

RESEARCH

Open Access



# Cytokine and human leukocyte antigen (HLA) profile for graft-versus-host disease (GVHD) after organ transplantation

Xinhua Chen<sup>1,2†</sup>, Xueqin Meng<sup>1,2†</sup>, Yuning Xu<sup>1,2</sup>, Haiyang Xie<sup>1,2</sup>, Shengyong Yin<sup>1,2</sup>, Hongchun Li<sup>3</sup>, Liming Wu<sup>1,2\*</sup> and Shusen Zheng<sup>1,2\*</sup>

## Abstract

**Background:** Graft-versus-host disease (GVHD) after liver and kidney transplantation has high mortality and causes diagnostic challenges. This study aims to describe the cytokine and human leukocyte antigen (HLA) profile in the GVHD after liver and kidney transplantation.

**Methods:** A high-throughput detection kit was applied and altogether 18 different cytokines were tested simultaneously. GVHD patients included 23 post-liver transplantation patients; 22 post-renal transplantation patients; The control patients include 22 hepatocellular carcinoma (HCC) patients without transplantation and 20 healthy controls. Their HLA characters were compared.

**Results:** The full spectrum of cytokines was present. The inflammatory markers were activated significantly in liver transplantation. The level of inflammatory markers in liver transplantation was higher than that in renal transplantation, HCC or healthy controls. GVHD was associated with the HLA characters; HLA characters are involved in liver GVHD occurrence and act as risk factors.

**Conclusion:** Our findings confirmed that the inflammatory cytokines play a pathogenic role in GVHD and can be used as early diagnostic markers. The HLA mismatch acts as a risk factor in liver transplantation to predict GVHD occurrence.

**Keywords:** Cytokine, Human leukocyte antigen (HLA), Graft-versus-host disease (GVHD), Transplantation, Multiplex immunoassay, High-throughput

## Background

Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death in the world [1, 2] and China accounts for almost half of HCC cases [2]. The liver transplantation is the curative treatment for HCC but post-transplantation graft-versus-host disease (GVHD) has caused mortality [3, 4]. The cytokine and HLA profile of GVHD can help understand the

inflammatory background and the risk factors [5, 6]. The full profile of cytokine in the cytokine storms post GVHD is still lacking. In this study, we present a high-throughput cytokine exam for GVHD after liver and kidney transplantation. The association with HLA was also analyzed.

## Methods

### Patients

Altogether, 87 patients were included: 23 HCC patients with liver transplantation (LT); 22 renal transplantation (RT); 22 HCC without transplantation (HCC); and 20 healthy controls (control). Patients underwent organ transplant between January 2004 and December 2014.

\*Correspondence: wlm@zju.edu.cn; shusenzheng@zju.edu.cn

<sup>†</sup>Xinhua Chen and Xueqin Meng contributed equally to this manuscript

<sup>2</sup>The Department of Hepatobiliary Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qingchun Road, Hangzhou 310003, Zhejiang, China

Full list of author information is available at the end of the article

### Measurement of cytokines by multiplex immunoassay

The multiple cytokines were measured by Multiplex Immunoassay Kit (Affymetrix, CA, USA). Briefly, 25  $\mu$ L serum and standards were incubated with pre-mixed beads coated with antibodies. After washing, plates were incubated with the detection antibody and the reaction revealed with streptavidin–phycoerythrin. The beads were analyzed on a Luminex system (Bio-Rad, USA) for the concentration of the following 18 cytokines: IL-10, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-9, GM-CSF, IFN- $\gamma$ , IL-1b, IL-12, P70, IL-13, IL-18, IL-2, IL-4, IL-5, IL-6, TNF- $\alpha$ .

### Pathological confirmation of the skin biopsy

The pathological slides of the biopsy were reviewed and the pathological figures to support the diagnosis of GVHD was present. The skin sample was stained and read by two independent pathologists. The hematoxylin–eosin (H&E) was stained for the confirmation of GVHD.

### The anti-HLA antibody measurement

The HLA antibodies were measured by Luminex technology (One Lambda, Inc. Canoga Park, CA, USA). According to the manufacturer's manual book, HLA-specific antibodies were identified using immune beads coated with purified HLA antigens. HLA antibodies in patient's serum were bound to the antigens coated on the beads. The complex was then stained with R-phycoerythrin-conjugated goat anti-human IgG. The fluorescent emission of R-phycoerythrin was measured and then analyzed by HLA analysis software (Canoga Park, CA, USA.). All adjusted and normalized reactions that were above 500 were considered positive.

### Statistical analysis

The present data are expressed as mean  $\pm$  SD. For statistical comparison of values, Student's *t* test was used. *P* values less than 0.05 were deemed to indicate statistical significance. The SPSS one-way analysis of variance (ANOVA) was used to determine whether there were any statistically significant differences.

## Results

### The clinical presentation

The four groups are age-, sex-, and storage-time-matched. The typical symptoms were skin lesions. The liver function was not affected and the liver damage was not obvious. A nonspecific skin basal vacuolar changes, dyskeratosis in the epidermis were found. Diarrhea was the most common complaints due to the absorptive function loss caused by lymphocyte infiltration and destruction of the intestinal mucosa (Table 1).

### Screening of cytokines

Among all the 18 screened cytokines, three cytokines IL-12, IL-18 and IFN- $\gamma$  showed a significant increase, and the diagnostic value varied from 0.5 (IL2) to 1.22 pg/mL (IL-18). The Luminex immunoassay measures cytokines in pg levels. Multiple cytokines can be tested in a single run by using a small volume of serum sample (Fig. 1).

### HLA identification

HLA haplotype is shown in Fig. 2. There were donor–recipient HLA-mismatching in liver-transplanted patients. Renal transplantation (RT) requires a strict matching, so there was no HLA mismatching status in any of kidney case in our study. All of the 22 cases of renal transplantation (Fig. 2) had no mismatches at HLA-A, HLA-B, and HLA-DR loci. HLA-typing showed that the liver donor shared a single antigen with the patient, but the donor was heterozygous at other loci (A2, A24, B13, B46, DR12). Although liver transplantation (LT) dose not require a strict HLA compatibility as renal transplantation. Our result verified that the use of the HLA-mismatching donor can result in the risk of developing GVHD after LT.

### The pathology of skin lesion biopsy

The biopsy from skin on right thigh of GVHD patient was shown (Fig. 3a). The pathology showed epidermal atrophy, excessive keratosis and parakeratosis in the epidermis (Fig. 3b) with significant dermal fibrosis and collagen (Fig. 3c). The lymphocytic infiltration was seen but not significant (Fig. 3d). The dermal perivascular inflammatory cell infiltration was found invading into the epithelium (Fig. 3b). Diagnosis: skin squamous cell dyskeratosis associated with dermal chronic inflammatory cell infiltration.

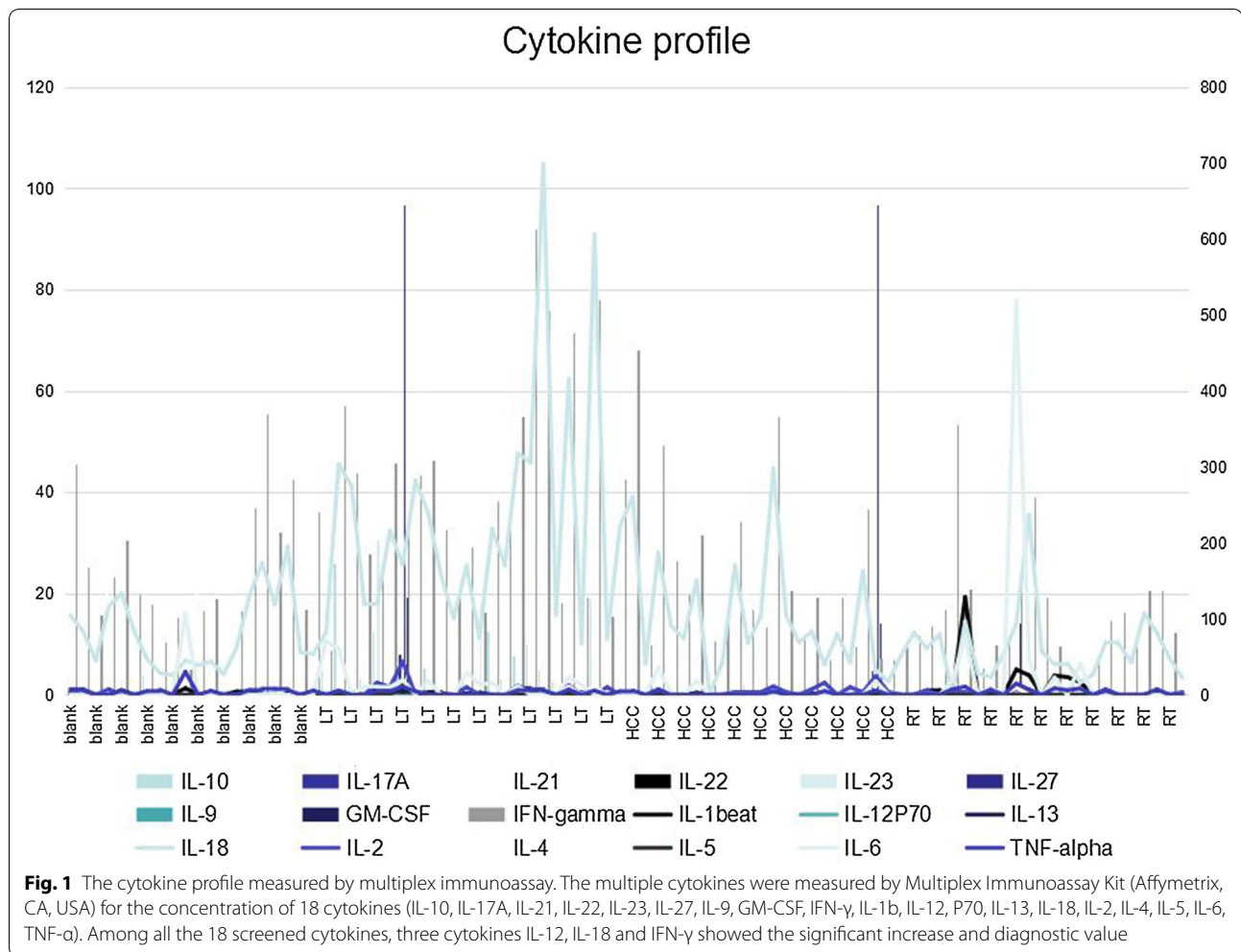
Different from kidney transplantation, which requires a strict donor–recipient HLA matching to improve long-term prognosis, liver transplantation can tolerate the HLA-mismatching to some degree. The incompatibility between donor and recipient might inhibit the host immune function. Whether HLA-B matching and the other HLA loci may impact long-term survival in liver transplantation is still debating [12, 13]. We use it as a risk factor rather than an absolute evaluation biomarker.

## Discussion

There are three basic requirements formulated by Billingham in 1966 for the development of GVHD: the graft must contain immunologically competent cells; the recipient must express tissue antigens that are not present in the transplant donor; and the recipient must be incapable of mounting an effective response to eliminate

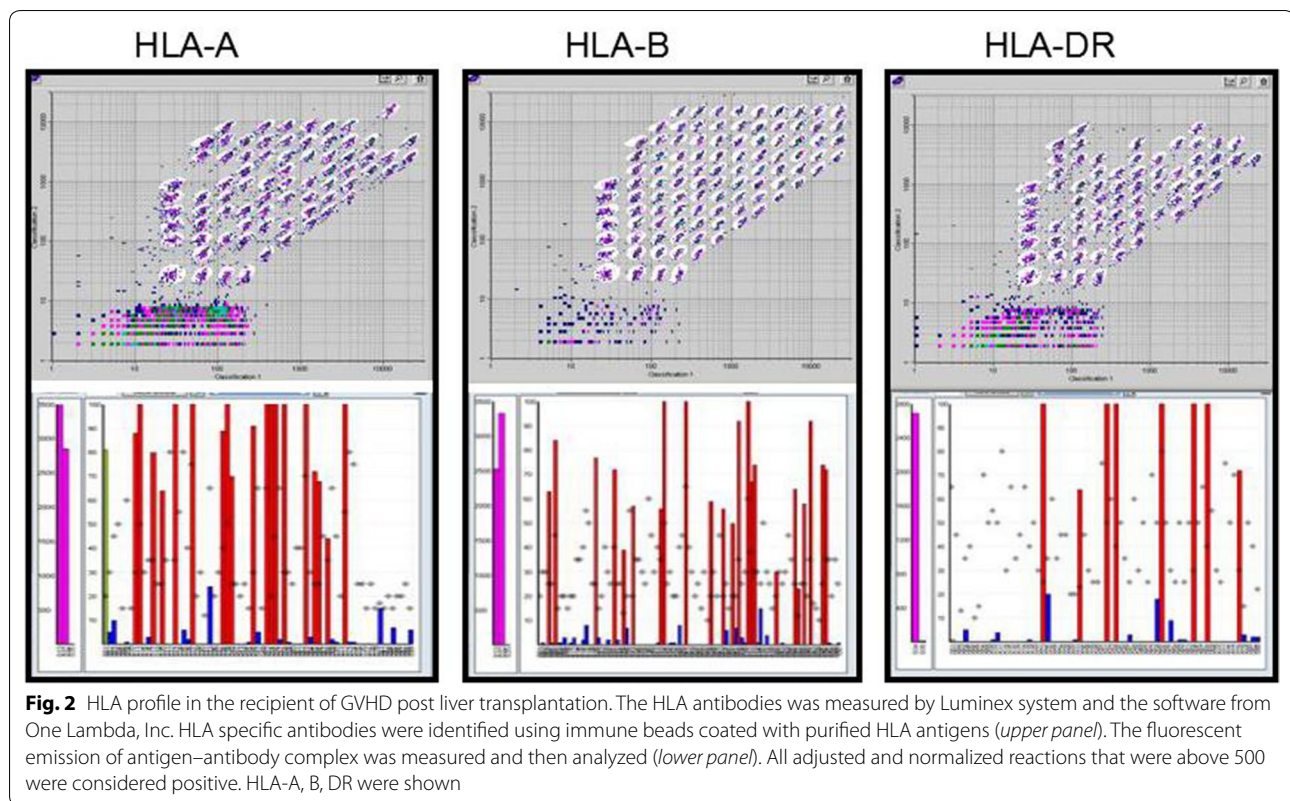
**Table 1** The clinical characteristics of patients

	Liver transplantation	Renal transplantation	HCC	Control
Number of patients	23	22	22	20
Recipient age (years)	54 ± 19	49 ± 12	58 ± 9	51 ± 21
Male/female	13/10	10/12	13/9	10/10
Time to onset (days)	253 ± 39	192 ± 51	/	/
BMI	22 ± 2.1	19 ± 3.4	20 ± 2.8	23 ± 3.2
AST (U/L)	56 ± 4	61 ± 2	45 ± 4	52 ± 3
ALT (U/L)	35 ± 2	28 ± 1	31 ± 3	31 ± 2
Total bilirubin (mg/dL)	0.95 ± 0.11	0.95 ± 0.16	0.95 ± 0.23	0.95 ± 0.37
Direct bilirubin (mg/dL)	0.45 ± 0.09	0.35 ± 0.14	0.47 ± 0.15	0.30 ± 0.17
Leukocytes (mil/mm <sup>3</sup> )	9.6 ± 2.1	4.8 ± 1.7	3.6 ± 0.4	4.5 ± 1.1
Haemoglobin (g/dL)	12 ± 2	11 ± 3	10 ± 2	14 ± 2



the transplanted cells [7–9]. Our results proved that GVHD post liver transplantation involved HLA mismatch and inflammatory cytokine release.

Examining the levels and interplay of cytokines can help to identify GVHD biomarkers and medical intervention targets [10]. However, the complicated interactions



of cytokine network in GVHD can only be understood when the full cytokine profile is present [11]. Multiplex immune-assays can detect multiple cytokines with a small amount of patient's serum (only 25  $\mu$ L) with good parallel among different cytokines. The multiplex luminex immunoassay can simultaneously detect antibody activity to 18 antigens in a single 96-well plate. The exam can be performed automatically in a centralized laboratory. The results indicate that after GVHD onset, an exaggerated response of cytokines IL2, IL18, IFN- $\gamma$  may predispose to GVHD. They can be used to predict the occurrence of GVHD in transplant recipients. High levels of the three cytokines above correlated with the development of GVHD. The results suggest that measurement of IL2, IL18 and IFN- $\gamma$  is of predictive value. Beside IL2, IL18 and IFN- $\gamma$ , the baseline of other 15 cytokines were also present (IL-10, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-9, GM-CSF, IL-1b, IL-12P70, IL-13, IL-4, IL-5, IL-6, TNF- $\alpha$ ). These results together provide a full cytokine profile and further substantiate the complex cytokine cascade that is initiated by GVHD.

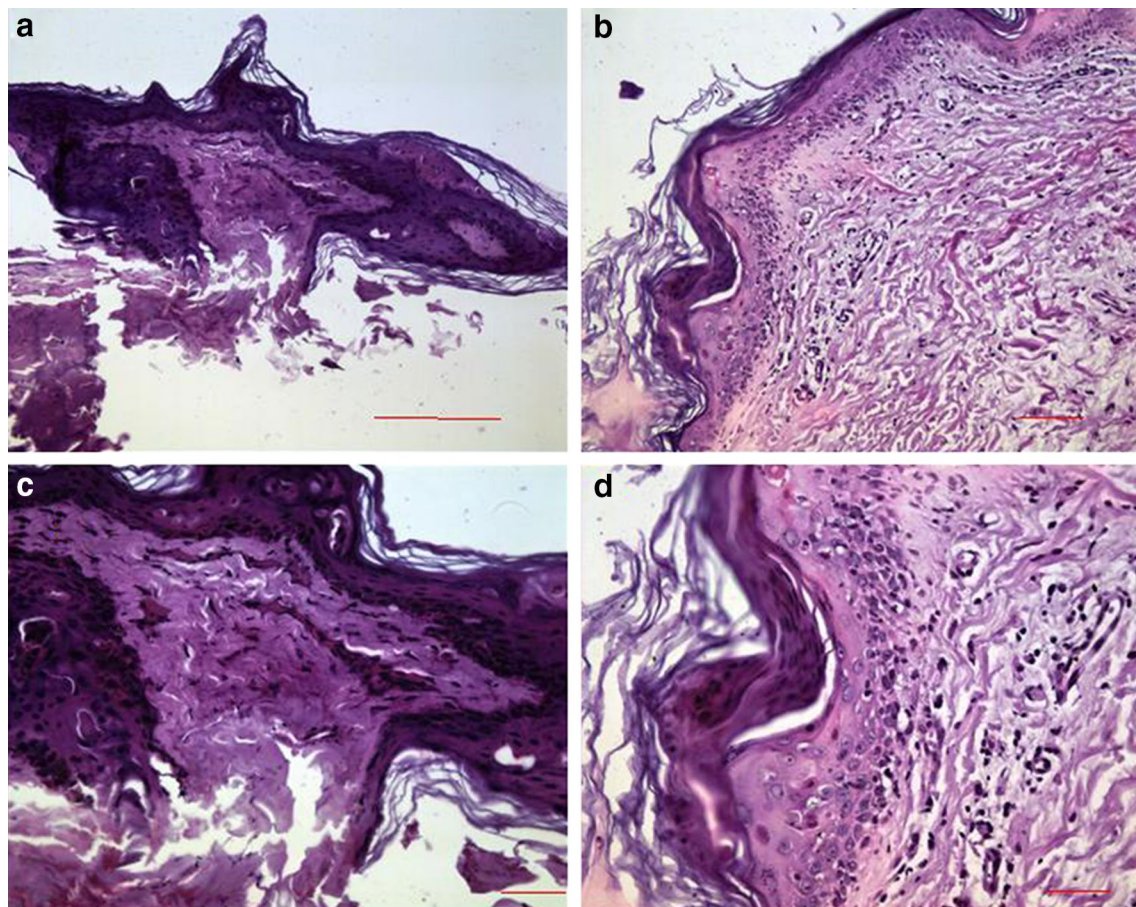
Another notable finding in our study is the cytokine difference between GVHD post liver transplantation and renal transplantation. The GVHD and cytokine situation in liver transplantation and renal transplantation is quite different. IL2, IL18 and IFN were all lower in RT group than other

groups. To address this difference, we have to add an additional HLA-typing experiment to explain the reason. The renal transplantation requires a very strict HLA matching. So, all renal transplantation cases were HLA-matched. The GVHD in renal transplantation and inflammation response were not as high as that in liver transplantation. The strict HLA match in renal transplantation might be the possible reason. We added a typical case of LT to show its HLA-typing. HLA-mismatching allows patient to survive in liver transplantation without acute rejection. But it did increase the risk of GVHD in long term.

The immune mechanism on GVHD involves T cells and B cells. However, it is becoming increasingly focused on host-derived molecules that result from tissue incompatibility, e.g., HLA-mismatching [12]. Our result showed that HLA contribute to the occurrence of GVHD and then the rejection reaction was so wide that it caused skin damage. An improved understanding of these pathways may reveal novel therapeutic targets to decrease GVHD and increase long-term survival.

One major risk factor for the development of GVHD after LT was determined: human leukocyte antigen (HLA) matching between the donor and the recipient; Donor-recipient compatibility for HLA-B was identified as a significant risk factor for the development of GVHD. An accurate diagnosis of GVHD depends on histological and





**Fig. 3** The pathology of skin lesion biopsy. The biopsy from skin on the right thigh (a). The pathology showed epidermal atrophy, excessive keratosis and parakeratosis in the epidermis (b) with significant dermal fibrosis and collagen (c). The lymphocytic infiltration was seen but not significant (d). The inflammatory cells infiltrate and invade into the epithelium (b). Diagnosis: skin squamous cell dyskeratosis associated with dermal chronic inflammatory cell infiltration

immuno-histochemical proof [13]. In our study, the pathological in skin include lymphocyte infiltration, basal cell vacuolization and the keratinocyte resembling. The differential diagnosis excluded the bacterial, viral and fungal causes of gastrointestinal symptoms. Then, the patients were given standard treatment of increased immune suppression and antibody preparations. Immune suppressants are a necessary right after transplant surgeries, preventing the rejection-caused new organ function failure. But the long term use of immune suppressants can cause many side effects such as infection, cancer, diabetes, GVHD compared to the general population. The proper withdrawal or minimize the use of immune suppression can help the immune system reconstitute itself.

### Summary

Our study provides a comprehensive platform for GVHD diagnosis. The HLA-mismatching works as a risk factor for the physician to suspect the high possibility of

occurrence. Cytokine profile can be used for early diagnosis. Skin biopsy can be used as confirmation.

### Conclusion

Our findings confirmed that the inflammatory cytokines play a pathogenic role in GVHD and can be used as early diagnostic markers and future therapeutic targets. The HLA mismatch acts as risk factor in liver transplantation to predict GVHD occurrence.

### Abbreviations

HLA: cytokine and human leukocyte antigen; GVHD: graft-versus-host disease; HCC: hepatocellular carcinoma.

### Authors' contributions

XC designed the experiment and drafted the manuscript. XM carried out the HLA studies and collected the data, YX carried out the immunoassays. HX, SY, HL participated in the design of the study and performed the statistical analysis. LW and SZ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

## Author details

<sup>1</sup> Key Laboratory of Combined Multi-organ Transplantation, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Ministry of Public Health, Hangzhou, China. <sup>2</sup> The Department of Hepatobiliary Surgery, The First Affiliated Hospital, Zhejiang University School of Medicine, 79 Qingchun Road, Hangzhou 310003, Zhejiang, China. <sup>3</sup> Key Laboratory of Hepatobiliary Disease in Shenzhen, Shenzhen 518112, China.

## Acknowledgements

Authors thank Miss Lin Zhang for collecting clinical information and Dr. Ke Sun for providing pathology slides.

## Competing interests

The authors declare that they have no competing interests.

## Availability of data and material

Please contact author for data requests.

## Ethics approval and consent to participate

This comparative study of a retrospective cohort was approved by the Ethical Committee of the First Affiliated Hospital of Zhejiang University.

## Funding

Natural Science Foundation of China (81372425, 81572954, 81401319, 81421062, 91542205), Zhejiang Science Project (2013T301-15, LY13H180003, 2013C37002, 2016C33145), SanMing Project of Shenzhen City.

Received: 1 March 2016 Accepted: 30 September 2016

Published online: 12 October 2016

## References

- Xu X, Lu D, Ling Q, Wei X, Wu J, Zhou L, Yan S, Wu L, Geng L, Ke Q, Gao F, Tu Z, Wang W, Zhang M, Shen Y, Xie H, Jiang W, Wang H, Zheng S. Liver transplantation for hepatocellular carcinoma beyond the Milan criteria. *Gut*. 2016;65(6):1035–41.
- Tanaka M, Katayama F, Kato H, Tanaka H, Wang J, Qiao YL, Inoue M. Hepatitis B and C virus infection and hepatocellular carcinoma in China: a review of epidemiology and control measures. *J Epidemiol*. 2011;21(6):401–16.
- Chaib E, Silva FD, Figueira ER, Lima FR, Andraus W, D'Albuquerque LA. Graft-versus-host disease after liver transplantation. *Clinics*. 2011;66(6):1115–8.
- Green T, Hind J. Graft-versus-host disease in paediatric solid organ transplantation: a review of the literature. *Pediatr Transplant*. 2016;20(5):607–18.
- Mihailă RG. Liver graft versus host disease after allogeneic peripheral stem cell transplantation: update on etiopathogenesis and diagnosis. *Rom J Intern Med*. 2016;54(2):83–92.
- Magenau J, Runaas L, Reddy P. Advances in understanding the pathogenesis of graft-versus-host disease. *Br J Haematol*. 2016;173(2):190–205.
- Leveque-El Mouttie L, Koyama M, Le Texier L, Markey KA, Cheong M, Kuns RD, Lineburg KE, Teal BE, Alexander KA, Clouston AD, Blazar BR, Hill GR, MacDonald KP. Corruption of dendritic cell antigen presentation during acute GVHD leads to regulatory T-cell failure and chronic GVHD. *Blood*. 2016;128(6):794–804.
- Elfeki MA, Pungpapong S, Genco PV, Nakhleh RE, Nguyen JH, Harnois DM. Graft-versus-host disease after orthotopic liver transplantation: multivariate analysis of risk factors. *Clin Transplant*. 2015;29(12):1063–6.
- Shulman HM, Cardona DM, Greenson JK, Hingorani S, Horn T, Huber E, Kreft A, Longrich T, Morton T, Myerson D, Prieto VG, Rosenberg A, Treister N, Washington K, Ziemer M, Pavletic SZ, Lee SJ, Flowers ME, Schultz KR, Jagasia M, Martin PJ, Vogelsang GB, Kleiner DE. NIH, consensus development project on criteria for clinical trials in chronic graft-versus-host disease: II. The 2014 pathology working group report. *Biol Blood Marrow Transplant*. 2015;21(4):589–603.
- Hellwig Y, Yoo YE, Reß ML, Andres O, Braun M, Schlegel PG, Wöfl M. Fulminant skin GvHD with a cytokine pattern resembling of cytokine release syndrome successfully treated with multimodal immunosuppression including tocilizumab. *Pediatr Blood Cancer*. 2015;62(11):2033–5.
- Thiant S, Moutouou MM, Leboeuf D, Guimond M. Homeostatic cytokines in immune reconstitution and graft-versus-host disease. *Cytokine*. 2016;82:24–32.
- Uchiyama H, Kayashima H, Matono R, Shirabe K, Yoshizumi T, Ikegami T, Soejima Y, Matsuura T, Taguchi T, Maehara Y. Relevance of HLA compatibility in living donor liver transplantation: the double-edged sword associated with the patient outcome. *Clin Transplant*. 2012;26(5):E522–9.
- Murali AR, Chandra S, Stewart Z, Blazar BR, Farooq U, Ince MN, Dunkelberg J. Graft versus host disease after liver transplantation in adults: a case series, review of literature, and an approach to management. *Transplantation*. 2016. PMID: 27495762 (Epub ahead of print).

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at  
www.biomedcentral.com/submit

