Research Article

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CXCL10/CXCR3 signaling mobilized-regulatory T cells promote liver tumor recurrence after transplantation

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Background & Aims: Liver graft injury and tumor recurrence are the major challenges of liver transplantation for the patients with hepatocellular carcinoma (HCC). Here, we aimed to explore the role and mechanism of liver graft injury mobilizing regulatory T cells (Tregs), which lead to late phase tumor recurrence after liver transplantation.

Methods: The correlation among tumor recurrence, liver graft injury and Tregs mobilization were studied in 257 liver transplant recipients with HCC and orthotopic rat liver transplantation models. The direct roles of CXCL10/CXCR3 signaling on Tregs mobilization and tumor recurrence were investigated in *CXCL10*$^{-/-}$ and *CXCR3*$^{-/-}$ mice models with hepatic IR injury.

Results: Clinically, patients received the graft with graft weight ratio (GWR) <60% had higher HCC recurrence after liver transplantation than the recipients with GWR ⩾60% graft. More circulating Tregs and higher intragraft TLR4/CXCL10/CXCR3 levels were detected in recipients with GWR <60% graft. These results were further validated in rat transplantation model. Foxp3^{+} cells and expressions of TLR4, CXCL10, TGFβ, CTLA-4 and CD274 were increased in rat liver tumor tissues from small-for-size graft group. In mouse model, the mobilization and recruitment of Tregs were decreased in *TLR4*$^{-/-}$, *CXCL10*$^{-/-}$ and *CXCR3*$^{-/-}$ mice compared to wild-type mice. Moreover, less CXCR3^{+} Tregs were recruited into liver in *CXCL10*$^{-/-}$ mice after hepatic IR injury. The knockout of *CXCL10* and depletion of Tregs inhibited tumor recurrence after hepatic IR injury.

Conclusion: CXCL10/CXCR3 signaling upregulated at liver graft injury directly induced the mobilization and intragraft recruitment of Tregs, which further promoted HCC recurrence after transplantation.

Lay summary: There were positive correlation among tumor recurrence, circulating Tregs and liver graft injury after human transplantation for HCC patients. The knockout of *CXCL10* decreased hepatic recruitment of CXCR3^{+} Tregs and late phase tumor recurrence after hepatic IR injury.

Cancer

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancy in the world and the second cause of cancer-related mortality [1]. Liver transplantation is an effective therapeutic modality for the treatment of HCC patients and offers higher long term survival prospects compared to liver resection and local ablation [2]. Living donor liver transplantation (LDLT) has been developed as an alternative choice to overcome the critical shortage of liver grafts from deceased donors, and to shorten the waiting time and decrease waitlist mortality [2]. With the accumulation of liver transplantation for HCC patients, liver tumor recurrence after transplantation has become an important issue affecting the outcomes of liver transplantation [3]. The current opinions regarding the difference in HCC recurrence between LDLT and deceased donor liver transplant (DDLT) remains controversial [4,5]. Several liver transplantation centers have reported that the tumor recurrence after LDLT is higher compared to DDLT [6–8]. The liver graft from living donor is usually small-for-size for the recipient, and is associated with a

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Abbreviations: HCC, hepatocellular carcinoma; IR, ischemia reperfusion; LDLT, Living donor liver transplantation; TLR4, Toll-like receptor 4; CXCL10, C-X-C motif chemokine 10; Tregs, regulatory T cells; SFS, small-for-size; OLT, orthotopic liver transplantation; CD279, the programmed death-1 receptor; MFI, mean fluorescence intensity.

higher incidence of acute-phase liver graft injury [9]. Several studies have demonstrated that liver graft injury promoted tumor cell adhesion, invasion, and angiogenesis [10–12]. However, the mechanism of liver graft injury promoting late phase tumor recurrence after transplantation has not been well defined.

Liver graft injury is typified as an amplified and deleterious inflammatory response [13]. A multitude of different cytokines and chemokines as well as immune cells are involved in these inflammatory process. Toll-like receptor 4 (TLR4) has been demonstrated to initiate the inflammatory response in hepatic ischemia reperfusion (IR) injury via the activation of IRF3 mediated MyD88-independent signal pathway [14,15]. C-X-C motif chemokine 10 (CXCL10), a downstream product of TLR4-IRF3 signaling, was also proven to be responsible for inflammatory response during hepatic IR injury [16]. Recent research showed that CXCL10 signaling contributes to melanoma growth and metastasis through regulation of cellular adhesion, invasion and migration properties [17]. Our previous paper also demonstrated that CXCL10 was overexpressed in small-for-size liver graft and promoted HCC invasiveness via regulating endothelial progenitor cells and macrophages [12,18]. Furthermore, CXCL10 can recruit more regulatory T cells (Tregs) trafficking to the liver through their surface receptor CXCR3 [19,20]. However, the role and mechanism of CXCL10/CXCR3 signaling in regulating Tregs mobilization and late phase tumor recurrence after liver transplantation are unclear.

Tregs, were first proposed in 1970 as the term for suppressor T cells, which play important roles in maintaining immune tolerance and controlling inflammatory diseases [21,22]. Tregs exert their suppressive effects either via cell contact dependent manner by membrane-bound molecules or through contact independent manner mainly by release of inhibitory cytokines [23]. Tregs can ligate CD80/CD86 on effector T cells and DCs through CTLA-4, and result in suppression of effector T cells function and T cells deletion [24]. Secretion of inhibitory cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10 can inhibit T cells proliferation and differentiation as well as suppress macrophages activation and dendritic cell (DC) maturation [25,26]. In addition, the programmed death-1 receptor (CD279 or PD-1) and its ligand (CD274 or PD-L1) also play important roles in promoting Tregs development and enhancing Tregs function [27,28]. Recently, Tregs are considered to play critical roles in the induction of transplant tolerance and prevent allograft rejection. Adoptive transfer of Tregs can regulate the rejection response of naïve T cells, prevent allograft rejection and prolong graft survival [29,30]. This negative regulatory activity, however, can also suppress the immune responses against tumors and thereby promote tumor growth and progress. In the cancer patients, Tregs are significantly mobilized to the peripheral blood and accumulate in tumor regions [31,32]. Furthermore, the high correlation between frequency of Tregs in cancer patients and their mortality were found in different cancers [32,33]. The Tregs isolated from the tumor have potent immunosuppressive activity against effector immune cell response and thereby help tumor escape from the host immunosurveillance [34]. However, the effect and mechanism of Tregs in tumor recurrence after transplantation is still unknown. Therefore, strategies aimed to deplete Tregs or to functionally inactivate Tregs is an interesting approach for increasing anti-cancer immunity thereby suppressing tumor growth and recurrence.

In this project, we aimed to study the role and mechanism of acute-phase liver graft injury mobilizing Tregs, which lead to late phase tumor recurrence after liver transplantation. The correlation among liver graft injury, inflammatory cytokines/chemokines, Tregs mobilization, and tumor recurrence were investigated in both human and rat liver transplantation. The direct roles of CXCL10/CXCR3 signaling on mobilization and recruitment of Tregs together with tumor recurrence were further explored in *CXCL10*$^{-/-}$ and *CXCR3*$^{-/-}$ mice model with hepatic IR injury.

Materials and methods

Patients and specimens

From 1995 to 2013, 257 patients with HCC received liver transplantation in Department of Surgery, Queen Mary Hospital, The University of Hong Kong, were included in current study. The graft weight ratio (GWR) is the liver graft weight divided by the estimated standard liver weight of the recipient [9]. According to GWR above or below 60%, they were categorized into GWR $\geqslant$60% group (large liver graft, n = 107) and GWR <60% group (small-for-size liver graft, n = 150). The human liver graft biopsy samples were collected at 2 h after portal vein reperfusion and blood samples were collected at day 0, day 7, 1 month, 3 months, 6 months and 12 months after transplantation. The study protocol was approved by the Institutional Review Board of the University of Hong Kong (IRB approval number: UW_11-099). Informed consent was obtained from each patient.

Animal models

Rats and C57BL/6 mice were purchased from the Laboratory Animal Unit, The University of Hong Kong (HKULAU). The *TLR4*, *CXCL10*, and *CXCR3* knockout mice were described in previous paper [12,35]. The study had been licensed according to Animal Ordinance Chapter 340 by the Department of Health, Hong Kong Special Administrative Region (ref.: (14–613) in DH/HA&P/8/2/3 Pt. 63).

Rat orthotopic liver transplantation models

Orthotopic rat liver transplantation model using whole graft or small-for-size graft (about 50%) was established [36]. Blood and liver samples were collected at day 1, 3 and 5 respectively after liver transplantation to analyze Tregs mobilization and intragraft recruitment. To detect Tregs and expressions of inflammatory cytokines/chemokines in recurrent tumors, a rat liver transplantation model with tumor recurrence was also used as described in our previously paper [10]. Liver graft samples and liver tumor tissues were collected at day 14 after liver transplantation for further analyses.

Mouse model with major hepatectomy and hepatic IR injury

TLR4, *CXCL10* and *CXCR3* knockout mice and wild-type mice were subjected to major hepatectomy (left and caudate lobes) and partial hepatic IR injury (45 min ischemia of right and triangle lobes), which mimics transplantation with small-for-size liver grafts [37]. Blood and liver samples were collected at day 1, 3 and 5 after reperfusion to analyze Tregs mobilization and intragraft recruitment.

In order to explore the effect of CXCL10 and Tregs on tumor recurrence, mouse HCC cells (Hepa1-6, 1×10^6/in 100 μl) were injected through portal vein after reperfusion in *CXCL10*$^{-/-}$ mouse, to mimic the clinical scenario of circulating tumor cells homing to the graft after liver transplantation. In the group of Tregs depletion, the anti-mouse CD25 antibody (250 μg) was injected into peritoneal at day 1 before operation, and day 5 after hepatic IR injury in wild-type mouse, respectively. Liver tumor tissues were collected at day 14 after the operation.

Detection of circulating Tregs by flow cytometry

The numbers of circulating and hepatic Tregs were detected by flow cytometry. The details of flow cytometry were described in our previous paper [38]. Cells were stained with PE-Cy5-conjugated anti-CD4 (eBioscience, San Diego, CA) and PE-conjugated anti-CD25 (BD Pharmingen, San Diego, CA), and then permeabilized with fixation/permeabilization working solution and incubated with FITC-conjugated anti-Foxp3 (eBioscience, San Diego, CA). The matched isotype controls were also prepared at the same time. Furthermore, the expressions of

CTLA-4, CD274, CD279, and CXCR3 in $CD4^+$ $CD25^+$ Tregs were also detected by flow cytometry. The expression levels of CTLA-4, CD274, and CD279 in liver Tregs were expressed by mean fluorescence intensity (MFI).

Isolation of spleen $CD4^+$ $CD25^+$ Tregs

The spleen $CD4^+$ $CD25^+$ Tregs were purified from C57BL/6 and *CXCL10*$^{-/-}$ mice using a $CD4^+$ $CD25^+$ Tregs isolation kit (Miltenyi Biotec, GmbH, Germany), according to the manufacturer's recommendation. The $CD4^+$ $CD25^+$ Tregs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with anti-CD3 (2.5 μg/ml), anti-CD28 (2.5 μg/ml) and IL-2 (500 U/ml). 3 days later, the expressions of CTLA-4, CD274 and CD279 in spleen Tregs were detected by flow cytometry.

Detection of gene expression by real-time RT-PCR

RT-PCR was done with a modified version of a previous method. Gene expression levels were expressed as the folds relative to the normal liver [37].

Statistical analysis

The Chi-square test was carried out for the analysis of clinical correlation among graft size clinical features and tumor recurrence after liver transplantation. The correlation among the intragraft expressions of TLR4, CXCL10, CXCR3 and Tregs were analyzed by Spearman correlation. All the comparisons for the study in animals were performed using Student *t* test. $p < 0.05$ was considered statistically significant. Calculations were performed by using the SPSS computer software version 16.

Results

The tumor recurrence rate was higher in HCC recipients with small-for-size liver graft

From 1995 to 2013, 257 patients with HCC received liver transplantation in our liver transplantation center. HCC recurrence occurred in 39 recipients after liver transplantation. The clinical parameters related to tumor recurrence were summarized in Table 1. Among these characteristics, the recurrence of HCC was more frequent in the patients with advanced TNM stage ($p = 0.003$), beyond Milan and University of California San Francisco (UCSF) criteria ($p = 0.007$ and $p = 0.000$ respectively), venous infiltration ($p = 0.000$), higher serum alphafetoprotein (AFP) ($p = 0.001$) and positive HBsAg ($p = 0.027$). In addition, tumor recurrence was also related to GWR ($p = 0.028$) (Table 1). According to GWR, the HCC recipients were categorized into GWR $\geqslant$60% group (large liver graft, n = 107) and GWR <60% group (small-for-size liver graft, n = 150). The comparison of the clinical-pathological characteristics between the HCC recipients in GWR $\geqslant$60% and GWR <60% group were listed in Table 2. Significant higher cumulative recurrence (29/150 (19.3%) *vs.* 10/107 (9.3%), $p = 0.028$) was found in the HCC recipients with small-for-size liver graft after transplantation (Table 2). The percentage of patients with advanced TNM stage, high AFP levels, and beyond Criteria of Milan or UCSF were similar between GWR $\geqslant$60% and GWR <60% groups (Table 2). However, the recipients in small-for-size liver graft group had higher venous infiltration (24/102 *vs.* 57/149, $p = 0.014$) and less hepatitis B Virus (HBV) positive patients (95/107 *vs.* 117/150, $p = 0.025$) (Table 2).

The circulating Tregs were higher in the recipients with small-for-size liver graft and HCC recurrence after human transplantation

Tregs can suppress the immune responses against tumors and thereby promote tumor growth. In order to investigate the mechanism of HCC recurrence after liver transplantation, we analyzed the mobilization of Tregs at different time points after human liver transplantation. Before transplantation, there was no difference of circulating Tregs between the recipients with large liver graft and small-for-size liver graft. The patients received small-for-size liver graft had more circulating Tregs at day 7, 1 months and 3 months after transplantation than those with large liver graft (Fig. 1A). Furthermore, more circulating Tregs were also found in patients with HCC recurrence at 1 month, 3 months and 6 months after transplantation than those without HCC recurrence (Fig. 1B).

Intragraft expressions of inflammatory cytokines/chemokines were increased in the recipients with small-for-size liver graft and HCC recurrence after human transplantation

In order to further explore the mechanism of Treg mobilization after liver transplantation, we compared the expressions of cytokines/chemokines between two groups. Our results indicated that there were significantly higher intragraft TLR4, CXCL10 and CXCR3 expression levels at 2 h after reperfusion in small-for-size liver graft compared with large liver graft (Fig. 2A). The expression of CD274 was also upregulated in the small-for-size liver graft (Supplementary Fig. 1A). Furthermore, the intragraft expressions of TLR4, CXCL10 and CXCR3 were also significantly higher in the recipients with HCC recurrence compared to those without HCC recurrence (Fig. 2B). There were positive correlations among the intragraft expressions of TLR4, CXCL10, CXCR3 and CD274 after transplantation (Supplementary Figs. 1C and 2). Importantly, there were positive correlation among the circulating Tregs and the intragraft expressions of TLR4, CXCL10, and CXCR3 after human transplantation (Fig. 2C).

Association among inflammatory cytokines/chemokines expressions, Tregs recruitment and tumor recurrence in rat orthotopic liver transplantation (OLT) models

We further compared the circulating mobilization and hepatic recruitment of Tregs between whole liver graft and small-for-size liver graft in rat OLT models. The recipient rats with small-for-size liver graft possessed approximately 2-fold increase of circulating Tregs at day 5 after transplantation *vs.* whole liver graft (Fig. 3A). Consistently, the number of Tregs recruited to small-for-size liver graft was higher than that in whole liver graft (Day 1: 268 cells *vs.* 139 cells/per 10^5 cells, $p = 0.04$; Day 5: 1019 cells *vs.* 480 cells/ per 10^5 cells, $p = 0.02$) (Fig. 3B). Furthermore, compared to whole liver graft group, intragraft mRNA expression level of *TLR4* was significantly elevated in small-for-size liver graft on day 3 and 5 after rat transplantation (Supplementary Fig. 3A). Our previous study also reported that the expressions of CXCL10 and CXCR3 were significantly elevated in small-for-size liver graft after rat transplantation [12].

We already reported greater tumor burden in small-for-size liver graft at day 14 after transplantation compared with whole liver graft in a rat OLT model with tumor recurrence [10]. In current study, we also found that there were more Foxp3 positive cells (8.7 cells *vs.* 4.7 cells/HPF, $p = 0.004$) in tumors developed in small-for-size liver graft (Fig. 3C). After recruitment and homing of Tregs to the tissue, Tregs exerted their suppressive effects either via cell contact dependent manner by membrane-bound molecules or through contact independent manner mainly by

Table 1. Analyses of risk factors for HCC recurrence after liver transplantation.

	Number	Recurrence N = 39	Non-recurrence N = 218	*p* value
Sex				0.264
Male	215	35	180	
Female	42	4	38	
Age				0.019*
≥55 years	143	15	128	
<55 years	114	24	90	
TNM stage[a]				0.003*
Early stage (I-II)	101	7	94	
Advanced stage (III-IV)	150	31	119	
Milan criteria[a]				0.007*
Within criteria	164	18	146	
Beyond criteria	88	21	67	
UCSF[a]				0.000*
Within criteria	191	19	172	
Beyond criteria	61	20	41	
Venous infiltration[a]				0.000*
Absent	170	15	155	
Present	81	23	58	
HBsAg				0.027*
Negative	45	2	43	
Positive	212	37	175	
AFP level				0.001*
≥20 ng/ml	130	29	101	
<20 ng/ml	127	10	117	
GWR				0.028*
≥60%	107	10	97	
<60%	150	29	121	

[a]Total number less than 257 due to missing data; *p <0.05.

Table 2. Clinico-pathological Characteristics of recipients with GWR ⩾60% and GWR <60% group.

Clinico-pathological features	Number	GWR ≥60% N = 107	GWR <60% N = 150	*p* value
Sex				0.611
Male	215	91	124	
Female	42	16	26	
Age				0.256
≥55 years	143	64	79	
<55 years	114	43	71	
TNM stage[a]				0.802
Early stage (I-II)	101	42	59	
Advanced stage (III-IV)	150	60	90	
Milan criteria[a]				0.868
Within criteria	164	67	97	
Beyond criteria	88	35	53	
UCSF[a]				0.926
Within criteria	191	77	114	
Beyond criteria	61	25	36	
Venous infiltration[a]				0.014*
Absent	170	78	92	
Present	81	24	57	
HBsAg				0.025*
Negative	45	12	33	
Positive	212	95	117	
AFP level				0.071
≥20 ng/ml	130	47	83	
<20 ng/ml	127	60	67	
One year recurrence				0.083
Recurrence	18	4	14	
Non-recurrence	239	103	136	
Cumulative recurrence				0.028*
Recurrence	39	10	29	
Non-recurrence	218	97	121	

[a]Total number less than 257 due to missing data; *p <0.05.

release of inhibitory cytokines. Both in tumor and non-tumor tissues, the expressions of TLR4, CXCL10, CD274 and IL-10 were higher in small-for-size liver graft than those in whole liver graft (Fig. 3D and Supplementary Fig. 3B). Consistently, the expressions of TGF-β and CTLA-4 were also increased in tumor tissues from small-for-size liver graft (Supplementary Fig. 3B). Furthermore, the expressions of TGF-β, CTLA-4, CXCL10, CXCR3, CD274 and CD279 were upregulated in tumor tissues compared to non-tumor tissues (Fig. 3D and Supplementary Fig. 3B).

The knockout of CXCL10/CXCR3 signaling reduced the mobilization and recruitment of Tregs

Both the clinical and animal studies showed the correlations among CXCL10/CXCR3 signaling, Tregs mobilization and tumor recurrence after liver transplantation. We then proceeded to investigate the direct role of CXCL10/CXCR3 signaling on Tregs mobilization and recruitment in mouse hepatic IR injury model using *TLR4*$^{-/-}$, *CXCL10*$^{-/-}$ and *CXCR3*$^{-/-}$ mice. A significant decrease of Tregs mobilization in circulation was observed in *TLR4*$^{-/-}$ mice compared to wild-type mice after hepatic IR injury (Day 3: 940 cells *vs.* 510 cells/per10^5 PBMC, $p = 0.04$; Day 5: 1134 cells *vs.* 541 cells/per10^5 PBMC, $p = 0.02$) (Fig. 4A). In order to further determine whether the mobilization and recruitment of Tregs after liver injury was dependent on the CXCL10/CXCR3 signaling, *CXCL10*$^{-/-}$ mice and *CXCR3*$^{-/-}$ mice hepatic IR injury model were applied. The circulating and hepatic Tregs were decreased both in *CXCL10*$^{-/-}$ and *CXCR3*$^{-/-}$ mice after hepatic IR injury (Fig. 4B and C). Before hepatic IR injury, there was no difference of circulating and hepatic Tregs among these knockout mice and wild-type mice.

Furthermore, we also investigated the expressions of TLR4, CXCL10 and CXCR3 in mouse liver after hepatic IR injury and major hepatectomy. The expressions of TLR4, CXCL10 and CXCR3 were significantly elevated in small liver remnant from wild-type mice at day 1, 3 and 5 after hepatic IR injury and liver resection (Supplementary Fig. 4A–C). The knockout of *TLR4* decreased the hepatic expressions of CXCL10 and CXCR3. The expressions of TLR4 and CXCR3 were also downregulated in the small liver remnant of *CXCL10*$^{-/-}$ mice after hepatic IR injury and major hepatectomy (Supplementary Fig. 4A–C). Furthermore, the expressions of CD274, CTLA-4 and IL-10 were reduced in *TLR4*$^{-/-}$, *CXCL10*$^{-/-}$ and *CXCR3*$^{-/-}$ mice compared to wild-type mice (Supplementary Fig. 4D–F). However, there was no significance in TGF-β expression among these knockout and wild-type mice.

The knockout of CXCL10 decreased hepatic recruitment of CXCR3$^+$ *Tregs after hepatic IR injury*

Since CXCL10 recruits cells via its cognate receptor CXCR3, the cells recruited must express CXCR3. Therefore, we next

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determined whether CXCR3$^+$ Tregs were recruited into the liver after hepatic IR injury. Before hepatic IR injury, there was similar CXCR3$^+$ Tregs in liver between *CXCL10*$^{-/-}$ mice and wild-type mice. The knockout of *CXCL10* resulted in a significant decrease in the number of hepatic CXCR3$^+$ Tregs compared with wild-type mice (Day 1: 226 cells *vs.* 64 cells/per10^5 NPCs, $p = 0.03$; Day 3: 152 cells *vs.* 35 cells/per10^5 NPCs, $p = 0.01$; Day 5: 324 cells *vs.* 50 cells/per10^5 NPCs, $p = 0.00$) (Fig. 5A). On the contrary, the number of circulating CXCR3$^+$ Tregs was higher in *CXCL10*$^{-/-}$ mice at day 1 and day 3 after hepatic IR injury compared with wild-type mice (Fig. 5B). It indicated that knockout of *CXCL10* significantly suppressed hepatic recruitment of CXCR3$^+$ Tregs.

The knockout of CXCL10 did not change the expressions of CTLA-4, CD274 and CD279 on Tregs

The association and functional study showed that CXCL10/CXCR3 signaling may contribute to Tregs mobilization and recruitment after liver transplantation. We further investigated the role of CXCL10/CXCR3 signaling on Tregs function both *in vivo* and *in vitro*. CTLA-4, CD274 and CD279 expressed in Tregs and play important roles in maintaining and enhancing Tregs function. Firstly, we detected the expressions of CD274, CD279 and CTLA-4 in Tregs recruited to liver after hepatic IR injury. The expression levels of CD274, CD279 and CTLA-4 in liver Tregs after hepatic IR injury were similar in *CXCL10*$^{-/-}$ mice and wild-type mice (Fig. 5C–E). Furthermore, we also examined the role of CXCL10 signaling on the function of the primary spleen Tregs from *CXCL10*$^{-/-}$ and wild-type mice. There were comparable percentages of CD274$^+$, CD279$^+$ and CTLA-4$^+$ spleen Tregs between *CXCL10*$^{-/-}$ group and wild-type group (Supplementary Fig. 5).

The knockout of CXCL10 and depletion of Tregs inhibited tumor recurrence after hepatic IR injury

In order to further explore the effects of CXCL10 and Tregs on late phase tumor recurrence, mouse hepatic IR injury model with tumor development was applied. Compared to wild-type mice, the knockout of *CXCL10* was significantly inhibited tumor development after hepatic IR injury (Fig. 6A and B). At 14 days after hepatic IR injury, hepatic replacement area by tumor was about 60.3% in wild-type mice when compared with 39.6% in *CXCL10*$^{-/-}$ mice. Furthermore, we also examined the effect of Tregs depletion on tumor development after hepatic IR injury. The depletion of Tregs by CD25 monoclonal antibody treatment was confirmed (Supplementary Fig. 6). The depletion of Tregs significantly inhibited tumor development after hepatic IR injury (Fig. 6A and B).

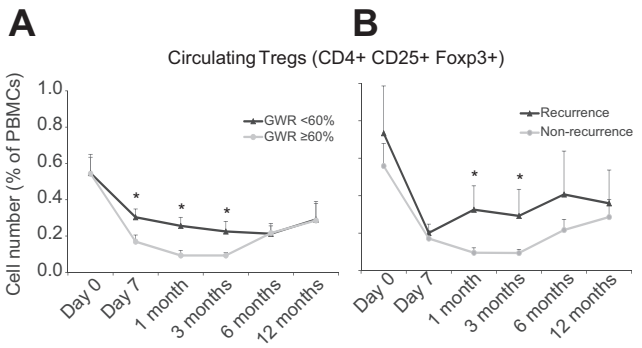

Fig. 1. The circulating Tregs were higher in patients with small-for-size liver graft and HCC recurrence after human transplantation. The circulating Tregs were detected at day 0, day 7, 1 month, 3 months, 6 months, and 12 months by flow cytometry. (A) The patients received small-for-size liver graft (GWR <60%) had more circulating Tregs at day 7, 1 months and 3 months after transplantation than patients with large liver graft (GWR ⩾60%). (B) More circulating Tregs were found in patients with HCC recurrence at 1 month, 3 months and 6 months after transplantation than patients without HCC recurrence. (*p <0.05.)

Discussion

Although liver transplantation is an effective therapeutic modality for the treatment of HCC patients, HCC recurrence after liver transplantation remains a critical issue [2]. The rate of HCC recurrence was significantly higher following LDLT compared to DDLT [7,8]. However, the precise mechanisms of tumor recurrence after liver transplantation haven't been clearly explored. Therefore, it is worthwhile to reveal the molecular mechanism of tumor recurrence after transplantation and to identify the potential therapeutic target for new treatment.

In this study, we explored for the first time the potential mechanism of Tregs in bridging liver graft injury and tumor recurrence after liver transplantation. In clinical cohort, 15.2% of the HCC recipients occurred tumor recurrence after liver transplantation. The recurrence of HCC was associated with advanced TNM stage, beyond Milan and UCSF criteria, venous infiltration, higher serum AFP and positive HBsAg. In addition, tumor recurrence was also related to GWR. The patients received small-for-size liver graft had significantly higher HCC recurrence incidence than those with large liver graft after transplantation. Our clinical outcome was similar to those with higher recurrence rates following LDLT compared to DDLT from other transplantation centers [7,8]. Importantly, similar proportion of the recipients with advanced TNM stage, high AFP levels, and beyond Criteria of Milan and UCSF was found between small-for-size graft and large graft. It suggested that small-for-size liver graft itself might be an independent risk factor and contribute to tumor recurrence after liver transplantation.

It has been reported that Tregs play important roles in tumor immunity [33]. There are more Tregs both in circulation and tumor tissues of the patients bearing tumor [39,40]. Tregs help tumor cells to escape from the host immunosurveillance by their potent immunosuppressive activity against effector immune cell response. Depletion and inhibition of Tregs promote development of tumor immunity and inhibit tumor growth [41,42]. In our clinical study, more circulating Tregs were found in the patients implanted with small-for-size liver graft and those developed HCC recurrences after transplantation. Consistently, there were more circulating and hepatic Tregs in small-for-size liver graft after rat transplantation. Furthermore, Foxp3 positive cells and expressions of TGF-β, CTLA-4 and CD274 were also increased in the tumor tissues from small-for-size graft group compared to whole graft group. In mouse model, the knockout of *CXLC10* also decreased the hepatic expressions of IL-10, CTLA-4, and CD274. Furthermore, the knockout of *CXCL10* and depletion of Tregs inhibited tumor recurrence after hepatic IR injury. Tregs can secret inhibitory cytokines such as TGF-β and IL-10, which can inhibit T-cell proliferation and differentiation as well as suppress macrophage activation and DC maturation [26,33]. CTLA-4-expressing Tregs can suppress effectors T cells

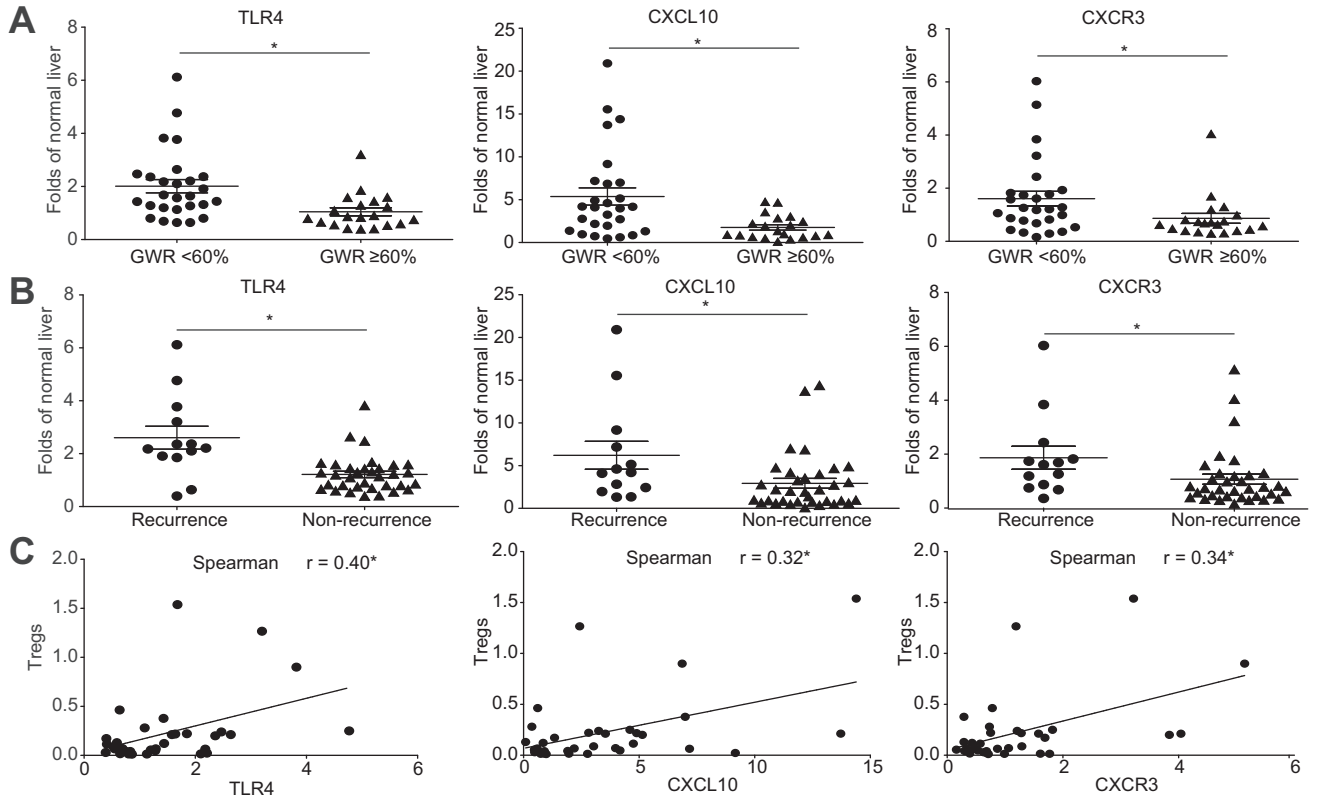

Fig. 2. Expressions of inflammatory cytokines/chemokines were increased in patients with small-for-size liver graft and HCC recurrence after human transplantation. Intragraft TLR4, CXCL10 and CXCR3 expression levels were detected at 2 h after liver transplantation. (A) There were significantly higher expressions of TLR4, CXCL10 and CXCR3 in small-for-size liver graft group compared with large graft group. (B) The post-transplant expressions of TLR4, CXCL10 and CXCR3 were significantly higher in the recipients with HCC recurrence compared to patients without HCC recurrence. (C) There were positive correlation among Tregs mobilization and the expressions of TLR4, CXCL10, and CXCR3 after human liver transplantation. (*p <0.05.)

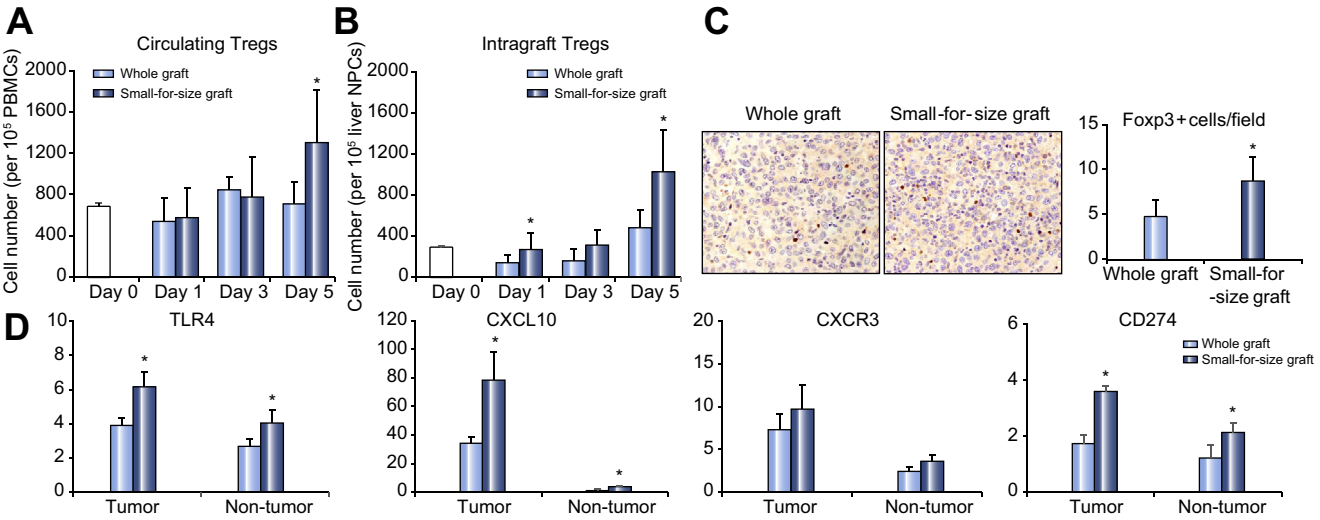

Fig. 3. Association among inflammatory cytokines/chemokines expressions, Tregs recruitment and intragraft tumor recurrence in rat orthotopic liver transplantation models. (A) The recipient rats with small-for-size liver graft possessed significantly more circulating Tregs at day 5 after transplantation *vs.* whole liver graft. (B) The number of Tregs recruited to small-for-size liver graft was higher than that in whole liver graft. (C) There were more Foxp3 positive cells in tumor tissues developed in small-for-size liver graft. (D) Both in tumor and non-tumor tissues, the expressions of TLR4, CXCL10 and CD274 were higher in small-for-size liver graft than those in whole liver graft. (N = 3–5/group; *p <0.05.)

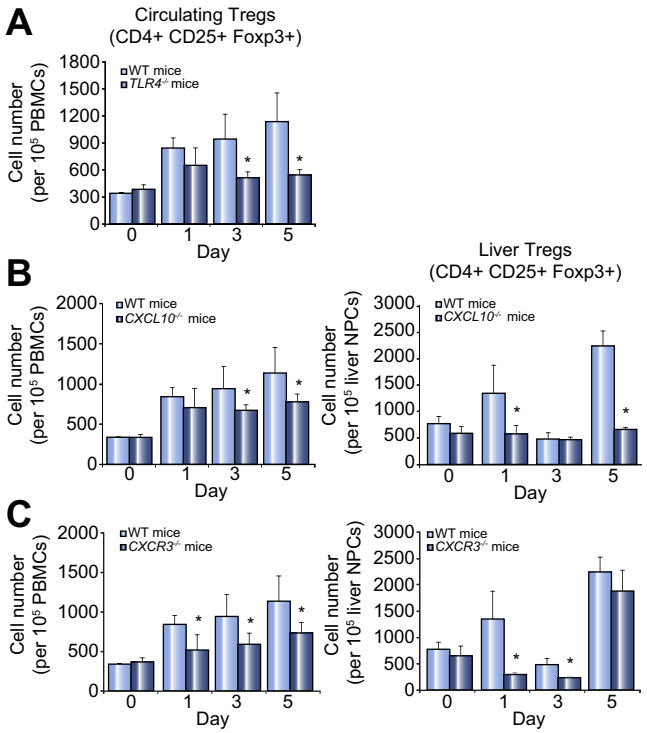

Fig. 4. The knockout of *CXCL10*/*CXCR3* signaling reduced the mobilization and recruitment of Tregs. (A) A significant decrease of Tregs mobilization in circulation was observed in $TLR4^{-/-}$ mice compared to wild-type mice after hepatic IR injury. (B) The circulating and hepatic Tregs were decreased in $CXCL10^{-/-}$ mice after hepatic IR injury compared to wild-type mice. (C) The knockout of *CXCR3* reduced the mobilization and recruitment of Tregs after hepatic IR injury. (N = 3–5/group; $^{*}p$ <0.05.)

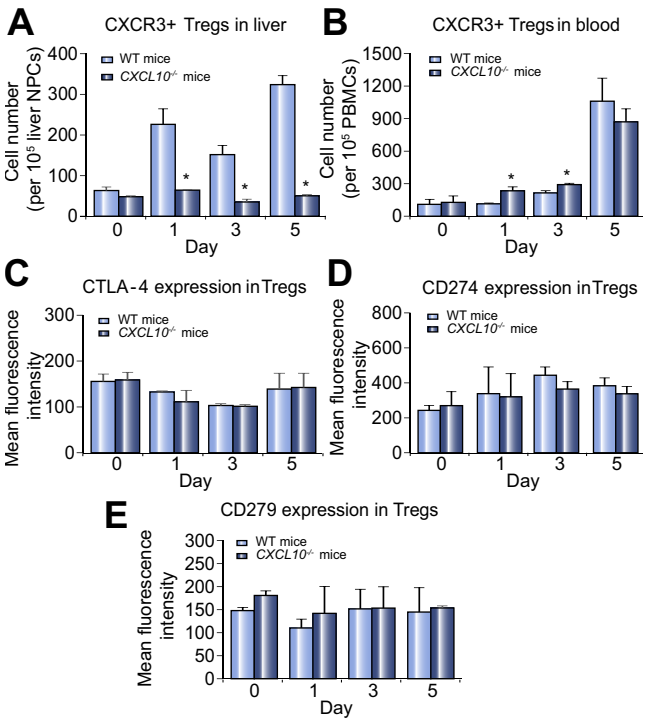

Fig. 5. The knockout of *CXCL10* decreased the hepatic recruitment of $CXCR3^{+}$ Tregs after hepatic IR injury. (A) The knockout of *CXCL10* resulted in a significant decrease in the number of hepatic $CXCR3^{+}$ Tregs compared to wild-type (WT). (B) The number of circulating $CXCR3^{+}$ Tregs was higher in $CXCL10^{-/-}$ mice at day 1 and day 3 after hepatic IR injury compared to WT mice. (C) The expression levels of CTLA-4, CD274 and CD279 in liver Tregs after liver IR injury were similar between $CXCL10^{-/-}$ mice and WT mice. (N = 3–5/group; $^{*}p$ <0.05.)

by ligating CD80, resulting in the inhibition of T-cell proliferation and cytokine production [43]. In addition, both CD274 and CD279 expressed in Tregs and play important roles in promoting Tregs development and enhancing Tregs function [27]. These results indicated that the mobilization of Tregs by small-for-size graft injury may contribute to HCC recurrence after liver transplantation. The effects of CTLA-4, CD279 and its ligands on Tregs function and their inhibition of anti-tumor immunity need to further studies.

The liver graft from a living donor is usually small-for-size for the recipient, and is associated with a higher incidence of acute-phase graft injury [9]. Several studies have demonstrated that small-for-size liver graft injury has serious inflammatory response and higher expressions of inflammatory cytokines/chemokines [9,16]. In this study, we found that the expressions of TLR4, CXCL10 and CXCR3 were significantly increased in small-for-size liver graft after transplantation both in human and rat. Importantly, the expressions of TLR4, CXCL10 and CXCR3 were correlated with the frequency of circulating Tregs. Recently research showed that CXCL10 can recruit more Tregs trafficking to the liver through their surface receptor CXCR3 after natural killer (NK) cells activation [19]. CXCR3 is expressed on Tregs and mediates Tregs mobilization [20,44]. However, the effect of CXCL10/CXCR3 in regulating Tregs mobilization and recruitment after liver transplantation has not been clearly explored. We investigated for the first time the direct roles of TLR4, CXCL10 and CXCR3 on mobilization and recruitment of Tregs using $TLR4^{-/-}$, $CXCL10^{-/-}$ and $CXCR3^{-/-}$ mice underwent hepatic IR injury and major hepatectomy. Significant less circulating mobilization and hepatic recruitment of Tregs were found in $TLR4^{-/-}$, $CXCL10^{-/-}$ and $CXCR3^{-/-}$ mice. Our results also showed that the number of $CXCR3^{+}$ Tregs recruitment into the liver was decreased in $CXCL10^{-/-}$ mice after hepatic IR injury. These results indicated that liver graft injury upregulated intragraft expressions of TLR4 and CXCL10, which further recruited the $CXCR3^{+}$ Tregs into the liver. We also investigated the effect of CXCL10 signaling in the function of Tregs through *in vitro* and *in vivo* experiment. Our results showed that the expression levels of CD274, CD279 and CTLA-4 were similar in liver Tregs after hepatic IR injury and primary Tregs from spleen between $CXCL10^{-/-}$ group and wild-type group. These results suggested that CXCL10 may only contribute to Tregs mobilization and recruitment but do not change Tregs function. Several studies have demonstrated that other immune cells such as B cells, NK cells and effector T cells can also express CXCR3 and mediates these cells mobilization [45,46]. The effect of CXCL10 on other immune cells mobilization and their roles in liver tumor recurrence after transplantation will be definitely worthwhile for further studies.

In conclusion, we first defined the correlation among liver graft injury, Tregs and late phase tumor recurrence after liver transplantation. Post-transplant enhanced CXCL10/CXCR3 signaling in acute-phase liver graft directly induced the mobilization and recruitment of Tregs, which further promoted liver tumor growth and recurrence after transplantation. Therefore, targeting at CXCL10/CXCR3 signaling may attenuate acute-phase liver graft

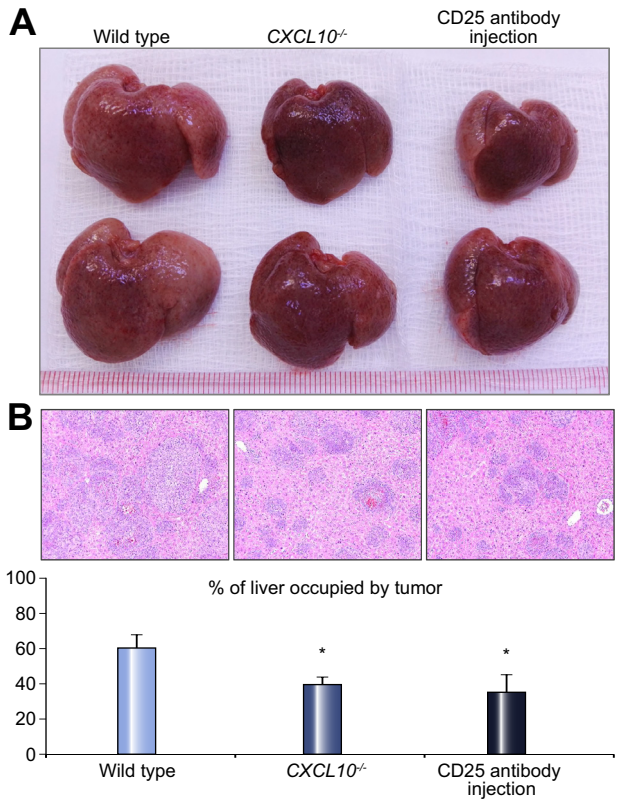

Fig. 6. The knockout of *CXCL10* and depletion of Tregs inhibited tumor recurrence after hepatic IR injury. (A) The knockout of *CXCL10* and depletion of Tregs significantly inhibited tumor development at 14 days after hepatic IR injury and (B) Decreased the percentage of hepatic replacement area by tumor. (N = 3–4/group; $^{*}p$ <0.05.)

injury induced Tregs mobilization and recruitment, and then prevent late phase tumor recurrence.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

Study concept and design: CX Li, CC Ling, CM Lo, K Man; Acquisition of data: CX Li, Y Shao, CC Ling, XB Liu, YY Ma; Analysis and interpretation of data: CX Li, Y Shao; Drafting of the manuscript: CX Li; Critical revision of the manuscript for important intellectual content: K Man; Obtained funding: CM Lo, K Man; Administrative, technical, or material support: X Qi, KTP Ng, XC Li, A Xu, Y Zhai, OWH Yeung, H Liu, J Liu, QS Liu, XX Yang, YF Lam; Study supervision: CM Lo, K Man.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2016.05.032.

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Author names in bold designate shared co-first authorship

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