

Cytokine profile of food-allergic post-liver transplant children is identified by high levels of IL-5 and low IL-10 secretion from patients' peripheral blood mononuclear cells

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Abstract: Severe allergic reaction to food following liver transplantation is a well-known phenomenon. However, the mechanisms underlying this phenomenon are not yet elucidated. This study aimed to reveal the nature of the immune response in post-transplanted allergic patients and compare them to non-allergic transplanted as well as allergic and non-allergic control subjects, with focus on cytokine milieu. Post-liver transplant patients with and without allergic reactions as well as food-allergic but otherwise healthy and healthy non-allergic control patients were recruited. We reviewed patient records and routine laboratory tests and assayed subjects' PBMCs, studying cytokine secretion profile in response to different stimuli. Post-transplant patients with food allergy showed a unique cytokine profile in response to various stimuli, with extremely elevated IL-5, low IL-10 secretion, and somewhat higher IFN- γ . T regulatory cell number was not significantly different among the groups of patients and controls. Immune response of food-allergic post-liver transplant patients is identified by a unique cytokine profile when compared to allergic but otherwise healthy individuals.

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Acquired food allergy after liver transplantation is a well-described clinical phenomenon. These allergic reactions may be life-threatening, and the majority of reported cases are in young children (1). The mechanism leading to the development of these reactions has been investigated during the last few years and is yet to be elucidated.

It is proposed that among its many functions, the liver is responsible for the elimination of dietary antigens from portal blood, thereby preventing sensitization. In support of this idea, several studies in murine models have suggested that the presentation of an antigen in the hepatic microenvironment leads to tolerance (2, 3).

As for human studies, most were concentrated on descriptions of the clinical and routine laboratory features of patients with organ transplant and food allergy. These studies showed higher prevalence of clinically significant food allergy in liver transplant vs. kidney and other organ transplant (4). Also, sensitization, manifested by blood eosinophilia and specific IgE, was documented in up to 50% of patients in a study by Granot et al. (5), with more than 10% of children experiencing clinically evident allergic reactions. An important finding was the increased prevalence of allergic reactions and sensitization in tacrolimus compared to cyclosporine-treated patients. This, in turn, raised the possibility of an allergy-promoting effect by tacrolimus (6) supported by the agents' effect on T regulatory cells (7). Nevertheless, a recent study of kidney-transplanted adults receiving tacrolimus or cyclospor-

Abbreviations: LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin.

ine revealed a significant higher sensitization rate in tacrolimus-treated patients, but although there was a higher prevalence of clinically evident allergic reactions in the tacrolimus group, the difference was not statistically significant from that of cyclosporine-treated group (8).

Boyle et al. (9) described in 2005 a case of a split liver graft: the child recipient developed food allergy while on tacrolimus, whereas the adult did not. This suggests that host-specific factors play an important role in the development of food allergy after liver transplantation. From these clinical studies and the murine models, there are several suggested mechanisms for this phenomenon: Th1 vs. Th2 imbalance caused by immunosuppression (6), calcineurin suppression of T regulatory cells (10), and increase in gut permeability by tacrolimus leading to desensitization (11). And finally, there could be an interference with normal function of the liver itself contributing to the evolution of sensitization and allergic reactions as suggested by murine models and clinical studies (2, 3, 12).

In this study, we show that post-transplant food allergy phenomenon has a unique cytokine milieu differentiating it from food-allergic reactions in otherwise healthy children.

Patients and methods

We studied the records of 15 liver-transplanted patients followed in the liver transplant clinic. Eight patients had documented history of severe allergic reactions to food, most of them to multiple food allergens. Five of the allergic patients and four clinically non-allergic transplanted children agreed to actively participate in our study. We recruited as control groups four matched food-allergic but otherwise healthy and five healthy non-allergic children. Healthy non-allergic children were those undergoing elective surgeries or other minor procedures and were recruited with parental or legal guardian consent. Information on the history of atopic diseases was collected by a detailed questionnaire. Accordingly, we enrolled only healthy children with no evidence of any atopic disease. In the group of allergic but otherwise healthy children, allergies to milk, egg, and sesame were found. One patient was allergic to both milk and egg. In transplanted patients, underlying disease leading to transplantation, immunosuppressive regimen, and tacrolimus trough levels, during the six-month period prior to our laboratory assay and all other routine laboratory tests, were collected from medical records. Serum specific antibodies to a wide panel of food allergens were measured by Immulite 2000 (Siemens, Erlangen, Germany), and skin prick test with standardized allergen extracts (ALK-Abello, Hørsholm, Denmark) was performed during allergy clinic visits. However, because sensitization as reflected in specific antibodies in the serum and positive skin tests is quite prevalent in post-transplanted children (5), and to much lesser degree also in healthy population, we included in the group of allergic post-transplant patients only those with documented clinical reactions. Institutional Helsinki Committee approval was granted.

Cell purification and culture

Freshly isolated PBMCs were isolated by Ficoll (Ficoll-Paque; GE Healthcare Bioscience AB, Uppsala, Sweden) density gradient centrifugation from whole blood samples in sodium heparin. Cells were cultured in RPMI supplemented with 10% FCS at 37 °C and 5% CO₂.

Cell activation

PBMCs at a concentration of 2×10^6 cells/mL were activated by a non-specific T-cell stimulus, PHA, and stimuli with LPS for 24 h and allergen-specific stimuli such as casein and ovalbumin for 72 h.

Cytokine concentration in cell medium

Cytokine concentration in growth media was measured by ELISAs. These included IFN- γ , IL-2, IL-4, IL-5, and IL-10 (eBioscience, San Diego, CA, USA). ELISAs were carried out according to the manufacturer's instructions, and all samples were tested in duplicate and fitted to assay standard curve.

T regulatory lymphocyte immunophenotyping

CD4 and CD4+CD25+ FOXP3+ T regulatory cell numbers were measured by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA) with antibodies against CD4, CD25, FOXP3, and the respective isotype control (eBioscience).

Results

The records of our liver transplant patients were reviewed, patients older than 12 yr were excluded, and we clinically assessed 15 patients after liver transplant followed in our clinic, seven without evidence for allergic reaction or sensitization, and eight allergic or sensitized (six and two, respectively). Of these, five clinically allergic and four non-allergic patients agreed to participate in our study by giving a blood sample for the biological assays. Patients' clinical reactions were described in medical records ranging from urticarial rash, edema, to anaphylaxis manifesting with facial and/or upper airway edema, and wheezing requiring treatment with automatic adrenaline injector, EpiPen. These reactions were recorded in all patients at some point in time. Among all transplanted patients, none had a history of food allergy prior to transplant, and one patient had family history of asthma. Milk, sesame, egg, peanuts, and nuts were common types of allergenic foods. Four of the clinically allergic transplanted patients had multiple food allergy; one patient had allergic reactions to milk, sesame, nuts, egg, and almonds, one had clinical reactions to almonds and tomatoes, one reacted to milk, egg, sesame, peanuts, and fish, and one reacted to peanuts, nuts, and soy. Children with food allergy but otherwise healthy and

healthy control matched subjects were recruited from our pediatric ambulatory care unit. Patients' mean age was six and a half yr, ranging from 2 to 11.5 yr, and spanning a range of 1–10 yr from transplant. Healthy control children's (food-allergic and non-allergic) mean age was seven and a half yr, ranging from 4 to 12 yr.

Patients transplanted early in their life exhibited higher incidence of severe food-allergic reactions

In our cohort of transplant patients, the leading diagnosis prior to transplant was biliary atresia, in both allergic (five of eight) and non-allergic patients (four of seven), and other causes were autoimmune hepatitis, neonatal hepatitis, hepatoblastoma, familial intrahepatic cholestasis, and idiopathic acute liver failure (Table 1). Comparison of patients' age at transplant revealed that the median age for transplant in patients with severe allergic reactions was 0.5 yr, all patients were under one and a half yr at the time of transplant, and as in the non-allergic group, their median age was two yr with all children older than one yr at transplant (Table 1). Comparison of patients' means revealed a statistically significant difference between the two groups (Fig. 1).

Tacrolimus levels do not correlate with current allergic status or patient's cytokine profile

In an attempt to evaluate the impact of current immunosuppression therapy with tacrolimus on

Table 1. Characteristics of liver transplant patients: age at transplant, current age, and etiology leading to transplant in non-allergic and allergic patients

	Patient	Age	Age at transplant in years	Primary disease
Non-allergic	1	11.5	8	Biliary atresia
	2	6.5	2	Autoimmune hepatitis
	3	4.5	4	Hepatoblastoma
	4	11.5	7	HCC, FIHC
	5	11	1	Biliary atresia
	6	11	1	Biliary atresia
	7	7	1	Biliary atresia
Allergic		Median	2	
	1	9	0.5	Liver failure UK
	2	9.5	0.5	Biliary atresia
	3	7.5	0.5	Liver failure UK
	4	6	1	Biliary atresia
	5	2	0.5	Biliary atresia
	6	3	0.5	Neonatal hepatitis
	7	3.5	1.5	Biliary atresia
	8	4	1.5	Biliary atresia
		Median	0.5	

FIHC, familial intrahepatic cholestasis; HCC, hepatocellular carcinoma; UK, unknown.

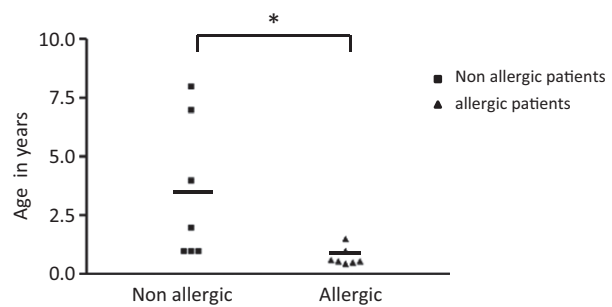


Fig. 1. Young age at transplant correlates with higher prevalence of allergic reactions. Comparison of mean age of non-allergic and allergic patients revealed a statistically significant difference, * $p = 0.0362$.

our laboratory assay and to the current allergic status of our patients, we collected data of consecutive drug trough levels obtained during clinic visits over the six-month period before conducting the assays. Mean level for non-allergic patients was 3.77 ± 0.9 and for allergic patients 4.82 ± 1.05 with a p-value of 0.47. There was no significant difference in tacrolimus levels between the allergic and non-allergic group of patients during the six months preceding performance of assays (Fig. 2).

Liver transplant allergic and non-allergic patients display a unique cytokine profile

We measured the concentration of a panel of cytokines secreted from PBMCs in response to various stimuli in liver transplant patients with food allergy, in post-transplant patients without food allergy, and in allergic but otherwise healthy children as well as healthy non-allergic individuals. We measured the levels of IL-2, IL-4, IL-5, IL-10, and IFN- γ in all patients' and

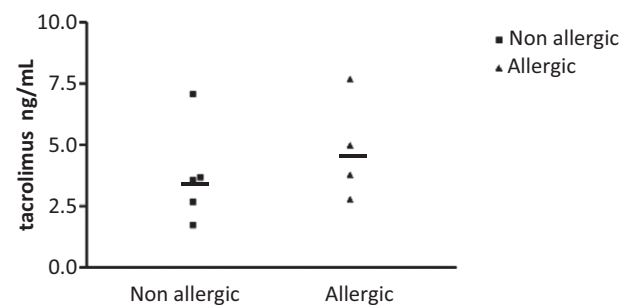


Fig. 2. Tacrolimus concentration in patient's serum. Mean of consecutive drug trough levels obtained during clinic visits over the six-month period before conduction of laboratory assays. Comparison between allergic and clinically non-allergic transplanted patients revealed a non-significant difference, p-value of 0.47.

controls' PBMCs after various stimulations. Measurement of IFN- γ concentrations showed that both groups of transplanted patients regardless of their allergy status exhibited an increase in IFN- γ secretion after stimulation with PHA as compared to healthy controls and healthy allergic individuals (Fig. 3). The levels were especially higher in non-allergic transplanted children compared to healthy allergic children. This trend was evident also in allergic transplanted PBMCs in comparison with healthy allergic children, although not reaching statistical significance.

Measurement of IL-5 levels revealed a significant difference ($p < 0.05$) of nearly fourfold difference in median levels between allergic transplanted patients and healthy individuals (Fig. 4). These high levels of IL-5 were not found in non-allergic post-transplant children or in healthy children regardless of their allergy status.

As for IL-10, there was a clear difference between transplanted and non-transplanted children regardless of them being allergic. It appears that healthy controls both allergic and not allergic showed higher secretion of IL-10 in response to stimulation with LPS as compared to transplanted children (Fig. 5). This was even more evident when measuring response after three days of incubation of PBMCs with ovalbumin. In this setting, liver transplant children's cells failed to show an increase from basal levels, while healthy individuals and healthy allergic patients exhibited a notable response to the expo-

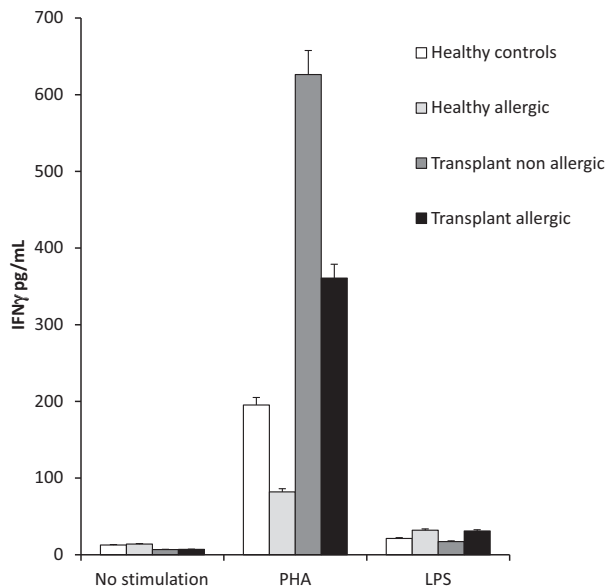


Fig. 3. IFN- γ secretion from PBMCs of transplanted non-allergic and transplanted allergic patients is higher than that from control patients' cells, but is not statistically significant, $p = 0.052$.

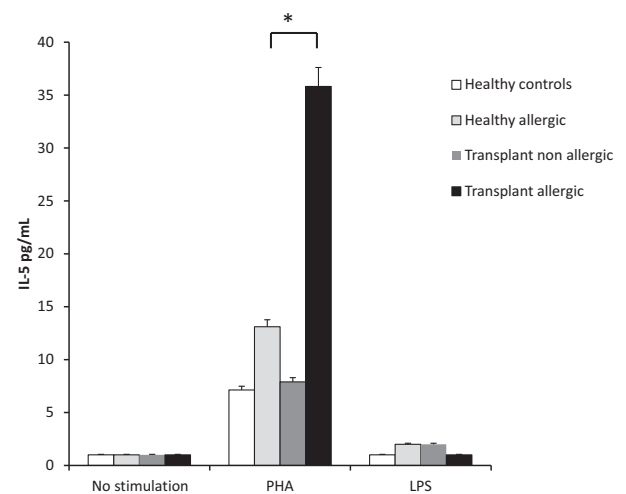


Fig. 4. IL-5 secretion from PBMCs of transplanted allergic patients is significantly higher than that from all other control groups tested: healthy non-allergic and healthy allergic patients. *Significant difference, $p = 0.016$.

sure with higher concentration of IL-10 in the medium ($p = 0.02$ between healthy and post-transplant patients). In our experimental system, the levels of IL-2 and IL-4 were barely detectable and the concentrations measured were insignificant.

T regulatory cell numbers in patients after liver transplant do not differ significantly from healthy individuals

The observation of differences in IL-10 levels led us to examine whether there is a difference in T

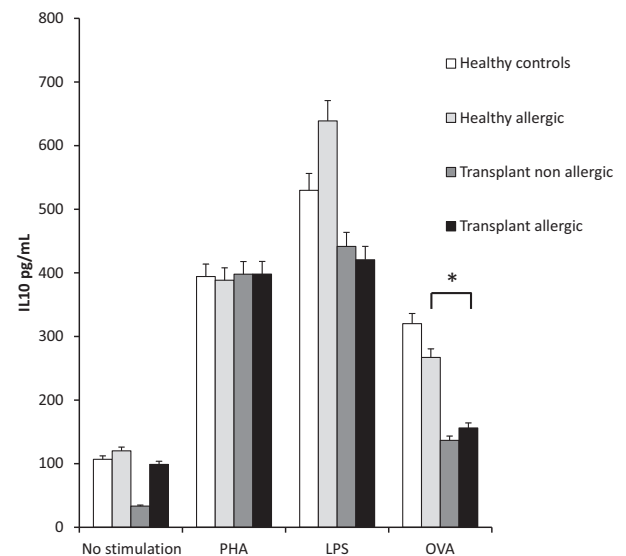


Fig. 5. IL-10 secretion from PBMCs of healthy non-allergic, and healthy allergic patients, is higher than that of transplanted allergic patients in response to general stimulation, but more pronounced in response to specific allergen, ovalbumin. *Significant difference, $p = 0.024$. OVA, ovalbumin.

regulatory CD4+CD25+FOXP3+ numbers between transplanted and healthy control patients. This in turn would serve as a putative mechanism for abnormal immune-allergic reactions. Using flow cytometry analysis to quantify T regulatory cell number by measuring the CD4+CD25+FOXP3+ cells in our assay, we could not detect major differences between healthy controls and transplanted patients (allergic and non-allergic). Mean CD4+CD25+FOXP3+ T regulatory cell percentage of the lymphocytes gated before stimulation in healthy allergic and transplanted patients was 0.65% and 0.34%, respectively ($p = 0.2604$), rising up to a mean of 2.3% and 3.17%, respectively, after PHA stimulation in healthy and transplanted children with no statistical significance ($p = 0.647$).

Discussion

Severe food-related allergic reaction in children undergoing liver transplantation is a known but poorly understood phenomenon. In this study, we have identified some unique features of patients with severe food-allergic reactions following liver transplant. One of the common assumptions so far was that this phenomenon is mainly related to the immunosuppressive treatment, in particular tacrolimus. A recent large-scale study by Gruber et al. (8) comparing kidney transplant patients treated with tacrolimus to those treated with cyclosporine revealed that although tacrolimus-treated group had higher rate of sensitization to food allergens manifested by positive skin test or specific IgE antibodies than cyclosporine-treated patients, the rates of clinical allergic reactions were not statistically significant between the groups. This is in keeping with previous reports from our group showing higher sensitization in patients treated with tacrolimus vs. cyclosporine (5). In our current study, where all patients are treated with tacrolimus, we could not find any correlation between tacrolimus serum levels and patients' current clinical allergic status, or to our laboratory studies of patients cells, such as cytokine secretion or T regulatory cell numbers. Therefore, it may well be that tacrolimus contributes to sensitization potential but certainly is not the sole mechanism underlying this phenomenon. It is possible that certain individuals are more susceptible to tacrolimus effects in terms of sensitization and allergic reactions; however, this is yet to be proven. A significant finding in our study, which lends further credibility to previous observations (12), is that earlier age at transplantation is associated with higher inci-

dence of clinically severe food allergy, and while older children may reveal sensitization potential, reflected by specific IgE levels to food antigens (4), they appear less likely to develop clinical reactions. This finding is supported by the paradigm claiming that tolerance is evolving during the first months of life and that TH1/TH2 balance is tipped toward TH2 responses at this age. Recent studies addressing the issue of exposure to antigens during the first year emphasize the role of timing of food introduction in acquiring tolerance to food antigens, suggesting a early window of opportunity that, if missed, may lead to higher rates of allergic reactions (13, 14).

A study of PBMC responses to different stimuli revealed that the cytokine milieu of liver transplant patients differs from that of healthy controls mainly in their lower IL-10 levels and higher IFN- γ levels; this may reflect deficiency in regulatory activity in all liver transplant patients regardless of their allergy status.

In contrast to previous reports, we could not detect a significant difference in T regulatory cell numbers between any of these groups. Alas, the difference in IL-10 level is suggestive of a functional defect in T regulatory cells activity. This has been shown to be important in non-transplanted food-allergic patients (15, 16) and may be even more significant in post-transplanted children. This issue will be assessed in future studies.

When looking at the group of allergic liver transplant patients, the most striking finding is a significantly higher level of IL-5, in comparison with non-allergic transplant patients, as well as healthy individuals (both allergic and non-allergic). Although healthy allergic children exhibited as expected higher secreted levels of IL-5 in comparison with non-allergic children (did not reach statistical significance), this was not comparable to the level measured in post-transplant allergic individuals. Increased IL-5 is a hallmark of allergic disease but usually more accentuated in eosinophilic gastrointestinal diseases rather than immediate hypersensitivity, and IgE mediated-type reactions (17). This is an interesting finding as the liver plays an important role as a gatekeeper to antigens entering from the digestive system. It remains to be further explored and established whether IL-5 could serve as a predictive marker in these patients.

In summary, our study shows that young age at transplant is an important contributor to the evolution of severe allergic reaction and that transplanted patient's cells secrete in response to stimuli the constellation of extremely high IL-5 level and low IL-10 level in comparison with

non-allergic and allergic but otherwise healthy children. These findings suggest an immune mechanism unique to liver transplant itself, rather than non-specific immunosuppression.

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Authors' contributions

Amit Nahum: Involved in study design, laboratory work, interpretation, and drafting of the manuscript. Avivit Brenner: Involved in laboratory work and data collection. Ettie Granot: Involved in data collection and critical review and drafting of the manuscript.

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