

Raised immunoglobulin A and circulating T follicular helper cells are linked to the development of food allergy in paediatric liver transplant patients

R. De Bruyne¹, P. Gevaert², M. Van Winckel¹, N. De Ruyc², A. Minne³, D. Bogaert^{3,4}, S. Van Biervliet¹, S. Vande Velde¹, F. Smets⁵, E. Sokal⁵, F. Gottrand⁶, J. Vanhelst⁷, B. Detry^{8,9}, C. Pilette^{8,9}, B. N. Lambrecht^{4,10,11} and M. Dullaers⁴

¹Department of Pediatric Gastroenterology, Hepatology and Nutrition, Princess Elisabeth Children's Hospital, Ghent University Hospital, Ghent, Belgium,

²Upper Airways Research Laboratory, Department of Otorhinolaryngology, Ghent University Hospital, Ghent, Belgium, ³Department of Pediatrics, Princess Elisabeth Children's Hospital, Ghent University Hospital, Ghent, Belgium, ⁴Clinical Immunology Research Laboratory, Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium, ⁵Service de Gastro-entérologie et Hépatologie Pédiatrique et Institut de Recherche Expérimentale et Clinique, Cliniques Universitaires Saint-Luc, Université Catholique de Louvain, Brussels, Belgium, ⁶Inserm U995, Faculty of Medicine, CIC-PT-9301, Department of Pediatric Gastroenterology, Hepatology and Nutrition, Hospital Jeanne de Flandre, CHRU Lille, University Lille2, Lille, France, ⁷Centre d'Investigation Clinique de Lille-PT-1403-Inserm-CH&U, Inserm U995, Faculty of Medicine, University Lille2, Lille, France, ⁸Pole of Pneumology, ENT and Dermatology, Institute of Experimental and Clinical Research, Cliniques Universitaires Saint-Luc, Université Catholique de Louvain, Brussels, Belgium, ⁹Walloon Excellence in Lifesciences and Biotechnology (WELBIO) Institute, Brussels, Belgium, ¹⁰Laboratory of Immunoregulation, VIB Inflammation Research Center, Ghent, Belgium and ¹¹Department of Pulmonary Medicine, Erasmus MC, Rotterdam, The Netherlands

Clinical & Experimental Allergy

Summary

Background Post-transplant food allergy (LTFA) is increasingly observed after paediatric liver transplantation (LT). Although the immunopathology of LTFA remains unclear, immunoglobulin (Ig) E seems to be implicated.

Objective To study humoral and cellular immunity in paediatric LT patients in search for factors associated with LTFA, and compare with healthy controls (HC) and non-transplant food-allergic children (FA).

Methods We studied serum Ig levels in 29 LTFA, 43 non-food-allergic LT patients (LTnoFA), 21 FA patients and 36 HC. Serum-specific IgA and IgE against common food allergens in LTFA, IgA₁, IgA₂ and joining-chain-containing polymeric IgA (pIgA) were measured. Peripheral blood mononuclear cells were analysed by flow cytometry for B and T cell populations of interest.

Results Serum IgA and specific IgA were higher in LTFA compared to LTnoFA. LTFA patients had the highest proportion of circulating T follicular helper cells (cTfh). The percentage of cTfh correlated positively with serum IgA. Unique in LTFA was also the significant increase in serum markers of mucosal IgA and the decrease in the Th17 subset of CXCR5⁺ CD4⁺ cells compared to HC. Both LT patients exhibited a rise in IgA⁺ memory B cells and plasmablasts compared to HC and FA.

Conclusions LT has an impact on humoral immunity, remarkably in those patients developing FA. The increase in serum markers of mucosal IgA, food allergen-specific IgA and cTfh cells observed in LTFA, point towards a disturbance in intestinal immune homeostasis in this patient group.

Keywords children, food allergy, immunoglobulin A, liver transplantation, polymeric IgA, secretory IgA, specific IgA, T follicular helper cells, regulatory T cells

Submitted 24 October 2014; revised 14 January 2015; accepted 16 January 2015

Correspondence:

Ruth De Bruyne, Department of Pediatric Gastroenterology, Hepatology and Nutrition, Princess Elisabeth Children's Hospital, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium.

E-mail: ruth.debruyne@ugent.be

Cite this as: R. De Bruyne, P. Gevaert, M. Van Winckel, N. De Ruyc, A. Minne, D. Bogaert, S. Van Biervliet, S. Vande Velde, F. Smets, E. Sokal, F. Gottrand, J. Vanhelst, B. Detry, C. Pilette, B. N. Lambrecht and M. Dullaers, *Clinical & Experimental Allergy*, 2015 (45) 1060–1070.

Introduction

Food allergy (FA) is increasingly reported after paediatric liver transplantation (LT) ([1–4]). The underlying pathogenesis remains unresolved. In our experience,

liver transplant-associated FA (LTFA) is seen in 28% of children [5]. Young age at time of transplantation and immunosuppression with tacrolimus are associated with LTFA [3, 5]. Immunoglobulin (Ig) E seems implicated in the immunopathology as most patients present

IgE-mediated signs, positive skin prick testing and/or elevated specific IgE for the responsible food antigen(s) [5]. The type of Ig produced by B cells is heavily influenced by help from T helper 2 lymphocytes providing cognate costimulatory molecules and cytokines for Ig class switching [6]. Tacrolimus, a calcineurin antagonist, inhibits nuclear factor of activated T cells (NFAT) signalling and T cell activation [7, 8]. The current knowledge on the influence of calcineurin inhibition on humoral immunity remains limited, and no data are available on serum Ig levels other than IgE in LTFA.

IgA is thought to play a protective role in the context of FA mainly through the function of mucosal IgA. As neutralizing antibody, IgA contributes to mucosal homeostasis by limiting the uptake of antigens from the gut, hence preventing primary sensitization or triggering of allergic reactions by food antigens [9]. Eighty per cent of the body's plasma cells are found in the gut mucosa, the largest effector organ of humoral immunity. In the small intestine, these are primarily IgA-producing cells (80%). In humans, serum IgA is predominantly IgA₁, while mucosal IgA includes a substantial fraction of IgA₂ [9, 10]. Most mucosal plasma cells produce dimers and larger polymers of IgA containing joining ('J')-chain. The 'J-chain' facilitates binding of polymeric IgA (pIgA) to the polymeric immunoglobulin receptor (pIgR). This receptor exports pIgA across the epithelial layer into the intestinal lumen [9]. At the apical membrane of the epithelial cell, the proteolytic fragment 'secretory component' (SC) is cleaved of the pIgR to release pIgA containing 'J-chain' as well as SC into the gut [11].

T follicular helper cells (Tfh), a CD4⁺ subset which provides help to B cells undergoing isotype switching

and affinity maturation in germinal centres (GC), play an essential role in the generation of high-affinity IgA and maintenance of the mucosal barrier [12]. The quality of intestinal IgA critically depends on the number and the nature of Tfh in Peyer's patches [13].

To investigate the mechanism of LTFA and the potential role of IgA, we measured levels of IgA₁, IgA₂ and 'J-chain'-containing pIgA as indirect serum markers of mucosal immunity as well as specific IgA and IgE against common food antigens. Furthermore, peripheral blood mononuclear cells (PBMC) of LTFA patients were analysed by flow cytometry in comparison with age-matched liver transplant patients without food allergy (LTnoFA) and food-allergic patients (FA) and healthy controls (HC), focusing on Ig expression in B cells, regulatory T cell (Treg) and circulating Tfh phenotypes.

Methods

Study population

The study population consisted of four groups of children (0–16 years): 29 LTFA, 43 LTnoFA, 21 FA patients and 36 HC. Demographic data and the indications for LT are shown in Table 1. Table S1 summarizes the clinical features, the responsible food allergens and the diagnostic parameters in LTFA patients. In the FA group, IgE-mediated and mixed IgE-mediated FA was seen in, respectively, 16 and 5 of 21 children. Data regarding immunosuppressive treatment in LTFA and LTnoFA patients are shown in Table S2. We also analysed serum Ig in renal transplant patients and children with chronic parenchymal (non-immune mediated) liver disease.

Table 1. Demographic features and indication for liver transplantation

	LTFA	LTnoFA	FA	HC
Number (sex)	29	43	21	36
	16 M	23 M	16 M	18 M
Age at blood test (y)*	6.5 (1.5–14.9)	8.79 (0.9–15.3)	6.67 (2.5–15.7)	5.08 (0.5–13.7)
Age at LT (y)*	0.86 (0–3)	2.55 (0–15)	NA	NA
Time interval LT – diagnosis FA (m)*	9 (1–155)	NA	NA	NA
Follow-up after LT (y)*	5.7 (1.6–13.1)	4.2 (0.3–15.2)	NA	NA
Indication LT				
EHBA	21/29	19/43	NA	NA
ALF	3/29	6/43		
PFIC	1/29	4/43		
A-1-AT deficiency	–	2/43		
IEM	1/29	5/43		
Alagille syndrome	1/29	1/43		
Hepatoblastoma	–	4/43		
Other causes	2/29	2/43		

A-1-AT, alpha-1-antitrypsin deficiency; ALF, acute liver failure; EHBA, extrahepatic biliary atresia; FA, food-allergic patients; HC, healthy controls; IEM, inborn errors of metabolism; LT, liver transplantation; LTFA, patients with liver transplant-associated food allergy; LTnoFA, liver transplant patients without food allergy; M, male; m, months; NA, not applicable; PFIC, progressive familial intrahepatic cholestasis; y, years.

*Median (range).

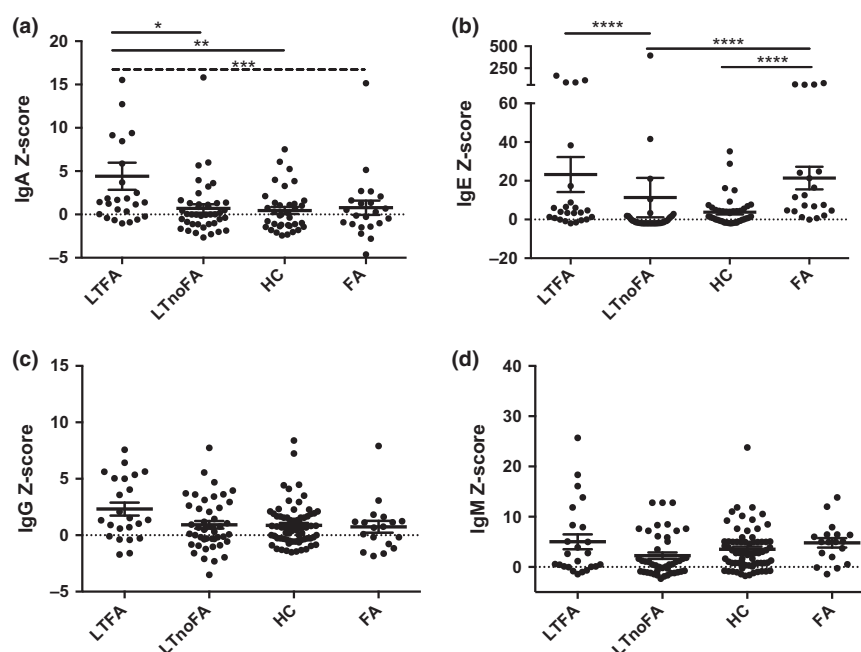


Fig. 1. serum IgA is elevated in LTFA patients. Scatter dot plots (with mean and standard error of the mean) of Z-scores for serum immunoglobulin A (a), IgE (b), IgG (c) and IgM (d) in patients with liver transplant-associated food allergy (LTFA), liver transplant patients without food allergy (LTnoFA), food-allergic patients (FA) and healthy controls (HC). Mann-Whitney *U*-test was used to compare groups mutually with Bonferroni post-test. **P* = 0.003; ***P* = 0.002; ****P* = 0.02; *****P* < 0.001.

Results are shown in Figure S1. For flowcytometric analysis of PBMC, we equalled out a likely age effect on the cell populations tested by matching each LTFA patient (median age 6.92 years; range 2.5–14.9 years) with a peer of the same age (difference < 12 months) in the LTnoFA (7.6 years; 2.3–14.9 years) and HC (6.1 years; 2.5–14.2 years) group. In the FA group, only 21 age-matched individuals could be included (6.1 years; 1.5–13.8 years).

Liver transplant-associated FA and LTnoFA patients were recruited within the departments of paediatric gastroenterology of Cliniques Universitaires Saint-Luc (Brussels, Belgium), Hospital Jeanne de Flandre (Lille, France) and Ghent University Hospital (Ghent, Belgium). HC consisted of children admitted for minor day care surgery (urology, ophthalmology, dental care) under general anaesthesia at Ghent University Hospital without any previous history or symptoms of atopy or FA. FA patients were recruited in the paediatric gastroenterology clinic of Ghent University Hospital.

The diagnosis of FA was confirmed by a paediatric gastroenterologist and based on convincing clinical signs followed by a clear response to elimination (disappearance of signs after withdrawing of the food allergen from the diet) and provocation (re-occurrence of signs when food antigen is reintroduced in diet). Supervised (mostly open) food challenge was performed when clinical history and clinical features

were not unequivocal. Only patients with typical IgE-mediated (signs within minutes to hours of ingestion of foods) or mixed IgE-mediated (signs with delayed/subacute onset in the presence of positive skin prick testing and/or serum-specific IgE) signs were included in the study. Skin prick testing (prick to prick) was performed with fresh products according to standard guidelines [14]. Histamine chloride and sodium chloride (0.9%) were used as positive and negative controls, respectively. Specific IgE was measured by the ImmunoCAP assay (Phadia®, Uppsala, Sweden). Informed consent was obtained from the parents and/or patients. An extensive doctor's questionnaire was taken from the parents to obtain detailed information regarding medical antecedents, family history, diet history and history of atopic signs (in case of LT patients, both prior to and post-transplant). Additional clinical data and the results of the diagnostic tests were collected from the medical records. Exclusion criteria were as follows: recent (< 3 months before visit) history of infection or acute cellular rejection, recent treatment with high-dose steroids, recent immunizations or a previous history of (non-allergic) immune-mediated diseases. In the LTnoFA and HC groups, patients with asthma, allergic rhinitis or atopic dermatitis were also excluded. The study was approved by the ethics committee of Ghent University Hospital, the Cliniques Universitaires Saint-Luc and Hospital Jeanne de Flandre.

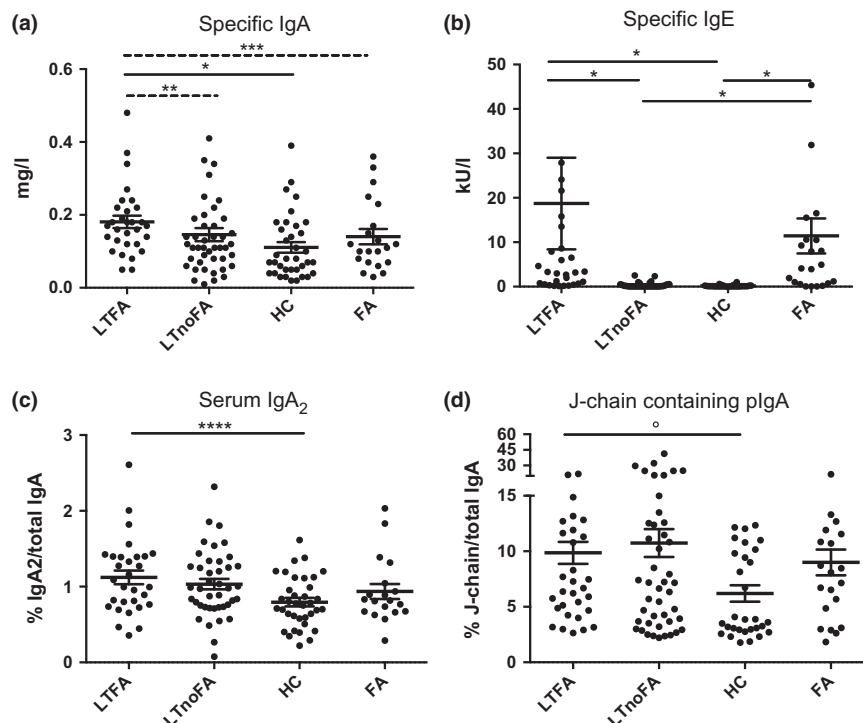


Fig. 2. food allergen-specific IgA and the proportions of IgA₂ and J-chain-containing polymeric IgA on total IgA are elevated in LTFA sera. Scatter dot plots (with mean and standard error of the mean) of specific IgA (a) and IgE (b) serum concentrations against a panel of food allergens (egg white, cow's milk, fish, wheat, peanut and soya bean) and of the percentage of serum IgA₂ (c) and serum joining-chain (J-chain)-containing polymeric IgA (pIgA) (d) of total IgA concentration in patients with liver transplant-associated food allergy (LTFA), liver transplant patients without food allergy (LTnFA), food-allergic patients (FA) and healthy controls (HC). Mann-Whitney *U*-test was used to compare groups mutually with Bonferroni post-test. **P* < 0.001; ***P* = 0.02; ****P* = 0.04; *****P* = 0.002; °*P* = 0.003.

Serum immunoglobulin levels

Total serum IgA, IgE, IgG and IgM concentrations were determined by nephelometry (Behring Nephelometer Analyzer II, Siemens AG, Munich, Germany). Serum-specific IgA and IgE levels against a mix of the most frequently encountered food allergens in LTFA, containing egg white, cow's milk, fish, wheat, peanut and soya bean (fx5 CAP, Phadia), were measured by the UniCAP system (Phadia, Thermo Fisher Scientific, Uppsala, Sweden). IgA₁ and IgA₂ were measured by ELISA in serum (human IgA₁ and IgA₂ ELISA Southern Biotech, Birmingham, AL, USA), all according to the manufacturers' guidelines. Human reference serum (Bethyl, Montgomery, TX, USA) containing known amounts of IgA₁ and IgA₂ was used as standard.

Western blot for 'J-chain'-containing pIgA

Serum samples (dilution, 1 : 50) were heated at 100°C for 5 min, loaded in a SDS-12% PAGE gel and run at 100 V for 15 min, then at 180 V for 45 min. Proteins were transferred onto nitrocellulose membrane (Thermo Fisher, Waltham, MA, USA) at 0.18 A for 2 h at RT.

The membrane was blocked with 5% BSA (Sigma-Aldrich, Saint-Louis, MO, USA) in TBS with 0.1% Tween-20 (Sigma-Aldrich) for 1 h at RT, washed and incubated with anti-human J-chain rabbit Ab (made in house) overnight at 4°C. Membranes were then incubated for 1 h at RT with secondary anti-rabbit immunoglobulin G (IgG)-HRP linked (Cell Signalling, Danvers, MA, USA). Immunoreactive bands were revealed by chemiluminescence (GE Healthcare, Pittsburgh, PA, USA) and detected by Chemidoc XRS apparatus (Bio-Rad, Hercules, CA, USA). Quantification was made by QUANTITY ONE software (Bio-Rad). Results were expressed in arbitrary units, as corrected for a standard of purified human J-chain, and then as a ratio on total IgA levels.

Flow cytometry

Peripheral blood mononuclear cells were freshly isolated by Ficoll-Hypaque density gradient and cryopreserved at -150°C for collective analysis. Thawed PBMC were stained with fixable viability dye 506 (eBioscience, San Diego, CA, USA) and fluorescently labelled monoclonal antibodies using manufacturers'

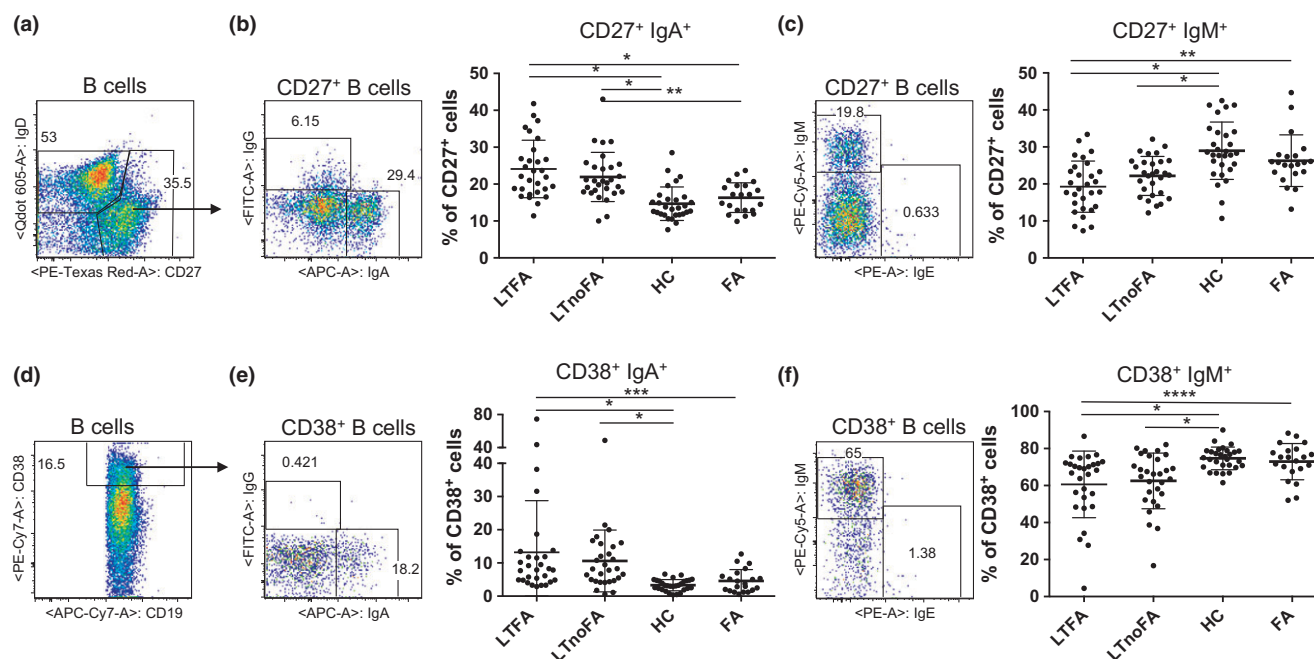


Fig. 3. increased frequency of IgA⁺ B cells and decreased frequency of IgM⁺ B cells in liver transplant patients. PBMC were gated on alive CD19⁺ lymphocytes for B cells. Gating strategy for CD27⁺ memory B cells and CD38⁺ B cells are shown. (a, d). Gating strategies and frequency for IgA⁺ (b, e) and IgM⁺ (c, f) cells in CD27⁺ and CD38⁺ B cell populations in age-matched patients with liver transplant-associated food allergy (LTFA), liver transplant patients without food allergy (LTnoFA), food-allergic patients (FA) and healthy controls (HC). Wilcoxon-matched-pairs signed rank test was used to compare groups mutually with Bonferroni post-test. * $P < 0.001$; ** $P = 0.004$; *** $P = 0.002$; **** $P = 0.005$.

instructions. Following monoclonal antibodies were used: CD138-FITC, CD3 PerCP-Cy5.5, CD123-PE, CD56-PE-Cy5, CD8-PE-Cy7, BDCA1-Alexa Fluor 647, HLA-DR-Alexa Fluor 700, CD19-APC-Cy7, CD11c-Pac Blue, CD20 –PerCP-Cy5.5, IgE-PE, CD278-FITC (ICOS), CD45RO PE-Cy5, CCR6-PECy7, CD25-APC-Cy7, CD4-Pac Blue (all from Biolegend, San Diego, CA, USA); CD38-PE-Cy7, CD274-PE (PD-L1), Foxp3-APC (all from eBioscience); IgG-FITC, IgM-PE-Cy5, IgD-Brilliant Violet 605, CXCR3-Brilliant Violet 605, CD27-PE-TxRed, CXCR5-Biotin, Streptavidin-PE CF594 (all from BD, Becton, Dickinson and company, Franklin Lakes, NJ, USA); CD14-PE-TxRed (Caltag, Buckingham, UK); IgA-APC (Miltenyi Biotec Inc., San Diego, CA, USA). The cells were acquired on a BD LSR Fortessa and the data analysed with FLOWJO (LLC, Ashland, OR, USA) software.

Statistics

Data were analysed in Prism 6 for Mac OS X (Graphpad Software, La Jolla, CA, USA). Z-scores were calculated for serum Ig values to correct for age-dependent reference ranges. Kruskal–Wallis test was used to compare groups ($P < 0.05$ statistically significant) followed by Mann–Whitney *U*-test to compare groups mutually and Bonferroni post-test for multiple

comparisons ($P < 0.008$ statistically significant). To compare the flow cytometric data between the age-matched subjects, Wilcoxon-matched-pairs signed rank test was used with Bonferroni post-test ($P < 0.008$ statistically significant). For correlation analysis, Spearman's test was used ($P < 0.05$ statistically significant).

Results

Serum IgA, food allergen-specific IgA and IgE and serum markers for mucosal IgA are increased in LTFA

Serum IgA levels were specifically increased in those patients diagnosed with *de novo* food allergy (LTFA) compared to those without food allergy (LTnoFA) ($P = 0.003$) and healthy controls (HC) ($P = 0.002$), whilst serum IgA did not differ significantly between LTnoFA, HC and food-allergic children (FA). Serum IgE was, as expected, higher in LTFA and FA patients ($P < 0.001$). No difference was found in distribution of serum IgG and IgM across groups (Fig. 1). Furthermore, serum food allergen-specific IgA was raised in LTFA compared to HC ($P < 0.001$), LTnoFA ($P = 0.02$) and FA ($P = 0.04$). Understandably, LTFA and FA patients had significantly higher specific IgE levels than LTnoFA and HC (all $P < 0.001$) (Fig. 2).

As IgA was increased in LTFA, we looked at serum markers of mucosal IgA. We found that the ratio of serum IgA₂ and J-chain-containing pIgA on total IgA were higher in LTFA compared to HC ($P = 0.002$; $P = 0.003$) (Fig. 2).

IgA⁺ memory B cells and IgA⁺ plasmablasts are increased at the expense of their IgM⁺ counterparts in liver transplant patients

To check whether elevated serum IgA levels resulted from an increase in IgA-producing B cells, we collected and stained PBMC to evaluate the surface Ig expression of CD19⁺ B cells. We looked at the surface expression of IgA, IgE, IgG and IgM on CD27⁺ memory B cells and CD38⁺ plasmablasts. The percentage of IgA⁺ memory B cells was significantly higher in the total group of liver transplant patients (LT) compared to HC (both $P < 0.001$) and FA ($P < 0.001$; $P = 0.004$). We found a concomitant decrease in percentage of IgM⁺ memory B cells in LT compared to

HC ($P < 0.001$) and in LTFA patients compared to FA ($P = 0.004$), suggesting an increase in IgM to IgA class switching. The percentage of IgA⁺ plasmablasts was also higher in LT than HC ($P < 0.001$) and in LTFA than FA patients ($P = 0.002$) and was equally reflected by a decrease in IgM⁺ plasmablasts in LT compared to HC ($P < 0.001$) and LTFA compared to FA ($P = 0.005$) (Fig. 3). No difference was seen in IgG⁺ and IgE⁺ memory B cells and plasmablasts between the groups.

CD45RO⁺ CD4⁺ memory T cells are increased in liver transplant patients

Because B cell differentiation and class switching are processes heavily influenced by helper T cells, we also studied T helper cell populations in the peripheral blood of age-matched patients and healthy controls. No difference was seen in percentage of CD4⁺ T cells within alive T lymphocytes between the groups. CD45RO⁺ CD4⁺ memory cells,

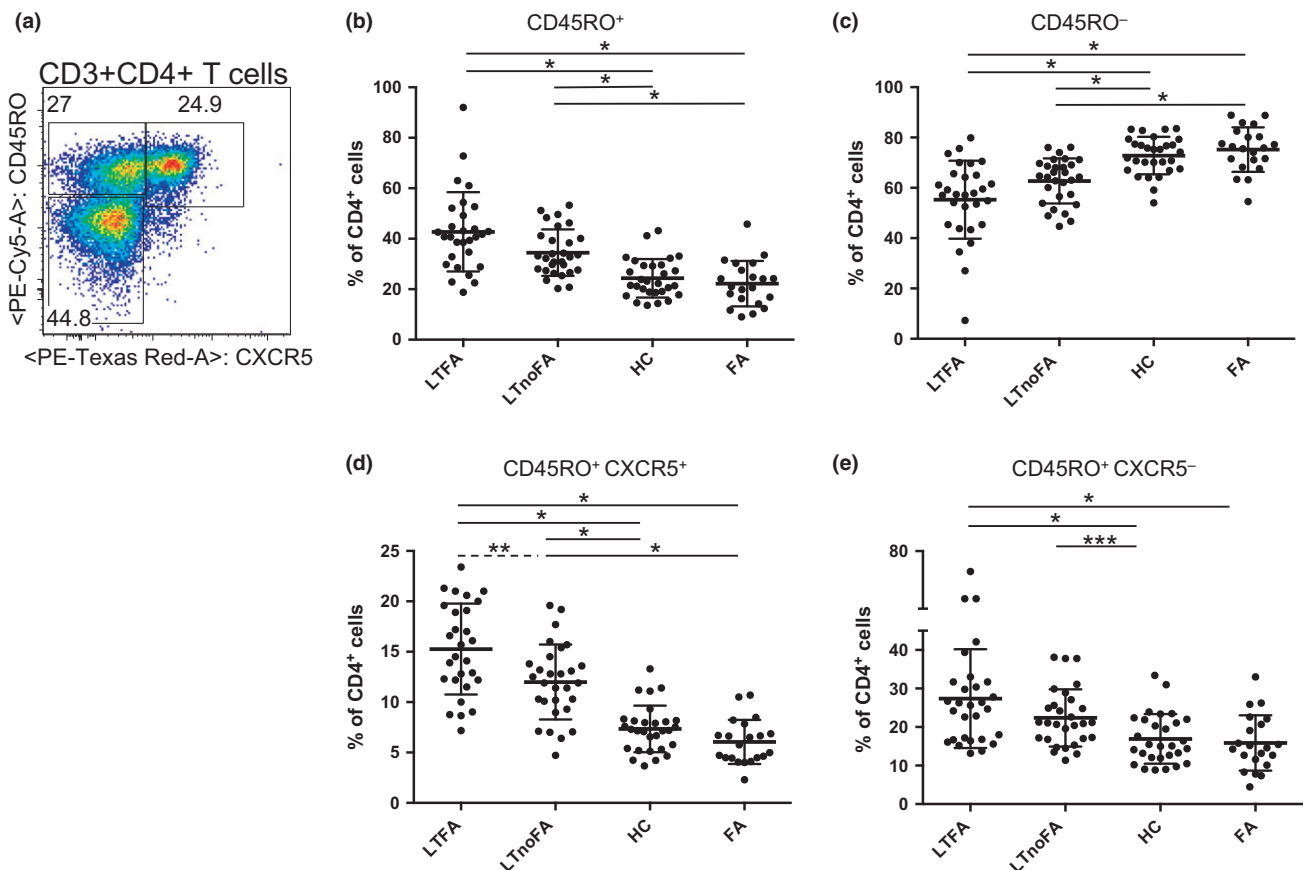


Fig. 4. increased frequencies of memory CD4⁺ T cells and T follicular helper cells in LTFA. PBMC were gated on alive CD3⁺CD4⁺ T cells for T helper cells. Gating strategy (a) and frequencies of CD45RO⁺ (b), CD45RO⁻ (c), CD45RO⁺ CXCR5⁺ (d) and CD45RO⁺ CXCR5⁻ (e) cells of total CD4⁺ T cells in age-matched patients with liver transplant-associated food allergy (LTFA), liver transplant patients without food allergy (LTnoFA), food-allergic patients (FA) and healthy controls (HC). Wilcoxon-matched-pairs signed rank test was used to compare groups mutually with Bonferroni post-test. * $P < 0.001$; ** $P = 0.03$; *** $P = 0.006$.

however, were significantly higher in the total LT group than in HC and FA ($P < 0.001$). Reciprocally, the percentage of naive $CD45RO^- CD4^+$ cells was decreased in LT patients compared to HC and FA ($P < 0.001$) (Fig. 4).

LTFA patients have the highest proportion of $CD45RO^+$ CXCR5⁺ cells within the $CD4^+$ T cell compartment

Within the $CD45RO^+$ T cell compartment, we looked at CXCR5⁺ circulating Tfh (cTfh) and CXCR5⁻ non-follicular T helper cells and their Th1 (CXCR3⁺ CCR6⁻), Th2 (CXCR3⁻ CCR6⁻) and Th17 (CXCR3⁻ CCR6⁺) subsets. We found an increase in cTfh in all LT patients compared to HC and FA ($P < 0.001$). Within the LT group, cTfh were higher in LTFA than in LTnoFA patients ($P = 0.03$). Also more non-follicular memory T helper cells were found in LT patients compared to HC ($P < 0.001$; $P = 0.006$) but these cells were not different between LTFA and LTnoFA (Fig. 4). Furthermore, within cTfh, all LT had a decrease in Th1 ($P < 0.001$) in favour of Th2 cells ($P = 0.007$) compared to HC, which was not observed in the non-follicular T helper cells. Th17 cells, on the contrary, were decreased in the non-follicular memory T cells in LTFA compared to HC ($P = 0.003$) but not in cTfh (Fig. 5).

Natural and induced regulatory $CD4^+$ T cells are decreased in liver transplant patients

Treg play an important role in the induction and maintenance of tolerance for food allergens. We therefore looked at $CD25^+ foxp3^+$ Treg within the naive $CD45RO^-$ and memory $CD45RO^+ CXCR5^+$ and $CXCR5^-$ T cells. Natural ($CD45RO^-$) and induced $CD45RO^+ CXCR5^-$ Treg and T follicular regulatory cells (Tfr) were decreased in all LT patients compared to HC. All P -values are shown in Fig. 6.

Serum IgA levels correlate positively with cTfh frequency

We analysed the correlation between IgA Z-scores and the percentage of Tfh cells in all subjects. A significant positive correlation was found between both variables ($r = 0.4027$; $P < 0.0001$) (Fig. 7). No correlation was found between percentage of Tfh cells and IgE, IgM and IgG Z-scores. Furthermore, no correlation was found between IgA Z-scores and IgE Z-scores, serum food allergen-specific IgA and percentage of IgA₂. The serum IgA Z-scores correlated positively with the frequencies of $CD27^+$ and $CD38^+ IgA^+$ B cells ($r = 0.3$, $P = 0.002$; $r = 0.2$, $P = 0.03$, respectively).

Of note, we also compared $CD8^+$ T cells, $CD14^+$ monocytes, $CD138^+$ plasma cells, $CD3^- CD14^- CD56^+$

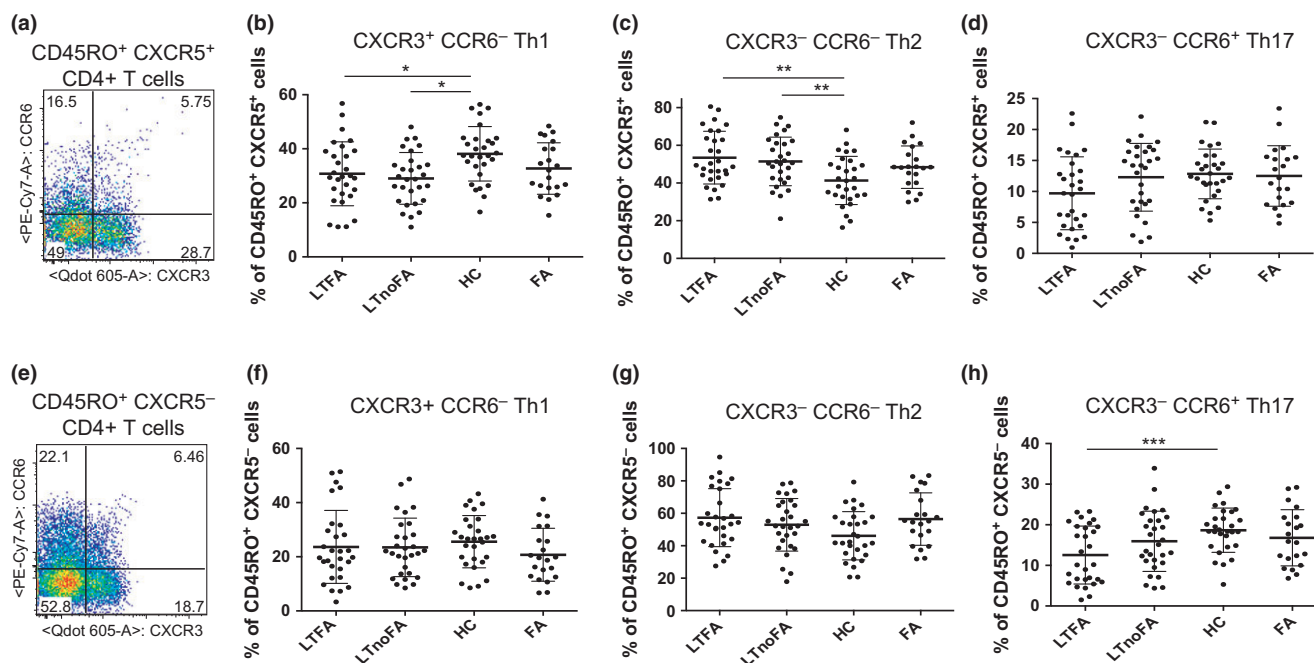


Fig. 5. shift in $CD4^+$ T helper cell subsets in liver transplant patients. PBMC were gated on alive $CD3^+ CD4^+ CD45RO^+$ T cells as shown in Fig. 4. Gating strategies (a, e) and frequencies for CXCR3⁺ CCR6⁻ Th1 (b, f), CXCR3⁻ CCR6⁻ Th2 (c, g) and CXCR3⁻ CCR6⁺ Th17 (d, h) subsets within $CD45RO^+$ cTfh CXCR5⁺ and CXCR5⁻ T cells in age-matched patients with liver transplant-associated food allergy (LTFA), liver transplant patients without food allergy (LTnoFA), food-allergic patients (FA) and healthy controls (HC). Wilcoxon-matched-pairs signed rank test was used to compare groups mutually with Bonferroni post-test. * $P < 0.001$; ** $P = 0.007$; *** $P = 0.003$.

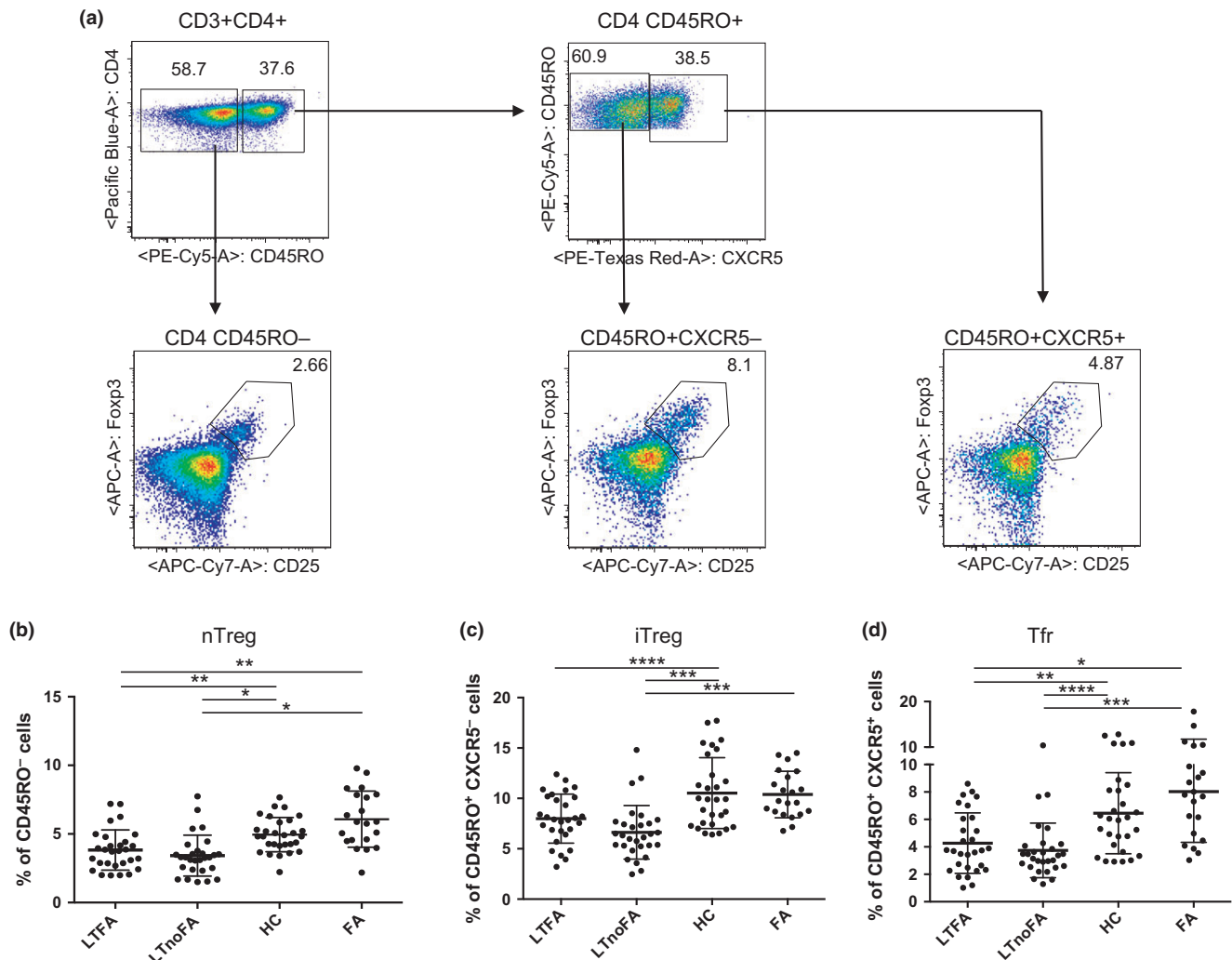


Fig. 6. decreased regulatory T cells in liver transplant patients. PBMC were gated on alive CD3⁺CD4⁺ T cells. Flow plots demonstrating gating strategy for CD45RO en CXCR5 (a). Frequency of natural Treg (nTreg) (b), induced CXCR5⁻ Treg (iTreg) (c) and T follicular regulatory cells (Tfr) (d) in age-matched patients with liver transplant-associated food allergy (LTFA), liver transplant patients without food allergy (LTnoFA), food-allergic patients (FA) and healthy controls (HC). Wilcoxon-matched-pairs signed rank test was used to compare groups mutually with Bonferroni post-test. * $P = 0.001$; ** $P = 0.003$; *** $P < 0.001$; **** $P = 0.002$.

NK cells, CD3⁻ CD14⁻ CD19⁻ CD56⁻ HLA DR⁺ CD11c⁺ myeloid and CD123⁺ plasmacytoid dendritic cells. In these subsets, we did not find any differences between the four groups.

Discussion

We investigated humoral and cellular immunity in paediatric LT patients with and without FA compared to HC and FA patients in search for factors that could be associated with LTFA.

When comparing LTFA with LTnoFA patients, serum IgA and food allergen-specific IgA levels as well as serum IgE and food allergen-specific IgE levels are higher in the LTFA group with LTFA patients

having a higher proportion of cTfh than LTnoFA patients. Unique in LTFA is also the significant increase in serum markers of mucosal IgA and the decrease in the Th17 subset of CXCR5⁻ CD4⁺ cells compared to HC. Both LTFA and LTnoFA patients exhibited a rise in IgA⁺ memory B cells and plasmablasts, CXCR5⁺ circulating Tfh [15] and CXCR5⁻ non-follicular CD4⁺ cells and a decrease in the Treg compartment compared to HC and FA. The decrease in Treg cells likely puts LT patients more at risk to develop FA. Furthermore, the percentage of cTfh, which is highest in LTFA patients, correlates positively with serum IgA.

The rise in serum IgA in LTFA seems to be in conflict with the protective role of IgA in FA [9] and in allergy

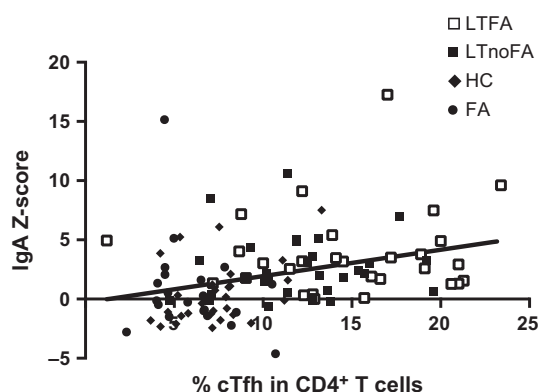


Fig. 7. serum IgA levels correlate with frequency of cTfh. IgA Z-score vs. the percentage of circulating T follicular helper cells (cTfh) of total CD4⁺ T cells in age-matched patients with liver transplant-associated food allergy (LTFA), liver transplant patients without food allergy (LTnoFA), food-allergic patients (FA) and healthy controls (HC). Spearman's test was used for correlation analysis ($r = 0.4027$; $P < 0.0001$).

in general [16, 17] as selective IgA deficiency is associated with an increased prevalence of atopy and FA [18].

An increase in serum IgA could result from defective intestinal IgA secretion with back leaking of IgA into the circulation or from increased IgA production. The latter may ensue from induction of IgA class switching or stimulation of the longevity and/or Ig production in plasma cells.

The increased proportions of serum IgA₂ and 'J-chain'-containing pIgA, observed in LTFA, could fit with defective secretory IgA defence. Frossard et al. showed in a mouse model of FA that serum IgA was strongly increased in anaphylactic mice. Sensitized mice exhibited increased β -lactoglobulin-specific IgA levels in serum but not in faeces, in contrast to tolerant mice that had increased levels in faeces but not in serum [19]. A disturbed integrity of the gut membrane or impaired transepithelial transport of IgA can lead to a deficiency in secretory IgA and a subsequent rise in serum IgA. In analogy with our findings, pIgR-deficient knock-out mice present a remarkable increase in preferentially dimeric serum IgA [20]. These mice have a strong predisposition for anaphylaxis, lack secretory IgA and exhibit aberrant mucosal leakiness with increased uptake of food proteins and commensal bacteria [21]. Treatment with tacrolimus, known to be associated with LTFA [1, 5, 22], increases intestinal permeability [23, 24] and might hence affect secretory immunity.

We found an increase in IgA⁺, with a concomitant decrease in IgM⁺, memory B cells and plasmablasts in LT patients (without any difference in surface IgG⁺ and IgE⁺), which might be suggestive of IgM to IgA class switching. Furthermore, the proportion of blood cTfh is

increased in LT and is highest in LTFA patients. These cells share functional properties with Tfh from secondary lymphoid organs as they can induce naive and memory B cells to become Ig-producing cells [15]. cTfh can be further subdivided in three subsets: cTfh1 (CXCR3⁺ CCR6⁻), cTfh2 (CXCR3⁻ CCR6⁻) and cTfh17 (CXCR3⁻ CCR6⁺) cells [15]. LT patients were found to have a shift towards the cTfh2 subset with a decrease in the cTfh1 subset, leaving the percentage of cTfh17 cells unaltered. Within the CXCR5⁺ compartment CXCR5⁺ foxp3⁺ CD25⁺ T follicular regulatory cells (Tfr) tune the GC responses by limiting the number and the quality of Tfh cells and selection of the GC B cells, thereby preventing unwanted production of autoantibodies and autoimmune destruction [25–27]. We found a decrease in circulating Tfr in LT patients, which can contribute to an increase in cTfh and IgA-producing GC B cells. We indeed found a positive correlation between serum IgA and the percentage of cTfh and not for the other serum Ig. This observation is indicative for a T cell dependent, rather than innate, IgA induction, taking place in GC.

Besides the increase in cTfh, also CXCR5⁻ CD4⁺ cells were increased in LT patients. Within this compartment, Th1 and Th2 cells were not different from HC but Th17 cells were decreased in LTFA compared to HC. Th17 cells have been implicated in a number of autoimmune diseases but their role in IgE-mediated allergic disorders remains unclear. One study is available investigating Th17 responses in human FA providing the first evidence of a systemic impairment in Th17 responses in FA children [28]. A reduced proportion of IL-17 producing CD4⁺ T cells and an impaired IL-17 response in CD4⁺ T cells in PBMC stimulated *in vitro* with both peanut and control antigens was seen in peanut-allergic children. The impairment in Th17 response was unique to FA and not seen in atopic patients without FA.

Treg play a primary role in controlling adaptive immune responses and maintaining tolerance to self-antigens and harmless non-self-antigens, including food-borne antigens [29]. Several studies suggest deficiencies in allergen-specific Treg in allergic diseases [30–34]. Both natural and induced CD4⁺ CD25⁺ foxp3⁺ Treg were found to be decreased in all LT compared to HC.

The changes observed in LTFA are not encountered in FA patients, which is indicative that LTFA patients represent a unique population.

The strength of this study lies in the investigation of a considerably large, well-defined group of paediatric LTFA patients. In the LTnoFA and HC groups, we excluded patients with asthma, allergic rhinitis or atopic dermatitis. In the LTFA and FA groups, asthma and allergic rhinitis did not occur, however, respectively, 9 and 6 children also suffered from atopic dermatitis. Therefore,

the observed differences might not be ascribed to FA only, the presence of atopic dermatitis in the same patients might also play a role. Because of the retrospective nature of this study, any observed difference might be either cause or consequence of LTFA. However, the results presented here are a solid basis for future prospective studies on the pathogenesis and risk factors for LTFA. As LTFA significantly affects the daily life of a substantial proportion of paediatric LT patients, there is a need to review our current immunosuppressive regimens and consider more 'Treg friendly' drugs such as rapamycin, an mTOR inhibitor, which has been shown to facilitate the development and survival of Treg [35, 36].

In conclusion, LT has an impact on humoral immunity. Serum IgA is significantly increased in LTFA. The exact mechanism and the precise link with LTFA need to be further elucidated. The increase in serum markers of mucosal IgA, food allergen-specific IgA and circulating Tfh cells point towards a disturbance in intestinal immune homeostasis.

Acknowledgements and funding

P Gevaert, M Dullaers, R De Bruyne received a grant as resp. senior clinical investigator, postdoctoral fellow and clinical PhD fellow by the Flemish Scientific Research Board (FWO Vlaanderen). The research was supported by a personal grant of FWO to M Dullaers (FWO14/KAN/019). Catherine Lombard and Julia Versavau (Cliniques Universitaires Saint-Luc, Brussels, Belgium) for their assistance in inclusion and data collection. Prof Dr Karim Vermaelen for use of the laboratory infrastructure. Veronique De Backer for her laboratory assistance. Dr Marc Coppens, head of the Surgical Day Care Unit (Department of Anesthesia, Ghent University Hospital, Ghent, Belgium) and his team for the contribution in the recruitment of healthy controls.

Conflict of interest

The authors declare no conflict of interest.

References

- Boyle RJ, Hardikar W, Tang MLK. The development of food allergy after liver transplantation. *Liver Transpl* 2005; 11:326–30.
- Levy Y, Davidovits M, Cleper R, Shapiro R. New-onset post-transplantation food allergy in children—is it attributable only to the immunosuppressive protocol? *Pediatr Transplant* 2009; 13:63–9.
- Noble C, Peake J, Lewindon PJ. Increase in de novo allergies after paediatric liver transplantation: the Brisbane experience. *Pediatr Transplant* 2011; 15:451–4.
- Ozbek OY, Ozcay F, Avci Z, Haberal A, Haberal M. Food allergy after liver transplantation in children: a prospective study. *Pediatr Allergy Immunol* 2009; 20:741–7.
- De Bruyne R, Dullaers M, Van Biervliet S *et al.* Post-transplant food allergy in children is associated with liver and not with renal transplantation: a monocentric comparative study. *Eur J Pediatr* 2013; 172:1069–75.
- Smits HH, Gloudemans AK, Nimwegen MV *et al.* Cholera toxin B suppresses allergic inflammation through induction of secretory IgA. *Mucosal Immunol* 2009; 2:331–9.
- Dumont FJ. FK506, an immunosuppressant targeting calcineurin function. *Curr Med Chem* 2000; 7:731–48.
- Macian F. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 2005; 5:472–84.
- Berlin MC. Mucosal antibodies in the regulation of tolerance and allergy to foods. *Semin Immunopathol* 2012; 34:633–42.
- Brandtzaeg P. The gut as communicator between environment and host: immunological consequences. *Eur J Pharmacol* 2011; 668(Suppl. 1):S16–32.
- Johansen F-E, Braathen R, Brandtzaeg P. The J chain is essential for polymeric Ig receptor-mediated epithelial transport of IgA. *J Immunol* 2001; 167:5185–92.
- Winstead CJ. Follicular helper T cell-mediated mucosal barrier maintenance. *Immunol Lett* 2014; 162:39–47.
- Kawamoto S, Tran TH, Maruya M *et al.* The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. *Science* 2012; 336:485–9.
- Verstege A, Mehl A, Rolinck-Werninghaus C *et al.* The predictive value of the skin prick test weal size for the outcome of oral food challenges. *Clin Exp Allergy* 2005; 35:1220–6.
- Morita R, Schmitt N, Bentebibel S-E *et al.* Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity* 2011; 34:108–21.
- Pilette C, Durham SR, Vaerman J-P, Sibille Y. Mucosal immunity in asthma and chronic obstructive pulmonary disease: a role for immunoglobulin A? *Proc Am Thorac Soc* 2004; 1:125–35.
- Pilette C, Nouri-Aria KT, Jacobson MR *et al.* Grass pollen immunotherapy induces an allergen-specific IgA2 antibody response associated with mucosal TGF- β expression. *J Immunol* 2007; 178:4658–66.
- Janzi M, Kull I, Sjöberg R *et al.* Selective IgA deficiency in early life: association to infections and allergic diseases during childhood. *Clin Immunol* 2009; 133:78–85.
- Frossard CP, Hauser C, Eigenmann PA. Antigen-specific secretory IgA antibodies in the gut are decreased in a mouse model of food allergy. *J Allergy Clin Immunol* 2004; 114:377–82.
- Shimada S, Kawaguchi-Miyashita M, Kushiro A *et al.* Generation of polymeric immunoglobulin receptor-deficient mouse with marked reduction of secretory IgA. *J Immunol* 1999; 163:5367–73.
- Johansen F-E, Pekna M, Norderhaug IN *et al.* Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory compo-

- nent-deficient mice. *J Exp Med* 1999; **190**:915–22.
- 22 Lykavieris P, Frauger E, Habes D, Bernard O, Debray D. Angioedema in pediatric liver transplant recipients under tacrolimus immunosuppression. *Transplantation* 2003; **75**:152.
 - 23 Gabe SM, Bjarnason I, Tolou-Ghamari Z *et al*. The effect of tacrolimus (FK506) on intestinal barrier function and cellular energy production in humans. *Gastroenterology* 1998; **115**:67–74.
 - 24 Favre L, Spertini F, Corthésy B. Secretory IgA possesses intrinsic modulatory properties stimulating mucosal and systemic immune responses. *J Immunol* 2005; **175**:2793–800.
 - 25 Kato LM, Kawamoto S, Maruya M, Fagarasan S. Gut TFH and IgA: key players for regulation of bacterial communities and immune homeostasis. *Immunol Cell Biol*. Nature Publishing Group; 2014; **92**:49–56.
 - 26 Linterman MA, Pierson W, Lee SK *et al*. Foxp3⁺ follicular regulatory T cells control the germinal center response. *Nat Med* 2011; **17**:975–82.
 - 27 Chung Y, Tanaka S, Chu F *et al*. Follicular regulatory T cells expressing Foxp3 and Bcl-6 suppress germinal center reactions. *Nat Med* 2011; **17**:983–8.
 - 28 Dhuban KB, d'Hennezel E, Ben-Shoshan M *et al*. Altered T helper 17 responses in children with food allergy. *Int Arch Allergy Immunol*. Karger Publishers; 2013; **162**:318–22.
 - 29 Sakaguchi S. Naturally arising Foxp3-expressing CD25⁺ CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005; **6**:345–52.
 - 30 Akdis M, Verhagen J, Taylor A *et al*. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med*. Rockefeller Univ Press; 2004; **7**:199.
 - 31 Bellinghausen I, König B, Böttcher I, Knop J, Saloga J. Regulatory activity of human CD4⁺ CD25⁺ T cells depends on allergen concentration, type of allergen and atopy status of the donor. *Immunology*. Blackwell Science Ltd; 2005; **116**:103–11.
 - 32 Ling EM, Smith T, Nguyen XD *et al*. Relation of CD4⁺ CD25⁺ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet* 2004; **363**:608–15.
 - 33 Smith M, Tourigny MR, Noakes P, Thornton CA, Tulic MK, Prescott SL. Children with egg allergy have evidence of reduced neonatal CD4⁺CD25⁺(+)CD127(lo/–) regulatory T cell function. *J Allergy Clin Immunol* 2008; **121**:1460–6–1466.e1–7.
 - 34 Schaub B, Liu J, Höppler S *et al*. Impairment of T-regulatory cells in cord blood of atopic mothers. *J Allergy Clin Immunol* 2008; **121**:1491–1499.e13.
 - 35 Askar M. T helper subsets & regulatory T cells: rethinking the paradigm in the clinical context of solid organ transplantation. *Int J Immunogenet* 2014; **41**:185–94.
 - 36 Battaglia M, Stabilini A, Miglia vacca B, Horejs-Hoeck J, Kaupper T, Roncarolo M-G. Rapamycin promotes expansion of functional CD4⁺ CD25⁺ FOXP3⁺ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* 2006; **177**:8338–47.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Serum Ig in paediatric renal transplant and chronic liver disease patients in comparison with the other study groups.

Table S1. Responsible food antigens, clinical features and diagnostic parameters in food allergic liver transplant patients.

Table S2. Current immunosuppressive treatment at the time of blood test.