Title

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Journal

Transplantation, 105(9)

ISSN

0041-1337

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Publication Date

2021-09-01

DOI

10.1097/tp.0000000000003488

Peer reviewed

Published in final edited form as:

Transplantation. 2021 September 01; 105(9): 1989–1997. doi:10.1097/TP.0000000000003488.

Ischemia-Reperfusion Injury in Allogeneic Liver Transplantation: A Role of CD4 T Cells in Early Allograft Injury

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Abstract

Background: A major discrepancy between clinical and most experimental settings of liver ischemia-reperfusion injury (IRI) is the allogenicity.

Methods: In the current study, we first established a murine model of allogeneic orthotopic liver transplantation (allo-OLT) with extended cold ischemia time (18h). Roles of CD4 T cells in the pathogenesis of ischemia reperfusion injury (IRI) in liver allografts was determined using a depleting anti-CD4 Ab. The clinical relevance of CD4 as a marker of liver IRI was analyzed retrospectively in 55 liver transplant patients.

Results: CD4 depletion in both donors and recipients resulted in the most effective protection of liver allografts from IRI, as measured by serum transaminase levels and liver histology. CD4 depletion inhibited IR-induced intra-graft neutrophil/macrophage infiltration and pro-inflammatory gene expressions. Quantitative RT-PCR analysis of human liver biopsies (2h postreperfusion) revealed that post-, rather than pre-, transplant CD4 transcript levels correlated positively with pro-inflammatory gene expression profile. When we divided patients into sub-groups according to intra-graft CD4 levels, the high-CD4 cohort developed a more severe hepatocellular damage than that with low-CD4 levels.

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Authorship

SK, RWB, JWK-W and YZ designed the experiments;

SK, KK and KN, HH, RAS, EFR, and FMK performed the experiments; SK, TI and DWG analyzed the data; SK, DWG, JWK-W and YZ wrote the manuscript.

Disclosure

The authors declare no conflicts of interest.

Conclusions: CD4 T cells play a key pathogenic role in IRI of allogeneic liver transplants and intra-graft CD4 levels in the early postreperfusion phase may serve as a potential biomarker and therapeutic target to ameliorate liver IRI and improve OLT outcomes.

Introduction

Orthotopic liver transplantation (OLT) remains the standard life-saving treatment for end-stage liver diseases. Ischemia-reperfusion injury (IRI), an inevitable consequence of solid organ transplantation, is a major cause of early graft dysfunction/failure and contributes to acute and chronic rejection.[1] The current donor organ shortage has led to more frequent use of organs from extended criteria donors, which however often results in inferior clinical outcomes,[2] due in part to increased susceptibility of marginal organs to IR-stress. Thus, targeting hepatic IRI will not only improve graft survival and clinical outcomes but may also increase the donor pool available for liver transplantation.

Experimental studies have revealed that liver IRI is driven by an innate immune-dominated inflammatory response, which occurs immediately after reperfusion regardless of foreign Ags. Ischemia-induced metabolic stress causes the initial tissue damage, which generates danger associated molecular patterns (DAMPs), which then activate pattern recognition receptors (PRRs), such as toll-like receptor 4 (TLR4), to initiate the inflammatory response.[3,4] As the dominant innate immune cells in the liver, macrophages are critical players in the mechanism of hepatic IRI by producing inflammatory mediators, such as tumor necrosis factor (TNF) α, interleukin (IL)-6 and C-X-C motif chemokine (CXCL) 10.[5–9] Thus, inhibition of innate immune activation is one of the most effective means to control liver IRI in experimental models.

Interestingly, putative Ag-specific T cells are also involved in innate inflammatory tissue damage due to IR-stress. The initial observation that systemic immunosuppression (CsA, FK506) attenuated hepatocellular damage, provided indirect evidence for potential roles of T cell activation in the mechanism of IRI.[3] In liver partial warm IRI model, CD4 T cells are essential for pro-inflammatory immune activation.[10,11] while CD8 and NK cells are relatively dispensable.[12] Livers in both CD4-deficient mice and WT mice depleted of CD4 T cells are protected from IRI. However, there were also reports that liver IRI was reduced in CD4 deficient mice[13] and that nonconventional γδT cells were in fact involved in the disease process by secreting IL-17 to recruit/activate neutrophils.[14,15] Recently, donor graft CD4 T cells were found essential in a mouse syngeneic liver IRI-OLT model.[16] Mechanistically, costimulatory molecule interactions, such as CD28-B7 and CD154-CD40, critical for T cell activation, also play important roles in liver inflammatory response against IR-stress via either T cell-derived inflammatory cytokines[11,17] or reverse-costimulation[12] or T cell-platelet/endothelium interactions.[11] Additionally, the negative costimulation pathway PD-1 was also involved in the pathophysiology of liver IRI.[18]

The overwhelming majority of experimental studies on liver IRI utilize models of either partial warm ischemia in situ or syngeneic transplantation after cold storage, which lack the key clinical component of organ transplantation, i.e., allogenicity. This major discrepancy between animal models and the clinical setting may hamper our appreciation of the disease

pathogenesis and prohibit from identifying appropriate therapeutic targets. In particular, the question of whether and how allo-Ag/alloimmunity may affect tissue inflammatory responses against IR remains to be determined.

In this study, we first established a murine allogeneic IRI-OLT model and then determined the role of CD4 T cells in liver IRI in this true clinical-mimicking Tx settings. As a key component of alloimmunity and graft rejection, whether CD4 T cells play a role in early graft injury due to ischemia remains unclear. The clinical relevance of intragraft CD4 and liver IRI was determined by analyzing 55 human OLT patients. Our results indicate that CD4 T cells are critical for the pathogenesis of IRI in liver allografts, and that intragraft CD4 phenotype is an essential determinant in the innate immune-driven IRI in liver Tx patients. Thus, CD4 T cells may serve as a potential therapeutic target in the management of liver sterile inflammation and hepatocellular function in OLT patients.

Materials and Methods

Animals

C57BL/6 and Balb/c male mice at 6–8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were housed in UCLA animal facility under specific pathogen-free conditions, received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” (NIH publication 86–23 revised 1985).

Mouse allogeneic orthotopic liver transplantation (allo-OLT)

We have developed and validated a syngeneic mouse model of ex-vivo hepatic cold storage followed by OLT.[19,20] For allo-OLT, Balb/c donor livers were stored in UW solution at 4°C for 18h prior to transplantation into C57BL/6 recipients. Animals were treated with CD4-mAb (GK1.5, BioXCell, West Lebanon, NH) or control IgG (20mg/kg i.v. based on preliminary study, Figure S1A) for 2 consecutive days prior to allo-OLT. Liver grafts and serum samples were collected at 6h and 24h postreperfusion. The sham group underwent the same procedures except for OLT.

Serum transaminase assay

Mouse serum alanine transaminase (ALT) and asparate transaminase (AST) levels were measured with Infinity™ ALT and AST Liquid Stable Reagent (ThermoFisher Scientific, Rockford, IL) and validated with Validate® GC3 (Maine Standards Company, LLC, ME).

Liver histology and immunohistochemistry

Formalin-fixed paraffin-embedded liver sections (5μm) were stained with hematoxylin and eosin. The severity of IRI was graded using Suzuki’s criteria.[21]

The mouse liver infiltrating macrophages and neutrophils were detected with rat anti-CD11b Ab (BD Biosciences, San Jose, CA) and rat anti-Ly6G Ab (Bio-Rad, Hercules, CA), respectively. Signals were visualized with secondary Alexa Fluor 488 anti-rat IgG. Results were scored semi-quantitatively by blindly counting the number of positive cells in 10 HPF/section (x400).

TdT-mediated dUTP nick and labeling (TUNEL) assay

Cell death in formalin-fixed paraffin-embedded human and mouse OLT sections (5μm) was detected by the Apop Tag Plus Peroxidase in Situ Apoptosis Kit (Millipore, Temecula, CA). Results were quantitated by counting the number of positive cells in 10 HPF/section (x400). Lower power images (x200) were shown.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis

RNA was extracted from human and mouse liver tissue samples using RNAse Mini Kit (Qiagen, Germantown, MD). A total of 5.0μg of RNA was reverse-transcribed into cDNA (complementary DNA). Quantitative PCR was performed using DNA Engine with Chrome 4 Detector (JA Research, Waltham, MA). The primers sequences are listed in Table S1. The expression of the target gene was normalized to the housekeeping HPRT in mouse and β-actin in human.

Enzyme-linked immunosorbent assay (ELISA)

Serum concentrations of TNF-a and MCP1 were measured with ELISA kits (Thermo Fisher Scientific) according to the manufacturer's protocol.

Flow cytometry

Liver nonparenchymal cells (NPCs) were isolated after perfusion with collagenase, as previously described.[22,23] In brief, liver was perfused with collagenase (MilliporeSigma Co., St. Louise, MO) solution using peristatic pump via inferior vena cava and graft samples were pressed against a 100um nylon cell strainer, NPCs were isolated with 33% Percoll (GE Healthcare Bio-Sciences, Uppsala, Sweden) and lysed with ACK lysing buffer (Quality Biological, Inc. Gaithersburg, MD). Isolated liver NPCs were first incubated with anti-mouse CD16/32 antibody (clone:93 BioLegend, San Diego, CA) to block Fc-mediated nonspecific Ab binding. Cells were then stained with the fluorochrome-conjugated antibodies; CD25-APC, CD3-BV785, CD4-FITC, CD4-BV605, CD40-PerCP-Cy5.5, CD80-PE/Cy7, CD86-BV605, Ly6G-PE (BioLegend), and F4/80-eFluor450 (eBioscience, San Diego, CA). For intracellular cytokine staining, cells were fixed, permeabilized, and incubated with the fluorochrome-conjugated antibodies; interferon (IFN) γ-BV711, interleukin (IL)-17-BV510, IL-4-PE/Dazzle594 (BioLegend) in PBS with 0.2% bovine serum albumin and True-Nuclear Transcription Factor Buffer Set (BioLegend) according to the manufacture's instruction ion. Multi parameter flow cytmetric analysis was performed using a LSRFortessa X-20 SORP (BD Bioscience, San Jose, CA) and results were analyzed using BD FACSDiva software (BD) at the UCLA Jonsson Comprehensive Cance Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility (UCLA, CA).

Clinical liver transplant study

The UCLA Institutional Research Board (IRB #13–000143 and 18–000216) approved the clinical study. Fifty-five adult primary OLT recipients were recruited under an IRB protocol (13–000143; May 2013 – August 2015). The demographic data and clinical parameters of recipients and donors are shown in Table S2. Routine standard of care

and immunosuppressive therapy were administered as specified by UCLA liver transplant protocols. Study data were collected and managed using REDCap electronic data capture tools. Donor organs, procured using standardized techniques, were perfused with and stored in cold University of Wisconsin (UW) solution (ViaSpan; Bristol-Meyers Squibb Pharma). Cold ischemia time was defined as the time between the perfusion of the donor liver with UW solution and its removal from cold storage. Recipient venous blood was collected within the hour prior to the transplant and on postoperative days 1–14 (POD 1–14). Protocol Tru-Cut needle biopsies (Bx) were obtained from the left hepatic lobe after liver cold storage at back table (prior to put-in) and intra-operatively approximately 2h after portal reperfusion (prior to the abdominal closure) and snap-frozen.

Statistical analysis

In mouse experiments, group comparisons were performed using Student's t-test. For human data, continuous values were analyzed by Kruskal-Wallis test with Dunn's posttest, chronological changes were analyzed by random-effects general least squares (GLS) regression and categorical variables by Fisher's exact test. Spearman's correlation coefficient (r) was used to evaluate the strength of relationship between variables. When correlating CD4 and clinical outcomes, patients were divided into 3 groups according to their CD4 levels to obviate assumptions regarding linearity given the relatively small number of transplants. R (The R Foundation for Statistical Computing, version 3.2.2) for Fisher's exact test, STATA for random-effects GLS regression and GraphPad Prism 5 for Mac (GraphPad Software, Inc., La Jolla, CA) was used for the other analyses, respectively. Given the exploratory nature of this study elucidating factors impacting relationships between CD4 and IRI, p-values were not corrected for multiple comparisons, and values of <0.05 were considered statistically significant.

Results

CD4 depletion ameliorates IRI in allo-OLTs.

To study the mechanism of liver IRI in a true clinical experimental setting, we established a murine allogeneic OLT model. Balb/c liver grafts were first stored in UW solution for 18h and transplanted orthotopically into MHC-fully mismatched C57BL/6 recipients (Figure 1A). Liver IRI was monitored continuously for 24h post-OLT by measuring sALT levels. Similar to our findings in a murine syngeneic OLT model,[24] the sALT level peaked at 6h in allogeneic OLTs and declined rapidly to near baseline levels at 24h postreperfusion (Figure S1A).

To determine roles of CD4 T cells in the allogeneic liver IRI, we utilized CD4-depleting mAb GK1.5, which has been shown to protect livers from IRI in a partial warm ischemia model.[12] Interestingly, although CD4 depletion in either donor or recipient alone showed trends of protection, the treatment in both donor and recipient was required to achieve consistent and maximum protection of allogeneic OLTs from IRI (Figure S1B). Compared with control Ig, GK1.5 treatment resulted in lower sALT and sAST levels at 6h (Ctrl-Ig=8538±3794/ 10581±3146 U/L, n=11 vs. GK1.5=3088±2239/ 5184±2590 U/L, n=9, P <0.05; Figure 1B), and better preserved liver histological architecture at both 6h and 24h,

with lower Suzuki's scores (Ctrl-Ig=6.6±2.2 and 6.2±1.5 vs. GK1.5=3.1±1.1 and 3.2±1.0, n=4–5/gr, P <0.05, Figures 1B and 1C). The hepatocellular damage was also evaluated by TUNEL staining. The frequency of TUNEL+ cells was reduced in GK1.5-treated OLTs at both 6h and 24h post reperfusion (Ctrl-Ig=312.1±44.6 and 113.4±15.8 vs. GK1.5=53.2±27.6 and 2.8±3.6 per HPF, n=4–5/gr, P <0.05, Figure 1D).

The depletion of CD4 T cells in allo-OLT recipients was confirmed by FACS analysis. Numbers of CD3+CD4+ T cells in both peripheral blood lymphocytes (PBLs) and liver NPCs were significantly lower in GK1.5 vs. Ctrl-Ig treated mice at 6h postreperfusion (Ctrl-Ig=13.2±0.7% and 6.5±1.5% vs. GK1.5=2.4±1.5% and 0.2±0.2%, n=4/gr, P <0.05, Figure S2A). This resulted in decreases in the expression of IFNγ, IL-4 and CD25, (Ctrl-Ig=13.0±5.4, 10±3.8 and 6.7±0.6% vs. GK1.5= 5.9±1.2, 5.1±0.9 and 3.4±1.3%, n=4/gr, P <0.05, Figure S2B), but not IL-17, in intragraft CD3+ cells.

CD4 depletion inhibits pro-inflammatory IRI response in a mouse allo-OLT.

We next evaluated the effect of anti-CD4 treatment on liver inflammatory immune response against IR-stress in allo-OLT. Administration of GK1.5 decreased intrahepatic expression of TNFα, IL-1β, CCL2, CXCL1, and CXCL10, but increased expression of TGF-β, IL-4 and CD163, as compared with controls (P <0.05, n=6–8/gr, Figure 2A). To analyze whether GK1.5 preferentially depleted specific CD4 subsets, we measured the expression of T cell transcriptional factors. Interestingly, only FoxP3 level was reduced in the CD4-depleted livers, but not Tbet and RORγ (Figure S3). The relative ratio of Tbet/FoxP3 was not altered by the Ab-treatment. Serum cytokine/chemokine levels were also measured, and results showed that both TNF-a and CCL2 levels were significantly lower in GK1.5- vs. control-treated hosts (Figure 2B). To test whether CD4 regulated graft infiltration of neutrophils and macrophages in allo-OLTs, we performed immunohistochemistry analysis of Ly6G and CD11b positive cells in liver tissue sections. Indeed, CD4 depletion decreased the frequency of both cell types at 6h postreperfusion (HPF: Ly6G: Ctrl-Ig=37.6±16.5 vs. GK1.5=21.8±13.7; CD11b: Ctrl-Ig=38.5±13.5 vs. GK1.5=19.8±4.8, n=5–6/gr, P <0.05; Figure 3A and 3B). These results indicate that CD4 T cells promote liver inflammatory response against IRI in mouse allo-OLT.

CD4 depletion inhibits IR-induced co-stimulatory molecule expression in a mouse allo-OLT.

The upregulation of costimulatory molecules on APCs is critical for their ability to stimulate T cell activation. To determine whether CD4 T cells regulate IR-induced costimulatory molecule expression, we measured T cell costimulatory molecules in GK1.5 vs. Ctrl-Ig-treated allo-OLT at 6h of reperfusion. CD4 depletion reduced transcript levels of both positive and negative costimulatory molecules in IR livers, including CD28, CD40L, as well as PD-1 and CTLA-4 (n=8/gr, P <0.05; Figure 2). The expression of CD40, CD80, and CD86 in liver NPCs was analyzed by FACS. CD4 depletion inhibited the upregulation of these costimulatory molecules in the general APC population and F4/80^{high} macrophages (Ctrl-Ig: CD40, 43.0±6.4 and 20.4±4.1%; CD80, 51.8±15.8 and 7.4±0.7%; CD86, 49.5±7.3 and 51.3±5.7 vs. GK1.5: CD40, 23.9±8.9 and 11.4±2.7%; CD80, 22.9±7.6 and 5.0±0.4%; CD86, 34.1±6.5% and 43.5±3.5%; n= 4/gr; P <0.05, Figure 3D and 3E). Thus, CD4

depletion inhibits the upregulation of costimulatory molecules in APCs by IR-stress in mouse allo-OLT.

Liver graft CD4 levels correlate with pro-inflammatory phenotype and hepatocellular damage in human OLT.

To investigate the potential functional relevance of CD4 T cells and liver IRI in clinical transplantation, we analyzed retrospectively the correlation between CD4 level and pro-inflammatory gene programs in human OLT biopsy specimens (n=55). Posttransplant liver biopsies were obtained prior to the abdominal closure (ca. 2h after reperfusion) and gene expressions were measured by qRT-PCR. As shown in Figure 4, intra-graft CD4 levels were positively correlated with the expression of TLR4 (r=0.879, $P<0.001$), CXCL10 (r=0.741, $P<0.001$); CD68 (r=0.768, $P<0.001$, macrophage marker), cathepsin G (r=0.771, $P<0.001$, neutrophil marker), CD80 (r=0.887, $P<0.001$) and CD86 (r=0.841, $P<0.001$). As CD4 is expressed in many non-T cell types in human, we also determined the relationship between CD4 and other T cell markers. Indeed, post-Tx CD4 levels were positively correlated with CD3 (r=0.834, $P<0.001$) and T cell costimulatory molecule CD28 (r=0.939, $P<0.001$) in OLT recipients. Interestingly, analysis of intra-graft CD4 levels prior to OLT (pre-Tx CD4) showed no correlation with the post-Tx pro-inflammatory gene program (Figure S4). These results indicate that both donor and recipient CD4 are associated with hepatic inflammatory immune response against IRI in human OLT recipients.

To evaluate the potential impact of intra-graft CD4 levels on clinical outcomes, we divided human OLT samples into high- (n=19), med- (n=17), and low- (n=19) CD4 expression groups (Figure 5A). The medium group as adopted due to the clustering of CD4 levels at the median and better separation of high and low cohorts. The patients' demographic data and clinical parameters are summarized in Table S2. The CD4 level did not correlate with graft cold ischemia time, donation status, ABO differences, MELD score, or intraoperative blood transfusion. Sex, body weight, body mass index (BMI), and preoperative transaminase of donor and recipient also have no correlation with CD4 levels. However, the low-CD4 patients tended to be younger compared with med-CD4 cohort ($P<0.05$) and the heterogeneity of recipient primary etiology suggested that low-CD4 group has a more uniform etiology distribution, whereas high-CD4 has an unimodal etiology distribution due to HCV ($P<0.05$; Table S2). In the high-CD4 patients, intrahepatic mRNA levels coding for TLR4; CXCL10, and CD68/Cathepsin G were significantly ($P<0.05$) higher, as compared with those in the low-CD4 cohort (Figure 5B). As to the OLT outcome, high-CD4 patients developed more severe hepatocellular damage, with elevated serum AST levels ($P<0.05$; Figure 5C) as compared with low-CD4 cohorts. Representative TUNEL staining results showed increasing numbers of apoptotic/necrotic cells were associated with higher CD4 expression (Figure 5D). The 7-day posttransplant clinical data showed significant differences only in the ALT level, but not AST, bilirubin, INR or EAD among these CD4 groups (Figure S5).

Discussion

This translational study aimed to analyze the role of CD4 T cells in liver IRI in a true clinical setting, i.e., allogeneic OLT with extended cold ischemia. In the experimental arm, our results provide functional evidence that CD4 T cells facilitate allogeneic liver IRI. Indeed, pretreatment with a depleting anti-CD4 mAb, in both donors and recipients, markedly reduced hepatocellular damage and inhibited hepatic pro-inflammatory responses against IR-stress by suppressing the infiltration and activation of macrophages/neutrophils. Importantly, disruption of CD4 signaling reduced the upregulation of costimulatory molecules, such as CD80, CD86, and CD40 in liver APCs, which may subsequently interfere with allo-immune activation and allograft rejection cascade. In the clinical arm, retrospective analysis of OLT patients reveals strong association between intra-graft CD4/CD3 transcript levels and hepatic pro-inflammatory phenotype at 2h post-OLT. Post- but not pre-Tx CD4 levels are positively correlated with the expression of a selective subset of innate immune molecules, including key costimulatory molecules critical for alloreactive T cell activation. To the best of our knowledge, this is the first report to document both experimentally and clinically the correlation between intragraft CD4 and liver inflammatory immune activation early after Tx in the allogeneic setting.

The mechanism of IR-mediated hepatocellular damage has been studied in syngeneic OLT models. Surprisingly, there are only a few reports addressing directly roles of CD4 T cells. In rats, adenoviral mediated gene transfer of CD40Ig in the grafts protected livers from IRI possibly by inhibiting T cell activation via the interference with costimulatory CD40–40L signaling.[3] The blockade of chemokine receptor CXCR3 also diminished CD4 T cell graft infiltration and protected livers against IRI.[25] In mice, depletion of donor CD4 T cells was sufficient to protect liver isografts from IRI.[16] In our study, although depletion of either donor or recipient's CD4 confers some protection of liver allografts from IRI, depletion of both donor and recipient's was required to achieve maximum and consistent protection. We have found that majority of intragraft CD4+ cells at 6h after allo-OLT were recipient origin (>80%, data not shown), which strongly suggests that recipient circulating CD4 T cells are involved in the inflammatory response against IR-stress. As to the types of CD4 T cells depleted by the Abs, we have found in FACS analysis that CD25+ Treg was similarly eliminated as CD25- T cells (data not shown). The PCR measurement of T-cell transcriptional factors in liver revealed that there were no significant changes in the levels of Tbet and RORg. Only FoxP3 levels was lower in the GK1.5-treated livers. Thus, the liver protection is the result of total CD4 depletion, rather than the relative sparing of Treg vs. Teff.

CD4 T cells can promote innate immune activation by 2 mechanisms, i.e., inflammatory cytokines (e.g. IFNγ) or co-stimulatory molecule signaling (e.g. CD28). Indeed, co-stimulation blockade is effective in inhibiting liver pro-inflammatory immune response against IR-stress.[26] While co-stimulatory pathways are essential for productive T cell activation by APCs, the signaling can be bi-directional that activated T cells also facilitate innate immune responses of APCs. We have tested the hypothesis in a murine liver partial warm ischemia model that activated CD4 T cells promote liver innate immune activation by reverse-signaling through CD154-CD40. Indeed, livers were protected from IRI following

CD154 blockade in WT mice or in nude mice reconstituted with CD154-deficient CD4 T cells.[26] Most importantly, anti-CD40 mAb agonist readily restored liver IRI in otherwise IRI-resistant CD4 KO mice.[12] Our current data in the murine allo-OLT model show that CD4 depletion results in lower levels of IFN-γ, CD28 and CD154 expression in liver allografts, and inhibition of CD40, CD80/86 induction in liver APCs.

There are certain limitations in our clinical analysis. The small biopsy sample size limited us to perform only qRT-PCR. As CD4 expression in humans is not limited to T cells, the measurement of CD4 transcripts is obviously neither specific nor sufficient to quantitate CD4 T cells in OLTs. Although intragraft CD3 and CD4 levels were correlated, immunohistological staining of CD4/CD3 vs. CD16 with morphological analysis in biopsies would be necessary to differentiate CD4 T cells from other cells, such as macrophages, in the graft and specifically quantify their relationship with hepatic IRI. When we compared patients with high vs. low intragraft CD4 levels, CD4 high cohort was clearly associated with enhanced inflammatory gene expression and worse OLT outcomes.

Our results specified that it was the post-, rather than the pre-Tx, CD4 levels which were correlated with liver inflammatory immune activation in clinical OLT. Thus, both infiltrating and preexisting CD4+ cells are involved in the mechanism of IRI in human liver transplant patients. This is consistent with our finding in the mouse allogeneic OLT model that the depletion of both donor and recipient's CD4 provided the best protection from IRI. Of note, there were no obvious differences in patient MELD scores or graft cold ischemia time between the high and low CD4 expression groups. Interestingly, the low-CD4 patient cohort was younger, despite the fact that CD4 count declines with age due to thymic involution.[27] Although recipient age is a risk factor of graft failure and dysfunction,[28] these young CD4-low patients developed less severe hepatic inflammatory injury early after Tx. Therefore, intragraft CD4 levels represent a novel indicator of liver IRI independent of ischemia time and patient status (e.g., MELD scores and age). It is noted that significantly higher percentage of HCV patients were present in the high-CD4 cohort. Chronic HCV patients are reported to have increased numbers of hepatic CD4+ T-lymphocytes as compared with chronic HBV patients.[29] It will be interesting to determine in a larger cohort whether primary etiology would affect hepatic CD4 expression in Tx patients.

To translate the finding into clinical practice, we may perform biopsies during operation prior to the abdominal closure. As the measurement of intragraft CD4 transcripts is indicative of early allograft injuries, we may choose to treat or monitor those high liver CD4 patients accordingly. From the therapeutic point of view, the emerging normothermia machine perfusion platform provides us the opportunity to modify or deplete donor CD4 T cells prior to transplantation.[30,31] Fully humanized T cell-targeted Abs or CD4 depleting Abs are readily available[32,33] and may be used to depleting CD4 in Tx recipients. Thus, intragraft CD4+ T cells may represent not only as a diagnostic but also therapeutic target in the management of IR-induced liver inflammatory injury and graft rejection in OLT recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Flow cytometry was performed in the UCLA Jonsson Comprehensive Cancer Center (JCCC) and Center for AIDS Research Flow Cytometry Core Facility that is supported by National Institutes of Health awards P30 CA016042 and 5P30 AI028697, and by the JCCC, the UCLA AIDS Institute, the David Geffen School of Medicine at UCLA, the UCLA Chancellor's Office, and the UCLA Vice Chancellor's Office of Research. We thank Damla Oncel (undergrad UCLA student) for immunohistochemical stain assistance; and Justine Aziz for helping with clinical data collection.

Funding

This work was supported by NIH Grants P01 AI120944 (YZ and JKW).

Abbreviations:

ALT	Alanine transaminase
ANOVA	analysis of variance
AST	aspartate transaminase
BMI	body mass index
Bx	biopsy
CXCL	C-X-C motif chemokine
DAMP	danger associated molecular pattern
HCC	hepatocellular carcinoma
IFN	interferon
IL	interleukin
IRI	ischemia-reperfusion injury
NPCs	non parenchymal cells
OLT	orthotopic liver transplantation
PBLs	peripheral blood lymphocytes
PRR	pattern recognization receptors
TCR	T-cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
TLR	toll-like receptor

TUNEL	TdT-mediated dUTP nick and labeling
UW solution	University of Wisconsin solution

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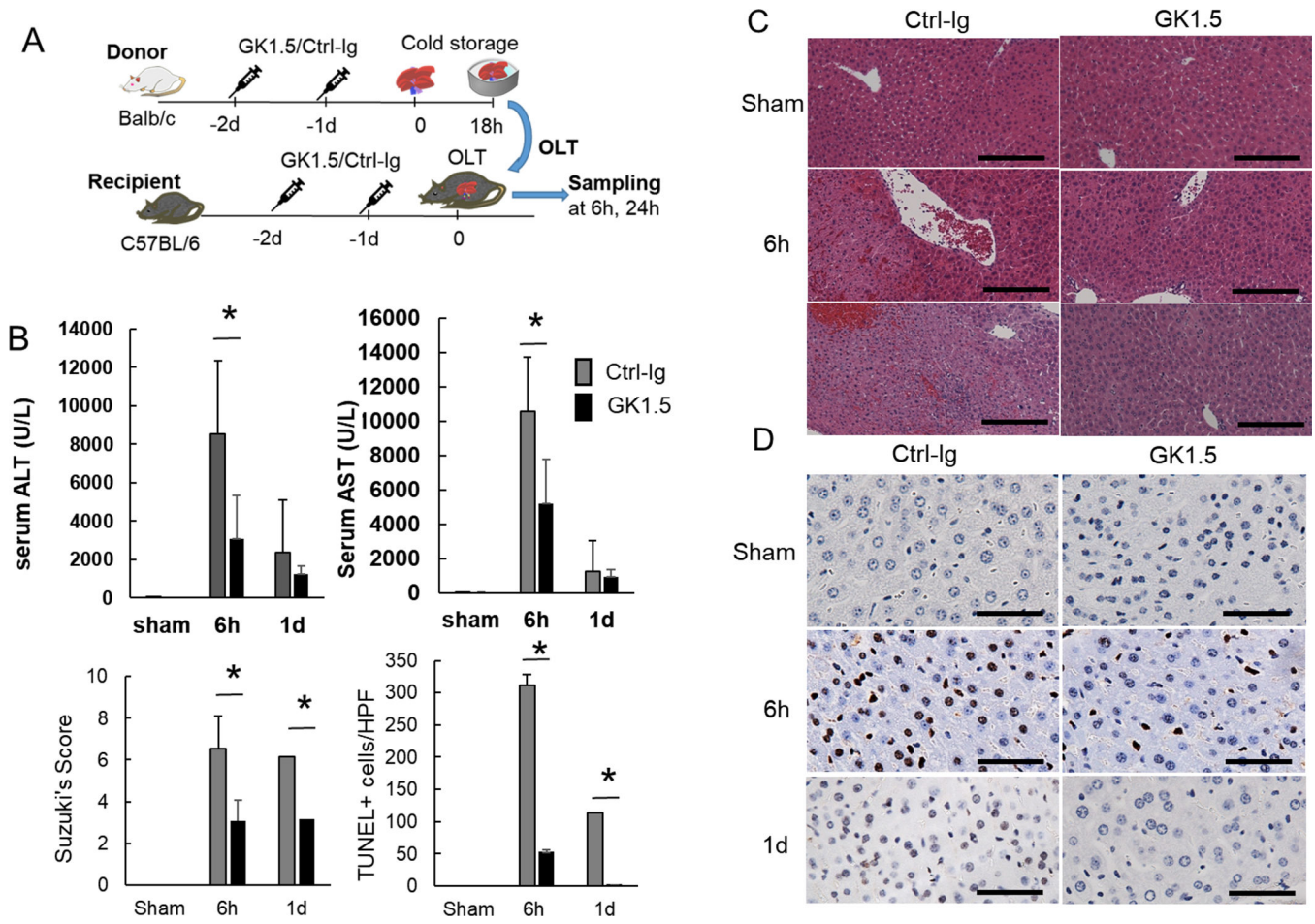

Figure 1. Anti-CD4 mAb (GK1.5) treatment attenuates hepatic IRI in mouse allo-OLT.

Both donor and recipient mice were treated with 2 consecutive doses of GK1.5 or control Ig (20mg/kg, i.v.) prior to OLT. Allogeneic Balb/c liver grafts were transplanted to C57/BL6 recipients after 18h cold storage in UW solution. Liver and serum samples were collected at 6h and 1d post-OLT. (A) The experimental scheme was shown. (B) Average levels of serum ALT/AST (U/L, n= 9/group at 6h and 4 at 1d/ group), Suzuki's histological grading of liver IRI, and quantification of TUNEL+ cell/HPF of different OLT groups were shown. (C) Representative H&E (x100) staining of liver allografts. Scale bar=200μM. (D) Representative TUNEL (x400) staining of liver allografts. Scale bar=100μM. Data shown as mean±SD, n=5/group, $*P<0.05$.

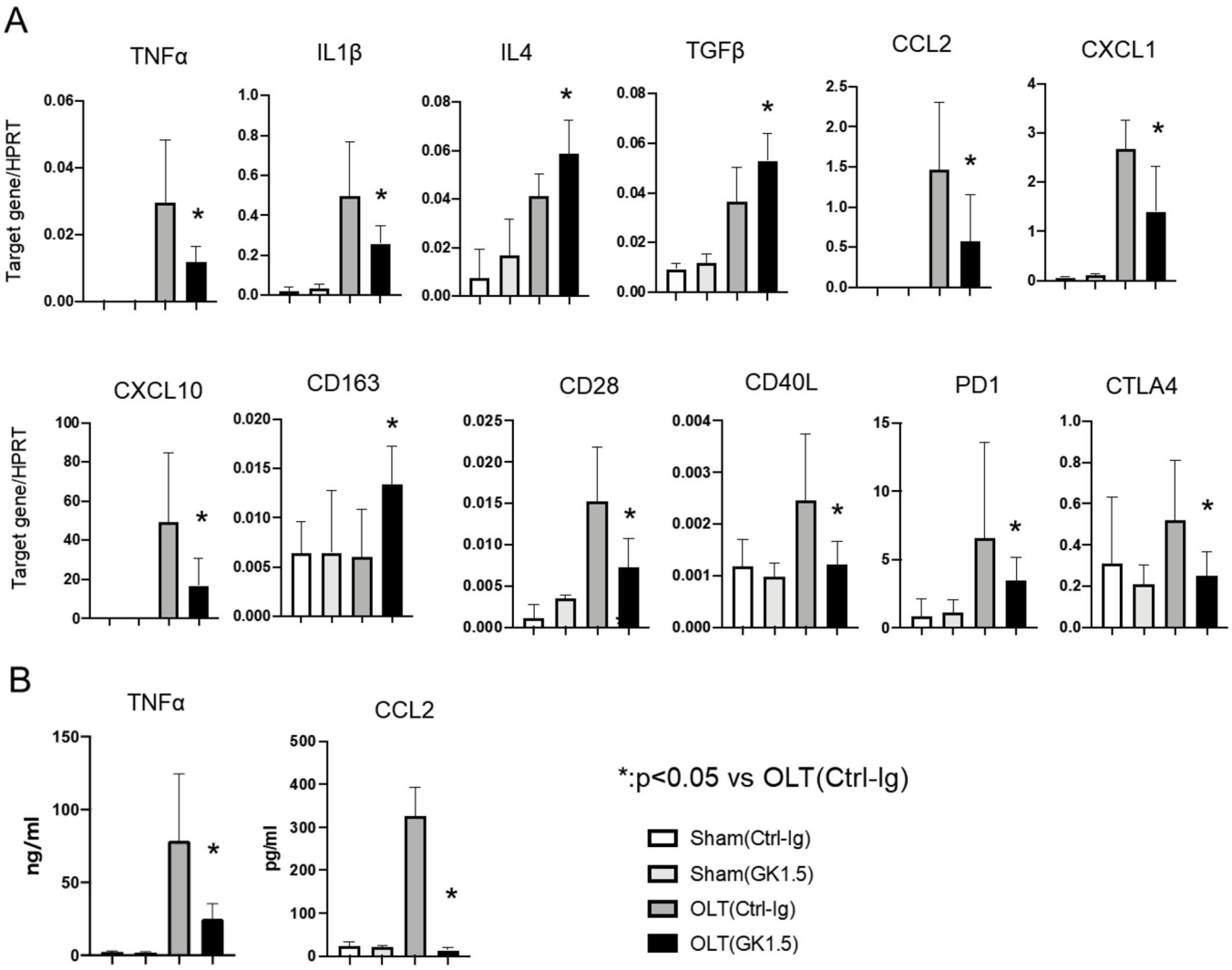

Figure 2. Intragraft gene expressions in mouse allo-OLT.

Allogeneic Balb/c liver grafts were transplanted to C57/BL6 recipients treated with either control Ig or GK1.5, as described in the Materials and Methods. Liver allografts and serum samples were harvested at 6h post Tx. (A) Intragraft gene expressions were analyzed by quantitative RT-PCR. Average rations of target gene/HPRT in different OLT groups were plotted. (B) Serum TNF-a and CCL-2 levels were determined by ELISA. Data shown as mean±SD, n=6–8/group, $*P<0.05$.

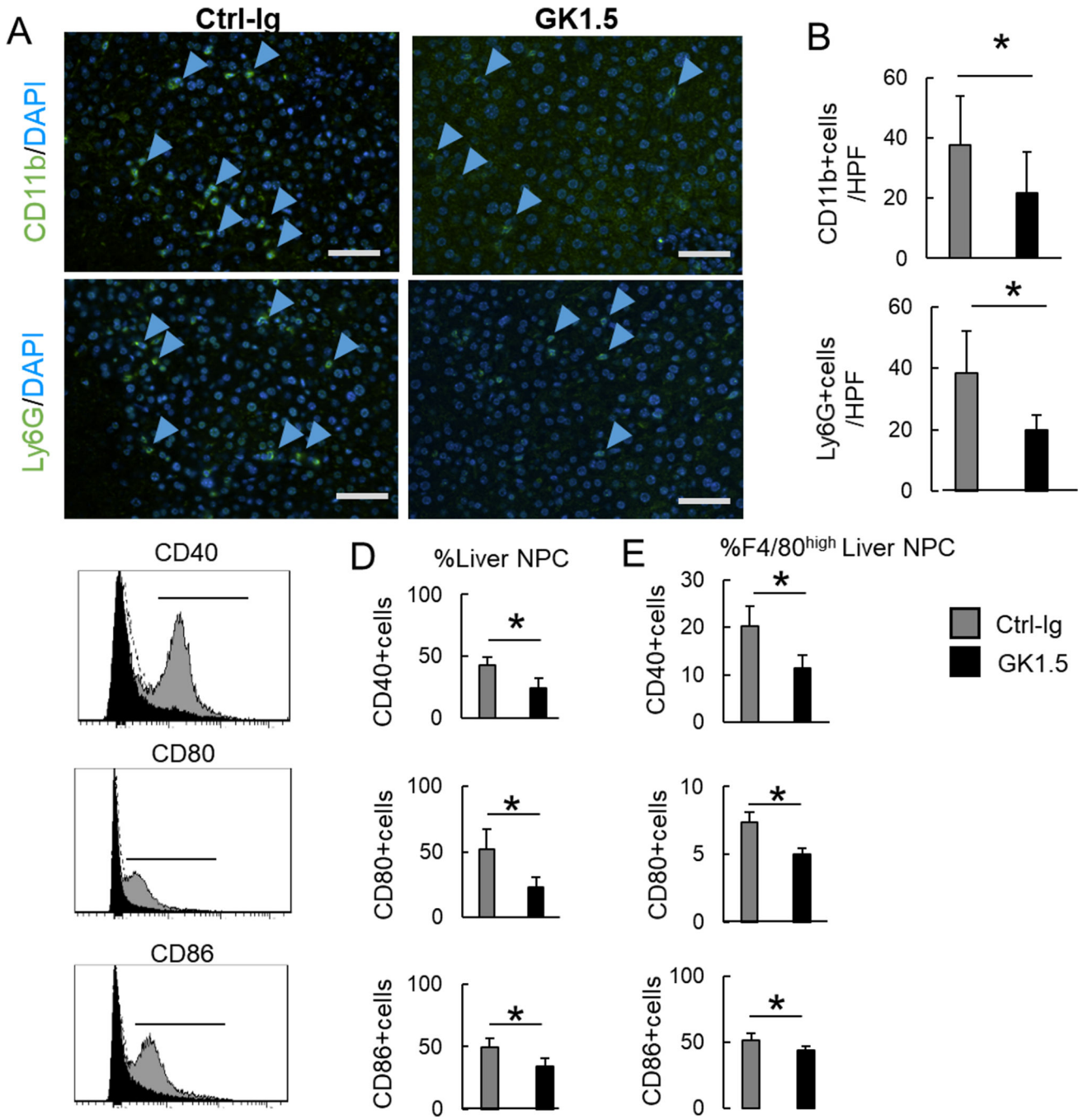

Figure 3. Anti-CD4 mAb inhibits inflammatory immune response in mouse allo-OLT.

Liver allografts from GK1.5 or control Ig treated recipients were sampled at 6h postreperfusion, as described in the Materials and Methods. (A) Representative immunohistochemical staining of liver infiltrating CD11b+ (macrophage) and Ly6G+ (neutrophil) cells in liver allografts (x200, scale bar=100μM). Blue allows indicate positively stained cells. (B) Quantification of CD11b+ and Ly6G+ cells/HPF. Data was shown as mean±SD. n=5–6/group, $*P<0.05$. (C) Representative FACS histogram of CD40, CD80, and CD86 positive cells in liver NPCs were shown. (D) Average % of CD40, CD80, and

CD86 positive cells in total liver NPCs or in F4/80^{high} Ly6G^{low} population from recipients treated with GK1.5 or control Ig were plotted. Data was shown as mean±SD. n=4/ group (*P <0.05, Student t-test).

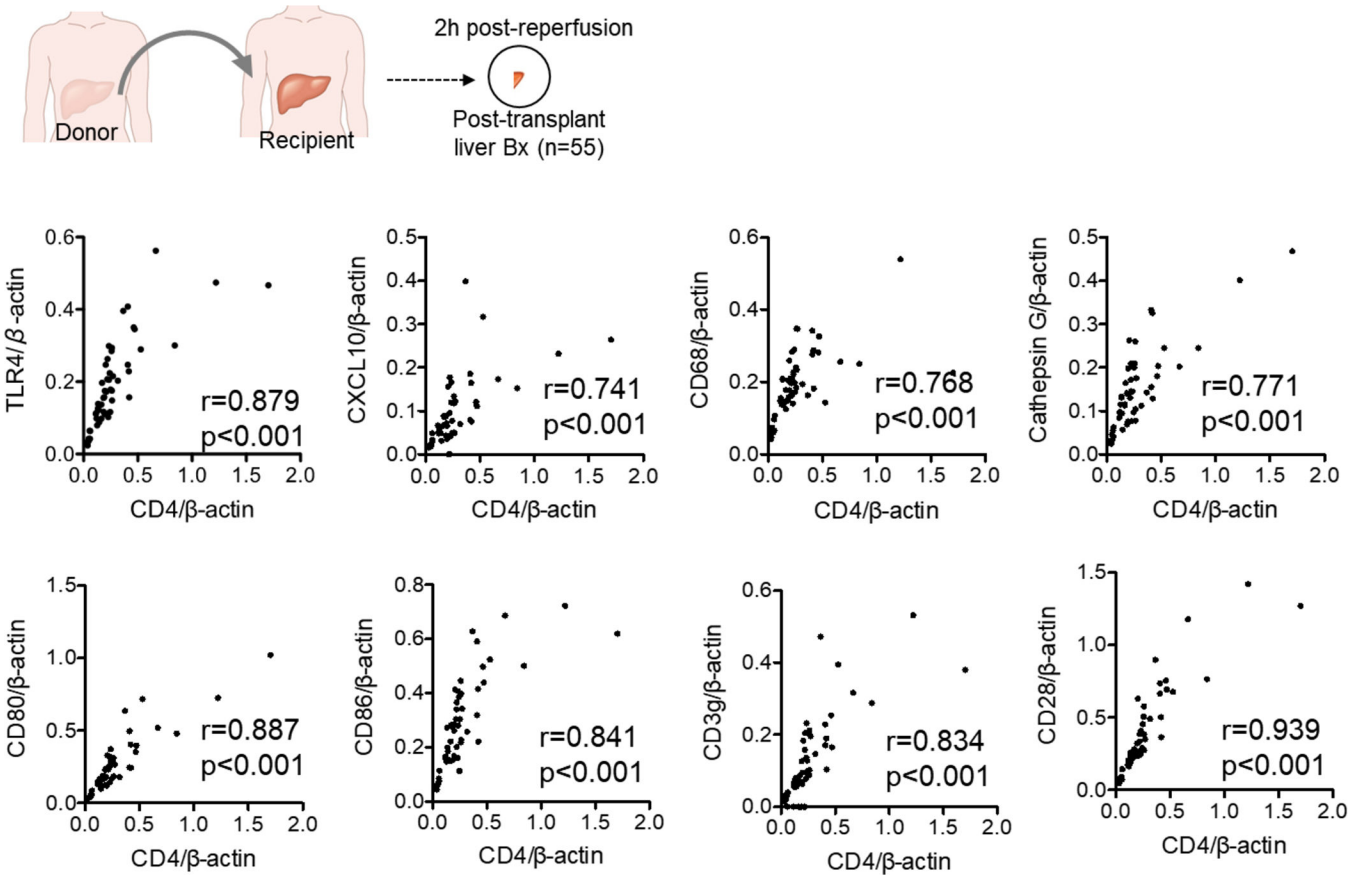

Figure 4. Post-OLT intragraft CD4 levels correlate with pro-inflammatory phenotype in human OLT.

Posttransplant liver biopsy samples, collected from 55 OLT patients, were analyzed by qRT-PCR. Average ratios of target gene/β-actin were used to plot the relationship between intra-graft CD4 and, TLR4, CXCL10, CD68, Cathepsin G, CD80, CD86, CD3g, and CD28-levels. r: Spearman's correlation coefficient.

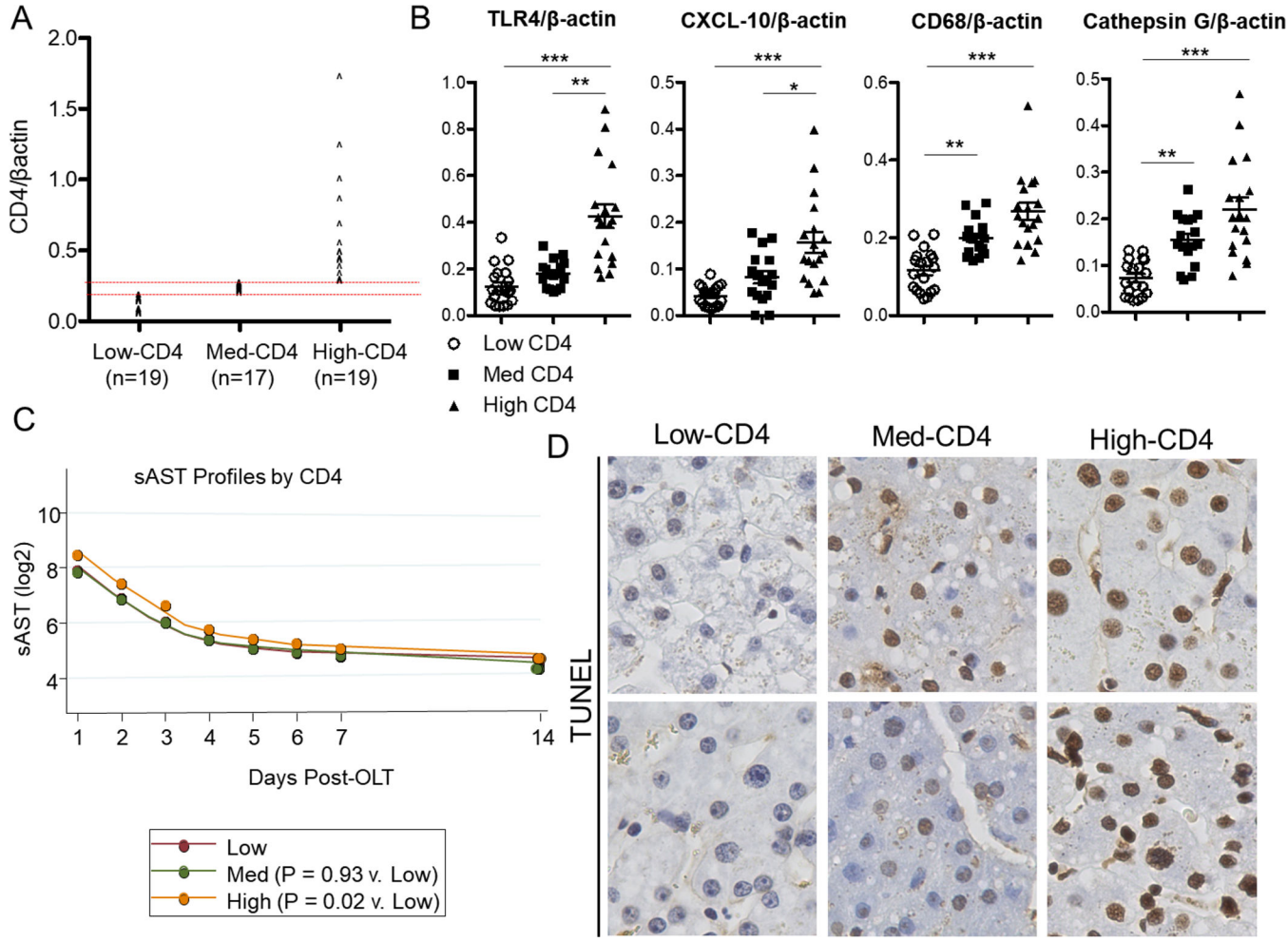

Figure 5. Intra-graft CD4 levels and clinical OLT outcomes.

(A) Fifty-five OLT patients were divided into CD4-low (n=19), -med (n=17), and -high (n=19) expression groups according to intragraft CD4 levels in biopsies collected at 2h post Tx. (B) Intragraft gene expressions of TLR4, CXCL10, CD68, and Cathepsin G in CD4 -low, -med, vs. -high groups were plotted. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Kruskal-Wallis test with Dunn's posttest). Data was shown in dot plots with mean±SEM. (C) Serum AST levels at postoperative day 1 to 14. Purple, green, and orange closed circle and line indicate the low-, med-, and high-CD4 patients, respectively. Data indicates means. (D) Representative TUNEL staining of OLT biopsies of CD4 -low, -med and -high groups (2 samples for each group). Original magnification, x400.