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Heme Oxygenase-1 Dictates Innate – Adaptive Immune Phenotype in Human Liver Transplantation

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Abstract

Liver transplantation (LT) has become the standard of care for patients with end-stage liver disease and those with hepatic malignancies, while adaptive immune-dominated graft rejection remains a major challenge. Despite potent anti-inflammatory and cytoprotective functions of heme oxygenase-1 (HO-1) overexpression upon innate immune-driven hepatic ischemia reperfusion injury, its role in adaptive immune cell-driven responses remains to be elucidated. We analyzed human biopsies from LT recipients (n=55) to determine putative association between HO-1 levels and adaptive/co-stimulatory gene expression programs in LT. HO-1 expression negatively correlated with innate (CD68, Cathepsin G, TLR4, CXCL10), adaptive (CD4, CD8, IL17) and co-stimulatory (CD28, CD80, CD86) molecules at the graft site. LT recipients with high HO-1 expression showed a trend towards improved overall survival. By demonstrating the association between graft HO-1 levels and adaptive/co-stimulatory gene programs, our study provides important insights to the role of HO-1 signaling in LT patients.

Introduction

Liver transplantation (LT) has become the standard of care for patients with end-stage liver disease and those with hepatic malignancies (1). Despite improved survival rates due to a progress in surgical techniques and post-operative patient management, nearly 30% of LT recipients lose their grafts within 5 years (2, 3). In convert with innate immune cells, adaptive immune system plays a central role in the pathogenesis of LT rejection and dysfunction (2). Co-stimulation, provided by the interaction between co-stimulatory molecules expressed on T-cells and antigen presenting cells such, as macrophages or dendritic cells, is required for T cell alloreactivity and ultimate graft rejection (3). Indeed, the blockade of CD28-CD80/86 costimulation has led to calcineurin inhibitor-free

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immunosuppression in clinical kidney transplantation (4), whereas, the efficacy of CD80-CD86 blockade in the liver transplant patients awaits compelling evidence with a larger patient cohort (5). With preventive strategies leaving much room for clinical LT improvement, better understanding of pathogenic and tolerogenic mechanisms underlining allograft rejection is of paramount importance.

Heme oxygenase-1 (HO-1; HMOX1), a heat shock protein (hsp32) catalyzing the conversion of heme into biliverdin, carbon monoxide, and iron, exerts anti-oxidative and anti-inflammatory functions (6). A plethora of studies have reported its immunoregulatory functions in innate immune cells, such as macrophages and neutrophils, while we and others demonstrated potent anti-inflammatory and cytoprotective functions of HO-1 against hepatic ischemia reperfusion injury (IRI) in preclinical LT models (7) (8) (9) (10). Indeed, a therapeutic HO-1 inducing approach using ex-vivo genetically modified HO-1 overexpressing macrophages ameliorated IRI in a mouse LT model (9). In parallel, we reported that post-transplant HO-1 expression negatively correlated with the severity of early liver damage in LT patients (9) (11). Although experimental evidence indicates HO-1 may modulate adaptive immune responses (12) (13) and promote engraftment (14), its relevance in clinical LT remains to be elucidated.

In this study, we analyzed hepatic biopsies from fifty-five LT patients for putative crosstalk between HO-1 and adaptive immune gene expression programs. By demonstrating, for the first time, the association between graft HO-1 levels and adaptive/co-stimulatory immune phenotype, our study provides important insights as to the role of HO-1 in LT patients.

Materials and Methods

Clinical liver transplant study

Fifty-five adult primary liver transplant (LT) recipients were recruited under IRB protocol (13–000143) between May 10, 2013 and April 6, 2015. Patients provided informed consent prior to their participation in the study. The demographic data and clinical parameters of recipients and donors are shown in Table S1. Routine standard of care and immunosuppressive therapy was administered as specified by UCLA liver transplant protocols. Study data were collected and managed using REDCap electronic data capture tools hosted at UCLA (15). All donor organs were perfused with and stored in cold University of Wisconsin solution (ViaSpan; Bristol-Meyers Squibb Pharma, Garden City, NY). Cold ischemia time was defined as the time from the perfusion of the donor with preservation solution to the removal of the liver from cold storage. Protocol Tru-Cut needle biopsies (Bx) were obtained intra-operatively from the left lobe approximately 2 h after portal reperfusion (prior to surgical closing of abdomen) and snap-frozen (Fig. 1).

Quantitative RT-PCR analysis

RNA was extracted from LT biopsies using RNAse Mini Kit (Qiagen, Germantown, MD). (16). A total of 5.0μg of RNA was reverse-transcribed into cDNA. Quantitative PCR was performed using QuantStudio 3 (Applied Biosystems, Foster City, CA) (17) (18). The

primers sequences are listed in Table S2. The expression of the target gene was normalized to the housekeeping β-actin.

Statistical Analysis

Group comparisons were performed using Mann-Whitney U test for continuous values and Fisher's exact test for categorical variables, respectively. Spearman's correlation coefficient (r) was used to evaluate the strength of linear relationship between variables. The cumulative graft survival rate was analyzed by Kaplan-Meier method, and differences between groups were compared using a log-rank test. JMP for Windows 8.0 (SAS Institute, Cary, NC) was used for statistical analyses. A p-value of <0.05 was considered statistically significant.

Results

HO-1 levels at the graft site negatively correlate with innate immune gene signature in LT

We have reported HO-1 modulates innate immune responses in preclinical LT models (7) (8) (9) (10). To verify the clinical relevance of our findings, we now collected human hepatic Bx from LT recipients (n=55) at 2 h after reperfusion, and screened them by RT-PCR (Fig. 1). Graft HO-1 expression levels correlated negatively with mRNA coding for CD68 (a macrophage marker, r=−0.2802, p=0.0383, Fig. 2A) and Cathepsin G (a neutrophil marker, r=−0.4804, p=0.0002, Fig. 2B), indicating increased HO-1 expression was associated with diminished innate immune cell infiltration in human LT. Post-reperfusion HO-1 levels also correlated negatively with the expression of TLR4 (a key receptor to sense initial cell damage/prime inflammatory cascade, r=−0.4706, p=0.0003, Fig. 2C) and CXCL10 (a key pro-inflammatory chemokine, r=−0.4768, p=0.0002, Fig. 2D). Hence, increased HO-1 in human LT was accompanied by inhibition of innate immune gene expression program at the graft site.

HO-1 levels at the graft site negatively correlate with adaptive immune gene expression in LT

We next aimed to evaluate putative association between graft HO-1 and adaptive immune gene signatures. HO-1 levels correlated with mRNA coding for CD4 (r=−0.3600, p=0.0069, Fig. 3A) and CD8 (r=−0.5031, p<0.0001, Fig. 3B), indicting increased HO-1 levels coincided with decreased T-cell infiltration at the graft site. We have reported that IL17 was one of the prominent T-lymphocyte cytokines to switch from an innate to adaptive immune activation in human LT (19). As shown in Fig. 3C, post-reperfusion graft HO-1 levels correlated negatively with IL17 mRNA expression (r=−0.5384, p<0.0001). These data indicate increased HO-1 was associated with suppressed adaptive immune signature in human LT.

HO-1 levels at the graft site negatively correlate with T cell co-stimulatory gene expression in LT

Having demonstrated the association between graft HO-1 and innate/adaptive immune gene signature, we next focused on the graft costimulatory program in LT. Post-reperfusion HO-1 expression correlated negatively with CD28 costimulatory T cell receptor (r=−0.4582, p=0.0004, Fig. 4A), and its CD80 (r=−0.5149, p<0.0001, Fig. 4B) and CD86 (r=−0.4978,

p=0.0001, Fig. 4C) co-stimulatory innate immune cell ligands. Thus, increased HO-1 levels in LT correlated with diminished CD80/CD86-CD28 co-stimulation phenotype.

Increased HO-1 levels at the graft site trend towards improved survival

With a newly found association between graft HO-1 and adaptive immune gene expression program, we next asked whether graft HO-1 levels correlate with LT patient survival. LT recipients were divided into low HO-1 (n=28) and high HO-1 (n=27) expression groups, based on the RT-PCR-assisted mRNA levels at 2 h post-reperfusion. There was no correlation between HO-1 grouping and donor age, gender, weight, body mass index (BMI), cold ischemia time and donation status (donation after cardiac death [DCD] or donation after brain death [DBD]). There was no relationship between HO-1 classification and recipient age, gender, weight, BMI, disease etiology, prevalence of hepatocellular carcinoma (HCC), ABO compatibility, or model for end-stage liver disease (MELD) score. There was no correlation between HO-1 grouping and surgical factors, including intra-operative blood loss and operation procedure time (Table S1). Consistent with Fig. 2 data, LT with increased HO-1 expression had decreased mRNA levels coding for CD68 (p=0.0549, Fig. S1A), Cathepsin G (p=0.0015, Fig. S1B), TLR4 (p=0.0029, Fig. S1C) and CXCL10 (p=0.0011, Fig. S1D). Moreover, consistent with Fig. 3/4 data, LT with increased HO-1 expression pattern showed decreased mRNA levels coding for CD4 (p=0.0241), CD8 (p=0.0008), IL17 (p=0.0007), CD28 (p=0.0047), CD80 (p=0.0013), and CD86 (p=0.0026) (Fig. 5B). Interestingly, patients with increased HO-1 levels at the graft site trended towards better, albeit not significant, survival at 2-years, as compared to low HO-1 group (91.2% and 83.5%, respectively; p=0.4076, log-rank test, Fig. 5C).

Discussion

A number of studies have shown a critical contribution of HO-1 to mitigate innate immune-driven IR-hepatocellular damage (7) and the ability of HO-1 to directly modulate macrophage and neutrophil activation in vitro (7) (8) (11). Indeed, ex-vivo genetically modified HO-1 overexpressing macrophages mitigated IRI in a mouse LT model (9). In parallel, increased HO-1 expression in human LT was associated with attenuated hepatocellular injury post-reperfusion (9). However, in addition to early innate immune-driven IRI, subsequent LT rejection response remains a clinical problem during which adaptive immune cells dominate the local inflammatory injury. Several recent studies indicate HO-1 may control adaptive immune responses in mice, with HO-1 expressing dendritic cells promoting regulatory T cells and alleviating airway inflammation (13) or inhibiting co-stimulatory signals and improving cardiac allograft survival (14). With HO-1 predominantly expressed by macrophages, both in mouse and human LT (8) (20), others have shown that myeloid-specific HO-1 deficiency augmented CD80/CD86 expression, increased CD4+/CD8+ T-cell infiltration, and enhanced frequency of IL17-producing CD4+ T-cells in a murine autoimmune encephalomyelitis model (12). Here, we screened clinical LT biopsies and identified negative association between HO-1 expression and adaptive (CD4, CD8, IL17, Fig. 3) and co-stimulatory (CD28, CD80, CD86, Fig. 4) immune markers, all supporting the notion that graft HO-1 can indeed regulate co-stimulatory signaling at macrophage – T cell interface, and modulate adaptive alloreactivity. In parallel to decreased

expression of adaptive immune markers, high HO-1 expression LT group showed better, albeit not statistically different, overall survival (Fig. 5C). We assume that our current patient cohort (n=55) was not large enough to detect survival differences between low vs. high HO-1 expression groups; while assessing HO-1 levels at 2 h post-reperfusion may have been too early to predict late rejection crises. Being aware of the limitations of our study and unable to recognize causality and influence of HO-1 on LT survival, future animal experiments as well as clinical trials with larger patient cohorts are warranted.

Although aging and obesity are well-known poor-prognostic factors in LT, there was no correlation between graft HO-1 levels and donor/recipient demographic parameters, including age and BMI in our study (Table S1). On the other hand, human HO-1 gene expression is modulated by two functional polymorphisms in the gene promoter. First, a short $(GT)_n$ repeat polymorphism has been associated with enhanced transcriptional HO-1 activity (21). Indeed, a short $(GT)_n$ repeat in the kidney graft was accompanied by a favorable post-transplant renal function and survival (22, 23). Second, A(−413)T single nucleotide polymorphism (SNP) has also been identified as a functionally relevant variation of the HO-1 gene, while A-allele rather than T-allele of this SNP correlated with a higher promotor activity (24). In addition, post-reperfusion graft HO-1 level is influenced by genetic background of both donor and recipient (11). Since the decisive factors of graft HO-1 level in clinical LT remain to be elucidated and several lines of HO-1 inducing therapies now await clinical application (25) (26), future comprehensive studies are desired for perspective personalized LT management.

In conclusion, to the best of our knowledge, this is the first study to document the association between hepatic HO-1 expression and co-stimulatory and adaptive immune gene expression program, in LT patients. Since graft rejection remains a clinical problem, our findings add important mechanistic insights and highlight a novel therapeutic potential to target graft HO-1 phenotype in LT recipients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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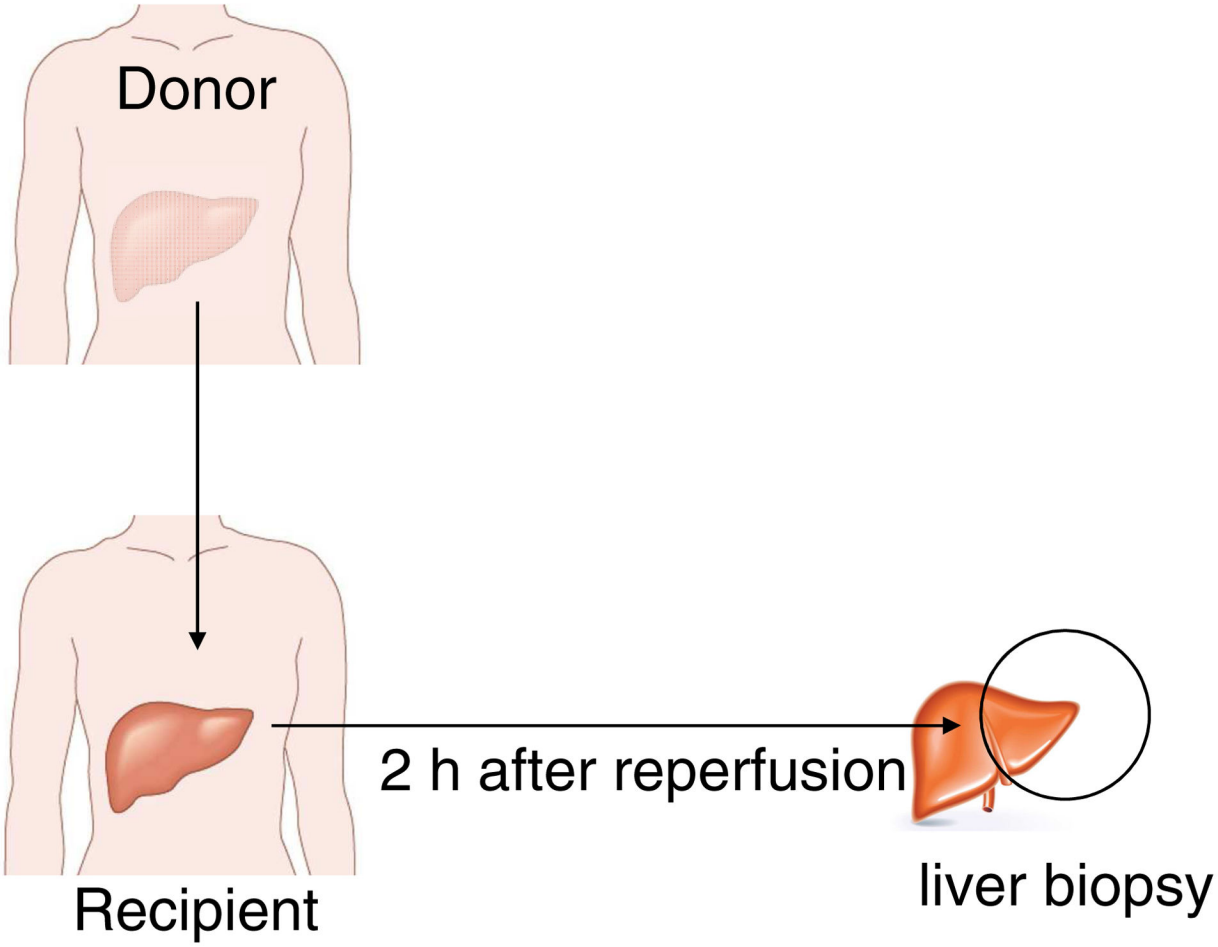

Figure 1: Post-reperfusion liver biopsy (Bx) collection in human liver transplant (LT).
Hepatic Bx samples were collected from fifty-five human LT cases approximately 2 h after portal reperfusion (prior to surgical closing of abdomen) under IRB protocol.

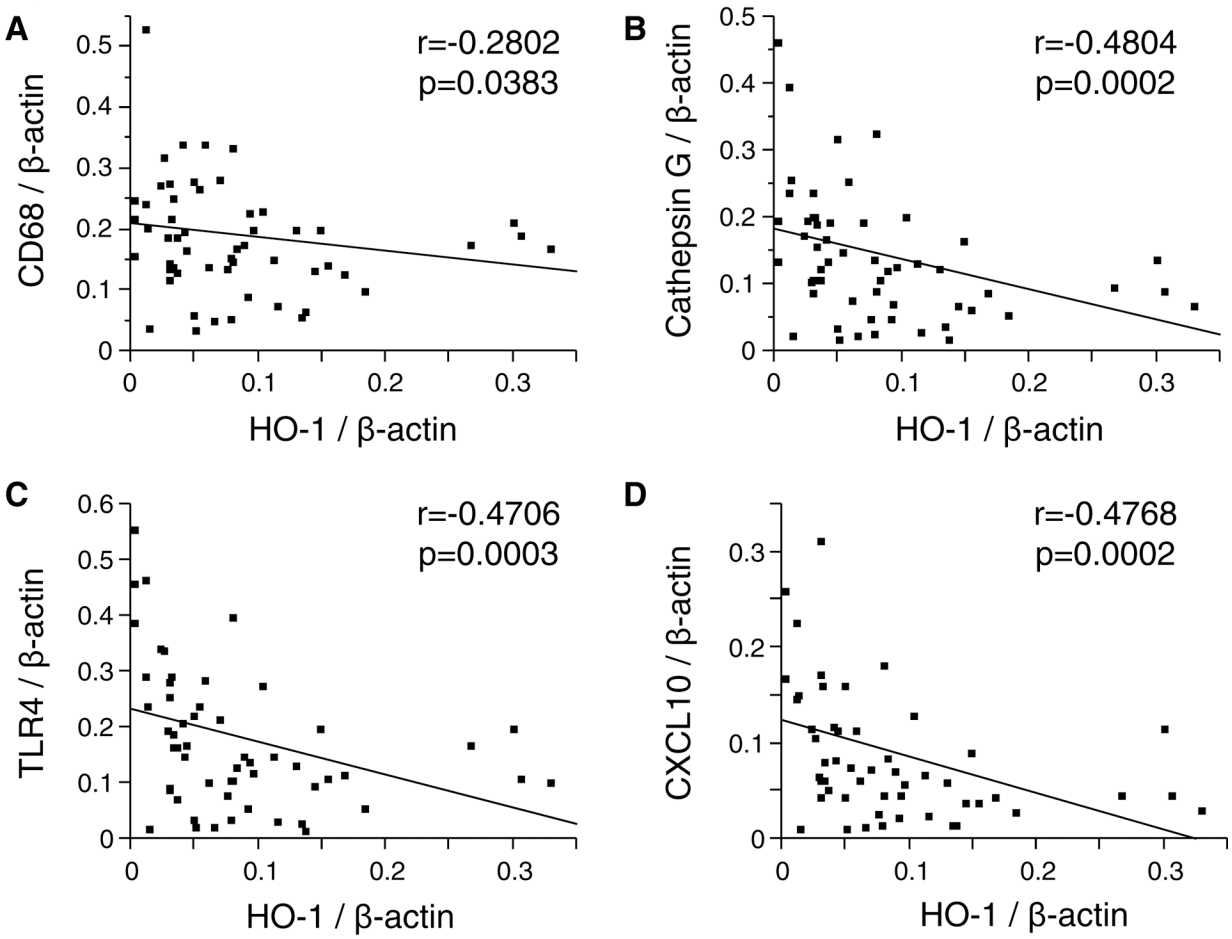

Figure 2: Heme oxygenase-1 (HO-1) levels correlate with innate immune gene expression program in LT.

Clinical Bx samples were collected at 2 h after portal reperfusion from fifty-five LT patients. mRNA levels of HO-1, CD68, Cathepsin G, TLR4 and CXCL10 were analyzed by RT-PCR with β-actin normalization. Relationship between HO-1 and: **(A)** CD68; **(B)** Cathepsin G; **(C)** TLR4. **(D)** CXCL10. r: Spearman's correlation coefficient.

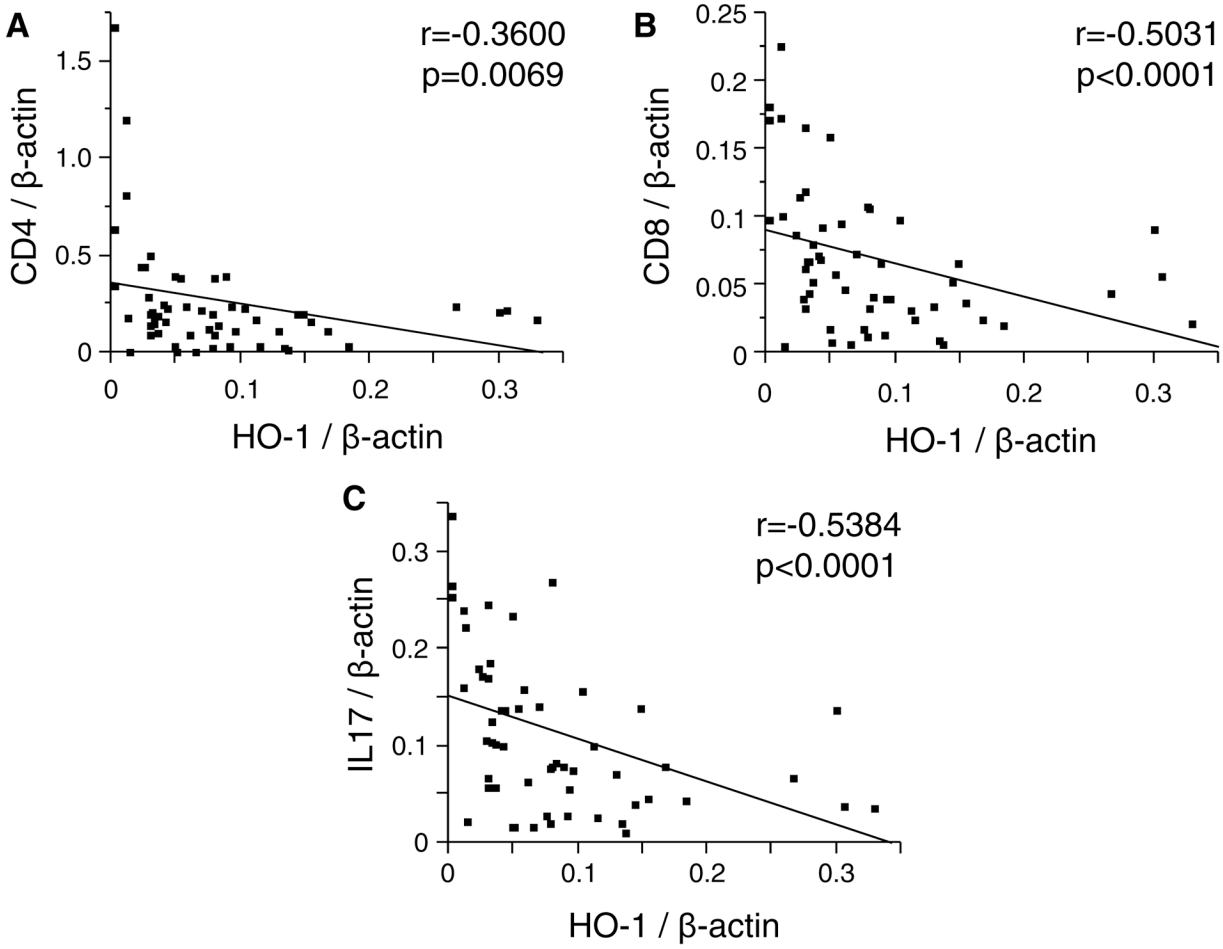

Figure 3: HO-1 levels correlate with adaptive immune gene expression in LT.

Clinical Bx samples were collected at 2 h after portal reperfusion from fifty-five LT patients. mRNA levels of HO-1, CD4, CD8, and IL17 were analyzed by RT-PCR with β-actin normalization. Relationship between HO-1 and: **(A)** CD4; **(B)** CD8; **(C)** IL17. r: Spearman's correlation coefficient.

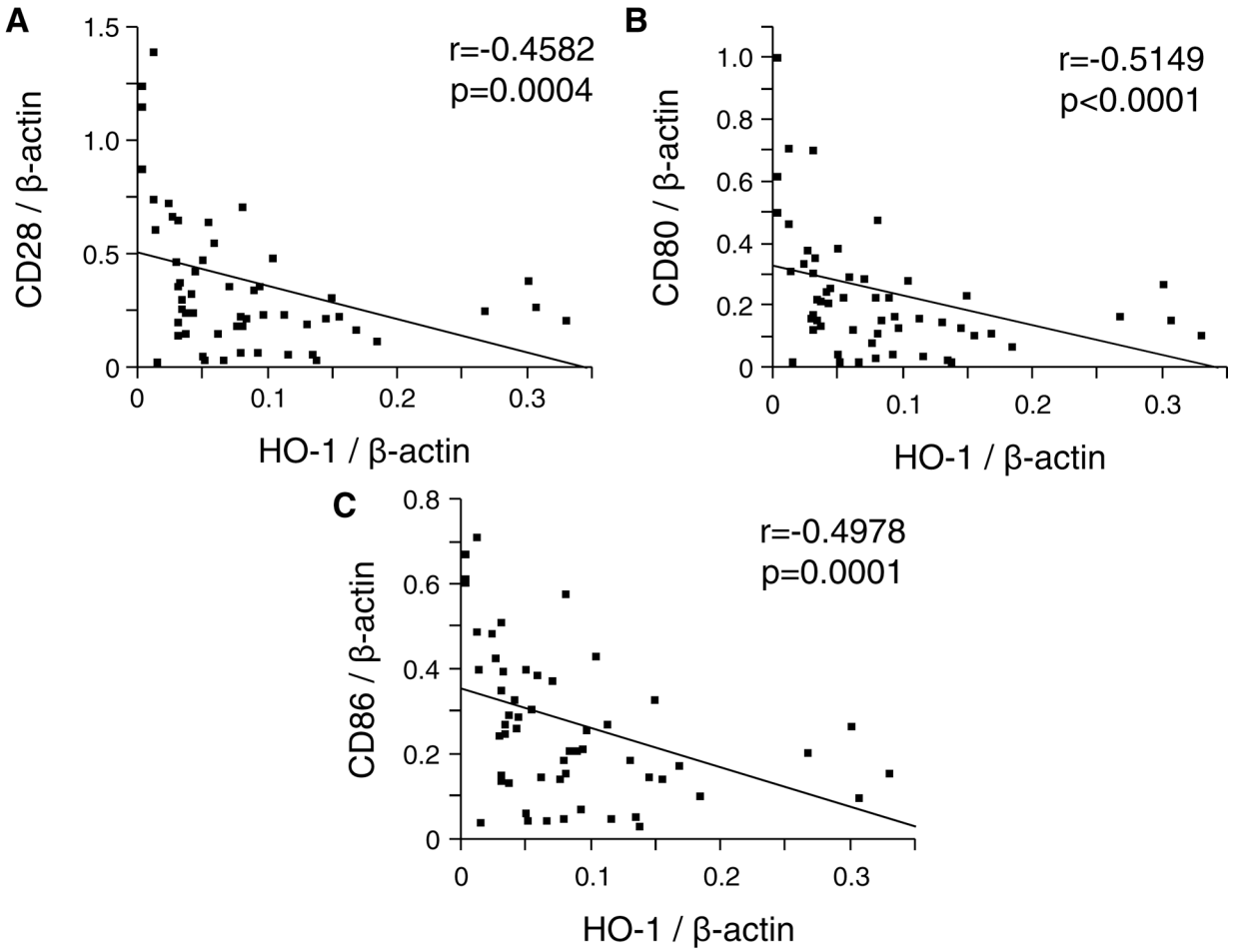

Figure 4: HO-1 levels correlate with co-stimulatory gene expression in LT.

Clinical Bx samples were collected at 2 h after portal reperfusion from fifty-five LT patients. mRNA levels of HO-1, CD28, CD80 and CD86 were analyzed by RT-PCR with β-actin normalization. Relationship between HO-1 and: **(A)** CD28; **(B)** CD80; **(C)** CD86. r: Spearman's correlation coefficient.

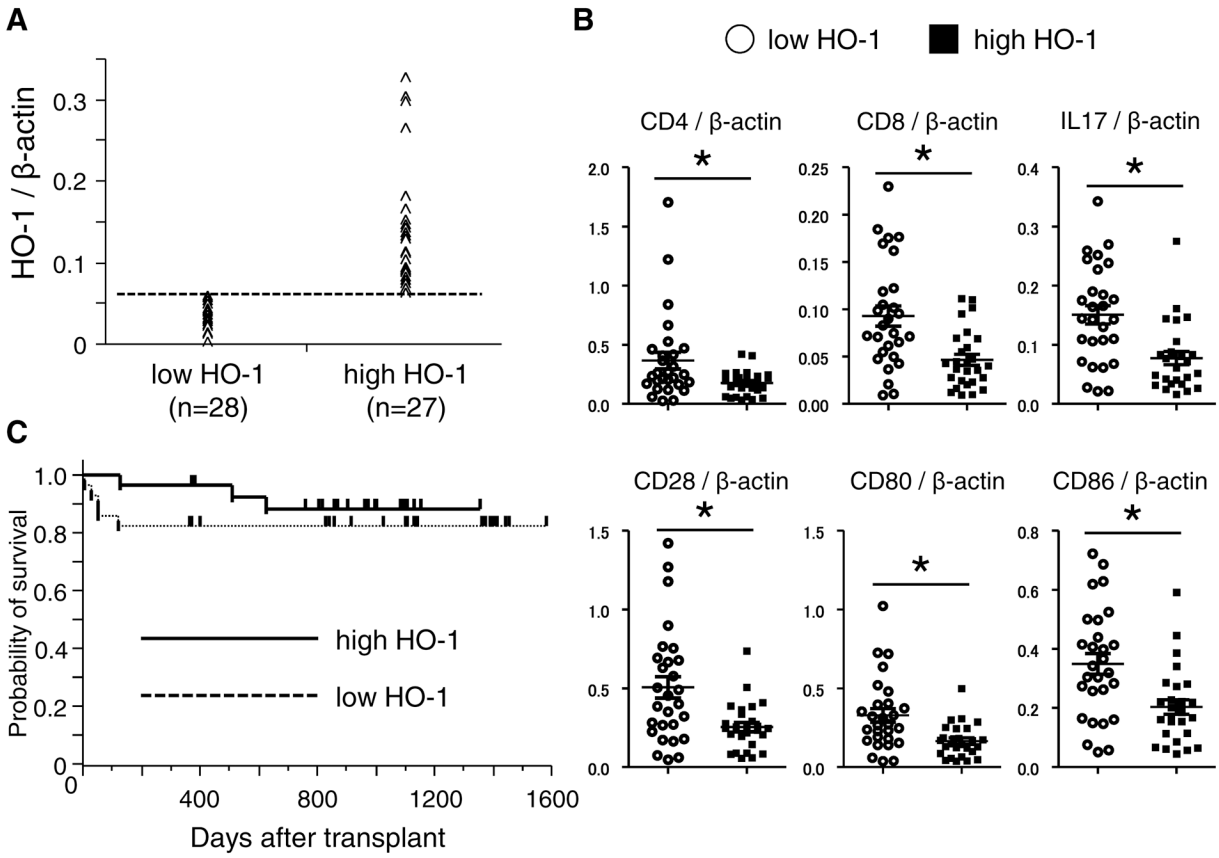

Figure 5: Adaptive immune gene expression and survival in low vs. high HO-1 expression LT groups.

(A) Bx samples collected 2 h after reperfusion from fifty-five LT patients were analyzed by RT-PCR. Based on mRNA level of HO-1 with β-actin normalization, LT cases were classified into low HO-1 (n=28) and high (n=27) groups. **(B)** RT-PCR assisted detections of mRNA levels coding for CD4, CD8, IL17, CD28, CD80 and CD86 with β-actin normalization. **(C)** The cumulative probability of patient survival (Kaplan-Meier method). Dotted line indicates low HO-1, while solid line indicates high HO-1 groups. *: $p<0.05$ (Mann–Whitney U test).