T cell CEACAM1 – TIM-3 crosstalk alleviates liver transplant injury in mice and humans

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Abbreviations used in this paper: sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; CC1, carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1); DAMPs, danger-associated molecular patterns; EAD, early allograft dysfunction; FBS, fetal bovine serum; HMGB1, high morbidity group protein B1; HO-1, heme-oxygenase-1; HPF, high-power field; HRP, horseradish peroxidase; IFN-γ, interferon-γ; IκBα , inhibitory subunit of NF-κB alpha; IRI, ischemia-reperfusion injury; ITIM, immune receptor inhibitory motifs; LDH, lactate dehydrogenase; LSECs, liver sinusoidal endothelial cells; NF-κB, nuclear factor kappa B; OLT, orthotopic liver transplantation; NLRP-3, Nod-like receptor pyrin domain containing 3; PBLs, peripheral blood lymphocytes; POD, postoperative day; PRRs, pattern recognition receptors; PtdSer, phosphatidylserine; ROS, reactive oxygen species; RT-PCR, reverse-transcription polymerase chain reaction; T-bet, T-box protein expressed in T cells; TIM-3, T cell immunoglobulin domain and mucin domain-containing protein 3; TLR, Toll-like receptor; TNF-α, tissue necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UW, University of Wisconsin; VCL, vinculin; WT, wild type.

Abstract

Background&Aims: CEACAM1 (CC1) acts through homophilic and heterophilic interactions with TIM-3, which regulates innate immune activation in orthotopic liver transplantation (OLT). We investigated whether $CD4^+$ T cell-dependent CC1-TIM-3 crosstalk may affect OLT outcomes in mice and humans.

Methods: Wild-type (WT) and CC1-deficient (CC1KO) mouse livers were transplanted into WT, CC1-deficient (CC1KO), or T cell TIM-3 transgenic (TIM-3Tg)/CC1KO double mutant recipients. $CD4^+$ T cells were adoptively transferred into T/B cell-deficient Rag2KO recipients, followed by OLT. The perioperative liver-associated CC1 increase was analyzed in fifty human OLT patients.

Results: OLT injury in WT livers deteriorated in CC1KO compared to CC1-proficient (WT) recipients. The frequency of $TIM\text{-}3^+CD4^+$ T cells was higher in WT than CC1KO hosts. Reconstitution of Rag2KO mice with CC1KO-T cells increased NF-κB phosphorylation and OLT damage compared to recipients repopulated with WT-T cells. T cell TIM-3 enhancement in CC1KO recipients (WT→TIM3Tg/CC1KO) suppressed NF-κB phosphorylation in Kupffer cells and mitigated OLT injury. However, TIM-3-mediated protection was lost by pharmacologic TIM-3 blockade or an absence of CC1 in the donor liver (CC1KO→TIM-3Tg/CC1KO). The perioperative CC1 increase in human OLT reduced hepatocellular injury, early allograft dysfunction, and cumulative rejection rate.

Conclusion: This translational study identifies T cell-specific CC1 signaling as a therapeutic means to alleviate OLT injury by promoting T cell intrinsic TIM-3, which in turn interacts with liver-associated CC1 to suppress NF-κB in Kupffer cells. By suppressing peritransplant liver damage, promoting T cell homeostasis, and improving OLT outcomes, recipient CC1 signaling serves as a novel cytoprotective sentinel.

Keywords: T cells; CEACAM1; T cell immunoglobulin domain and mucin domain-protein 3 (TIM-3); NF-κB; Liver transplantation

Introduction

Innate immune-dominated hepatic sterile inflammation following reperfusion drives liver ischemia-reperfusion injury (IRI) in orthotopic liver transplantation (OLT), contributing to early allograft dysfunction (EAD), acute rejection, and diminished long-term survival[1]. IR-induced hepatocellular injury triggers the release of danger-associated molecular patterns (DAMPs), which activate pattern recognition receptors (PRRs), such as Toll-like receptor (TLR) family, to initiate an inflammatory immune cascade[2,3]. However, consistent with others[4,5], we have documented that $CD4^+$ T cell activation can modulate IR-stress in OLT[6-8], confirming the pathogenic function of adaptive immunity in the mechanism of IRI-OLT in mice and humans.

T cell immunoglobulin domain and mucin domain-containing protein 3 (TIM-3) on the IFN-γ-producing activated Th1 cells was originally shown to regulate macrophage activation/function in the induction of autoimmune diseases[9]. It is currently known that in addition to $CD4^+$ T cells, regulatory T cells, myeloid cells, NK cells, and mast cells can express TIM-3[10]. We have shown that T cell TIM-3 signaling determines the severity of liver IRI in a TLR4-dependent manner and drives inhibitory regulation in OLT[11-13].

In addition to galectin-9, phosphatidylserine (PtdSer), and high morbidity group protein B1 (HMGB1)[14-16], carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1; CC1; *CD66a*) was recently discovered as a cell surface TIM-3 ligand[17]. Notably, CC1 acts through homophilic and heterophilic interactions with TIM-3. CC1–TIM-3 *cis*-interaction promotes the stability of mature TIM-3 on the cell surface, and both the *cis*- and *trans*-interactions drive the inhibitory TIM-3 functions. We have reported on the protective function of galectin-9 in IR-triggered OLT damage through T cell repression and the contribution of liver-associated CC1 in cold-stored grafts in mice and humans[12,18]. However, as the role of CC1–TIM-3 axis in the mechanism of innate immune-driven OLT injury remains to be elucidated, we hypothesized that by crosslinking with T cell-specific TIM-3, CC1 expressed by transplanted livers might have a protective role in OLT recipients.

In the present study, we used a clinically relevant mouse OLT model with extended liver cold storage, mimicking the “marginal” donor livers, and human liver transplant biopsies to investigate whether/how CC1-TIM-3 axis can regulate IRI-OLT in mice and humans. We demonstrated that T cell-specific CC1 enhanced TIM-3 expression on $CD4^+$ T cells, which in turn could interact with liver-associated CC1 to suppress the NF-κB in Kupffer cells and alleviate OLT injury. In the absence of CC1, TIM-3 expression on T cells and the ability to elicit its inhibitory regulation in OLT was diminished, suggesting CC1-TIM-3 crosstalk is critical for TIM-3 to protect OLT. Our clinical findings showed that increased recipient CC1 levels improved outcomes by suppressing acute liver injury and promoting T cell homeostasis in human OLT. This translational study provides the rationale for adjunctive strategies targeting $CD4^+$ T cell CC1 to alleviate hepatocellular injury in OLT patients.

Methods

Clinical liver transplant study

This study was approved by the UCLA Institutional Research Board (IRB #13-000143, 18-000216). Patients provided informed consent before they participated in the study. We performed a retrospective analysis of 60 adult patients who underwent OLT (May 2013-August 2015) and received routine standard of care and immunosuppressive therapy. Recipients who underwent re-transplantation were excluded. Donor livers, procured from donation after brain or cardiac death, were stored in the University of Wisconsin (UW) solution (Niaspan; Bristol-Meyers Squibb, Princeton, NJ). Pretransplant and posttransplant Tru-Cut needle biopsies from the left liver lobe were obtained after cold storage at the back table (before implantation) and about 2h after portal reperfusion (before abdominal closure). Hepatic biopsies were screened by qRT-PCR with β-actin normalization for CC1, CD154, CD28, IFN-γ, IL17, TLR2, TLR4, TLR9, CD68, Cathepsin G, heme-oxygenase-1 (HO-1), and by western blots with β-actin normalization for CC1 expression. Gene and protein expression of post-/pre-CC1 ratio was evaluated in 27 and 50

patients, respectively. Recipient blood was collected before transplant and at postoperative days (POD) 1–7. Liver injury was evaluated by serum aspartate aminotransferase (sAST) and alanine aminotransferase (sALT) levels. EAD was defined as described[1].

Animals

Wild type (WT) and Rag2-deficient (Rag2KO) mice were purchased (The Jackson Laboratory, Bar Harbor, ME). Global CC1-deficient (CC1KO) mice were provided by Dr. Maciej Kujawski (Beckman Research Institute, City of Hope, CA); originally generated by Dr. Nicole Beauchemin (McGill University, Montreal, Canada)[19]. T cell TIM-3 transgenic (TIM-3Tg) mice, provided by Dr. Vijay Kuchroo (Harvard University, Boston, MA), were generated by expressing full-length TIM-3 cDNA under control of human CD2 promoter[20]. We generated TIM-3Tg/CC1KO double mutant mice by breeding TIM-3Tg and CC1KO mice. All mice were at the C57BL/6 background and used at 8-11 weeks of age. Animals were housed in the UCLA animal facility under pathogen-free conditions and received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011). Their use was approved by UCLA Animal Research Committee (ARC #1999-094).

Mouse OLT model

We used a mouse model of hepatic cold storage and OLT, as described by our group[21]. To mimic marginal human OLT, donor livers were stored in UW solution (4°C/18h). Liver graft/serum samples were collected at 6h after reperfusion, the peak of hepatocellular damage in this model. To neutralize TIM-3, we used anti-TIM-3 mAb (BE0115, clone: RMT3-23, Bio X cell, Lebanon, NH) at 0.5 mg/mouse i.p. at 1 day prior to surgery[11]. The sham group underwent the same procedures except for OLT.

Statistics

For mouse experiments, comparisons between two groups were assessed with Student's *t*-test. For human data, continuous values were analyzed by Mann-Whitney U test and

categorical variables by Fisher's exact test. Spearman's correlation coefficient (*r*) was used to evaluate the strength of the linear relationship between variables. Cumulative rejection rates were evaluated using Kaplan-Meier methods, and the curves were compared using log-rank test. All *P* values were 2-tailed, and *P* less than 0.05 was considered statistically significant. GraphPad Prism 9 was used for statistical analyses.

Results

Recipient CC1 signaling mitigates the hepatocellular injury in IR-stressed mouse OLT

To investigate the role of recipient CC1 signaling in liver transplantation, we first used CC1KO mice as recipients of WT liver grafts in a clinically relevant model with extended donor organ cold storage[21]. IR-stressed OLT injury, assessed by Suzuki's histological score (sinusoidal congestion, hepatocellular vacuolization, and necrosis)[22] was significantly higher in CC1KO than WT recipients (CC1KO=7.9±0.8 vs. WT=5.2±0.5, $P<0.05$) at 6h after reperfusion (Figure 1A/B). Similarly, the frequency of TUNEL-positive cells, indicative of cellular death, was increased in CC1KO compared to WT counterparts (CC1KO=100.1±11.1 cells/HPF vs. WT=63.7±8.2 cells/HPF, $P<0.05$) (Figure 1A/B). In agreement with histological findings, sAST, sALT, and LDH levels were significantly elevated in CC1KO compared to WT hosts (AST=CC1KO 6678±842 vs. WT 3947±544, $P<0.05$; ALT=CC1KO 9663±959 vs. WT 5196±670, $P<0.01$; LDH=CC1KO 9841±1206 vs. WT 5776±816, $P<0.05$) (Figure 1C/D). Thus, CC1 signaling at the recipient site attenuated IRI-OLT.

While the mRNA levels coding for IFN-γ, IL-6, and IL-17 in OLT were significantly higher in CC1KO compared to WT hosts, the granzyme B and perforin 1 expression were comparable between CC1KO and WT recipients (Figure 1E). These indicates that $CD4^+$ T cell proinflammatory phenotype rather than $CD8^+$ T cells/NK cells contributed to the disruption of CC1 signaling in OLT recipients. At the same time, mRNA levels coding for TLR4, IL-1β, and TNF-α in OLT were

elevated in CC1KO compared to WT recipients (Figure 1F), suggesting recipient CC1-mediated signaling suppressed PRR activation/inflammatory cytokine program. We next evaluated the phosphorylation of inhibitory subunit of NF-κB alpha (IκBα) and NF-κB p65, a major downstream TLR4 signaling marker[23]. Phosphorylation of NF-κB p65/IκBα was markedly increased in OLT after disruption of host CC1 signaling (Figure 1G), suggesting that CC1 at the recipient site suppressed TLR4 and its downstream NF-κB pathway.

CC1 signaling enhances TIM-3 expression and suppresses proinflammatory signature in $CD4^+$ T cells

In addition to our previous finding that TIM-3 activation suppressed the NF-κB in IR-stressed liver[11], CC1 was discovered to promote cell surface TIM-3 expression through *cis*-interactions[17]. Therefore, we hypothesized that recipient CC1 signaling might suppress NF-κB pathway and OLT injury through crosslinking with TIM-3. We evaluated TIM-3 expression in PBLs immune cells, i.e., $CD4^+$ / $CD8^+$ T cells, monocytes ($CD11b^+Ly6G^-$), and neutrophils ($Ly6G^+$) from naive WT vs. CC1KO mice. Indeed, TIM-3 expression was observed selectively in $CD4^+$ T cells and monocytes ($CD11b^+Ly6G^-$) under homeostatic conditions (Figure 2A and Supplementary Figure 1A). Notably, most of TIM-3^+ cells co-expressed CC1 in WT $CD4^+$ T cells and monocytes (WT $CD4^+$ T cells: TIM-3^+CC1^+ 87.2±1.4% vs. TIM-3^+CC1^- 12.8±1.4%, P<0.0001; WT monocytes: TIM-3^+CC1^+ 98.8±0.5% vs. TIM-3^+CC1^- 1.2±0.5%, P<0.0001) (Figure 2B and Supplementary Figure 1B). Although CC1-deficient $CD4^+$ T cells also expressed TIM-3, the frequency of TIM-3^+ $CD4^+$ T cells was significantly higher in WT compared to CC1KO mice (WT=13.6±2.0% vs. CC1KO=7.3±0.9%, P<0.05) (Figure 2C). In contrast, the frequency of TIM-3^+ monocytes was similar in WT and CC1KO mice (WT=28.5±2.2% and CC1KO=24.2±3.0%, P=0.286) (Supplementary Figure 1C). While there was a trend for a higher frequency of TIM-3^+ cells in naive $CD8^+$ T cells from WT compared to CC1KO mice, only few TIM-3^+ cells could be detected at steady-state on $CD8^+$ T cells (WT=1.1±0.2% vs. CC1KO=0.5±0.2%, P=0.066) (Figure 2C). Similarly, TIM-3^+ cells were rarely observed in naive WT or CC1KO neutrophils (Supplementary

Figure 1C). Collectively, CC1 signaling efficiently enhanced TIM-3 expression in $CD4^+$ T cells even in their physiological steady-state, as described[17].

Most of TIM-3^+ cells in PBLs of mice subjected to liver IRI co-expressed CC1 in WT $CD4^+$ T cells (Figure 2D/E). Further, the frequency of activated TIM-3^+CD4^+ T cells was significantly higher in WT compared to CC1KO IR-stressed mice (WT=28.2±3.1% vs. CC1KO=14.5±1.7%, $P<0.01$) (Figure 2F). We confirmed that CC1 and TIM-3 were rarely seen on activated $CD8^+$ T cells (Figure 2F), suggesting CC1-TIM-3 signaling in $CD8^+$ T cells has little effect on acute liver IRI. Interestingly, the frequency of CC1 and TIM-3 expressing $CD4^+$ T cells significantly increased in WT hosts after liver IRI compared with naive counterparts (CC1: $P<0.05$, TIM-3: $P<0.01$) (Figure 2G/H). Moreover, the increase of TIM-3^+CD4^+ T cells was higher in WT than CC1KO recipients (WT: 13.58±1.99% - 28.21±3.11%, $P=0.0074$ vs. CC1KO: 7.32±0.93% - 14.46±1.74, $P=0.011$) (Figure 2H), supporting the notion that host CC1 signaling can enhance TIM-3 expression on T cells as reported[17].

We used qRT-PCR to evaluate T cell cytokines in purified $CD4^+$ T cell cultures. Disruption of CC1 signaling enhanced IFN-γ, T-bet, IL-6, IL-17, IL-22, and TNF-α in anti-CD3 and anti-CD28 stimulated as well as non-stimulated $CD4^+$ T cells (Figure 2D). Thus, *in vitro* CC1 signaling suppressed the inflammatory signature in $CD4^+$ T cell cultures.

$CD4^+$ T cell-specific CC1 protects OLT against IR-stress in the Rag2 KO mouse model

To elucidate the contribution of T cell-specific CC1 in OLT injury, we first repopulated Rag2KO mice (lacking mature T/B cells) with purified $CD4^+$ T cells from WT or CC1KO donors. These were then challenged with cold-stored WT liver grafts (Figure 3A). The purity of splenic $CD4^+$ T cells was over 95% (Figure 3B). The CD4/CC1 immunofluorescence staining confirmed reconstitution of Rag2KO mice with $CC1^+CD4^+$ T cells (Figure 3C). In contrast, while adoptively transferred $CD4^+$ T cells were readily detected in IR-stressed OLT, CD4/CC1 double-positive cells were absent following reconstitution with CC1-deficient $CD4^+$ T cells (Figure 3C). Functionally, transfer of WT $CD4^+$ T cells increased OLT injury in Rag2KO recipients, evidenced by Suzuki's

score (no $CD4^+$ T cells=2.8±0.4 vs. WT $CD4^+$ T cells=5.2±0.4, P<0.01), TUNEL+ cells (no $CD4^+$ T cells=23.4±3.8 cells/HPF vs. WT $CD4^+$ T cells=40.6±4.5 cells/HPF, P<0.05), and sAST/sALT (AST: no $CD4^+$ T cells=1555±157 vs. WT $CD4^+$ T cells=2578±406, P<0.05; ALT: no $CD4^+$ T cells=2453±437 vs. WT $CD4^+$ T cells=4279±390, P<0.05) (Figure 3D–G). Transfer of CC1-deficient $CD4^+$ T cells further exacerbated OLT damage in Rag2KO recipients compared to WT counterparts (CC1KO $CD4^+$ T cells Suzuki's score=7.2±0.7 vs. WT, P<0.05; TUNEL+ cells=58.0±5.2 cells/HPF vs. WT, P<0.05; ALT=7208±875 vs. WT, P<0.05) (Figure 3D–G). Thus, $CD4^+$ T cell-specific CC1 signaling was required for OLT hepatoprotection.

We screened for $CD4^+$ T cell-related cytokines and innate immune activation in IR-stressed WT livers transplanted into Rag2KO recipients. The reconstitution with $CD4^+$ T cells increased mRNA levels coding for IFN-γ (P<0.05), IL-6 (P<0.05), IL-17 (P=0.078), TLR4 (P=0.063), and IL-1β (P=0.084) in OLT (Figure 3H), confirming adoptively transferred $CD4^+$ T cells repopulated Rag2KO recipients. These gene expression levels increased further after transfer of CC1-deficient (CC1KO) compared to CC1-proficient (WT) $CD4^+$ T cells (IFN-γ, P<0.05; IL-6, P<0.05; IL-17, P<0.05; TLR4, P<0.05; IL-1β, P=0.064) (Figure 3H). Of note, unlike WT-T cells, adoptive transfer of CC1-deficient $CD4^+$ T cells significantly augmented NF-κB p65 phosphorylation in OLT compared to recipients of WT $CD4^+$ T cells (P<0.01, Figure 3I). Taken together, in addition to $CD4^+$ T cell-related inflammation, T cell-specific CC1 signaling suppressed TLR4 and NF-κB p65 activation in the OLT-Rag2KO mouse system.

Enhanced T cell TIM-3 signaling in CC1-deficient mice alleviates IRI and Kupffer cell NF-κB activation in OLT

To directly investigate the role of TIM-3 in CC1 signaling, we generated TIM-3Tg/CC1KO double mutant mice. We confirmed enhanced TIM-3 expression on their $CD4^+$ and $CD8^+$ T cells (but not $CD4^-$/$CD8^-$ cells), in parallel with profound CC1-deficiency (Supplementary Figure 2). We then evaluated IRI-OLT in groups of TIM-3Tg/CC1KO vs. TIM-3Tg/CC1KO + anti-TIM-3 Ab treated recipients, in addition to WT vs. CC1KO recipients of WT liver grafts. T cell-

specific TIM-3 enhancement significantly mitigated IRI-OLT in CC1KO recipients of WT livers (TIM-3Tg/CC1KO Suzuki's score=5.5±0.6 vs. CC1KO, P<0.05; TUNEL+ cells=69.1±6.1 cells/HPF vs. CC1KO, P<0.05; AST=4525±747 vs. CC1KO, P=0.077; ALT=6220±1140 vs. CC1KO, P<0.05) (Figure 4A–C). We confirmed that TIM-3 neutralization negated its inhibitory function in TIM-3Tg/CC1KO recipients (TIM-3Tg/CC1KO + anti-TIM-3 Suzuki's score=9.2±0.8 vs. TIM-3Tg/CC1KO, P<0.01; TUNEL+ cells=96.5±10.2 cells/HPF vs. TIM-3Tg/CC1KO, P<0.05; AST=7808±812 vs. TIM-3Tg/CC1KO, P<0.05; ALT=12959±853 vs. TIM-3Tg/CC1KO, P<0.001) (Figure 4A–C). Furthermore, proinflammatory mRNA levels in OLT of CC1KO recipients decreased with concomitant enhancement of T cell-specific TIM-3 signaling (IFN-γ, P<0.05; IL-6, P<0.05; IL-17, P=0.072; TLR4, P<0.05; IL-1β, P<0.05; TNF-α, P=0.091) (Figure 4D/E). Strikingly, augmented NF-κB p65 seen in CC1KO recipients was markedly suppressed by T cell-specific TIM-3 enhancement (in double mutants), whereas TIM-3 neutralization reversed NF-κB p65 suppression seen in TIM-3Tg/CC1KO hosts (Figure 4F). Collectively, T cell TIM-3 signaling alleviated IRI-OLT while suppressing TLR4 and NF-κB as well as $CD4^+$ T cell-associated inflammatory phenotype.

We next sought to identify the cell type that downregulated NF-κB due to T cell-mediated TIM-3 signaling. The NF-κB phosphorylation was comparable in $CD4^+$, $CD8^+$, and $CD11b^+$ cells obtained from WT, CC1KO, and TIM-3Tg/CC1KO mice, in both naive and activated conditions (Supplementary Figure 3). We then performed immunostaining in OLT and observed that NF-κB p65 was localized selectively in the nucleus of non-parenchymal cells in CC1-deficient and suppressed in TIM-3Tg/CC1KO hosts (Figure 4G). Subsequent immunofluorescence staining of NF-κB p65 and CLEC4F, a marker for Kupffer cells, revealed that the signal for NF-κB p65 phosphorylation overlapped with Kupffer cells in CC1KO recipients and was suppressed in TIM-3Tg/CC1KO recipients (Figure 4H). We confirmed NF-κB phosphorylation was absent in liver sinusoidal endothelial cells (LSECs), based upon stabilin-2 expression (Supplementary Figure 4). Thus, T cell-specific TIM-3 signaling suppressed Kupffer cell NF-κB signaling in OLT.

Liver-associated CC1 deficiency compromises T cell TIM-3 regulation in CC1-deficient recipients

To evaluate the role of CC1 as a TIM-3 ligand, we transplanted CC1-deficient livers into TIM-3Tg/CC1KO double mutant or CC1KO recipients (Figure 5A). In contrast to TIM-3Tg/CC1KO recipients of WT grafts (WT→TIM-3Tg/CC1KO) (Figure 4A-C), overexpression of T cell-specific TIM-3 failed to attenuate IRI-OLT in CC1 deficient OLT combinations (CC1KO→CC1KO or CC1KO→TIM-3Tg/CC1KO; Suzuki's score=8.5±0.9 vs. 8.8±0.8, *P*=0.790; AST=7020±1206 vs. 6083±1102, *P*=0.579; ALT=8322±1542 vs. 10361±1834, *P*=0.415) (Figure 5B/C). In agreement with these findings, Kupffer cell NF-κB p65 activation was not suppressed in TIM-3Tg/CC1KO recipients of CC1KO grafts (Figure 5D and Supplementary Figure 5A). Moreover, intra-graft inflammatory gene profile did not decrease despite TIM-3 enhancement in the absence of liver-associated CC1 (IFN-γ, *P*=0.281; IL-6, *P*=0.220; IL-17, *P*=0.563; TLR4, *P*=0.731; IL-1β, *P*=0.188; TNF-α, *P*=0.220) (Figure 5E/F). Notably, while NF-κB p65 was markedly suppressed in TIM-3Tg/CC1KO recipients in the presence of hepatic CC1, TIM-3 overexpression failed to suppress NF-kB in the absence of CC1 on the donor liver (Figure 5G). Taken together, although T cell-specific TIM-3 interacted with liver-associated CC1 to suppress NF-kB in Kupffer cells to mitigate IRI, CC1 signaling was critical to elicit TIM-3 inhibitory regulation in OLT. Consistent with these *in vivo* findings, significantly higher expression levels of IFN-γ, IL-6, IL-17, and IL-22 were recorded in $CD4^+$ T cell cultures from TIM-3Tg/CC1KO compared to TIM-3Tg mice (Supplementary Figure 5B), implying that TIM-3 enhanced-$CD4^+$ T cells lost their suppressive signature in the absence of CC1 signaling.

Enhanced perioperative CC1 levels promote anti-inflammatory phenotype in human OLT

Having shown the regulatory function of $CD4^+$ T cell CC1 in mouse OLT recipients, we next assessed its putative role in human liver transplant patients. Hepatic biopsies were collected after cold storage before liver implantation, and at 2h after portal reperfusion (Figure 6A). We therefore assessed the post-CC1 relative to the CC1 levels in the donor liver prior to

transplantation. The post-CC1 expression includes hepatocytes, LSECs, Kupffer cells and donor immune cells as well as infiltrating immune cells from the recipient. Since the protective effect of CC1 was a consequence of CC1 upregulation on the liver-associated cells, including infiltrating immune cells, at the time of the reperfusion injury, we used the ratio to normalize the levels between donors. We found that the post-/pre-CC1 ratio trended towards a negative correlation with mRNA levels coding for T cell activation marker CD154 (r=−0.3431, P=0.080) but exhibited a significant negative correlation with CD28 (r=-0.3840, P=0.048), an important co-stimulatory molecule (Figure 6B). Furthermore, the post-/pre-CC1 ratio showed a significant negative correlation with proinflammatory cytokines such as IFN-γ (r=−0.3877, P<0.05) and IL-17 (r=−0.4621, P<0.05) (Figure 6B) and TLR including TLR2 (r=-0.5171, P=0.006), TLR4 (r=-0.4505, P=0.018) and TLR9 (r=-0.5067, P=0.007). These results suggest that the perioperative CC1 increase suppressed the T and innate cell-related inflammatory immune signatures in human OLT. We further confirmed that the post-/pre-CC1 ratio in human OLT did not correlate with mRNA levels coding for CD4 (r=−0.2192, P=0.272) (Figure 6B). This suggests that the perioperative CC1 increase did not result from an increased frequency of graft-infiltrating $CD4^+$ T cells *per se*.

The TLR family, one of the best-characterized PRRs[24], such as TLR2, TLR4, and TLR9 were negatively correlated with post-/pre-CC1 ratio (r=−0.5171, P<0.01; r=−0.4505, P<0.05; and r=−0.5067, P<0.01, respectively) (Figure 6C). Further, CD68, a macrophage and activated Kupffer cell marker[25], trended towards a negative correlation with post-/pre-CC1 ratio (r=−0.3602, P=0.065) (Figure 6C). As PRRs are expressed predominantly in macrophages, including Kupffer cells and dendritic cells[6], these indicate a perioperative increase of CC1 modulates the innate immune response in addition to T cell activation in human OLT. Our previous studies demonstrated a role for T cell TIM-3 in neutrophil infiltration and TLR4 in OLT injury through its activities as a putative HO-1 repressor[11, 26]. Hence, we further evaluated cathepsin G (neutrophil marker) and HO-1 expression in relation to CC1 signaling. As expected, the post-/pre-CC1 ratio correlated negatively with cathepsin G (r=−0.4646, P<0.05) and positively with HO-1 gene

(r=−0.4921, P<0.01) (Figure 6D). Thus, by regulating adaptive and innate immune activation, a perioperative increase of CC1 could mitigate the inflammatory signature in human OLT.

Recipient CC1 signaling attenuates hepatocellular injury and rejection in human OLT

To elucidate the influence of enhanced perioperative CC1 signaling on human OLT outcomes, we classified our clinical cohort into “low” post-/pre-CC1 (n=25) and “high” post-/pre-CC1 (n=25) groups, according to the median of post-/pre-CC1 ratio determined by Western blot-assisted quantification of relative CC1 levels (cut-off=1.05, Figure 7A). Although donor age had a trend to be younger in the high than low post-/pre-CC1 group (39±3.5, range: 13–62 vs. 48±3.0, range: 19–67, P=0.053), the patients’ demographic data/clinical parameters did not reveal significant differences between recipient or donor groups (Supplementary Table 1A/B). Representative Western blots/case-related clinical parameters (case 1/2: low post-/pre-CC1, case 3/4: high post-/pre-CC1) are shown (Figure 7B). The high CC1 ratio patients showed comparatively decreased OLT damage, consistent with lower levels of AST (POD1–3, P<0.05) and ALT (POD1 P=0.072 and POD2 P<0.05) (Figure 7C). Further, case 2 patient in the low post-/pre-CC1 group experienced acute rejection at POD13 (Figure 7B). Immunofluorescence staining of CD4/CC1 suggested high post-/pre-CC1 ratio associated with increased $CC1^+$ T cell infiltration in OLT (Figure 7D). In addition, the incidence of EAD tended to be lower in the high compared with the low post-/pre-CC1 patient group, although the difference did not reach statistical significance (P=0.074) (Figure 7E). Strikingly, no patients in the high post-/pre-CC1 group suffered OLT rejection, leading to a significant difference in the cumulative rejection rate between the high vs. low post-/pre-CC1 group (P<0.05, Figure 7F). This indicates that the perioperative CC1 increase could be a potent modulator for adaptive immune response in human OLT. Collectively, recipient CC1 signaling may improve clinical outcomes by suppressing acute liver injury/T cell activation in human OLT.

Discussion

Our study documented the protective function of $CD4^+$ T cell CC1-TIM-3 signaling axis in mouse and human OLT recipients. First, we showed that by suppressing TLR4 and NF-κB, recipient and donor CC1 was essential to mitigate IR stress/tissue injury in mouse OLT. Then, we found that CC1 enhanced TIM-3 expression on $CD4^+$ T cells, while adoptive transfer of CC1-deficient $CD4^+$ T cells exacerbated IRI-OLT in immune-deficient mice lacking T/B cells. This corroborates our previous data that CC1 expression facilitates TIM-3 maturation within the secretory pathway and consequently its cell surface display[17]. Further, it is consistent with our prior observations that T cell TIM-3 signaling determines the severity of liver IRI[11]. Strikingly, the present study documented that by interacting directly with liver-associated CC1, T cell-specific TIM-3 suppressed liver Kupffer cell NF-κB p65 activation; this effect was negated by disruption of CC1 signaling in OLT recipients and required CC1 in the donor. This indicates TIM-3 requires CC1 signaling for its inhibitory function in IRI-OLT. Moreover, it indicates interactions between CC1 and TIM-3 in *cis* (as shown by a reduction of TIM-3 expression on $CD4^+$ T cells when CC1 is absent) and *trans* (based upon the dependence of allograft protection on T cell and liver expression of TIM-3 and CC1, respectively) are critical to these beneficial effects[17]. Consistent with these findings, in a clinical arm, recipient CC1 signaling alleviated acute liver injury, while enhanced perioperative CC1 mitigated the rejection incidence in OLT patients. Hence, activation of CC1 modulated innate and adaptive immune responses in OLT recipients.

In addition to promoting proinflammatory gene programs by regulating macrophage and dendritic cell activation through PRRs, NF-κB signaling may affect T cell differentiation/activation as well as cell survival through its anti-apoptotic functions[23, 27]. Thus, depending on the cell type, NF-κB may exert a dual role, by promoting inflammation to exacerbate IRI-OLT via innate immune cells, or by serving as homeostatic/cytoprotective sentinel via its anti-apoptotic function in hepatocytes/LSECs. Therefore, it was important to identify NF-κB expressing cells after disruption of recipient CC1 signaling. In contrast to comparable NF-κB p65 phosphorylation levels in

$CD4^+/CD8^+$ T cells, and $CD11b^+$ monocytes of WT and CC1-deficient mice, we found defective CC1 signaling caused NF-κB activation in Kupffer cells (but not LSECs), and this activation was suppressed by T cell-derived TIM-3. Although Kupffer cells are active players in acute liver injury through their production of reactive oxygen species (ROS) and inflammatory cytokines (e.g., IL-1β, TNF-α), they are also essential for homeostatic regeneration of the inflamed liver[28]. The precise role that CC1 (on Kupffer cell) and TIM-3 (on $CD4^+$ T cell) signaling plays in the acute and resolution phases of IRI-OLT is important to consider in future studies as is whether T cell-associated TIM-3 can induce cytoprotective events in the hepatocytes themselves.

In $CD4^+$ T cells and monocytes that express CC1 and TIM-3 at steady-state (Figure 2A and Supplementary Figure 1A), the majority of $TIM\text{-}3^+$ cells concomitantly expressed CC1 (Figure 2B and Supplementary Figure 1B), suggesting CC1 could promote TIM-3 expression and regulate signaling in CC1-proficient T cells. However, it is interesting that $CD4^+$ T cells and monocytes from CC1KO mice also expressed TIM-3 albeit at lower levels. In addition to CC1, TIM-3 on T cells is also regulated by other mediators such as T-bet, NFIL3, STAT3, IL-12, IL-27, and IL-35[10,29,30]. Such factors need to be considered in future studies in relation to the pathways identified in OLT injury[10].

Although not examined here, the functions of TIM-3 on T cells may be different depending on cell intrinsic (CC1) and extrinsic pathways. In the former instance, it is important to note that CC1 is expressed as two major classes of variants. These include CC1-long cytoplasmic tail domain variants that contain two immune receptor inhibitory motifs (ITIM) which provide inhibitory functions and those variants that contain a short cytoplasmic tail domain which lack ITIMs and can activate unique T cell functions[31,32]. $CD4^+$ T cells predominantly express CC1-long relative to short isoforms, leading to CC1 inhibitory signaling in T cell activation. However, the splice variant types that are expressed by liver-associated $CD4^+$ T cells is unknown as are the effects that they may have on TIM-3 expression/function. In a similar manner, understanding the expression of these two CEACAM1 variants in liver-associated cells may be important in

determining the responses to TIM-3-derived signals from $CD4^+$ T cells and warrants future investigation.

While our mouse model showed that TIM-3 enhancement reduced IRI-OLT in CC1KO recipients, it is interesting that three out of eight recipients showed comparatively high sAST/sALT levels (Figure 4C). This suggests that there are other factors, which regulate liver protection provided by TIM-3 and CC1. These might include host factors, such as genetic susceptibility or environmental determinants, such as the microbiome, given the importance of TLR signaling in our model. Regarding the former, it is important that TIM-3 and CC1 likely interact through amino acids in their respective membrane-distal IgV-domain associated GFCC'faces[17], which may be subject to genetic variation that might affect these interactions and their consequences[33]. It is interesting that genetic polymorphism has been described among the C57BL/6 inbred strain[34]. Thus, although we used a syngeneic OLT combination to investigate hepatocellular injury without the confounding effects of allo-rejection, future studies should consider genetic variation in the allograft model. Additionally, structural differences between mouse and human TIM-3 may impact their species-specific functions[33], although it is notable that our observations in human OLT donors were largely consistent with findings in the mouse models studied. Nonetheless, further studies need to consider genetic variation in TIM-3 and CC1 in donors and recipients and in relation to human OLT outcomes.

Although we focused on TIM-3 interactions with CC1, it is important to note that TIM-3 also engages other ligands such as galectin-9, PtdSer, and HMGB1[14-16]. For example, we have shown a protective function of galectin-9, which is produced by hepatocytes and Kupffer cells/macrophages, and suppresses T cell activation in OLT[12]. That said, our current study demonstrated that T cell TIM-3 enhancement failed to suppress IRI-OLT in the absence of CC1 signaling in the donor (Figure 5B–D). This indicates that CC1 is a particularly critical factor in converting T cell-associated TIM-3 into a cytoprotective response in the OLT. Future studies should therefore consider the role of galectin-9, PtdSer, and HMGB1 as modifiers of TIM-3-CC1

signals or whether or not these molecules may serve as a therapeutic target in CC1-depressed human OLT recipients. As we did not identify which liver-associated cells, e.g., hepatocytes, LSECs, Kupffer cells or all the liver cells, could interact with T cell TIM-3 through their CC1 expression, future studies using conditional gene knockout donor livers could provide answer to this question.

Although our clinical findings demonstrate that the perioperative levels of CC1 in human OLTs, derived from immune and possibly non-immune cells, negatively correlated with T and innate cell inflammatory signatures, some of these data did not show a statistical significance, possibly due to the limited number of patients. As such, perioperative increase of CC1 was associated with suppressed OLT injury, incidence of EAD, and cumulative rejection rate. As our results highlight the benefit of possibly recipient CC1 in protecting human OLT from IR-stress, future studies should address whether adjunctive enhancement of CC1-TIM-3 signaling in host $CD4^+$ T cells may impact clinical OLT outcomes. The intensity of $CD4^+$ T cell CC1 and TIM-3 could serve as a putative biomarker of IRI severity, and possibly OLT rejection. Based on our results, we need to validate whether CC1 and/or TIM-3 on $CD4^+$ T cells could alleviate hepatocellular injury and OLT rejection in a prospective clinical trial. If we identify the high-risk EAD and/or rejecting patients by evaluating the frequency of CC1/TIM-3-expressing $CD4^+$ T cells, subsequent treatment with IL-27 or IL-35 to enhance TIM-3 expression[29,30] could be a promising strategy in an otherwise low CC1/TIM-3-$CD4^+$ T cell clinical cohort. In addition, one may envision suppressing Kupffer cell NF-κB activation during *ex vivo* liver machine perfusion prior to transplantation into high-risk patients with low $CD4^+$ T cell CC1 levels. However, as pharmacologic or genetic inhibition of NF-κB exacerbated Nod-like receptor pyrin domain containing 3 (NLRP-3)-dependent inflammation in preclinical animal models and humans, possibly due to the loss of negative feedback regulation[35], the role of NF-κB signaling in Kupffer cell activation should be carefully investigated in human OLT setting.

In conclusion, we have documented T cell-specific CC1 expression and its corollary expression in the donor liver acts within a therapeutic pathway associated with alleviation of innate immune-driven OLT injury. In this mechanism, T cell CC1 promotes T cell intrinsic TIM-3 expression, which then interacts with liver-associated CC1 to suppress NF-κB activation in Kupffer and possibly other liver cells. Consistent with the ability of CC1 to exert TIM-3 inhibitory regulation in mouse OLT, recipient CC1 signaling was also associated with improved clinical outcomes by suppressing acute hepatic injury and promoting T cell homeostasis in OLT patients.

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Figure Legends

Figure 1. Recipient CEACAM1 signaling mitigates hepatic IRI and suppresses NF-κB p65 in mouse OLT. Mouse WT livers subjected to 18h of cold storage were transplanted into WT or CC1KO syngeneic recipients (n=6–8/group). OLT/serum samples were analyzed 6h post-reperfusion. The sham group (n=5) underwent the same procedures except for OLT. **(A)** Representative H&E and TUNEL staining. Scale bars=100μm. **(B)** Suzuki's histological grading of liver IRI and quantification of TUNEL+ cells/HPF. **(C)** sAST/sALT and **(D)** LDH levels (U/L). qRT-PCR–assisted OLT detection of **(E)** IFN-γ, IL-6, IL-17, Granzyme B/Perforin 1 and **(F)** TLR4, IL-1β, TNF-α (n=6–7/group). Data normalized to HPRT gene expression. **(G)** Western blot-assisted detection of p-IκBα, IκBα, p-NF-κB p65, NF-κB p65, vinculin (VCL). The relative intensity ratio of p-IκBα/IκBα and p-NF-κB p65/NF-κB p65 (n=4/group). Triangle: sham, white square: WT recipients, and black square: CC1KO recipients. Data shown as mean±SEM. **P*<.05, ***P*<.01, Student's *t*-test.

Figure 2. CEACAM1 signaling enhances TIM-3 expression and suppresses $CD4^+$ T cell inflammatory signature. (A) Representative (n=4/group) flow cytometry of CC1 and TIM-3 expression in PBLs from naive WT and CC1KO mice. $TIM\text{-}3^+$ ferquency in **(B)** WT $CC1^+$ and $CC1^-$ $CD4^+$ T cells; **(C)** WT and CC1KO $CD4^+$ and $CD8^+$ T cells. **(D)** Representative (n=4/group) flow cytometry of CC1 and TIM-3 expression in activated PBLs from WT and CC1KO mice subjected to hepatic IRI. $TIM\text{-}3^+$ frequency in **(E)** WT $CC1^+$ and $CC1^-$ $CD4^+$ T cells; **(F)** WT and CC1KO $CD4^+$ and $CD8^+$ T cells. **(G)** $CC1^+$ frequency in naive vs. post-IRI in WT $CD4^+$ T cells. **(H)** $TIM\text{-}3^+$ frequency in naive vs. post-IRI in WT and CC1KO $CD4^+$ T cells. **(I)** qRT- PCR–assisted detection of IFN-γ, T-bet, IL-6, IL-17, IL-22, and TNF-α in $CD4^+$ T cells from WT and CC1KO mice before and after stimulation (n=6/group). White square: WT; black square: CC1KO mouse. Data shown as mean±SEM. **P*<.05, *****P*<.0001, Student's *t*-test.

Figure 3. T cell-specific CEACAM1 signaling alleviates IRI-OLT and suppresses NF-κB p65 in Rag2KO mice. (A) WT livers after 18h of cold storage were transplanted into Rag2KO mice repopulated with $CD4^+$ T cells (10×10^6 i.v.) from WT vs. CC1KO mice, and analyzed 6h post-OLT (n=5/group). **(B)** Frequency of purified spleen $CD4^+$ T cells. **(C)** Representative CD4/CC1 staining. Arrows indicate $CD4^+$ T cells. Scale bars=100μm. **(D)** H&E and TUNEL staining in Rag2KO w/wo $CD4^+$ T cell reconstitution. Scale bars=100μm. **(E)** Suzuki's histological liver IRI grading. **(F)** TUNEL+ cells/HPF. **(G)** sAST/sALT (U/L). **(H)** qRT-PCR–assisted detection of IFN-γ, IL-6, IL-17, TLR4, IL-1β. Data were normalized to HPRT (n=5/group). **(I)** Western blot-assisted detection of p-NF-κB p65, NF-κB p65, β-actin. Relative p-NF-κB p65/NF-κB p65 intensity ratio (n=4/group). Wite circle: untreated Rag2KO, white and black squares: Rag2KO + WT or CC1-deficient $CD4^+$ T cells. Data shown as mean±SEM. **P*<.05, ***P*<.01, Student's *t*-test.

Figure 4. Enhanced T cell-specific TIM-3 alleviates IRI-OLT and suppresses Kupffer cell NF-κB p65 in CEACAM1-deficient recipients. WT livers after 18h of cold storage were transplanted into WT, CC1KO, T cell-specific TIM-3Tg/CC1KO, and TIM-3Tg/CC1KO + anti-TIM-3 Ab (n=6–8/group). OLT/serum samples were analyzed at 6h. **(A)** Representative H&E and TUNEL staining. Scale bars=100μm. **(B)** Suzuki's histological grading of liver IRI and quantification of TUNEL+ cells/HPF. **(C)** sAST/sALT (U/L). **(D)** qRT- PCR–assisted detection of IFN-γ, IL-6, IL-17 in OLT (n=6/group). Data normalized to HPRT gene expression. **(E)** qRT-PCR-assisted detection of TLR4, IL-1β, TNF-α in OLT (n=6/group). Data normalized to HPRT gene expression. **(F)** Western blot-assisted detection of CC1, p-NF-κB p65, NF-κB p65, and vinculin (VCL). The relative intensity ratio of p-NF-κB p65/NF-κB p65 (n=3–4/group). **(G)** Representative NF-κB staining in OLT. Arrows indicate nuclear NF-κB localization in non-parenchymal cells. Scale bars=100μm. **(H)** Representative CLEC4F (Kupffer cell) and p-NF-κB p65 staining. Arrowheads indicate Kupffer cells augmenting p-NF-κB p65. Scale bars=100μm (left panels) and 20μm (enlarged images). White square: WT, black square: CC1KO, purple square: TIM-

3Tg/CC1KO, red square: TIM-3Tg/CC1KO + anti-TIM-3. Data shown as mean±SEM. *P<.05, **P<.01, ***P<.01, Student's t-test.

Figure 5. Donor liver CC1 deficiency compromises T cell-specific TIM-3 regulation in CC1-deficient recipients. (A) CC1KO livers after 18h of cold storage were transplanted into CC1KO or TIM-3Tg/CC1KO mice. OLT/serum samples were analyzed at 6h (n=6/group). The sham group (n=5) underwent the same procedures except for OLT. **(B)** Representative H&E staining. Scale bars=100μm. **(C)** Suzuki's histological grading of liver IRI and sAST/sALT (U/L). **(D)** Representative CLEC4F (Kupffer cells) and p-NF-κB p65 staining in OLT. Arrowheads indicate p-NF-κB p65 positive cells. Scale bars=100μm. **(E)** qRT-PCR–assisted detection of IFN-γ, IL-6, IL-17 in OLT (n=6/group). Data were normalized to HPRT gene expression. **(F)** qRT-PCR-assisted detection of TLR4, IL-1β, TNF-α in OLT (n=6/group). Data were normalized to HPRT gene expression. **(G)** Western blot-assisted detection of CC1, p-NF-κB p65, NF-κB p65 and β-actin. The relative intensity ratio of p-NF-κB p65/NF-κB p65 (n=3/group). Black square: WT→CC1KO, purple square: WT→TIM-3Tg/CC1KO, gray square: CC1KO→CC1KO, pink square: CC1KO→TIM-3Tg/CC1KO OLT combination. Data shown mean±SEM. ***P<.001, Student's t-test.

Figure 6. Perioperative increase of CEACAM1 promotes anti-inflammatory phenotype in human OLT. (A) Pretransplant (after cold storage) and posttransplant (2h after reperfusion) hepatic biopsies were collected from OLT patients. Post-/pre CC1 ratios were analyzed at the gene (n=27) and protein (n=50) levels. Relationship between post-/pre-CC1 gene ratio and **(B)** CD154, CD28, IFN-γ, IL-17; and **(C)** TLR2, TLR4, TLR9, CD68; and **(D)** Cathepsin G and HO-1 gene expression with β-actin normalization. n=27, *P<.05, **P<.01; non-parametric Spearman's method.

Figure 7. Perioperative increase of CECAM1 attenuates hepatocellular injury and improves rejection-free human OLT survival. Post-/pre CC1 ratios were assessed by Western blots with β-actin normalization. **(A)** OLT patients were divided into low (n=25) and high (n=25) post-/pre-CC1 ratio groups, based on the median value of CC1 ratio (cut-off=1.05). **(B)** Representative Western blots and case-related clinical parameters (case 1/2: low post-/pre-CC1 ratio, case 3/4: high post-/pre-CC1 ratio). **(C)** sAST/sALT at POD 1−7. **(D)** Representative CD4/CC1 staining in OLT. Arrows: CC1-negative $CD4^+$ T cells and arrowheads: CC1-positive $CD4^+$ T cells. Scale bars=100μm. **(E)** Incidence of EAD. **(F)** The cumulative rejection rate (Kaplan-Meier method). The solid line indicates high and dotted line low post-/pre-CC1 ratio in human OLT. Data shown as mean±SEM. *P<.05, Mann-Whitney U test **(C)**, Fisher's exact test **(E)**, log-rank test **(F)**.