



# MAIT cell activation in adolescents is impacted by bile acid concentrations and body weight

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## Introduction

Bile acids (BAs) have received considerable interest due to their critical role in metabolic modulation. Dysregulated metabolism and signaling of BAs are suggested to play a role in several diseases, such as dyslipidemia, fatty liver disease, diabetes, obesity and atherosclerosis [1], as well as in inflammatory diseases [2]. The two primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), are generated by the liver hepatocytes

## Summary

Bile acids (BAs) are produced by liver hepatocytes and were recently shown to exert functions additional to their well-known role in lipid digestion. As yet it is not known whether the mucosal-associated invariant T (MAIT) cells, which represent 10–15% of the hepatic T cell population, are affected by BAs. The focus of the present investigation was on the association of BA serum concentration with MAIT cell function and inflammatory parameters as well as on the relationship of these parameters to body weight. Blood samples from 41 normal weight and 41 overweight children of the Lifestyle Immune System Allergy (LISA) study were analyzed with respect to MAIT cell surface and activation markers [CD107a, CD137, CD69, interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ ] after *Escherichia coli* stimulation, mRNA expression of promyelocytic leukemia zinc finger protein (PLZF) and major histocompatibility complex class I-related gene protein (MR1), the inflammatory markers C-reactive protein (CRP), interleukin (IL)-8 and macrophage inflammatory protein (MIP)-1 $\alpha$  as well as the concentrations of 13 conjugated and unconjugated BAs. Higher body weight was associated with reduced MAIT cell activation and expression of natural killer cell marker (NKP80) and chemokine receptor (CXCR3). BA concentrations were positively associated with the inflammatory parameters CRP, IL-8 and MIP-1 $\alpha$ , but were negatively associated with the number of activated MAIT cells and the MAIT cell transcription factor PLZF. These relationships were exclusively found with conjugated BAs. BA-mediated inhibition of MAIT cell activation was confirmed *in vitro*. Thus, conjugated BAs have the capacity to modulate the balance between pro- and anti-inflammatory immune responses.

**Keywords:** bile acids, body weight, conjugated bile acids, MAIT cell activation, MAIT cells

from cholesterol breakdown [3]. After conjugation with glycine or taurine, BAs are secreted to the bile and the lumen of the small intestine, where they absorb and digest dietary lipids, cholesterol and lipid-soluble vitamins [3]. In the gut, glycol- and tauro-CA and -CDCA are further processed by the microbiota, generating the secondary BAs deoxycholic acid (DCA) and lithocholic acid (LCA) [4]. The BAs are mainly (95%) reabsorbed in the distal ileum and returned to the liver by the enterohepatic circulation [5]. A minor fraction of BAs escapes

the enterohepatic circulation and reaches the systemic circulation where they regulate many processes, including lipid and glucose homeostasis, intestinal motility, inflammation and configuration and growth of the gut microbiome [6–9]. A direct impact of BAs on the function of innate immune cells such as monocytes, macrophages and granulocytes has been reported [10–12].

Mucosal-associated invariant T (MAIT) cells are a relatively new subset of innate-like T cells preferentially located in mucosal tissues of the gut, the lungs and the female genital tract [13–16]. In addition, MAIT cells circulate at high frequency in the human peripheral blood (1–8% of T cells) [17] and represent 10–15% of the entire hepatic T cell population [14,18–21]. Due to the fact that the liver provides an important second ‘firewall’ when intestinal mucosal defenses are broken or in the presence of systemic infection, MAIT cells placed here may have an important role in bacterial defense. In contrast to other immune cells in the liver, such as  $\gamma\delta$ T cells, for example [22], MAIT cells specifically respond to bacterial metabolites via their T cell receptor (TCR). This TCR has low diversity due to a semi-invariant TCR $\alpha$  chain (invariant V $\alpha$ 7.2 pairing to J $\alpha$ 33, J $\alpha$ 12 or J $\alpha$ 20 in humans) [23,24]. According to their restriction to the non-classical major histocompatibility complex class I-related (MR1) molecule [25,26], MAIT cells can only recognize a limited number of antigens, mainly metabolites of the bacterial riboflavin and folate pathway [27]. Upon activation, they produce proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  and cytotoxic molecules such as perforin and granzyme B [28,29]. Due to the high abundance of MAIT cells in the liver, we speculated that BAs may have a modulatory function on the bacterial activation of these cells. Furthermore, there are reports showing that obesity is associated with alterations in MAIT cell function and distribution in human adults [30,31] as well as with alterations in inflammatory markers such as C-reactive protein (CRP), macrophage inflammatory proteins (MIP)-1 $\alpha$  and interleukin (IL)-8 [32,33] and the expression of chemokine receptor (CXCR3). Thus, the relationship between BA concentrations, MAIT cell activation and inflammatory parameters with regard to weight was the focus of our present investigation. Therefore, we analyzed the MAIT cell frequency, the expression of CXCR3 and natural killer cell marker (NKp80) on these cells, their bacterial activation (production of CD107a, IFN- $\gamma$ , TNF- $\alpha$ , CD69, CD137), the inflammatory markers CRP, IL-8 and MIP-1 $\alpha$  and the serum concentration of 13 BAs in 15-year-old normal weight and overweight children of the Lifestyle Immune System Allergy (LISA) study. The impact of BAs on the modulation of MAIT cell activity was validated in *in-vitro* experiments.

## Materials and methods

### LISA study design

The LISA study was designed to investigate the influence of lifestyle and environmental factors on the immune system and the allergy risk in childhood as well as on the development of metabolic diseases. A total of 3097 newborns who were born between December 1997 and January 1999 in the four German cities of Munich, Leipzig, Wesel and Bad Honnef were engaged for this prospective birth cohort study. Only healthy term neonates of German descent were included. Newborn children whose mothers suffered from autoimmune disease or infectious disorders during pregnancy were excluded. The study design has been described in detail previously [34]. Children were followed-up regularly from birth to 15 years of age with clinical examinations and blood sampling. At the age of 15, blood samples were taken for the determination of several parameters and, in the subcohort from Leipzig, also for the isolation of peripheral blood mononuclear cells (PBMC). The present investigation is based on data gained from PBMC and is therefore restricted to the subcohort of Leipzig. All analyses were performed on overweight children ( $n = 41$ ) and a randomly selected normal weight control group of these children ( $n = 41$ ). Participation in the study was voluntary and informed written consent was given by the parents of all children. The study was approved by the Ethics Committees of the University of Leipzig (EK-BR-02/13-1).

### Blood sampling and PBMC isolation

Blood of study participants was obtained by venipuncture and prepared within 4 h for further analysis. In brief, heparinized blood samples were centrifuged (500 g, 10 min) and the cell-free supernatant (plasma) was collected and stored at  $-80^{\circ}\text{C}$  until analysis. The remaining cell pellet was diluted 1 : 1 with phosphate-buffered saline (PBS); PBMC were isolated by gradient centrifugation using Ficoll Paque Plus (GE Healthcare, Little Chalfont, UK) and cryopreserved until analysis. Serum was collected after clotting of whole blood and centrifugation at 2010 g for 10 min and  $4^{\circ}\text{C}$ . For the *in-vitro* bile acid assays, PBMC were isolated from buffy coats of healthy donors ( $n = 6$ ) using Ficoll Paque Plus and cryopreserved until analysis.

### *In-vitro* stimulation of PBMC

PBMC from the LISA study samples and from healthy donors were thawed and counted;  $4 \times 10^5$  PBMC were directly used for surface staining and  $1 \times 10^6$  living PBMC were seeded per well in 100  $\mu\text{l}$  culture medium within a 96-well U-bottomed (Greiner Bio-One, Frickenhausen, Germany)

cell-culture microplate. Culture medium composed of Iscove's modified Dulbecco's medium (IMDM) (GlutaMax supplement; Fisher Scientific, Schwerte, Germany) was supplemented with 10% fetal bovine serum (BSA; Biochrom, Berlin, Germany),  $\times 1$  penicillin–streptomycin solution (Biowest, Nuaille, France) and 50  $\mu\text{M}$   $\beta$ -mercaptoethanol (AppliChem, Darmstadt, Germany). Cells were allowed to rest overnight at 37°C and 5%  $\text{CO}_2$ . Thereafter, cells were stimulated with 30 bacteria per cell (BpC) of *Escherichia coli* (*E. coli*) for 6 h. After 2 h of *E. coli* stimulation, 10  $\mu\text{g/ml}$  brefeldin A (Sigma-Aldrich, St Louis, MO, USA) or in particular cases 2.5  $\mu\text{M}$  monensin A and phycoerythrin (PE) anti-human CD107a [lysosomal-associated membrane protein 1 (LAMP-1)] antibody (clone H4A3; BioLegend, San Diego, CA, USA) were added. For intracellular staining, cells were treated with  $\times 1$  BD FACS™ lysing Solution and  $\times 1$  BD FACS™ permeabilizing solution 2. For the *in-vitro* bile acid assays, PBMC from healthy donors ( $n = 6$ ) were pretreated with solvent [dimethylsulfoxide (DMSO; final concentration 0.95%) or various concentrations (50, 100, 200  $\mu\text{M}$ ) of the bile acids tauroolithocholic acid (sodium salt), tauroursodeoxycholic acid and glycochenodeoxycholic acid (sodium salt) (all from Cayman Chemical, Michigan, MI, USA) for 45 min prior to bacterial stimulation. The synthetic bile acid receptor agonists INT-747 (Biomol GmbH, Hamburg, Germany) and INT-777 (Cayman) were used at the same concentrations to activate the Farnesoid X receptor (FXR) and the G protein-coupled bile acid receptor (TGR5), respectively, directly.

#### Antibody staining and flow cytometry

After *in-vitro* stimulation and fixation, PBMC were transferred to V-bottomed plates and stained with Fixable Viability Dye eFluor™ 506 (eBioscience, Frankfurt/Main, Germany) for dead cell exclusion, followed by cell surface and intracellular staining with the antibodies given in Supporting information, Table S4. The samples were analyzed on a BD FACSCanto™ II cytometer provided with FACS Diva software version 8.0.1 (BD Biosciences, San Jose, CA, USA). Data were evaluated with FlowJo version 10.2 (FlowJo, Ashland, OR, USA) and Flowlogic Software (Miltenyi Biotec, Bergisch Gladbach, Germany).

#### *E. coli* preparation for PBMC stimulation

In order to obtain a starter culture of *E. coli*, 10 ml of LB Miller (Carl Roth GmbH, Karlsruhe, Germany) pH 7.5 were inoculated with cryoconserved *E. coli* DH5 $\alpha$  stock (Thermo Fisher Scientific, Waltham, MA, USA) under sterile conditions and grown overnight in a shaking incubator at 37°C and 175 rpm. Thereafter, the starter culture was diluted 1 : 50 in fresh LB Miller to obtain the main culture, which was incubated for an additional 16 h in the incubation shaker at 37°C and 175 rpm. After centrifugation for 20 min

at 2000 g and room temperature, the supernatant was discarded and the bacteria pellet was fixed using 1% formaldehyde (Thermo Fisher Scientific, USA) for 10 min at room temperature. The bacteria were then washed with PBS followed by centrifugation for 20 min at 2000 g and room temperature. Bacteria were counted using a Multisizer 3 Coulter Counter (Beckman Coulter, Indianapolis, IN, USA) and frozen as aliquots of  $3 \times 10^8$  bacteria at  $-80^\circ\text{C}$ .

#### Cell viability assay

In order to determine the impact of the bile acids TLCA, TUDCA and GCDCA on cell viability, the cell counting kit-8 (CCK-8; Sigma-Aldrich) was used. Specifically,  $2 \times 10^5$  PBMC per well were seeded in 100  $\mu\text{l}$  culture medium in a 96-well U-bottomed plate. Cells were stimulated as described above; 2 h before the end of the stimulation, CCK-8 reagent was added in accordance with the manufacturer's instructions. The absorbance was then measured by means of a microplate reader (Tecan, Männedorf, Switzerland) at 450 nm (detection wavelength) and 650 nm (reference wavelength).

#### Measurement of clinical parameters

High-sensitivity (hs)-CRP concentrations were measured in the serum samples of the study participants by means of the Roche (Mannheim, Germany) Tina-quant CRP (latex) high-sensitivity assay, according to the manufacturer's instructions.

The concentrations of total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglycerides were also measured in serum by means of homogenous enzymatic colorimetric methods on a Modular Analytics System from Roche Diagnostics GmbH Mannheim, according to the manufacturer's instructions. External controls were used in accordance with the guidelines of the German Society of Clinical Chemistry and Laboratory Medicine.

#### Measurement of cytokine concentrations

Concentrations of the cytokines IL-8 and MIP-1 $\alpha$  were measured in the plasma samples of study participants by means of flow cytometry using the BD CBA Human Soluble Flex Set system (Becton Dickinson, Heidelberg, Germany), according to the manufacturer's instructions. In brief, cytokine-specific antibody-coated beads were incubated with 25  $\mu\text{l}$  of plasma samples or standard solution for 1 h. Thereafter, samples were incubated with the corresponding PE-labeled detection antibodies for 2 h. After one washing step, samples were measured using flow cytometry. Analysis of data and quantification of cytokines was performed with FCAP Array™ software (Becton Dickinson, Heidelberg, Germany) on the basis of corresponding standard curves.

## Measurement of gene expression

The expression of the genes promyelocytic leukemia zinc finger protein (PLZF) and MR1 was measured via quantitative polymerase chain reaction (qPCR) in the LISA study samples as well as in the *in-vitro* bile acid assay samples. Total RNA was prepared from blood collected in PAXgene blood RNA tube by PAXgene blood RNA kit (Qiagen, Hilden, Germany). After cDNA synthesis, gene expression was measured with the 96.96 Dynamic Array (Fluidigm, San Francisco, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [forward: 5'-gctctctgctcctctgcttc-3', reverse: 5'-acgaccaaatccgtgactc-3', Universal Probe Library (UPL): 60] and glucuronidase beta (GUSB) (forward: 5'-cgccctgctctatctgattc-3', reverse: 5'-tccccacaggagtgtag-3', UPL: 57) were used as reference genes. The Ct values of GAPDH and GUSB were subtracted from the cycle threshold (Ct) values of PLZF (forward: 5'-caagaagtcagcctcaagca-3', reverse: 5'-cactcaaaggctctctacc-3', UPL: 78) and MR1 (forward: 5'-gctgctctgggtccattgt-3', reverse: 5'-gatggctcatttgcctc-3', UPL: 20) resulting in  $\Delta$ Ct values. Those values were then normalized to the highest value of all study samples, respectively. This leads to a relative gene expression, with high values corresponding to high gene expression (relative unit).

## Measurement of bile acid concentrations

The serum concentrations of BAs were measured as described previously [35]. In total, 20 bile acids were measured in the serum samples of the 15-year-old LISA children. The measurements were carried out with the Biocrates® bile acids kit (Biocrates Life Sciences AG, Innsbruck, Austria) on well sandwich filter plates and prepared according to the manufacturer's instructions. The liquid chromatography with tandem liquid chromatography with tandem mass spectrometry (LC-MS/MS) analysis carried out by means of multiple-reaction monitoring (MRM) acquisition using a Waters Acquity UPLC System coupled with QTRAP 5500 (AB Sciex, Darmstadt, Germany); the triple quadruple mass spectrometers (MS/MS) were operated with electrospray source in negative mode. Target bile acids were chromatographically separated on a reverse-phase column. Data processing was carried out with the provided quantitation method kit (Biocrates® bile acids kit).

## Statistical analysis

In the present investigation, the children of the LISA study were categorized into overweight and normal weight children. To this end, body mass index (BMI) Z-scores were calculated according to the World Health Organization (WHO) guidelines. Overweight was defined as a BMI Z-score above the 85th percentile of the entire LISA cohort in the 15th year of life (BMI Z-score > 88). The 5% of children with the lowest BMI Z-score (underweight, BMI Z-score < -1.69) were excluded from the study and the

remainder (between the 5th and 85th percentiles) was used as the normal weight group; 41 children were found to be overweight. Of the normal weight group, 41 children were randomly selected for the present investigation. Although all associations in this publication are calculated on the basis of the BMI Z-scores, the term 'body weight' is used instead throughout the whole publication for simplicity.

The  $\chi^2$  test was used for comparison between characteristics of the entire LISA cohort in the study center in Leipzig and the analyzed subgroups, both at the age of 15 years. The relationship between the analyzed parameters (inflammatory and MAIT cell markers, bile acids) and body weight, parental education, sex and season of blood sampling of the children was determined using the Mann-Whitney *U*-test. Odds ratios adjusted for sex, parental education, lifetime prevalence of asthma, hay fever and atopic dermatitis, as well as exposure to environmental tobacco smoke (ETS), were calculated in order to show the relationship between body weight and the analyzed parameters. Spearman's rank test was used to analyze the relationship between inflammatory markers, MAIT cell parameters and bile acid concentrations. Data are presented as heatmaps presenting the Spearman's correlation coefficients where significant *P*-values ( $P < 0.05$  and  $P < 0.0003$  after Bonferroni correction) were marked. To further substantiate this relationship, we used a linear regression model adjusted for body weight, sex, parental education, lifetime prevalence of asthma, hay fever and atopic dermatitis as well as exposure to ETS. For all *in-vitro* assays, the one-way analysis of variance (ANOVA) was used to analyze the significance of effects. Statistical analyses were performed using Statistica for Windows version 10.0 [Statsoft Inc. (Europe), Hamburg, Germany], GraphPad Prism version 7.04 (GraphPad Software, San Diego, CA, USA) and R version 3.4.3, package 'gplots'.

## Results

### Characteristics of the LISA study population

At the age of 15 years, 438 children participated in the LISA study in Leipzig. Blood was collected from 286 of these children. The characteristics of the selected study population ( $n = 82$ ) were comparable with those of the entire LISA cohort from Leipzig (Table 1). There were no significant differences in the groups regarding the lifetime prevalence of asthma, hay fever and atopic dermatitis, as well as the other selected characteristics. Inflammatory parameters (CRP, IL-8, MIP-1 $\alpha$ ), MAIT cells and BAs were assessed in the subgroup of overweight children ( $n = 41$ ) and in the randomly selected control group of normal weight children ( $n = 41$ ). BMI, BMI Z-scores and LDL in these groups were significantly different, whereas HDL,



**Table 1.** Characteristics of the analyzed subgroups and the entire Leipzig Lifestyle Immune System Allergy (LISA) cohort at the age of 15 years. Due to missing data, case number may vary for some variables

Parameters	Entire Leipzig LISA cohort <i>n</i> (%), <i>n</i> = 976	Analyzed subgroups		<i>P</i> -value*
		Overweight children <sup>4</sup> <i>n</i> (%), <i>n</i> = 41	Normal weight children <sup>5</sup> <i>n</i> (%), <i>n</i> = 41	
Sex of the child				
Male	482 (49.4)	25 (61.0)	21 (51.2)	0.208
Female	494 (50.6)	16 (39.0)	20 (48.8)	
Parental history of atopy <sup>1</sup>				
No	573 (58.7)	26 (63.4)	23 (56.1)	0.567
Yes	403 (41.3)	15 (36.6)	18 (43.9)	
Parental education <sup>2</sup>				
Low	71 (7.3)	1 (2.4)	1 (2.4)	0.287
Intermediate	309 (31.7)	12 (29.3)	12 (29.3)	
High	575 (58.9)	28 (68.3)	28 (68.3)	
At the age 15 y				
Blood collection <sup>3</sup>				
Summer	228 (23.4)	36 (87.8)	34 (82.9)	0.344
Winter	66 (6.8)	5 (12.2)	7 (17.1)	
Asthma				
No	747 (76.5)	32 (78.0)	35 (85.4)	0.235
Yes	118 (12.1)	9 (22.0)	6 (14.6)	
Atopic dermatitis				
No	303 (31.0)	29 (70.7)	27 (65.9)	0.301
Yes	217 (22.2)	12 (29.3)	14 (34.1)	
Hay fever				
No	757 (77.6)	36 (87.8)	34 (82.9)	0.407
Yes	92 (9.4)	5 (12.2)	7 (17.1)	
Smoking <sup>6</sup>				
No	380 (38.9)	35 (85.4)	37 (90.2)	0.742
Yes	36 (3.7)	5 (12.2)	4 (9.8)	

\**P*-value from  $\chi^2$  test for cross-relationship.<sup>1</sup>History of atopy is defined as: occurrence of asthma or atopic dermatitis or hay fever;<sup>2</sup>low, 9 years of schooling or less 'Hauptschulabschluss', intermediate, 10 years of schooling 'Mittlere Reife', high, 12 years of schooling or more 'Fach-)hochschulreife';<sup>3</sup>summer: April–October, winter: November–March;<sup>4</sup>definition of overweight: body mass index (BMI) *Z*-score > 0.88;<sup>5</sup>definition of normal weight: 0.88 > BMI *Z*-score > -1.69;<sup>6</sup>smoking: exposure to environmental tobacco smoke indoors.

**Table 2.** Body mass index (BMI), BMI *Z*-score and serum lipids [median, interquartile range (IQR)] in Lifestyle Immune System Allergy (LISA) study samples of 15-year-old children stratified by weight

	Weight		<i>P</i> -value
	Normal ( <i>n</i> = 41)	Overweight ( <i>n</i> = 41)	
BMI (kg/m <sup>2</sup> )	<b>19.82 (18.50–20.76)</b>	<b>23.94 (23.23–25.77)</b>	< <b>0.001</b>
BMI <i>Z</i> -score	<b>-0.03 (-0.63 to 0.28)</b>	<b>1.21 (1.01–1.61)</b>	< <b>0.001</b>
Total cholesterol (mM)	4.12 (3.80–4.36)	4.41 (3.72–4.84)	0.189
High-density lipoprotein (HDL) (mM)	1.41 (1.22–1.60)	1.34 (1.05–1.54)	0.132
Low-density lipoprotein (LDL) (mM)	<b>2.28 (2.04–2.53)</b>	<b>2.55 (1.88–3.05)</b>	<b>0.041</b>
Triglycerides (mM)	1.36 (0.86–1.66)	1.56 (0.95–1.86)	0.108

Significant differences (*P*-value < 0.05) between normal weight and overweight children are marked in bold.

total cholesterol and triglycerides were similar (Table 2). Data for MAIT cell and inflammatory markers as well as BAs in relation to body weight are given in Tables 3 and 4. The relationship to sex, season of blood sampling and parental education is shown in Supporting information, Tables S1 and S2.

## MAIT cells

The number of MAIT cells in the isolated PBMC was determined by surface staining of unstimulated PBMC. Furthermore, the expression of the NKp80 and CXCR3 was measured on the surface of these cells. In order to determine

**Table 3.** Inflammatory and mucosal-associated invariant T (MAIT) cell parameter [median, interquartile range (IQR)] in Lifestyle Immune System Allergy (LISA) study samples of 15-year-old children. Due to missing data, case number may vary for some variables. *P*-value shows differences between normal weight and overweight children

	All children	Weight		<i>P</i> -value
	( <i>n</i> = 78-82)	Normal ( <i>n</i> = 39-41)	Overweight ( <i>n</i> = 39-41)	
CRP (mg/l)	0.51 (0.32–0.87)	<b>0.40 (0.3–0.6)</b>	<b>0.66 (0.36–1.02)</b>	<b>0.016</b>
IL-8 (pg/ml)	11.04 (1.65–46.45)	13.24 (2.34–46.75)	10.97 (0.6–31.90)	0.365
MIP-1 $\alpha$ (pg/ml)	2.51 (0.1–4.45)	<b>3.32 (1.56–5.72)</b>	<b>1.56 (0.1–3.32)</b>	<b>0.021</b>
MR1 (rel. unit)	1.65 (1.52–1.77)	1.67 (1.53–1.79)	1.65 (1.52–1.71)	0.464
MAIT frequency (%)	2.46 (1.69–3.75)	2.47 (1.62–3.86)	2.45 (1.89–3.59)	0.97
CXCR3 <sup>+</sup> MAIT (%)	62.75 (34.97–76.57)	<b>51.69 (29.97–72.24)</b>	<b>67.05 (46.85–79.11)</b>	<b>0.027</b>
NKp80 <sup>+</sup> MAIT (%)	13.49 (8.87–24.66)	<b>22.5 (12.53–27.2)</b>	<b>11.51 (6.96–14.25)</b>	<b>&lt; 0.001</b>
CD107a <sup>+</sup> MAIT (%)	40.24 (26.61–51.61)	<b>46.06 (31.2–56.26)</b>	<b>36.41 (24.71–43.98)</b>	<b>0.013</b>
IFN- $\gamma$ <sup>+</sup> MAIT (%)	7.93 (4.59–12.03)	<b>10.48 (5.81–13.22)</b>	<b>5.99 (4.48–8.9)</b>	<b>0.019</b>
TNF- $\alpha$ <sup>+</sup> MAIT (%)	48.21 (40.9–54.45)	51.06 (40.85–56.51)	45.58 (41.2–53.12)	0.401
CD69 <sup>+</sup> MAIT (%)	72.51 (64.28–82.93)	72.64 (64.28–83.16)	71.02 (63.22–79.4)	0.583
CD137 <sup>+</sup> MAIT (%)	43.71 (35.25–50.9)	44.29 (36.46–55.09)	40.86 (33.71–49.84)	0.445
PLZF (rel. unit)	2.68 (2.28–3.06)	2.51 (2.20–2.88)	2.76 (2.36–3.10)	0.189

CRP = C-reactive protein; IL = interleukin; MIP =; MR1 = major histocompatibility complex class I-related gene protein; CXCR3 = chemokine receptor (CXCR3); NKp80 = natural killer cell marker; IFN = interferon; TNF = tumor necrosis factor; PLZF = promyelocytic leukemia zinc finger protein. Significant differences (*P*-value < 0.05) between normal weight and overweight children are marked in bold.

**Table 4.** Serum bile acids (BA) concentrations [median, interquartile range (IQR)] in Lifestyle Immune System Allergy (LISA) study samples of 15-year-old children. *P*-value shows differences between normal weight and overweight children

	All children	Weight		<i>P</i> -value
	( <i>n</i> = 82)	Normal ( <i>n</i> = 41)	Overweight ( <i>n</i> = 41)	
CA (nM)	4.6 (2.7–10.8)	3.7 (2.6–7.8)	5.7 (3.5–11.9)	0.091
CDCA (nM)	10.7 (4–33.9)	13.6 (2.9–44.3)	7.4 (4–14.3)	0.241
DCA (nM)	20 (8–35.6)	17.6 (4.1–36.2)	22.4 (8.9–28.1)	0.711
GCA (nM)	38.3 (20.7–71.3)	38.8 (18–75.6)	35.9 (21–70.1)	0.846
GCDCA (nM)	126.5 (53.5–199)	120 (44.3–202)	140 (82.8–196)	0.531
GDCA (nM)	31.5 (12.4–81.1)	31.5 (13.1–92.2)	31.4 (12.4–77.1)	0.982
GLCA (nM)	1.8 (0.8–3.1)	1.6 (0.6–3)	1.9 (1.1–3.1)	0.436
GUDDCA (nM)	11.1 (6.2–24.7)	11.7 (6.4–24.3)	10 (6–27.2)	0.867
TCA (nM)	6.4 (3.7–10.2)	7.3 (4.2–10.7)	5.5 (2.8–8.8)	0.1
TCDDCA (nM)	18.6 (6.7–33.9)	20.5 (4.7–34.8)	16.8 (8.5–31.4)	0.444
TDCA (nM)	5.5 (2.1–11.1)	5.9 (1.4–12.2)	4.6 (2.5–9.3)	0.864
TUDCA (nM)	1 (0.6–1.7)	0.8 (0.6–1.5)	1 (0.6–1.9)	0.755
UDCA (nM)	5.1 (0–12.9)	7.8 (0–17.3)	3 (0–9.1)	0.303

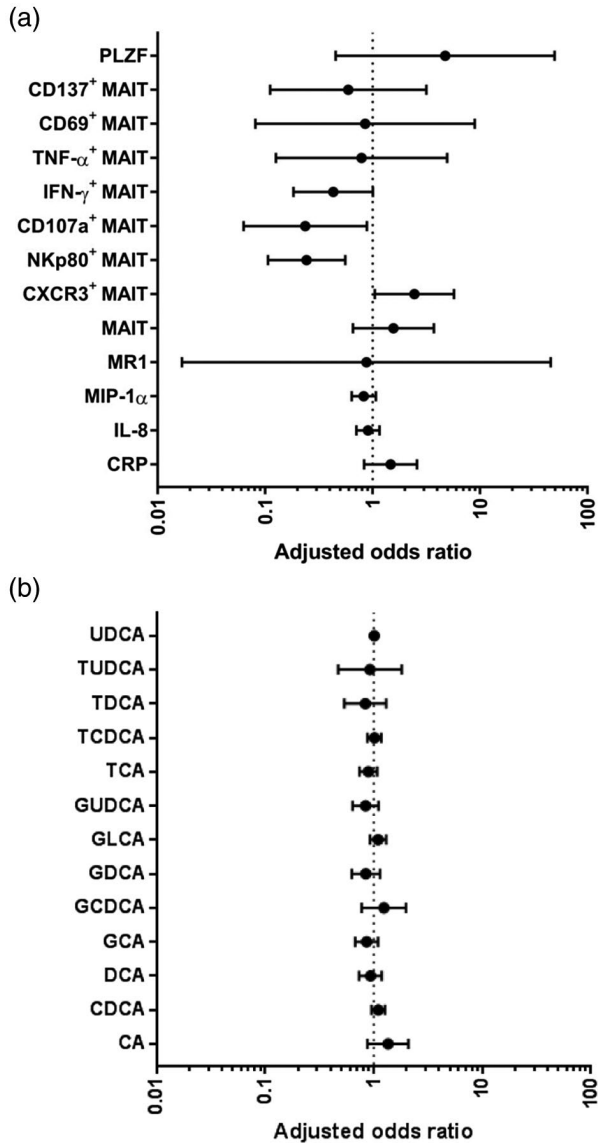
CA = cholic acid; CDCA = chenodeoxycholic acid; DCA = deoxycholic acid; GCDCA = glycochenodeoxycholic acid; GUDDCA = glyoursodeoxycholic acid; TCA = taurocholic acid; TCDDCA = taurodeoxycholic acid; TUDCA = tauroursodeoxycholic acid; UDCA = ursodeoxycholic acid.

the MAIT cell count in the isolated PBMC, cells were gated for CD3, CD8a, CD161 and TCR V $\alpha$ 7.2 expression (Supporting information, Fig. S1) and are given as percentage of CD3<sup>+</sup> T cells. The number of unstimulated MAIT cells [median, interquartile range (IQR)] was, on average, 2.46% (1.69–3.75). Of the unstimulated MAIT cells, 62.75% (34.97–76.57) and 13.49% (8.87–24.66) expressed CXCR3 and NKp80, respectively (Table 3). MAIT cell activation was assessed after stimulation with *E. coli* leading to the production (median, IQR) of CD69 (72.51%, 64.28–82.93), IFN- $\gamma$  (7.93%, 4.59–12.03), TNF- $\alpha$  (48.21%, 40.9–54.45), CD107a

(40.24%, 26.61–51.61) and CD137 (43.71%, 35.25–50.9) by MAIT cells (Table 3 and Supporting information, Fig. S1). In addition, the expression of the MAIT cell transcription factor PLZF and the MR1 molecule were analyzed in unstimulated blood samples via qPCR. The mRNA of both genes was detectable in all samples (Table 3).

Stratification by body weight revealed no difference in the number of unstimulated MAIT cells (Table 3). However, overweight children had significantly lower numbers of MAIT cells expressing NKp80 and higher amounts of MAIT cells expressing CXCR3 (Table 3). These associations remained

significant after adjustment for confounding factors (sex, season of blood sampling, parental education, lifetime prevalence of asthma, hay fever and atopic dermatitis, as well as ETS) (Fig. 1a). In addition, we found that overweight children had a lower amount of MAIT cells producing IFN- $\gamma$  and the degranulation marker CD107a after *E. coli* stimulation than normal weight children (Table 3). These associations remained significant after the adjustment for the confounding factors mentioned above (Fig. 1a).



**Fig. 1.** Relationship of inflammatory and mucosal-associated invariant T (MAIT) cell parameters (a) and bile acids (b) to weight in Lifestyle Immune System Allergy (LISA) study samples of 15-year-old children. Data are presented as odds ratios (ORs) with 95% confidence intervals (CI) adjusted for confounders [sex, season of blood sampling, parental education, lifetime prevalence of asthma, hay fever and atopic dermatitis, as well as exposure to environmental tobacco smoke (ETS)]. Data are presented as ORs with 95% CI.

Considering the distribution in other groups (sex, season of blood sampling, parental education), the amount of MAIT cells and MR1 expression were dependent on sex, being significantly higher in girls (see Supporting information, Table S1). No other significant associations were found with these groups.

### Bile acids in serum

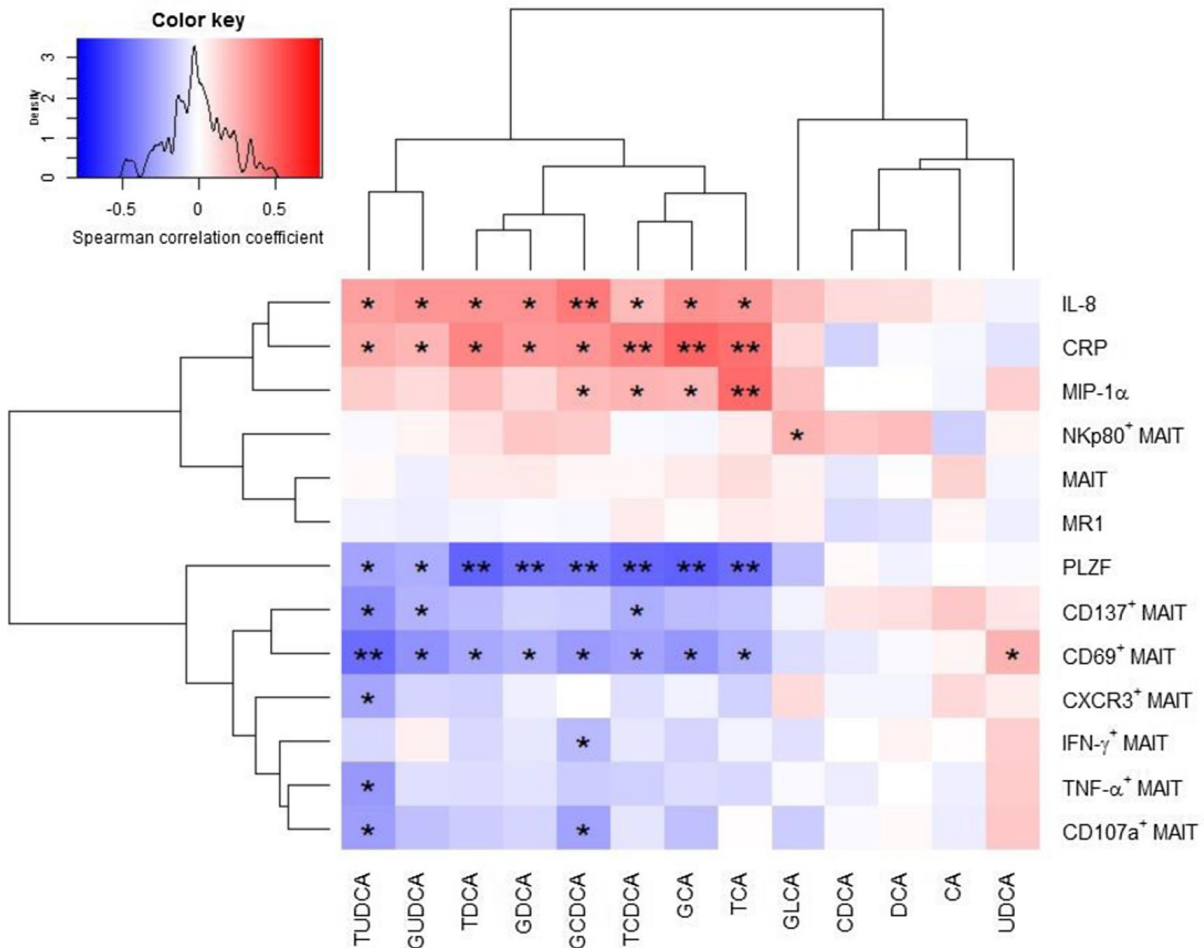
BA concentrations were analyzed in serum of the selected study population ( $n = 82$ ). Of the 20 BAs measured in serum, a total of 13 BAs (CA, CDCA, DCA, GCA, GCDCA, GDCA, GLCA, GUDCA, TCA, TCDCA, TDCA, TUDCA and UDCA) were reliably detected and retained for analysis (Table 4). In general, the BA concentrations were low, ranging from (median, IQR) 1 nM (0.6–1.7) for TUDCA to 126.5 nM (53.5–199) for GCDCA. Stratification by body weight revealed no association with BA concentrations (Table 4, Fig. 1b). Furthermore, it was evident that girls had higher serum concentrations of DCA and GDCA (see Supporting information, Table S2). Stratification by parental education revealed that GUDCA and TDCA and in trend GDCA, TCDCA and TUDCA were also more highly concentrated in the serum of children having parents with low/intermediate education level (see Supporting information, Table S2).

### Inflammatory parameters

The concentrations of the inflammatory markers CRP, IL-8 and MIP-1 $\alpha$  in the selected study population are shown in Table 3. Stratification by body weight revealed no significant differences of IL-8 concentration. Although the CRP concentration was higher in overweight children, while MIP-1 $\alpha$  was at a lower level (Table 3), these associations failed to reach significance after adjustment for the confounding factors mentioned above (data not shown).

### Association between BA concentrations, MAIT cells and inflammatory parameters

The associations between BA concentrations, MAIT cell and inflammatory parameters in the analyzed LISA subgroup ( $n = 82$ ) are presented in Fig. 2 as a heatmap of the Spearman's correlation coefficients. We observed a strong positive association between most conjugated BA serum concentrations and the inflammatory markers CRP, IL-8 and MIP-1 $\alpha$  (Fig. 2). In contrast, unconjugated BAs were not related to the concentration of inflammatory parameters (Fig. 2). We did not find any consistent association between the amount of unstimulated MAIT cells and BA concentrations (Fig. 2). However, GLCA levels were associated with a higher amount of NKp80<sup>+</sup> MAIT cells. This association remained significant after adjustment for confounding factors (Fig. 3). Regarding the function of MAIT cells, we found that



**Fig. 2.** Correlation between bile acids, inflammatory and mucosal-associated invariant T (MAIT) cell parameters in Lifestyle Immune System Allergy (LISA) study samples of 15-year-old children ( $n = 82$ ). The associations are presented as Spearman's correlation coefficients. Red and blue fields indicate positive and negative correlations, respectively. \* $P < 0.05$ , \*\*significant after Bonferroni correction ( $P < 0.0003$ ).

children with high levels of conjugated BA had, in general, lower numbers of MAIT cells responding to *E. coli* stimulation. We found a negative association between GCDCA and TUDCA and the amount of MAIT cells producing the degranulation marker CD107a (Fig. 2). These two BAs were also associated with lower numbers of IFN- $\gamma$ - and TNF- $\alpha$ -producing MAIT cells, respectively. Almost all conjugated BAs were negatively associated with the amount of CD69-producing MAIT cells after *E. coli* stimulation. GUDCA, TCDCA and TUDCA were also negatively associated with the amount of CD137<sup>+</sup> MAIT cells after *E. coli* stimulation (Fig. 2). The expression of the MAIT cell transcription factor PLZF was negatively correlated with all conjugated BA levels in serum, the strongest association being for GCA ( $R = -0.5$ ,  $P < 0.0001$ , Fig. 2). After correction for confounding factors, the associations of GCDCA, TDCA

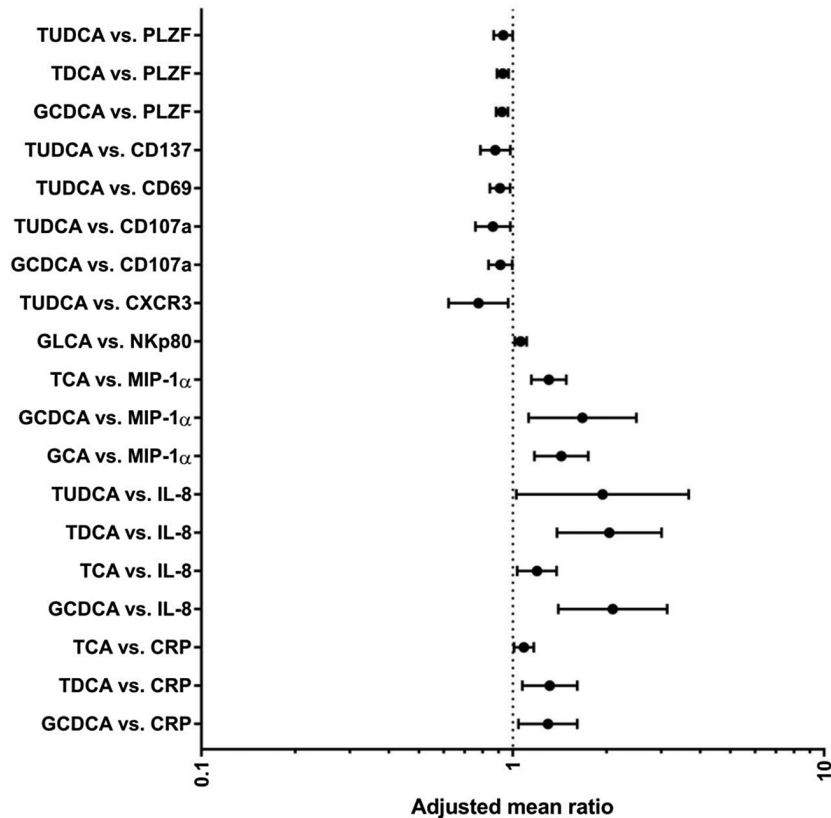
and TUDCA to PLZF expression still remained significant (Fig. 3).

Data stratified by body weight are presented in Fig. 4. The associations were similar in these groups but were, in general, weaker for overweight than for normal weight children (Fig. 4).

#### *In-vitro* bile acid assays

To validate our epidemiological findings, we performed *in-vitro* assays in order to assess the impact of selected BAs on MAIT cell activation (production of IFN- $\gamma$ , TNF- $\alpha$ , CD107a and CD69). Both GCDCA and TUDCA were chosen due to their significant associations with MAIT cell activation parameters in the LISA study (Fig. 2). In addition, GCDCA was at the highest concentration in our study (Table 4). Although TLCA was not quantifiable in the LISA study samples, this BA was tested *in vitro* as





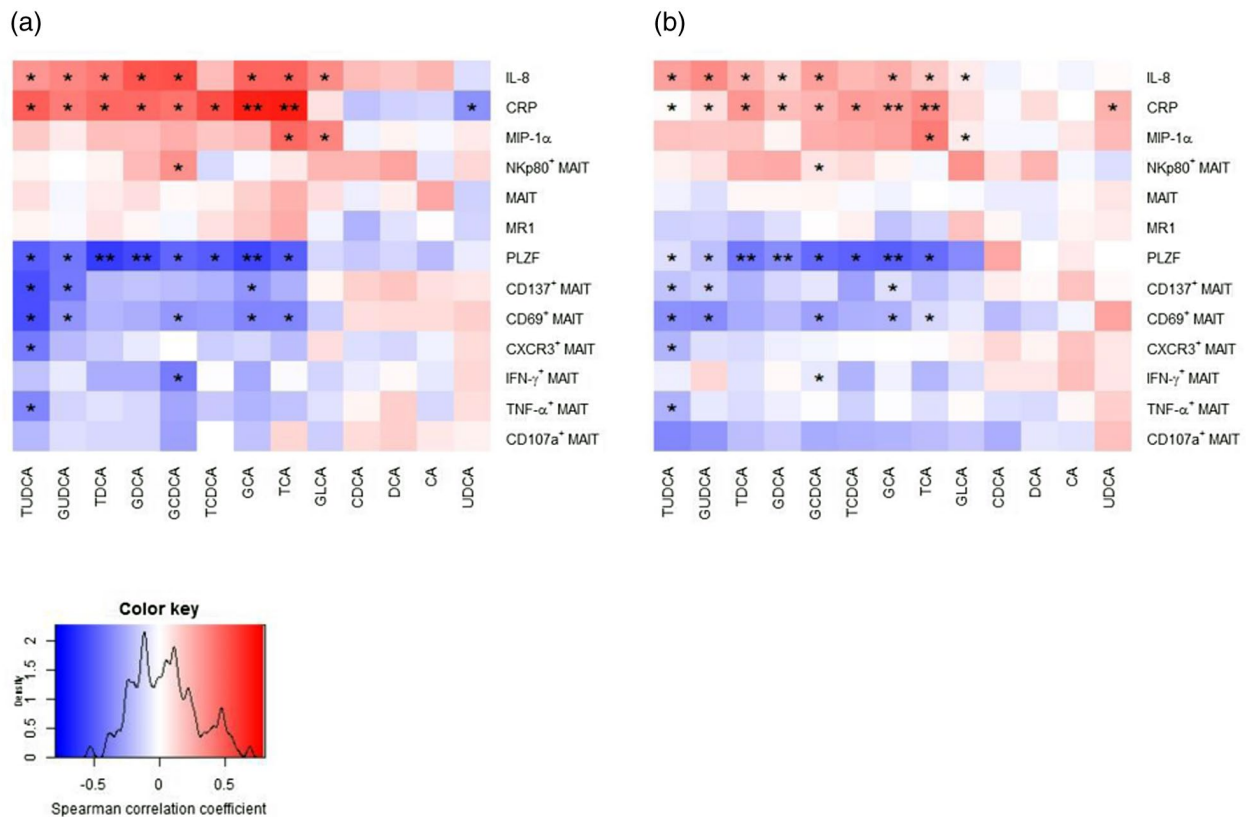
**Fig. 3.** Relationship between bile acids and inflammatory/ mucosal-associated invariant T (MAIT) cell parameters in Lifestyle Immune System Allergy (LISA) study samples of 15-year-old children ( $n = 82$ ). Data are presented as mean ratios (MR) with 95% confidence intervals (CI) adjusted for confounders [body weight, sex, season of blood sampling, parental education, lifetime prevalence of asthma, hay fever and atopic dermatitis, as well as exposure to environmental tobacco smoke (ETS)]. Data represent only the significant associations from Spearman's correlation in Fig. 2.

TLCA has already been described as possessing immunomodulatory properties [10]. The impact of TLCA, TUDCA and GCDCA was tested at three different concentrations, either alone or together with *E. coli*. The amount of IFN- $\gamma$ - and CD107a-producing MAIT cells after *E. coli* stimulation was significantly reduced in the presence of all three BAs (Fig. 5a,b). TLCA treatment caused a significantly dose-dependent reduction of TNF- $\alpha$ -producing MAIT cells upon *E. coli* stimulation (Fig. 5c). A trend towards a lower amount of TNF- $\alpha$ -producing MAIT cells after *E. coli* stimulation was observed in the presence of TUDCA and GCDCA (Fig. 5c). The amount of CD69-expressing MAIT cells was generally very high after *E. coli* stimulation (Supporting information, Fig. S2). However, not the amount but the mean fluorescence intensity (MFI) of CD69 was significantly lower after TLCA, and in trend also after GCDCA, treatment (Fig. 5d). As positive control for direct BA receptor activation, INT-747 (FXR agonist) and INT-777 (TGR5 agonist) were used and tested for their ability to modulate MAIT cell activation. The amount of TNF- $\alpha$ -producing MAIT cells as well as the MFI of CD69 expression in these cells were significantly reduced

by INT-777 and INT-747 in a dose-dependent manner (Fig. 6a,b). In both cases, the INT-747-induced inhibition was stronger than the INT-777-mediated effect. BAs, INT-747 and INT-777 did not induce the production of CD107a, INF- $\gamma$ , TNF- $\alpha$  or CD69 in the absence of *E. coli* at any concentration (data not shown).

#### Viability test

PBMC viability for the *in-vitro* BA assays was determined using a cell counting kit 8 (CCK8) for all tested BAs (TLCA, TUDCA and GCDCA) as well as for INT-747 and INT-777 alone and in the presence of *E. coli*. Sodium dodecyl sulfide (SDS) was used as a positive control for cell death and led to a strong cytotoxic effect of approximately 88% cell death (see Supporting information, Table S3). Treatment with 50  $\mu$ M TLCA and *E. coli* led to a significant decrease of cell viability, which was not observed at higher concentrations of TLCA (see Supporting information, Table S3). None of the other BAs or BA receptor agonists had an impact on PBMC viability, regardless of the concentration used and the presence or absence of *E. coli* (see Supporting information, Table S3).



**Fig. 4.** Correlation between bile acids, inflammatory and mucosal-associated invariant T (MAIT) cell parameters in Lifestyle Immune System Allergy (LISA) study samples of 15-year-old children stratified by weight. The associations are presented as Spearman's correlation coefficients. Red and blue fields indicate positive and negative correlations, respectively. (a) Normal weight children ( $n = 41$ ); (b) overweight children ( $n = 41$ ). \* $P < 0.05$ , \*\*significant after Bonferroni correction ( $P < 0.0003$ ).

## Discussion

The focus of the present investigation was on the association of BA serum concentrations with MAIT cells and inflammatory markers as well as on the impact of body weight on these parameters. In a subgroup of the 15-year-old children of the LISA study (41 normal weight and 41 overweight children) we measured the concentration of 13 BAs, inflammatory markers (CRP, MIP-1 $\alpha$  and IL-8), the amount of MAIT cells and the activation pattern of these cells after *E. coli* stimulation. In addition, we validated these findings in *in-vitro* assays.

The most important finding was that BAs positively associated with the inflammatory markers but were negatively related to the amount of activated MAIT cells and expression of the MAIT cell transcription factor PLZF. However, these relationships differed slightly between normal and overweight children, being weaker in overweight children. In general, most associations were found with conjugated BAs, leading to the hypothesis that the immunomodulatory function is restricted to conjugated BAs. The *in-vitro* assays revealed that higher concentrations of TLCA, TUDCA and GCDCA

indeed lowered the amount of IFN- $\gamma$ -, TNF- $\alpha$ - and CD107a-producing MAIT cells after *E. coli* stimulation. In addition, the expression level of the activation marker CD69 was reduced in these cells.

The association between high BA concentrations and high levels of CRP, IL-8 and MIP-1 $\alpha$  in plasma might reflect the fact that BA concentrations are linked to inflammatory gene expression in various cell types. For example, it was recently shown that BAs promote hepatic inflammation involving MIP-1 $\alpha$  and IL-8 [36,37]. Furthermore, CRP is used as an early marker for liver injury and cholestasis [38]. Moreover, BAs appear to induce the production of the inflammatory markers IL-6 and IL-8 in the airway epithelium [39]. Taken together, these studies demonstrate that hepatocytes or airway epithelial cells exposed to BAs might contribute to the chemokine milieu that is responsible for lymphocyte and neutrophil recruitment into these organs and, in turn, to the further and systemic exacerbation of inflammation. Thus, an increase (for any reason) of systemic BA concentrations may lead to chronic low-grade inflammation.

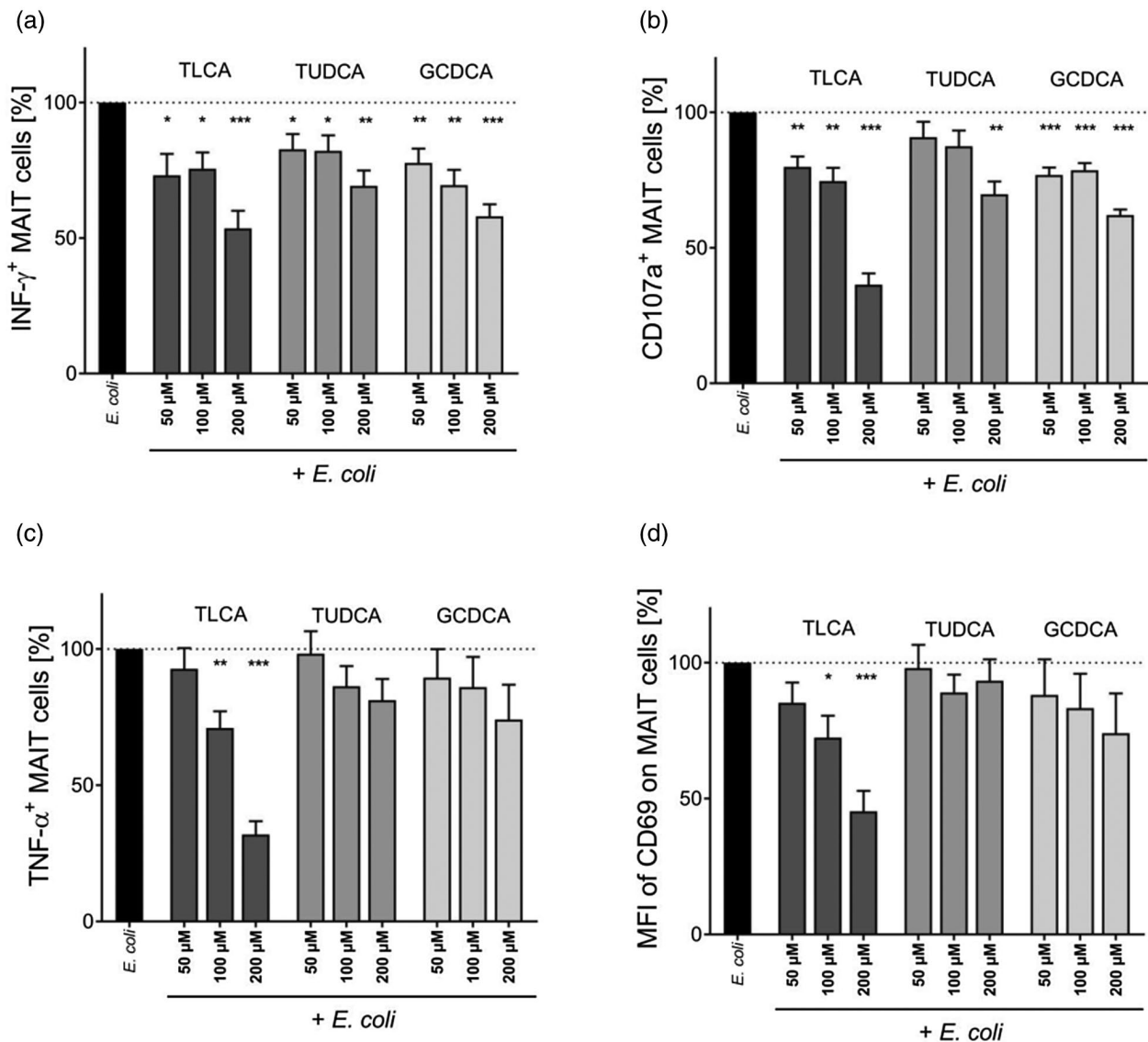
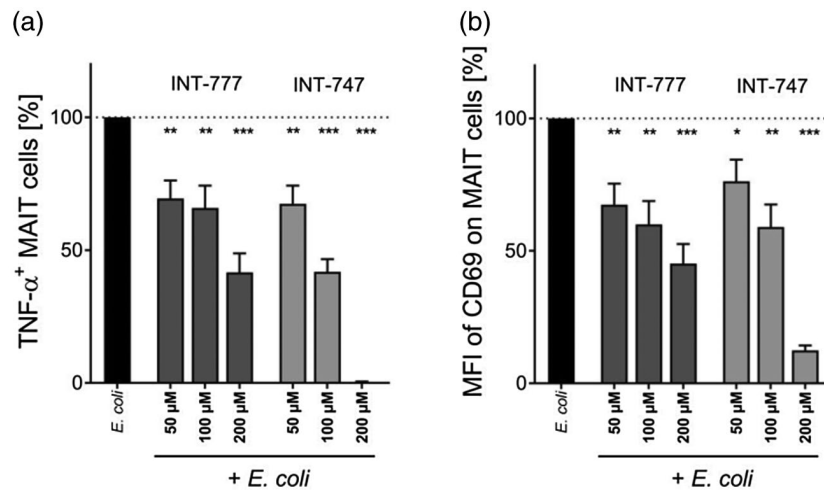


Fig. 5. Bile-acids-mediated inhibition of *Escherichia-coli*-induced cytokine and activation marker expression by mucosal-associated invariant T (MAIT) cells. PBMC were preincubated for 45 min with bile acids. *E. coli* was added for another 6 h before cells were stained for flow cytometry. Expression of all targets was normalized to *E. coli* stimulation alone (100%). Data are given as mean  $\pm$  standard error of the mean (s.e.m.) ( $n = 6$ ). (a) Amount of interferon (IFN)- $\gamma$ -producing MAIT cells; (b) amount of CD107a-producing MAIT cells; (c) amount of tumor necrosis factor (TNF)- $\alpha$ -producing MAIT cells; (d) mean fluorescence intensity (MFI) of CD69 expression on MAIT cells. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , one-way analysis of variance (ANOVA).

Regarding immune cells, an impact of BAs has been shown for monocytes and macrophages. These cells express the G-protein-coupled BA receptor Takeda G protein-coupled receptor 5 (TGR5), which can be activated by both conjugated and unconjugated BAs [40]. Activation of TGR5 in macrophages reduces proinflammatory cytokines while maintaining anti-inflammatory cytokine expression, thus promoting the development of an anti-inflammatory macrophage phenotype [12]. Furthermore, BAs inhibit the lipopolysaccharide (LPS)-induced expression of the proinflammatory cytokines TNF- $\alpha$  and IL-6

in primary human macrophages [10]. In contrast, it has been found that treatment of eosinophils with TCDCA and TUDCA induced the production of high amounts of IL-8 [11]. Thus, the functional influence of BAs on the immune system may vary depending on different immune cell types and conditions.

MAIT cells, an unconventional innate-like T cell type which is activated by bacterial metabolites, are found at high levels in the peripheral blood and are also highly abundant in the liver [15,17]. We therefore hypothesized that BAs might have the ability to modulate the effector



**Fig. 6.** INT-747- and INT-777-mediated inhibition of *Escherichia coli*-induced cytokine and activation marker expression by mucosal-associated invariant T (MAIT) cells. Peripheral blood mononuclear cells (PBMC) were preincubated for 45 min with bile acids. *E. coli* was added for another 6 h before cells were stained for flow cytometry. Expression of all targets was normalized to *E. coli* stimulation alone (100%). Data are given as mean  $\pm$  standard error of the mean (s.e.m.) ( $n = 6$ ). (a) Amount of tumor necrosis factor (TNF)- $\alpha$ -producing MAIT cells. (b) mean fluorescence intensity (MFI) of CD69 expression on MAIT cells. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , one-way analysis of variance (ANOVA).

function of these cells. Indeed, our results within the LISA study showed that conjugated BA concentrations were negatively related to the amount of activated MAIT cells, but not to the amount of unstimulated cells in the peripheral blood (Fig. 2). Similar to the reported studies on macrophages, we found that BAs were also negatively associated with the proinflammatory function of MAIT cells. Thereby TUDCA appeared to be the most important in connection with MAIT cell activation, being negatively associated with all measured markers. As yet it is not clear whether MAIT cells, like macrophages, express receptors for BAs. In our *in-vitro* assays, we observed an impact of TUDCA, TLCA and GCDCA on MAIT cell activation, resulting in a lower amount of MAIT cells producing IFN- $\gamma$ , TNF- $\alpha$ , CD107a and CD69 upon *E. coli* stimulation (Fig. 5a–d). Therefore, our results lead to the hypothesis that MAIT cells might express BA receptors. However, it has been reported that lymphocytes do not express TGR5 [41]. In contrast, the nuclear BA receptor FXR is expressed on CD4<sup>+</sup> and CD8<sup>+</sup> T cells [42]. In order to distinguish between TGR5- and FXR-mediated effects on MAIT cells, we directly activated both receptors with the synthetic specific agonists INT-777 and INT-747, respectively. We could show that both the TGR5 agonist INT-777 and the FXR agonist INT-747 had very strong impacts upon MAIT cell activation, intensively reducing the amount of TNF- $\alpha$ -producing MAIT cells and CD69 expression (Fig. 6a,b). However, the impact of INT-747 on MAIT cell activation was much stronger than the INT-777-mediated effect, especially at high concentrations (Fig. 6a,b). Although the exact mechanisms require additional

studies, this might implicate that MAIT cells react to BAs by expressing both TGR5 and Farnesoid X receptor (FXR), with FXR being of greater importance. It is noteworthy that the inhibition of MAIT cell activation by BAs was not a result of an impaired cellular viability, as indicated from the results of the viability assay performed (see Supporting information, Table S3).

In our study, high concentrations of almost all measured conjugated BAs correlated with low expression of the MAIT cell transcription factor PLZF and low expression of the activation markers TNF- $\alpha$ , IFN- $\gamma$ , CD107a, CD69 and CD137 by MAIT cells (Fig. 2). In addition to MAIT cells, PLZF is also expressed by invariant NK T (iNK T) cells and V $\delta$ 2  $\gamma$  $\delta$  T cells [22,43–45]. As PLZF mRNA levels were analyzed in whole blood in the present investigation, the signal determined might be influenced by these cell types. Considering that iNKT cells are 100-fold less frequent in the peripheral blood than MAIT cells [22,43], the impact of this cell type on PLZF levels can be expected to be less important. However, we cannot estimate the impact of V $\delta$ 2  $\gamma$  $\delta$  T cell frequency on PLZF expression in the whole-blood samples of our study. Whereas PLZF is down-regulated in most mature iNKT cells, it is expressed in late MAIT cell development, where it governs their final maturation step to generate functional MAIT cells and is maintained at high levels by these cells [46]. Thus, PLZF is probably necessary for MAIT cell function, as defined by cytokine secretion and degranulation.

The relationship between BAs, inflammatory markers and MAIT cells was similar in normal weight and overweight children (Fig. 4a,b). It is remarkable that the intensity of this relationship was stronger in normal weight children,



indicating that factors not yet known might impact this association in overweight children. In contrast to other research groups, we did not find a difference in the amount of MAIT cells in peripheral blood in relation to body weight [30,31]. In line with Magalhaes *et al.* [31], we showed that MAIT cells of overweight children are less prone to be activated by *E. coli* stimulation (Table 3). This may reflect an exhausted status of MAIT cells in overweight subjects, which could represent a transition towards cell death [31]. MAIT cell exhaustion has also been described in the context of hepatitis C-virus-infected patients [47,48]. In addition, overweight children had a lower amount of MAIT cells expressing NKp80 (Table 3). NKp80 has recently been shown to be critical for IFN- $\gamma$  production by NK cells [49]. This may explain the simultaneous observation of lower numbers of NKp80- and IFN- $\gamma$ -expressing MAIT cells in the overweight children in our study. Moreover, overweight children had a higher amount of MAIT cells expressing CXCR3 in our study. It has been published that CXCR3 contributes to T cell accumulation in adipose tissue of obese mice [50]. Due to the fact that MAIT cells are also found in human adipose tissue [30,31] it may be that MAIT cells expressing CXCR3 contribute to local inflammation in this tissue and perhaps also to the systemic expansion of this inflammation.

In our study, the amount of unstimulated MAIT cells and the BAs DCA and GDCA were at higher levels in girls (see Supporting information, Tables S1 and S2). These observations suggest that, at the age of 15, sex hormones might play a role in the observed findings. In contrast, it has already been shown that serum BAs are at lower levels in women [51]. This difference might be due to the participants' age of 15 in the present study, reflecting a disparity between adolescents and adults. In addition, we found elevated BA concentrations in children of parents with low/medium education. Thus, contrary to our initial assumption, mainly other factors than body weight appear to influence BA levels in 15-year-old children. Therefore, it is important to consider these influencing factors when performing epidemiological studies with BAs.

Taken together, our data show that BAs may have the capacity to influence the balance between pro- and anti-inflammatory immunity by modulating the immune response. Here, we show for the first time, to our knowledge, an association between BAs and MAIT cell activity by combining our epidemiological findings with the *in-vitro* data. Our results call for future studies regarding the underlying mechanism and further analysis of the role of MAIT cells in health and disease.

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## Disclosures

The authors have no financial conflicts of interest.

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### Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site.

**Fig S1.** Gating strategy for the identification of MAIT cells in LISA study samples of 15-year-old children ( $n = 82$ ).

**Fig S2.** Gating Strategy for the identification of MAIT cells in the *in vitro* bile acid assay.

**Table S1.** Inflammatory and MAIT cell parameter (median, IQR) in LISA study samples of 15-year-old children stratified by parental education, sex and season of blood sampling. Due to missing data, case number may vary for some variables.

**Table S2.** Serum bile acid concentrations in LISA study samples of 15-year-old children stratified by parental education, sex and season of blood sampling.

**Table S3.** PBMC viability in the presence of bile acids or bile acid analogs with and without *E. coli* stimulation. Values are presented as absorbance at 450 nm.

**Table S4.** Antibodies used for staining of the LISA study samples of 15-year-old children and the *in vitro* bile acid assay samples.