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CD39 deficiency in murine liver allografts promotes inflammatory injury and immune-mediated rejection

Osamu Yoshida [a], Lei Dou [a], Shoko Kimura [a], Shinichiro Yokota [a], Kumiko Isse [a,b], Simon C. Robson [c], David A. Geller [a,d], Angus W. Thomson [a,e,*]

[a] Starzl Transplantation Institute, Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[b] Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA
[c] Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA
[d] University of Pittsburgh Medical Center, Liver Cancer Center, Pittsburgh, PA, USA
[e] Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

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ABSTRACT

Adenosine triphosphate (ATP), an essential metabolic energy source, is released following cell apoptosis or necrosis. It acts as a damage-associated molecule pattern to stimulate innate immune cells. The ectonucleotidase CD39 regulates immune activation by hydrolysis of extracellular ATP. We have shown previously that CD39 expression by donor livers helps protect syngeneic grafts with extended (24 h) cold preservation time from ischemia reperfusion injury. Given its immune regulatory properties, we hypothesized that CD39 expression in donor livers might modulate transplant tolerance that occurs following mouse allogeneic liver transplantation (LTx). Livers from C57BL/6 (B6) wild-type (WT) or CD39 KO mice were transplanted into normal C3H recipients with minimal (approximately 1 h) cold ischemia. Serum alanine aminotransferase levels at day 4 post-LTx were significantly higher in animals given CD39KO compared with WT livers. Moreover, IFN-γ production by liver-infiltrating $CD8^+$ T cells at day 4 was significantly higher in CD39KO than in WT grafts. Furthermore, splenic T cells from CD39KO liver recipients exhibited greater proliferative responses to donor alloantigens than those from mice given WT grafts. By contrast, there was a concomitant significant reduction in the frequency of regulatory T cells (Treg) in CD39KO than in WT livers. Whereas WT liver allografts survived >100 days, no CD39KO grafts survived beyond 40 days (median survival time [MST]: WT: >100 days vs CD39KO: 8 days; $p < 0.01$). In addition, soluble CD39 administration significantly prolonged CD39KO liver allograft survival (MST: 27.5 days). These novel data suggest that CD39 expression in liver allografts modulates tissue injury, inflammation, anti-donor effector T cell responses and Treg infiltration and can suppress transplant rejection.

1. Introduction

The liver performs important metabolic functions, degrades toxic and waste products, and regulates immunity. Impairment of these functions, due to autoimmune liver disorders, viral hepatitis, alcohol or cancer can lead to end-stage liver disease, for which liver transplantation (LTx) is the only therapeutic option. The liver is regarded as a lymphoid organ with a unique constituency of immune cells [1–3] and exhibits inherent tolerogenic properties [3–5] These include oral and portal venous tolerance and allograft acceptance in rodents or pigs, without dependence on any immunosuppressive therapy [6,7]. Human liver transplant recipients have a relatively low susceptibility to rejection and achieve a comparatively high frequency of successful immunosuppressive drug withdrawal compared with recipients of other organs [5,8,9]. Although donor-derived leukocytes [10,11], donor-derived dendritic cells (DC) [12,13], regulatory T cells (Treg) [14,15] and expression of immune regulatory molecules, in particular B7-H1 [16] (=programed death ligand-1) and DNA-activating protein of 12 kD (DAP12) [17] on donor cells have been implicated as important factors that promote experimental allograft liver acceptance, mechanisms underlying liver transplant tolerance are still poorly understood.

Adenosine triphosphate (ATP) is essential for cell metabolism and is stored in the cell cytosol. Once released extracellularly, as the result of cell death or injury, extracellular (e)ATP acts as a damage-associated molecular pattern (DAMP) that activates innate immune cells through

Abbreviations: Ab, antibody; Ag, antigen; ALT, alanine aminotransferase; APC, antigen-presenting cell(s); ATP, adenosine triphosphate; DAMP, damage-associated molecular pattern; DC, dendritic cell(s); LTx, liver transplantation; MAMP, microbe-associated molecular pattern; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction; Treg, regulatory T cell(s); WT, wild-type.

* Corresponding author at: Starzl Transplantation Institute, University of Pittsburgh School of Medicine, 200 Lothrop Street, BST W1540, Pittsburgh, PA 15261, USA. Tel.: +1 412 624 6392; fax: +1 412 624 1172.
E-mail addresses: osamuyoishida@yahoo.co.jp (O. Yoshida), doul@upmc.edu (L. Dou), kimuras@upmc.edu (S. Kimura), yokotas@upmc.edu (S. Yokota), issek@upmc.edu (K. Isse), srobson@bidmc.harvard.edu (S.C. Robson), gellerda@upmc.edu (D.A. Geller), thomsonaw@upmc.edu (A.W. Thomson).

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its receptors, P2X and P2Y [18,19]. eATP not only activates eosinophils, neutrophils, macrophages and DC [18], but also recruits these immune cells as a 'find-me' signal [20]. eATP concentrations increase at sites of inflammation, contact hypersensitivity [21], tumor growth [22], liver injury [23] and graft-versus-host disease following bone marrow transplantation [24]. Furthermore, the eATP/P2 receptor axis is involved in the pathogenesis of organ allograft rejection [25] and P2X7 receptor deficiencies, or inhibition of P2X7 prolong mouse heart allograft survival [26].

CD39 is an ectonucleotidase and hydrolyzes eATP to maintain homeostatic eATP levels. eATP hydrolysis by CD39 regulates immune cell activation and recruitment [20,27]. Thus, CD39 contributes to the pathogenesis of infectious diseases [28], cancer [29–31], autoimmune disorders [32–34], and ischemia/reperfusion injury of the heart [35], kidney [36], intestine [37] and liver [38,39]. We have reported recently [40] that CD39 deficiency exacerbates liver injury after syngeneic LTx with 24 h cold organ storage. However, the role of CD39 in allogeneic organ transplantation has not been investigated.

Previously [40], we reported that cell surface costimulatory molecules, production of pro-inflammatory cytokines and T cell allostimulatory activity are augmented in CD39KO liver conventional myeloid dendritic cells (DC), that are regarded as key instigators and regulators of alloimmunity [41,42]. These observations suggest an enhanced potential of CD39KO liver DCs to stimulate host T cell responses and thus enhanced immunogenicity of CD39-deficient liver allografts. To evaluate the contribution of donor CD39 to liver allograft immunity and survival, we performed orthotopic mouse LTx with minimal cold ischemia time using B6 WT or CD39KO (B6 background) mice as donors and normal C3H mice as recipients. We examined allograft injury, the immune effector and regulatory T cell responses elicited and transplant survival. Our data suggest that CD39 expression by donor livers is important for the regulation of tissue injury, inflammation, anti-donor immunity and the suppression of liver allograft rejection.

2. Materials and methods

2.1. Mice

Male C57BL/6 (B6; H-2^b), and C3H ($H2^k$) mice (8- to 12-weeks old) were purchased from The Jackson Laboratory, Bar Harbor, ME. CD39KO mice (B6 background) were bred from pairs received from the Beth Israel Medical Center, Harvard University, Boston, MA. Animals were maintained in the specific pathogen-free Central Animal Facility of the University of Pittsburgh School of Medicine. Experiments were conducted under an Institutional Animal Care and Use Committee-approved protocol and in accordance with criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. Animals were fed a diet of Purina rodent chow (Ralston Purina, St. Louis, MO) and received tap water ad libitum.

2.2. Reagents

Complete culture medium comprised RPMI-1640 (BioWhittaker, Walkersville, MD) supplemented with 10% (v/v) fetal calf serum (Nalgene, Miami, FL), non-essential amino acids, L-glutamine, sodium pyruvate, penicillin–streptomycin, and 2-mercaptoethanol (all from Life Technologies, Gaithersburg, MD). ATP and soluble CD39 (Apyrase) were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Liver transplantation

Harvesting and orthotopic transplantation of the liver without hepatic artery reconstruction were performed as described initially by Qian et al. [7] with minor modifications [43]. Liver grafts (WT B6 or CD39KO) were perfused with University of Wisconsin solution via the portal vein, then transplanted into C3H recipients by anastomosis of the suprahepatic vena cava with a running 10–0 suture and by anastomosis of the portal vein and inferior vena cava using the cuff technique. The bile duct was connected via ligation over the stent. The cold ischemia period consistently lasted approximately 1 h. No immunosuppressive therapy was administered. Graft rejection was determined by host survival and confirmed histologically. In some recipients, apyrase (soluble CD39) was injected i.p. at days 0, 1, 3, 5, and 7 post-transplant and once a week after day 14.

2.4. ALT measurement

Serum alanine aminotransferase (ALT) levels were quantified as described [44].

2.5. Allograft histology

Hematoxylin and eosin-stained tissue sections were graded in a 'blinded' fashion by a transplant pathologist (KI) using the Banff schema for acute liver rejection [45].

2.6. Flow cytometry

Hepatic non-parenchymal cells (NPC) and spleen cells were treated with FcγR-blocking rat anti-mouse CD16/32 mAb (2.4G2) to prevent non-specific antibody (Ab) binding. They were then incubated for 30 min with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, APC-, PE-cyanin (Cy)5-, PE-Cy7- or Pacific Blue-conjugated monoclonal Abs (mAbs) to detect surface expression of CD3 (145-2C11) CD4 (GK1.5) or CD8 (53-6.7) (all eBioscience, San Diego, CA). For intracellular cytokine staining, cells were fixed with 4% paraformaldehyde and permeabilized using 0.1% saponin, then stained with anti-mouse IFN-γ Ab (XMG1.2) (BioLegend). For forkhead box p3 (Foxp3) staining, cells were fixed and permeabilized using Foxp3 Fix Perm kit (eBioscience) and stained with anti-Foxp3 mAb (FJK-16s) (eBioscience). Appropriate Ig isotype controls were obtained from BD Pharmingen (San Diego, CA). Flow analysis was performed using an LSR Fortessa flow cytometer (BD Biosciences) and results expressed as percent positive cells and mean fluorescence intensity (MFI).

2.7. Real-time reverse-transcription polymerase chain reaction (RT-PCR)

Messenger RNA (mRNA) expression was quantified by SYBR Green real-time RT-PCR using an ABI-Prism 7000 sequence detection system (PE Applied Biosystems, Foster City, CA) and primers specific for IFN-γ (F: 5′-CACGGCACAGTCATTGAAAG-3′; R; 5′-TTTTGCCAGTTCCTCCAG AT-3′) or β-actin (F: 5′-AGAGGGAAATCGTGCGTGAC-3′; R: 5′-CAATAG TGATGACCTGGCCGT-3′). The expression of IFN-γ was normalized to the expression of β-actin mRNA using the comparative cycle threshold method.

2.8. Anti-donor T cell responses

To assess anti-donor immune responses, T cell-depleted WT B6 splenocytes were used as stimulators and carboxyfluorescein succinimidyl ester (CFSE)-labeled recipient splenocytes as responders. Stimulator and responder cells were co-cultured at 1:1 ratio for 5 days. CFSE-labeled T cell proliferation was determined by flow cytometry [17].

2.9. Statistical analyses

The significances of differences between means were ascertained using the unpaired Student 't' test or Gehan–Breslow–Wilcoxon test using Prism version 5.00 (GraphPad Software, San Diego, CA). Values of $p < 0.05$ were considered significant.