmented production of IFN-γ by PBMC from controls, while stimulation with EmVF and PHA increased IFN-γ in AE patients.

MDC

Macrophage derived chemokine (MDC, CCL22) was released in higher amounts by PBMC from AE patients than by those from controls. This was observed in unstimulated cell cultures ($P = 0{\cdot}006$) and also following activation with EmMed ($P < 0{\cdot}002$), EmVes ($P < 0{\cdot}002$), EmVF ($P = 0{\cdot}02$) or PHA ($P < 0{\cdot}02$). Interestingly, PBMC from AE patients released more MDC in the presence of EmMed, EmVF or the mitogen PHA compared to their spontaneous production. In contrast, these antigens did not trigger PBMC from healthy controls to produce MDC, while EmVes even depressed MDC release when compared to unstimulated control cultures.

TARC

The spontaneous release of TARC (CCL17) by PBMC was reduced significantly in AE patients when compared to controls ($P = 0{\cdot}0001$). This depression was preserved after stimulation with EmVes ($P = 0{\cdot}009$), and also the combination with EmVF or *E. coli LPS* did not change TARC production.

Table 2. Fluorescein activated cell sorter (FACS) analysis of peripheral blood mononuclear cells (PBMC) isolated from alveolar echinococcosis patients (AE) and healthy controls (BS) after 24 h of *in vitro* cultivation with viable *Echinococcus multilocularis* vesicles (EmVes), vesicle fluid antigen (EmVF), *Escherichia coli* lipopolysaccharide (LPS) or without stimulation (medium). Mean values of gated cells as percentage ± s.e.m. from $n = 7$ AE patients or $n = 8$ BS are shown. Cell surface CD marker expression was compared between patients and controls by *t*-test and significant differences between groups (* < 0·05) are shown. For *t*-test analysis the data were stabilized by converting percentage values to arcsin values [arcsin(square root of values in percentage/100)].

	Medium		EmVes		EmVF		*E. coli* LPS	
	BS	AE	BS	AE	BS	AE	BS	AE
$CD4^+$ $CD28^+$	27·2 ± 6·5	46·5 ± 5·5	34·4 ± 5·5	53·9 ± 4·7*	32·4 ± 6·0	53·2 ± 4·5*	27·6 ± 6·8	46·2 ± 9·9
$CD8^+$ $CD28^+$	14·6 ± 3·3	15·4 ± 3·2	14·6 ± 3·2	16·5 ± 3·1	15·7 ± 4·4	15·4 ± 3·0	10·5 ± 3·6	16·7 ± 3·3
$CD4^+$ $CD25^+$	3·7 ± 1·4	7·7 ± 1·8	3·5 ± 1·4	26·1 ± 9·3*	6·0 ± 2·9	10·0 ± 2·5	4·9 ± 1·6	29·2 ± 8·1*

Although the TARC production was reduced in AE patients compared to controls, EmMed and EmVF stimulated TARC release by PBMC in AE patients, whereas EmVes reduced TARC release by PBMC from controls.

FACS analysis of $CD4^+$ $CD25^+$ and $CD28^+$ T cells

FACS analysis was performed using PBMC stimulated with EmVes, EmVF or *E. coli* LPS. The unspecific binding of isotype controls (IgG1 and IgG2α) was always less than 1% (data not shown). The spontaneous expression of $CD4^+$ $CD25^+$ was elevated in AE patients' PBMC compared to controls, and the contingent of $CD4^+$ $CD25^+$ cells increased three- to fivefold in AE patients' PBMC after stimulation with EmVes or *E. coli* LPS compared to healthy controls (Table 2). Neither activation with *E. multilocularis* antigens nor *E. coli* LPS could change the expression of $CD4^+$ $CD25^+$ cells in both groups (Fig. 1).

Stimulation of PBMC with *E. multilocularis* antigens (EmMed and EmVF) increased activated $CD4^+$ $CD28^+$ cells in AE patients' PBMC and in controls, while *E. coli* LPS did not (Table 2). AE patients' PBMC expressed spontaneously markedly more $CD4^+$ $CD28^+$ than control PBMC, as well as after antigen ($P < 0·05$) or mitogen stimulation. In contrast, the number of $CD8^+$ $CD28^+$ T cells was similar in both groups and antigen or mitogen stimulations showed no effect on $CD8^+$ $CD28^+$ expression.

Reduction of LPS-induced IL-12 and TNF-α by EmMed and EmVes

The kinetics of IL-12 and TNF-α production by PBMC after LPS stimulation was determined in the presence of EmMed or EmVes. EmMed and EmVes were able to reduce the LPS-induced cytokine release of IL-12 and TNF-α when compared to cell cultures stimulated only with LPS (Fig. 2). This reduction was observable after 24 h and lasted until 96 h of incubation.

Reduction of spontaneously released IL-12 by EmMed and EmVes in a dose-dependent manner

To analyse the effect of EmMed and EmVes on the spontaneous production of IL-12 from PBMC, different concentrations of EmMed (2–40%) or increasing numbers of vesicles (1–8) were added to PBMC cell cultures. EmMed reduced the spontaneous IL-12 production in a dose-dependent manner after 48 and 96 h of incubation. A single vesicle of *E. multilocularis* (diameter 2–3 mm) was sufficient to reduce the spontaneous IL-12 production below the detection limit after 48 h (Fig. 3).

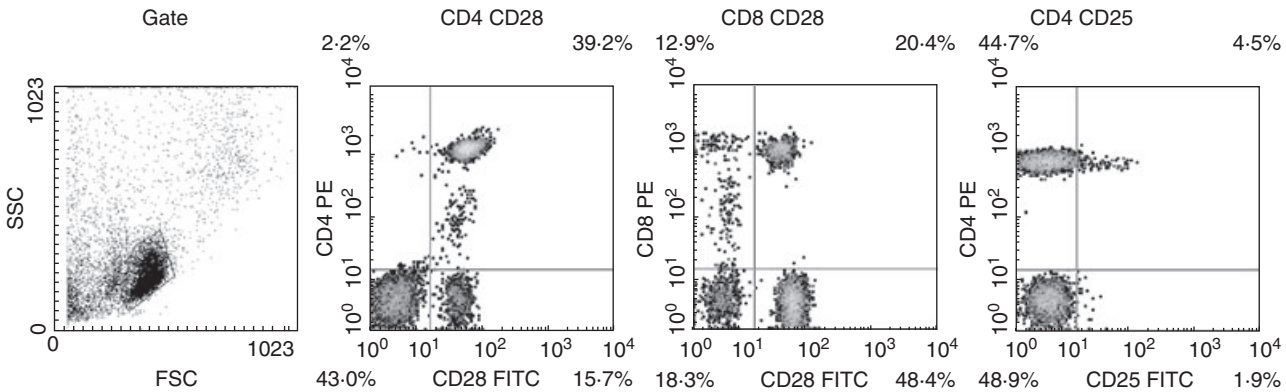

Fig. 1. Representative individual fluorescein activated cell sorter (FACS) analysis of peripheral blood mononuclear cells (PBMC) isolated from an alveolar echinococcosis patient after 24 h of *in vitro* cultivation. The plots show side scatter/forward scatter (FSC/SSC) gated lymphocytes as well as $CD4^+$ $CD28^+$, $CD8^+$ $CD28^+$ and $CD4^+$ $CD25^+$ cells.

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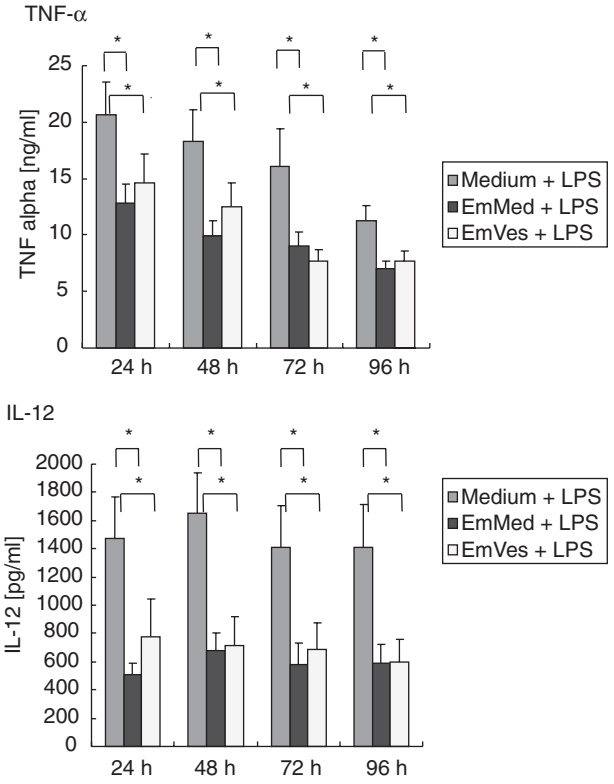

Fig. 2. Kinetics of tumour necrosis factor (TNF)-α (ng/ml + s.e.m.) and interleukin (IL)-12 (pg/ml + s.e.m.) production *in vitro* in the presence of *Escherichia coli* lipopolysaccharide (LPS) by peripheral blood mononuclear cells from healthy donors is shown. Cells were preactivated with 100 ng/ml *E. coli* LPS and spontaneous cytokine production (medium + LPS, $n = 15$ and $n = 13$) or in response to *Echinococcus multilocularis* metacestode culture supernatant (EmMed + LPS, $n = 11$) as well as viable vesicles (EmVes + LPS, $n = 8$) were quantified by enzyme-linked immunosorbent assay (ELISA) after 24, 48, 72 and 96 h of incubation. Dunnett's test was used for statistic analysis, and the data were stabilized by logarithmic transformation [log (pg/ml + 0·5)]. The level of significance was adjusted according to Bonferroni–Holm (16 tests; $*\alpha = 0{\cdot}0031$).

Discussion

Severe alveolar echinococcosis (AE) is associated with a general immune suppression [13,16], while Th2-type responses will favour parasite growth and disease progression, and dominant Th1-type cytokines and vigorous lymphocyte proliferation are linked with protection and abortive AE lesions [17,18]. Furthermore, *E. multilocularis*-derived molecules could inhibit and modulate cellular immune responses – not only by polarizing cytokine profiles [12], suppressing proliferation of blood cells [15] and the accessory cell function and the capacity of macrophages to present antigens [19], but also by impeding the generation of $CD4^+$ memory cells [20]. In the present study, we showed that *E. multilocularis* metacestode antigens promoted the expression of $CD25^+$ on $CD4^+$ T cells, i.e. suggesting a differentiation into regulatory T cells (Table 2). As CD25 is inducible on both Th1- as well as Th2-type helper cells, and is also present on other effector cell populations, e.g. activated B cells, dendritic cells and monocytes, further detailed studies should include CD25 high or low levels of expression together with forkhead box P3 (FoxP3) and intracellular cytokine profiling to support these results. The baseline expression of the IL-2 receptor CD25 on $CD4^+$ cells was elevated on PBMC from AE patients (Table 2) and stimulation with *E. multilocularis* vesicles further increased CD25 expression on T cells. Previously, $CD25^+$ $CD11b^-$ cells, identified morphologically as macrophages, were observed in the periparasitic cellular lining around *E. multilocularis* metacestodes [21]. Such cells were described as monocytes recruited to the intestine with an acquired profound inflammatory anergy, i.e. they will not produce inflammatory cytokines [22]; these observations add to our understanding of how *E. multilocularis* metacestodes may evade host immune surveillance. However, a probable explanation for a greater frequency of CD25 is more 'activated' CD4 cells – as observed for CD28. The higher numbers of activated $CD4^+$ $CD28^+$ T cells in AE patients in this study (Table 2) suggested that activated $CD4^+$ cells may be present around and within the periparasitic granulomas caused by *E. multilocularis* metacestodes. CD28 is a co-stimulator promoting T cell proliferation, cytokine production, T cell effector functions and

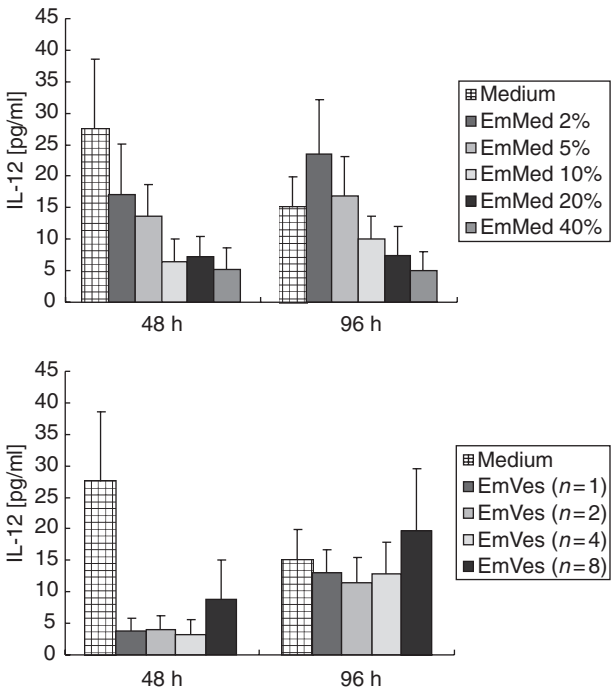

Fig. 3. *In vitro* production of interleukin (IL)-12 (pg/ml + s.e.m.) by peripheral blood mononuclear cells from healthy donors ($n = 6$). Spontaneous IL-12 release (medium) or in response to different concentrations of *Echinococcus multilocularis* metacestode culture supernatant (EmMed 2–40%) as well as increasing numbers of viable vesicles (EmVes 1–8) are shown.

antibody production. In addition, peripheral blood cells from AE patients secreted the CC chemokines MDC/CCL22 and TARC/CCL17 in response to *E. multilocularis* metacestode antigens –both attract monocytes, lymphocytes, eosinophils and basophils to numerous organs, including the liver, lung and dermal tissues [23]. MDC and TARC are implicated in chronic inflammatory skin disease and systemic sclerosis [24,25]; they were found elevated in helminth infection with *Paragonimus westermani* [26] and *Onchocerca volvulus* [27], and systemic neutralization of TARC diminished granuloma formation in mice challenged with *S. mansoni* eggs [28].

The extent to which MDC or TARC may contribute to metacestode growth or healing of AE lesions remains unknown. Our observations suggest that reduced levels of TARC, released by PBMC from AE patients after stimulation with *E. multilocularis* metacestode antigens (Table 1), may prevent the pathological features of lesions induced by infection with *E. multilocularis*. In contrast, the elevated MDC production by PBMC from AE patients (Table 1) may recruit effector cells into periparasitic tissues, thus supporting the development of efficient granulomas which restrain metacestode growth and dissemination and favour the outcome of infection. Clinical observations in AE patients and data from mass screenings have identified abortive cases where AE was cured spontaneously [3,4,8] and it remains to be determined how these chemokines contribute to clearance or parasite persistence.

As protection and abortive AE lesions were linked with dominant Th1-type cytokine responses [6,29,30], the elevated IFN-γ production by PBMC in AE patients following stimulation with metacestode culture supernatant or vesicle fluid antigen (Table 1) may slow progression of lesions and help to stop larval development [18]. Similarly, Shi *et al.* [9] have shown elevated IFN-γ concentrations in sera from AE patients irrespective of their stage of disease, and suggested that IFN-γ may have to act in synergy with other factors to slow parasite growth or to achieve curative effects.

The constantly diminished TNF-α release by PBMC in response to *E. multilocularis* antigens in patients from this study may indicate that metacestode vesicles will actively modulate the host immune responses, which may facilitate parasite survival. As necrosis and fibrosis are the main pathological features of echinococcosis, the cellular immune response, especially TNF-α synthesis, was considered responsible for clinical complications [21]. The AE patients studied here were with cured, stable or progressive disease and their PBMC secreted similar amounts of TNF-α in response to *E. multilocularis* antigens or PHA/*E. coli* LPS, except that the PHA-induced TNF-α release was stronger in cases with a progressive AE compared to those with a cured AE. An augmented production of TNF-α was seen in AE patients with severe tissue damage [9], while no TNF-α mRNA expression was detected in abortive lesions [21]. Therefore, the depressed TNF-α release, in response to *E. multilocularis* antigens, by cells from patients appears as an immune adaptation to prevent inflammatory host damage.

Furthermore, the Th1 type cytokine IL-12 was reduced in AE patients compared to controls (Table 1) and this has been observed previously in sera from AE patients at distinct states of infection [10]. Suppression of the cellular IL-12 production was mediated by *E. multilocularis* metacestode culture supernatant in a dose-dependent manner (Fig. 3), suggesting that excretory/secretory products released by *E. multilocularis* will mediate this effect. In addition, viable *E. multilocularis* vesicles depress IL-12 release by PBMC below the detection limit after 48 h of co-culture, and a single vesicle sufficed to mediate this effect, but after 96 h of co-culture these suppressive effects vanished. Therefore, *E. multilocularis* metacestode culture supernatant as well as viable vesicles diminished inflammatory cytokine releases of IL-12 and TNF-α after LPS stimulation after 24–96 h of co-culture (Fig. 2). This shows that *E. multilocularis* metacestodes have the potential to depress systemic inflammatory responses also induced by non-parasite-derived molecules. Parasite antigens with the capacity to inhibit TNF-α and other cytokines are present in a wide range of helminth parasites; ES-62, a phosphorylcholin-containing glycoprotein from *Acanthocheilonema viteae*, inhibits TNF-α, IL-6 and IFN-γ release [31]. Soluble egg antigens from *Schistosoma mansoni* [32], nematode cystatins [33] and the carbohydrate-rich molecules released into the parasite culture medium and contained in vesicles of *E. multilocularis* [15] have suppressive effects on mitogen [concanavalin A(ConA)] or crude parasite extract-induced cellular proliferation. Parasite extracts, and also entire parasites, have been studied intensively for their anti-inflammatory [32–34], anti-allergic [35,36] or anti-autoimmunological [31,37–39] potential, but *E. multilocularis* has received little attention, and therefore the robust anti-inflammatory capacity of metacestode-derived molecules merits further study.

In summary, proliferating metacestodes of *E. multilocularis* were found to depress proinflammatory cytokine responses even when PBMC were preactivated with LPS; they promoted $CD4^+$ $CD25^+$ T cell differentiation in AE patients and stimulated selectively Th2 type chemokine release. Such an immune modulatory capacity of *E. multilocularis* metacestodes may favour parasite growth and dissemination, limit adverse inflammatory and clinical effects to the host and may contribute to the often unnoticed progression of alveolar echinococcosis.

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