In vitro cytokine changes after pediatric liver transplantation

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Summary

Background: **Patients with chronic liver disease have been shown to have impaired immune statuses. Liver transplantation (LT) is the standard treatment for end-stage liver disease patients and immunosuppressive drugs are commonly used to prevent graft rejection. There is an increasing evidence of de novo food allergies post LT.**

Objective: **To investigate the cytokine response of peripheral blood mononuclear cells (PBMCs) of pediatric LT recipients before and six months after transplantation.**

Method: **PBMCs collected before and six months after LT were stimulated with phytohemagglutinin (PHA), beta-lactoglobulin (BLG), tacrolimus (Tac), dexamethasone (Dex), and a combination of BLG and Dex (B+D), BLG and Tac (B+T), BLG and Tac plus Dex (B+T+D). Culture supernatants were measured for IL-5, IFN-γ and IL-10. Blood for liver function tests, complete blood counts, total IgE and specific IgE (sIgE) to cow's milk were recorded.**

Results: **A total of five pediatric LT recipients were enrolled in the study. There were no food allergy cases. Total IgE and sIgE to cow's milk decreased significantly after LT. After transplantation, there was a significant increase in IL-5, IFN-γ and IL-10 in culture supernatants of PHA-stimulated PBMCs. Among different stimulations in post transplantation's PBMCs, the level of IL-5 significantly increased in B+D and B+T stimulated cells. However, this effect was suppressed with the combination of B+T+D. The level of IL-10 significantly increased in all conditions containing BLG both before and after transplantation.**

Conclusion: **There was an improvement of the *in vitro*- cytokine responses after liver transplantations. Immunosuppressive drugs used in post transplantation had an effect on the cytokine responses. *(Asian Pac J Allergy Immunol 2015;33:52-8)***

Keywords: *Liver transplantation, in vitro-cytokine, children, immunosuppressive drugs, chronic liver disease*

Introduction

The liver has a critical metabolic function. It also has an important immunologic function, such as removal of pathogens and antigens form the blood, and immunologic tolerance.[1, 2] Patients with acute and chronic liver disease have been shown to have reduced cellular immune responses.[3, 4]

Liver transplantation (LT) is currently a standard therapy for children with end-stage liver disease and irreversible acute life-threatening liver disease, including biliary atresia, fulminant liver failure and metabolic diseases.[5] The outcome of pediatric liver transplantation is excellent; however, a majority of post-LT children require long-term immunosuppressive drugs such as corticosteroids, calcineurin inhibitors, azathioprine, or mycophenolate mofetil.[6]

Several studies have demonstrated an impaired CD4 T-cell function, corroborated by a decreased IFN-γ gene expression in peripheral blood mononuclear cells (PBMCs) of LT recipients treated with calcineurin inhibitors.[7,8] However, there are only a limited number of studies that have evaluated patients' immune statuses before and after LT. In addition, there is increasing evidence of de novo food allergies after LT, and one of the most common causative foods is cow's milk.[9, 10] The primary objective of the current study was to investigate the immune response of pediatric LT recipients before

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and after transplantation using phytohemaglutin (PHA) and beta-lactoglobulin, the major cow's milk allergen. The secondary objectives were to investigate the changes in the specific IgE to cow's milk and clinical de novo food allergies, post LT.

Methods

Children who underwent primary, living-donor liver transplantation from December 2011 through November 2012 at Ramathibodi hospital, Mahidol University, Bangkok, Thailand, were prospectively enrolled in the study. Both donors' and recipients' histories of allergic diseases were recorded. The study protocol was approved by the Research Ethical Committee of Ramathibodi Hospital. All subjects' parents were provided written, informed consent.

Sample preparations

Blood samples were taken one day before and six months after the LT, or they would have been taken at the time of development of a clinical food allergy if one had occurred before six months. Peripheral blood mononuclear cells (PBMCs) were collected by Ficoll gradient centrifugation. Briefly, blood was layered on a Ficoll gradient and centrifuged, and the PBMC layer was collected with subsequent washing and centrifugation steps. Blood samples were also analyzed for complete blood count, liver function, and specific IgE to cow's milk (Immunocap, Phadia, Sweden).

PBMCs stimulated with PHA, beta-lactoglobulin (BLG), dexamethasone, and tacrolimus

Corticosteroids and tacrolimus are the drugs that are used in all patients for six months following a liver transplantation. In addition, cow's milk protein allergy is one of the common causes of food allergy developed post LT. In this context, PBMCs were cultured in 96 well plates at the concentration of 50,000 cells per well and stimulated in the following order: culture medium (10% FBS, RPMI-1640, and 1% Penicillin-Streptomycin), PHA (10 µg/mL), BLG (200 µg/ml), dexamethasone (10 nM), tacrolimus (Tac) (0.01 ng/ml) (all from Sigma-Aldrich, St. Louis ,USA), BLG (200 µg/ml) plus dexamethasone (10 nM) (B+D), BLG (200 µg/ml) and tacrolimus (0.01 ng/ml) (B+T), and the combination of BLG (200 µg/ml), dexamethasone (10 nM), and tacrolimus (0.01 ng/ml) (B+D+T) in 10% FBS, RPMI-1640, and 1% Penicillin-Streptomycin. Cells were incubated for 72 hours, and then the culture supernatant was collected.

Cytokine ELISA

The concentrations of interleukin 5 (IL-5), interleukin 10 (IL-10), and interferon gamma (IFN-γ) were measured using enzyme-linked immunosorbent assay (ELISA) by an Instant ELISA kit for the human IL-5, IL-10 and Platinum ELISA kit for Human IFN-γ (eBioscience, Vienna, Australia). Assays were performed according to the manufacturer's instructions; the lower limits of IL-5 and IL-10 detection were 0.66 pg/ml, and IFN-γ was 0.99 pg/ml.

Statistical analysis

Descriptive analysis was used to report the mean value and standard deviation (sd) of the data. Comparative analysis between pre and post LT was analyzed using a paired Student's t test or a Wilcoxon signed-rank test. ANOVA tests were used to compare the differences among the treatment groups. The differences with a p value less than 0.05 were considered statistically significant. Data were analyzed using SigmaPlot 12.

Results

A total of five children underwent LT from December 2011 to November 2012 at our institution. The indication for liver transplantation was biliary atresia in three cases, Allagile's syndrome in one case, and tyrosinemia in one case. The median Pediatric End stage Liver Disease (PELD) score was 18. All children received immunosuppressive drugs: tacrolimus and prednisolone for post-transplantation. Clinical characteristics of the recipients and donors are shown in Table 1. While none of the LT recipients had clinical food allergies or other allergies before transplantation, all LT recipients had evidence of cow's milk sensitivity (Table 2).

Changes of liver function test, peripheral blood white blood cell count, serum total IgE, sIgE to CM

Liver function test significantly improved after transplantation. There was no significant difference of peripheral blood white blood cell, neutrophil, lymphocyte and eosinophil counts between pre and post-LT. However, serum total IgE and specific IgE to cow's milk decreased significantly after transplantation (Table 2).

In-vitro cytokine changes after stimulation with PHA

Levels of IL-5, IL-10 and IFN-γ in culture supernatants from unstimulated PBMCs were low or

Table 1. Baseline characteristic of donors and LT recipients.

No	Recipient				Donor		
	sex	Age at LT (Mo)	Indication	PELD score	Relative	Age	Allergic disease
1	Female	24	Biliary atresia	18	Mother	35	No
2	Mae	25	Biliary atresia	14	Mother	26	No
3	Male	11	Biliary atresia	18	Father	31	No
4	Male	48	Allagile's syndrome	20	Mother	32	AR, asthma
5	Female	20	tyrosinemia	9	Mother	21	No

PELD = Pediatric End stage Live Disease, AR= allergic rhinitis

undetectable both in pre- and post-LT. After stimulation with PHA, only the level of IL-5 in culture supernatants from pre-LT PBMCs was significantly higher than those from unstimulated cells. In contrast to pre-LT, the levels of IL-5, IL-10 and IFN-γ in culture supernatants from post-LT PBMCs were significantly higher than those from unstimulated cells. In addition, the levels of IL-5, IL-10 and IFN-γ from post-LT PBMCs were significantly higher than those from pre-LT PBMCs (Figure 1).

In-vitro cytokine changes after PBMCs stimulated with BLG, dexamethasone and tacrolimus

IL-5 changes after PBMCs stimulations

There were no significant changes in the level of IL-5 in culture supernatants from pre-LT PBMCs stimulated with BLG, Dex, Tac, B+D,B+T, and B+T+D (Figure 2A) . In contrast to pre-LT, the level of IL-5 significantly increased in culture supernatants from post- LT PBMCs stimulated with B+T and B+D when compared to those from unstimulated PBMCs. However, the combination of B+T+D significantly decreased the level of IL-5 when compared to B+T (Figure 2B).

IL-10 changes after PBMCs stimulations

The level of IL-10 in culture supernatants from pre-LT PBMC stimulated with conditions that contained BLG (BLG, B+D, B+T, and B+T+D) was significantly higher than those from unstimulated PBMCs (Figure 3A). A similar result was also demonstrated in post LT. However, the levels of IL-10 in culture supernatants from post-LT PBMCs was significantly higher than those from pre-LT PBMCs (Figure 3B).

Table 2. Laboratory parameters of LT recipients before and post LT

	Pre-LT Mean (sd)	Post-LT 6 months Mean (sd)	*p* value
White blood cell count (cell/μL)	8828.3 (4024.3)	8000 (2485.5)	0.6
Blood neutrophil count (cell/μL)	4910.4 (2150.4)	3533.0 (1775..8)	0.14
Blood lymphocyte count (cell/μL)	3282 (2172.2)	3555.2 (868.9)	0.7
Blood eosinophil count (cell/μL)	123.84 (74.90)	159.98 (101.95)	0.6
Log Serum total IgE (KU/L)	2.58 (0.36)	1.48 (0.73)	**0.04**
Serum sIgE to cow's milk (KAU/L)	2.02 (0.5)	0.04 (0.02)	**0.001**
Liver function test			
AST (U/L)	149.20 (47.71)	30.40 (5.32)	**0.005**
ALT (U/L)	111.00 (67.78)	38.8 (3.89)	0.08
ALP (U/L)	600 (258.51)	276.6 (83.02)	**0.015**
GGT (U/L)	149.6 (36.52)	26.4 (10.5)	**0.001**
Total Bilirubin (mg/dL)	14.34 (10.3)	0.4 (0.07)	**0.04**
Direct bilirubin (mg/dL)	12.46 (9.38)	0.2 (0)	**0.04**
Albumin (g/L)	32.8 (9.9)	40.24 (2.6)	0.2

AST=aspartate aminotransferase, ALT=alanine transaminase, ALP=Alkaline phosphatase and GGT= gamma-glutamyl transferase

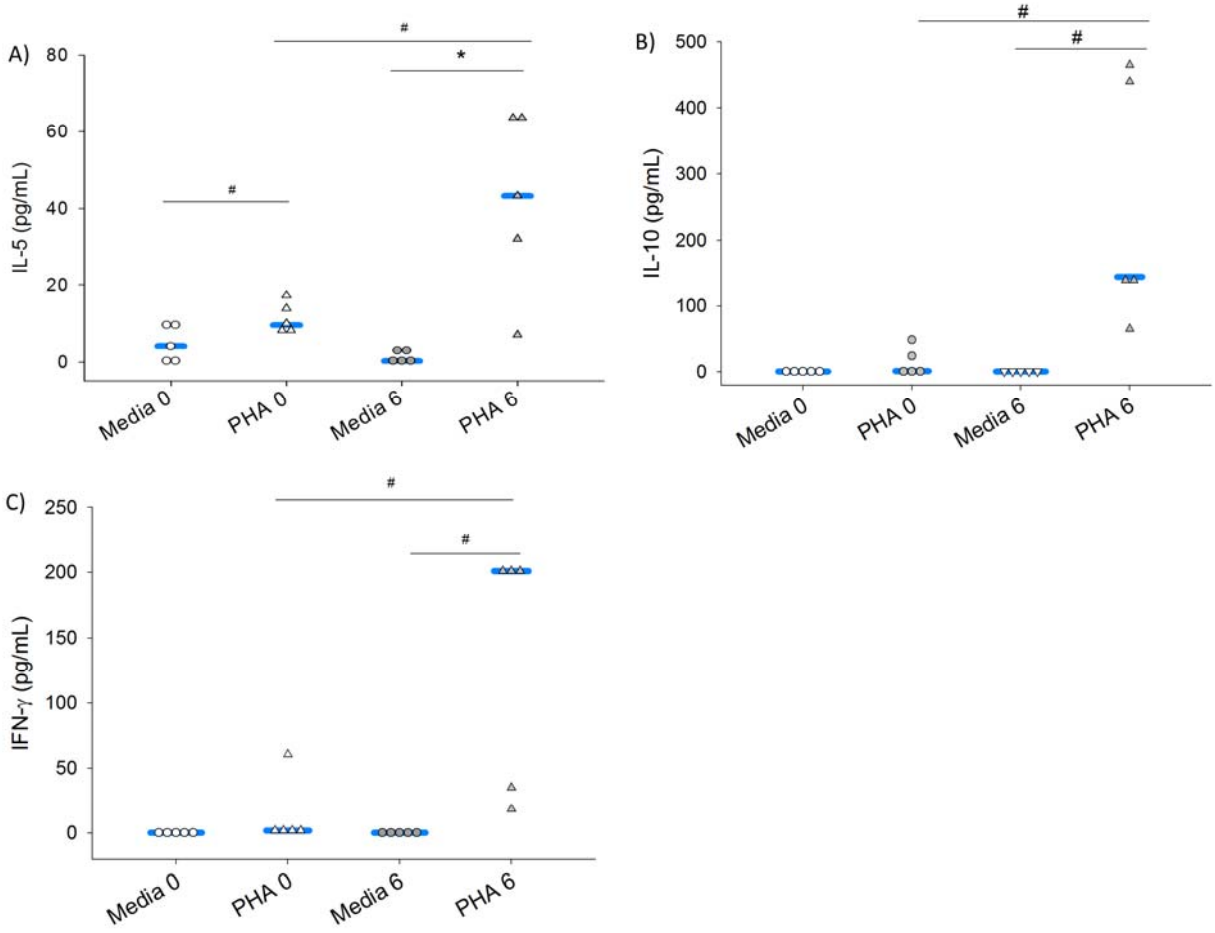

Figure 1. The levels of IL-5 (A), IL-10 (B) and IFN-γ (C) in culture supernatants of PBMCS stimulated with PHA and unstimulated cells comparing between before LT (0) and 6 months after LT (6). * represents $p < 0.01$, # represents $p < 0.05$.

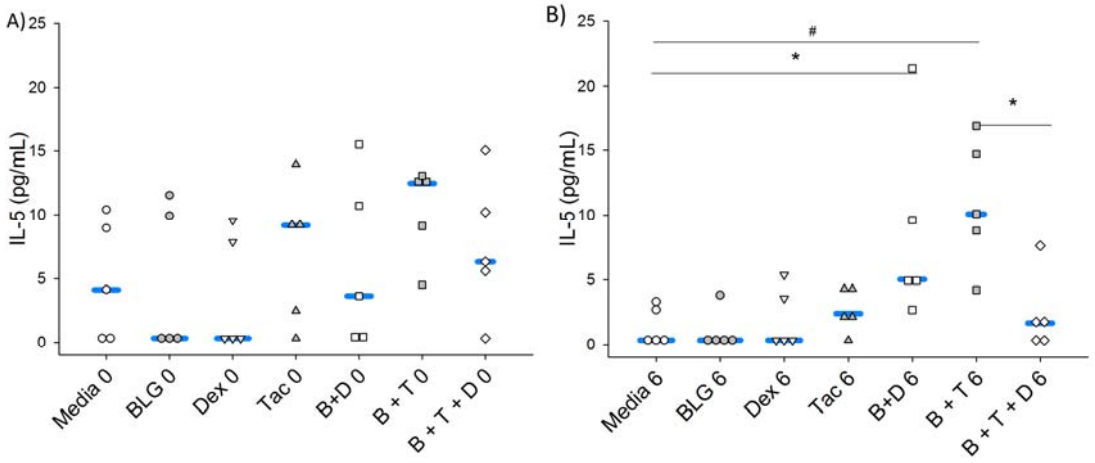

Figure 2. The level of IL-5 in culture supernatants of PBMCS stimulated with beta-lactoglobulin (BLG), tacrolimus (Tac), dexamethasone (Dex) and combination of BLG and Dex (B+D), BLG and Tac (B+T), BLG and Tac plus Dex (B+T+D) before LT (A) and 6 months after LT (B). * represents $p < 0.01$, # represents $p < 0.05$.

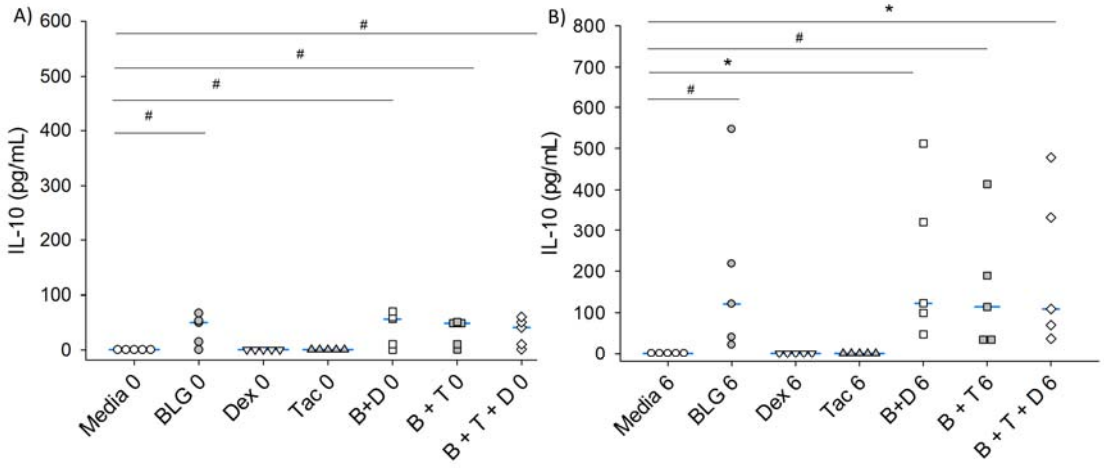

Figure 3. The level of IL-10 in culture supernatants of PBMCS stimulated with in beta-lactoglobulin (BLG), tacrolimus (Tac), dexamethasone (Dex) and combination of BLG and Dex (B+D), BLG and Tac (B+T), BLG and Tac plus Dex (B+T+D) before LT (A) and 6 months after LT (B). * represents $p < 0.01$, # represents $p < 0.05$.

IFN-γ changes after PBMCs stimulations

In contrast to IL-5 and IL-10, there were no significant changes in the levels of IFN-γ in culture supernatant from either pre-LT or post-LT PBMC stimulated with BLG, Dex, Tac, B+D,B+T, and B+T+D compared to those from unstimulated cells (Figure 4).

Discussion

Liver transplantation is the treatment of choice for children with end-stage liver disease. It has already been demonstrated that patients with chronic liver diseases have a defect in immune responses resulting in increased susceptibility to infections.[11] There is an increasing evidence of de novo food allergies in post-LT recipients, and cow's milk is one of the common causative agent.[12,13] While tacrolimus is widely used to prevent graft rejection after liver and kidney transplantation, recent studies have shown its association with de novo food allergies in the context of LT but not with renal transplantation.[9,14,15] A combination of calcineurin inhibitors (CNI) such as tacrolimus and corticosteroids are commonly used in the first three to six months post LT. Then, almost all LT recipients receive lifelong CNI to prevent graft rejection.[16] Several studies have shown that tacrolimus-based immunosuppression is associated with eosinophilia, an elevation of total and specific IgEs, food allergies, and eosinophilic gastrointestinal disorders.[13,17,18] It has been demonstrated that half of the de novo food allergies develop in the first six months following a liver transplantation.[14,19] In this context, we chose to evaluate the *in-vitro cytokine* changes at six months post LT. No food allergies within six months post LT were observed. This may be explained by the small number of LT patients enrolled in the study or by the differences in ethnicity of the studied group since genetic factors also have important influence on the development of allergy diseases. Follow ups after six months and more LT recipients need to be incorporated in subsequent studies to understand more precisely the patterns of occurence of de novo food allergy in LT recipients in our population.

The immunosuppressive drugs used in our institute's pediatric LT are tacrolimus and prednisolone for the first six months after transplantation. In addition, cow's milk protein allergy is the most common cause of de novo food allergy among pediatric LT recipients in our institute.[10] As a result, we chose to stimulate PBMCs with these two drugs and BLG, the major cow's milk allergens. The present study demonstrated the decrease of IL-5 in culture supernatants of PMBCs stimulated with tacrolimus plus BLG plus dexamethasone when compared to those stimulated with tacrolimus plus BLG. This may be explained by the insignificant increase of peripheral blood eosinophil after six months of LT compared to before transplantation in the present study, since all of our patients received a combination of prednisolone and tacrolimus over the first six months after transplantation. The type and combination of immunosuppressive agents is believed

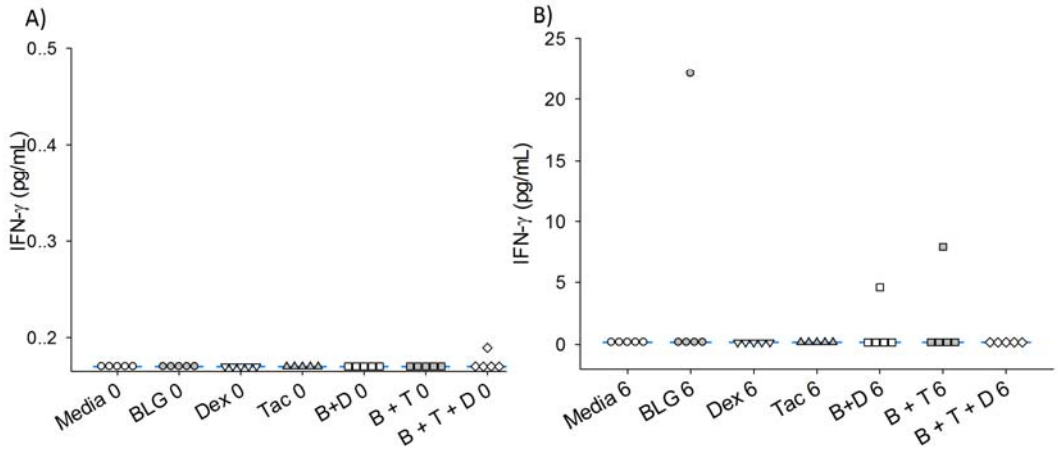

Figure 4. The level of IFN-γ in culture supernatants of PBMCS stimulated with beta-lactoglobulin (BLG), tacrolimus (Tac), dexamethasone (Dex) and combination of BLG and Dex (B+D), BLG and Tac (B+T), BLG and Tac plus Dex (B+T+D) before LT (A) and 6 months after LT (B). * represents $p < 0.01$, # represents $p < 0.05$.

to have an impact on the development of de novo food allergies post LT in our population.

The present study demonstrated an increase of IL-10, the major cytokine produced from regulatory T cells, in culture supernatants of post-LT PBMCs stimulated with conditions that contained BLG. Our *in-vitro* cytokine results may suggest that exposure to allergenic foods such as cow's milk can promote IL-10 production of PBMCs from LT recipients who do not have clinical food allergies. Consequently, cow's milk or cow's milk products should be consumed normally after LT since LT does not increase the risk of cow's milk allergy, but it may prevent food allergy by promoting the production of IL-10, the regulatory cytokine.

We also have shown an improvement of immune function of LT recipients as demonstrated by the increase in the level of *in-vitro* cytokines after PHA stimulation including IL-5 (Th2 cytokine), IL-10(regulatory T cell cytokine) and IFN-γ (Th1 cytokine) at six months after LT. This improvement of immune function was demonstrated even though all post-LT patients received corticosteroids and tacrolimus, which are T cell-suppressive drugs. Previous studies in adult liver transplantation recipients have shown a decrease in the level of IFN-γ in culture supernatant of PHA stimulated by PBMCs at 2, 4 and 8 weeks post LT when compared to unstimulated cells.[20] Since we did not evaluate the *in-vitro* cytokine changes earlier than six months, the improvements in immune functions after PHA stimulation may occur between two and six months after transplantation, based on our results and on previous studies' results.[20]

The limitations of the present study are the small sample size and the short duration between transplantation and follow-up. These limitations likely have contributed to our inability to observe food allergy cases developed after transplantation.

Conclusion

In conclusion, the present study has demonstrated an improvement of immune function of pediatric liver transplantation recipients at six months post LT in both Th1, and Th2 and regulatory T cells cytokine. Immunosuppressive drugs used post transplantation had an effect on the cytokine responses. A longer follow up period is needed to determine the time course effect of these drugs and de novo food allergy trends post LT.

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