

Liver Grafts from CD39-Overexpressing Rodents Are Protected From Ischemia Reperfusion Injury Due to Reduced Numbers of Resident CD4⁺ T Cells

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Ischemia-reperfusion injury (IRI) is a major limiting event for successful liver transplantation, and CD4⁺ T cells and invariant natural killer T (iNKT) cells have been implicated in promoting IRI. We hypothesized that hepatic overexpression of CD39, an ectonucleotidase with antiinflammatory functions, will protect liver grafts after prolonged cold ischemia. CD39-transgenic (CD39tg) and wildtype (WT) mouse livers were transplanted into WT recipients after 18 hours cold storage and pathological analysis was performed 6 hours after transplantation. Serum levels of alanine aminotransferase and interleukin (IL)-6 were significantly reduced in recipients of CD39tg livers compared to recipients of WT livers. Furthermore, less severe histopathological injury was demonstrated in the CD39tg grafts. Immune analysis revealed that CD4⁺ T cells and iNKT cells were significantly decreased in number in the livers of untreated CD39tg mice. This was associated with a peripheral CD4⁺ T cell lymphopenia due to defective thymocyte maturation. To assess the relative importance of liver-resident CD4⁺ T cells and iNKT cells in mediating liver injury following extended cold preservation and transplantation, WT mice depleted of CD4⁺ T cells or mice genetically deficient in iNKT cells were used as donors. The absence of CD4⁺ T cells, but not iNKT cells, protected liver grafts from early IRI. Conclusion: Hepatic CD4⁺ T cells, but not iNKT cells, play a critical role in early IRI following extended cold preservation in a liver transplant model. (HEPATOLOGY 2013;57:1597-1606)

schemia-reperfusion injury (IRI) is inherent to the transplant process. The duration of warm ischemia is relatively short for brain-dead heart-beating

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donors but more prolonged in states of donor cardiac arrest. Cold ischemia extends from organ procurement to engraftment and reperfusion. Donor organs are stored on ice to slow ischemic damage, but prolonged cold ischemia increases the risk of delayed graft function.¹ In the United States, the cold ischemic time during liver transplantation is usually between 6 and 10 hours.²

Noxious agents accumulate during ischemia and tissue injury is propagated when blood flow is reestablished due to ongoing inflammation and coagulation, which initiates a self-perpetuating cycle. In healthy tissue, adenosine triphosphate (ATP) is virtually absent from the extracellular milieu (1 to 10 nM range) but highly concentrated intracellularly (5 to 10 mM range). Upon cell injury or death such as initiated by IRI, ATP is quickly released and acts as a "danger signal" to propagate inflammation.³ CD39 is an ectonucleotidase that hydrolyses the nucleotides ATP and adenosine diphosphate (ADP) to adenosine monophosphate (AMP), which is converted into adenosine by CD73.^{4,5} CD39 has both thromboregulatory and immunoregulatory functions through modulating

Abbreviations: A2aR, adenosine A2a receptor; ALT, alanine transaminase; ATP, adenosine triphosphate; CD39tg, CD39 transgenic; IFN- γ interferongamma; iNKT, invariant NKT; IRI, ischemia-reperfusion injury; α GalCer, α -galactosylceramide; NK, natural killer cell; WT, wildtype.

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nucleotide concentrations in the extracellular space.^{4,6,7} The importance of CD39 as a modulator of IRI has been reported in different organs: deletion of CD39 rendered mice more susceptible to intestinal and cerebral IRI^{8,9} but paradoxically protected in a model of partial hepatic warm IRI.¹⁰ Conversely, soluble CD39 treatment protected mice from cardiac and renal IRI^{11,12} by augmenting adenosine levels. Adenosine has been shown to be protective in many models of IRI, and adenosine A2a receptor (A2aR) activation dampens liver injury in warm hepatic IRI models through natural killer T (NKT)-cell dependent mechanisms.^{13,14} Recently, we have shown that mice expressing CD39 are protected in a model of warm renal IRI through A2aR-dependent mechanisms and in a more clinically relevant syngeneic renal transplant model characterized by 5 hours of cold preservation.¹⁵

T cells, NK cells, and NKT cells are involved in the response to warm hepatic IRI. A direct effect of T cells, particularly CD4⁺ T cells, has been demonstrated; T cell-deficient and CD4-deficient mice are resistant to warm hepatic IRI, and T-cell reconstitution restores injury.¹⁶⁻¹⁹ NK and NKT cells are components of human and rodent liver lymphoid cell populations and play phase-specific roles in the course of IRI. Deletion of CD39 on NK cells inhibits their activation and protects in partial hepatic IRI by way of diminished interferon-gamma (IFN- γ) production.¹⁰ CD1d knockout (KO) mice, which lack NKT cells, are protected against warm hepatic IRI.^{14,20}

CD39 is widely expressed within the immune system including on activated B cells,²¹ skin-specific dendritic cells,⁷ a subset of CD4⁺ T cells (CD4⁺CD25⁺ regulatory T cells),²² and NKT cells.²³ Human CD39 transgenic (CD39tg) mice were generated to promote an anticoagulant and antiinflammatory milieu that could alleviate renal IRI.¹⁵ Herein we extend these observations using a clinically relevant model of orthotopic liver transplantation characterized by prolonged cold storage of the donor organ. Further, we explore putative mechanisms of protection through the immune phenotyping of these mice.

Materials and Methods

Animals. C57BL/6 wildtype (WT) mice were purchased from the Walter and Eliza Hall Institute (WEHI, Melbourne, Australia). CD39tg mice overexpressing CD39 under the control of the mouse H2K^b promoter (C57BL/6 background)²⁴ were backcrossed at least 10 generations. Invariant NKT KO mice (Jalpha281^{-/-} on a C57BL/6 background) were a kind gift from Prof. Mark Smyth (Peter MacCallum

Cancer Centre, Melbourne, Australia). Experimental mice were housed under specific pathogen-free conditions. All studies were approved by the Animal Ethics Committee of St. Vincent's Hospital following the *Australian Code of Practise for the Care and Use of Animals for Scientific Purposes*, 7th ed., 2004.

Isolation of Splenocytes, Thymocytes, and Liver Lymphocytes. Spleen and thymus were mechanically disrupted and red blood cell lysis buffer (eBioscience, San Diego, CA) was used on spleen preparations. Single cell suspensions were resuspended in phosphatebuffered saline (PBS) with 2% fetal calf serum (FCS) and counted before flow cytometric analysis.

The livers of anesthetized mice were perfused with PB) by way of the portal vein, harvested, and disrupted through a cell strainer. The resulting cell suspension was washed twice in PBS with 2% FCS and the cell pellet was resuspended in 25 mL of 37% isotonic Percoll (GE Healthcare, Buckinghamshire, UK) gradient and centrifuged. The cell pellet contained liver lymphocytes.

Flow Cytometry. Freshly obtained leukocytes were stained with the following antibodies: α-galactosylceramide (α-GalCer)/CD1d tetramer-PE (kind gift from Dr. Dale Godfrey, University of Melbourne, Australia), CD3-PerCPCy5.5, CD4-FITC, CD4-PeCy5, CD4-PerCP-Cy5.5, CD69-FITC, TCRb-PE, TCRb-PeCy5 (BD Biosciences, San Diego, CA), CD4-APC, CD8a-APC (eBioscience). Intracellular staining for IFN-γ-FITC and IL4-FITC (eBioscience) was performed using Cytofix/Cytoperm (BD Biosciences) reagents containing 1 mg/mL of Brefeldin A (Sigma, St Louis, MO) and following the manufacturer's instructions. Data acquired by FACSCalibur (BD Biosciences) were analyzed using FlowJo software (Tree Star, Ashland, OR).

In Vivo *iNKT Cell Functional Assay.* Mice were intraperitoneally injected with 5 mg of α GalCer (Alexis Biochemicals, Lausen, Switzerland) diluted in PBS/0.05% Tween (BDH, Radnor, PA) or vehicle only. Two hours postinjection, organs were harvested for flow cytometric analysis and serum was collected for IL-4 determination by enzyme-linked immunosorbent assay (ELISA) using the Mouse IL-4 ELISA Ready-Set-Go kit (eBioscience) following the manufacturer's instructions.

Cell Culture and Proliferation Assay. RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/mL penicillin, 100 mg/mL streptomycin (Invitrogen), and 50 mM 2-mercaptoethanol (Sigma) was used for T-cell culture. Splenocytes were labeled

with 5 mM 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE; Invitrogen) and stimulated with 5 mg/mL plate-bound anti-CD3 (clone 145-2C11) and 5 mg/mL anti-CD28 (clone 37.51) in suspension (WEHI, Melbourne, Australia). After 48 hours proliferation was assessed by flow cytometry. Viable CFSE-labeled T cells were gated using 7-AAD and CD4-APC or CD8-APC (BD Biosciences) staining and the percentage of dividing cells was determined using the FlowJo proliferation platform.

CD4 T-Cell Depletion. Liver donor mice were intraperitoneally injected for 3 consecutive days with 0.6 mg of monoclonal anti-CD4 (clone GK1.5) antibody. Donor livers were transplanted at day 6 and spleens were harvested to confirm depletion by flow cytometry using CD4-PerCP-Cy5.5 antibody (clone L3T4) (BD Biosciences).

Bone Marrow Adoptive Transfer. Four-week-old recipient mice were irradiated twice for 20 minutes with 550 rads, 2 hours apart. The donor bone marrow was extracted from tibia and femur and resuspended in sterile saline; 10^6 cells were injected into the tail vein of irradiated recipients. After 6 weeks of reconstitution, chimeric mice were sacrificed and organs and blood were harvested for flow cytometric analysis. Donor livers were used for orthotopic transplantation into WT recipient mice.

Orthotopic Mouse Liver Transplantation. Donor livers were subjected to prolonged cold storage in University of Wisconsin (UW) solution as described.²⁵ Male donors and recipients were used. Donor livers were dissected, the bile duct was cannulated, and the gall bladder was removed. The liver was perfused through the portal vein with UW solution and was excised. The liver graft remained in UW solution for 18 hours at 4°C.

Livers of recipient mice were removed and the graft liver was placed in the orthotopic position. The cuff technique was used for anastomosis of both the portal vein and the infrahepatic vena cava. The suprahepatic vein was anastomosed. The bile duct was reconnected through the stent. The anhepatic time was kept below 25 minutes. Six hours after liver transplantation, blood samples were collected from the inferior vena cava and liver grafts were harvested for analysis.

*Measurement of Serum Transaminase and IL-*6. Serum alanine transaminase (ALT) concentration was measured using a Chemistry Analyser AU2700 (Olympus, Tokyo, Japan). Serum IL-6 concentration was measured by flow cytometry using the mouse inflammation cytometric bead array (BD Biosciences) following the manufacturer's instructions. *Histological Procedures.* Liver grafts were immersed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Whole sections were analyzed and scored following Suzuki's method²⁶ by a histopathologist blinded to treatment groups. Apoptosis was assessed using rabbit anti-cleaved caspase-3 antibody [Asp175] (Cell Signaling Technology, Danvers, MA). Sections were analyzed and apoptosis scored according to percentage of central vein involvement by a histopathologist blinded to treatment groups.

Statistical Analysis. The computer software Graph-Pad Prism (v. 5, La Jolla, CA) was used for all statistical analyses and graphical representations, except for FACS plots and histograms, which were generated using FlowJo software. For statistical significance analyses, the unpaired Student t test was used to give the stated P values. P < 0.05 was considered significant.

Results

Donor Livers from Mice Overexpressing CD39 Are Protected Against IRI. To assess the impact of CD39 overexpression on hepatic IRI, donor livers (CD39tg and WT littermates) were subjected to 18 hours of cold ischemia and transplanted into WT recipient mice. At 6 hours post reperfusion, CD39tg livers were significantly protected against IRI compared to WT donor livers, as reflected by serum ALT concentrations of 10,018 U/L (±653) and 15,638 U/L $(\pm 1,513)$, respectively (Fig. 1A). The level of systemic proinflammatory cytokine IL-6 was also significantly reduced in recipients of CD39tg donor livers (2,396 $pg/mL \pm 438$) compared to WT livers (4,336 pg/mL) \pm 647) (Fig. 1B). Histologically, large necrotic areas were evident in WT donor livers, whereas the hepatic architecture of CD39tg donor livers was better preserved with well-defined, smaller, and nonconfluent necrotic areas. Hepatocellular damage was graded according to Suzuki's histological score²⁶ (Supporting Fig. 1A): five out of six WT donor livers showed mild to severe congestion and vacuolization associated with more than 30% hepatocyte necrosis (score 2 or greater) compared to only two out of six CD39tg livers (Fig. 1C). Further, there were significantly fewer central veins surrounded by cleaved caspase-3 positive cells in donor livers from CD39tg mice (Fig. 1D; Supporting Fig. 1B).

CD39 transgene expression in donor livers was confirmed by real-time polymerase chain reaction (PCR) (Supporting Fig. 2A) and flow cytometric analysis on donor liver lymphocytes (Supporting Fig. 2B). To



Fig. 1. CD39tg donor livers are protected against IRI following orthotopic liver transplantation. Serum ALT (A) and IL-6 (B) measured 6 hours after transplantation of CD39tg or WT donor livers into WT recipients. n = 6 per group, mean \pm standard error of the mean (SEM). **P < 0.01; *P < 0.05 by Student t test. (C) Histological assessment of donor liver damage by Suzuki's score. Symbols represent donor liver, means are shown, n = 6 per group. (D) Histological assessment of donor liver damage based on the percentage of central veins with cleaved caspase-3 positive cells. Symbols represent donor liver, means are shown, n = 4 WT and n = 6 CD39tg; ***P <0.001 by Student t test. (E) Percentages of WT or CD39tg bone marrow donor cells in thymus, spleen, and liver, 6 weeks after bone marrow adoptive transfer into WT or CD39tg recipient mice. Bars represent means \pm SEM, n = 6 per group. ***P < 0.001 by Student t test. (F) Serum ALT measured 6 hours after transplantation of WT (n = 5) or CD39tg (n = 6) donor livers from irradiated mice reconstituted with WT bone marrow. All recipients are WT mice. Data represent mean \pm SEM. ns, not significant by Student t test.

determine whether the protection observed was due to the expression of CD39 transgene on the liver parenchyma or on the resident hepatic immune cells, bone marrow adoptive transfer experiments were performed. Following sublethal irradiation, WT mice were reconstituted with bone marrow from CD39tg mice, thereby limiting CD39 expression to the hematopoietic cells within the circulation and resident within the liver. In the converse experiment, WT bone marrow was transplanted into irradiated CD39tg mice, limiting

CD39 overexpression to the liver parenchyma. Prior to liver transplantation experiments, reconstitution in the thymus, spleen, and liver was assessed. In all three organs reconstitution of CD39tg, but not WT, bone marrow was incomplete, which was particularly striking within the liver (Fig. 1E). Consequently, only WT and CD39tg livers reconstituted with WT bone marrow were used as donors for further liver transplantation studies. The serum ALT and IL-6 concentrations following prolonged cold ischemia and transplantation were not statistically different between the reconstituted WT and CD39tg donor liver, with 15,215 (±3,894) and 13,505 (±1,167) U/L, respectively, for ALT (Fig. 1F) and 6,709 (±2,296) and 9,725 (±4,020) pg/mL, respectively, for IL-6 (data not shown). These results suggest that overexpression of CD39 on liver parenchyma alone does not confer protection against hepatic IRI and implicates a mechanistic role for resident hepatic lymphocytes in this model.

CD39 Transgenic Mice Develop CD4⁺ T Cell and iNKT Cell Peripheral Lymphopenia. To investigate the poor reconstitution following CD39tg bone marrow transfer, hepatic leukocyte populations in unmanipulated mice were analyzed by flow cytometry. The CD4⁺ T cell, but not CD8⁺ T cell, population was significantly decreased in CD39tg livers compared to WT controls (Fig. 2A; Table 1). Analysis of hepatic invariant NKT (iNKT) cells with CD1d-tetramer showed profound deficiencies in CD39tg animals (Fig. 2B; Table 1). As about 80% of iNKT cells express CD4 on their surface,²⁷ the relative numbers of hepatic CD4⁺ and CD4⁻ iNKT cells among the resident lymphocytes was analyzed. Only CD4-expressing iNKT cells were deficient with 0.03×10^6 (±0.01) CD39tg compared to 0.33×10^6 (±0.05) WT CD4⁺ iNKT (Fig. 2C,D). Analysis of splenic lymphocyte populations showed similar deficiencies in CD4⁺ T cell and iNKT cell numbers (Fig. 2E,F; Table 1). Despite this, the proportion and number of regulatory T cells (Tregs), which also express the CD4 surface marker, were not deficient in the spleens from CD39 transgenic mice (Supporting Fig. 3A,B; Table 1). Splenic B cell and NK cell numbers were unaffected (Table 1).

 $CD4^+CD25^-$ (Responder/Effector) T Cells and iNKT Cells but Not Treg Cells from CD39tg Mice Are Functionally Deficient. The function of the remaining CD4⁺ T cells and iNKT cells in CD39tg mice was tested. CFSE-stained splenocytes were cultured for 2 days in the presence of anti-CD3 and anti-CD28. CD39tg CD4⁺ T cells, but not CD8⁺ T cells, were hypoproliferative compared to WT cells with 64% (±3) dividing cells versus 85% (±2) (Fig. 3A,B).



Fig. 2. $CD4^+$ T cell and iNKT cell lymphopenia in CD39tg liver and spleen. (A) Dot plots representative of CD4 and CD8 gating on liver lymphocytes from CD39tg and WT mice. Numbers in quadrants are representative of the proportion of cells among liver lymphocytes. (B) Proportion of iNKT cells defined by $CD3^+/CD1d$ -tetramer⁺. Numbers are representative of the proportion of cells among liver lymphocytes. (C) Proportions and (D) absolute numbers of iNKT cells expressing CD4 within the liver lymphocyte population. Representative proportions of cells among liver lymphocytes are indicated in quadrants. Absolute numbers data represent mean \pm SEM, n = 9 per group. ***P < 0.001; ns, not significant by Student *t* test. (E) Dot plots representative of CD4 and CD8 gating on total splenocytes from CD39tg and WT mice. (F) Proportion of iNKT cells among splenocytes.

The function of iNKT cells was determined *in vivo* 2 hours post stimulation with α GalCer. Both intracellular staining for IFN- γ and IL-4 on liver leukocytes and serum concentration of IL-4 showed unresponsiveness of CD39tg iNKT cells to α GalCer (Fig. 3C,D). The suppressive function of CD4-expressing CD39tg Treg

cells was similar to that of WT Treg cells as determined in a mixed lymphocyte reaction (Supporting Fig. 3C,D).

CD39 Transgenic Mice Have a T-Cell Thymic Development Deficiency. The peripheral lymphopenia, hypofunction, and poor reconstituting capacities

Liver Lymphocytes									
Mice		CD4 T cells		CD8 T cells		iNKT cells			
WT (n=9)		0.73 ± 0.10		0.27 ± 0.03		0.56 ± 0.11			
CD39tg (n=9)		0.30 ± 0.05		0.31 ± 0.04		0.18 ± 0.06			
P-value		0.002 **		0.52		0.007 **			
			Splenocytes						
Mice	CD4 T cells	CD8 T cells	Treg	iNKT cells	B cells	NK cells			
WT (n=9)	$20.4~\pm~1.7$	12.0 ± 1.0	1.56 ± 0.1	1.54 ± 0.14	60.1 ± 3.9	3.09 ± 0.2			
CD39tg (n=9)	13.7 ± 0.5	12.5 ± 0.8	1.64 ± 0.2	0.54 ± 0.09	68.6 ± 7.4	2.68 ± 0.1			
P-value	0.002 **	0.796	0.708	<0.0001 ***	0.545	0.062			

Table 1. Liver and Splenic Leucocyte Subsets (absolute numbers)

of CD4⁺ T cells and iNKT cells from CD39tg mice implicated a defect in thymopoiesis. CD39 transgene expression was confirmed in the thymus by histology and real-time PCR (not shown). Flow cytometric analysis confirmed a thymic maturation blockade at the double positive (DP) stage with an increased proportion of double negative (DN) thymocytes and a significant decrease of DP, CD4 single positive (SP) and CD8 SP thymocyte absolute numbers (Fig. 4A; Table 2). T cell receptor beta (TCR- β) rearrangement and expression of CD69 are hallmarks of T-cell development, but expression of both was virtually absent in



Fig. 3. CD39tg CD4⁺ T cell and iNKT cell functional deficiencies. CD4⁺ and CD8⁺ T-cell proliferation assays (A,B). (A) Histograms representative of cell division of CFSE stained CD4⁺ and CD8⁺ T cells from CD39tg and WT mice after 2 days of stimulation in vitro. (B) Bar represents the mean \pm SEM of the percentage of dividing cells determined using the FlowJo proliferation platform, n = 4 per group. **P < 0.01; ns, not significant by Student t test. iNKT cell in vivo functional assays (C,D). (C) Histograms represent intracellular staining for IFN- γ and IL-4 in WT and CD39tg liver lymphocytes 2 hours after vehicle or α GalCer injection. (D) Data represent mean \pm SEM of serum IL-4 measured by ELISA 2 hours after stimulation, n = 4 per group. ***P < 0.001 by Student *t* test.



Fig. 4. Maturation blockade in CD39tg thymi. (A) Dot plots representative of CD4 and CD8 gating on thymocytes from CD39tg and WT mice. Numbers in quadrants represent the mean \pm SEM of the proportion of cells among total thymocytes, n = 12 per group. ****P* < 0.001; **P* < 0.05; ns, not significant by Student *t* test. (B) Dot plots representative of CD69 and TCR- β on CD4⁺/CD8⁺ (DP) thymocytes from CD39tg and WT mice. Numbers in quadrants are representative of the proportion of cells among DP thymocytes. (C) Histograms representative of TCR- β expression on CD4 SP and CD8 SP thymocytes from CD39tg and WT mice. Data are representative of n = 6 per group (B,C).

CD39tg DP thymocytes (Fig. 4B). TCR- β expression was significantly decreased in CD4 SP and CD8 SP cells (Fig. 4C). The same immunodeficiency was observed in CD39tg crossed with A2a-receptor KO mice (not shown), suggesting that an A2a-receptor-independent mechanism was responsible for the thymic maturation blockade. POMMEY ET AL. 1603

 $CD4^+$ T-Cell Depletion but Not iNKT Cell Depletion Is Protective Against IRI in Liver Transplantation. To determine the relative importance of hepatic $CD4^+$ T cell versus iNKT cell deficiencies in protection from IRI, iNKT KO and CD4-depleted WT livers were transplanted into WT recipients. CD4 depletion was confirmed by flow cytometric analysis of the spleen of the donor at the time of liver harvesting (Fig. 5A). CD4-depleted (ALT 10,296 ± 1,376) but not iNKT KO donor livers (ALT 26,271 ± 2,231) (Fig. 5B) were protected to the level observed for CD39tg donor livers. This suggests that the resident hepatic CD4⁺ T cells (but not iNKT cells) are predominant mediators of early IRI following prolonged cold preservation and liver transplantation.

Discussion

Herein we have shown that the overexpression of CD39 in the donor mouse is protective against hepatic IRI triggered by extended cold preservation (18 hours) in a model of liver transplantation. Unexpectedly, protection did not appear to be due to elevated levels of CD39 in the liver parenchyma itself, because liver grafts from CD39tg mice reconstituted with a WT immune system prior to transplant were not protected, but rather to a reduction in CD4⁺ cells in the donor liver. CD39tg mice exhibited a selective CD4⁺ T-cell panlymphopenia encompassing CD4⁺ iNKT cells. CD4⁺ T-cell depletion of WT donor livers, but not the absence of iNKT cells, paralleled the level of hepatic protection observed for CD39tg livers. Together this suggests that the protection imparted by CD39 overexpression is a consequence of depletion of resident hepatic $CD4^+$ T cells.

In this study, a clinically relevant model of prolonged cold storage followed by orthotopic liver transplantation was adopted. In clinical transplantation, prolonged cold ischemia and reperfusion activate both the immune response and the interrelated coagulation system. There are several putative mechanisms by which CD39 overexpression may protect, given its role in modulating both of these systems. In this model, passenger leukocytes, which are donor-derived hepatic

Table 2. Thymocyte (absolute numbers)

Thymocytes									
Mice	Total	DN	DP	CD4 SP	CD8 SP				
WT (n=12) CD39tg (n=12)	$\begin{array}{c} 150 \pm 11.1 \\ 113 \pm 6.4 \end{array}$	$\begin{array}{c} 3.6 \pm 0.2 \\ 4.5 \pm 0.4 \end{array}$	$\begin{array}{c} 155\pm8.2\\ 118.9\pm8.2\end{array}$	$\begin{array}{c} 19.8\pm1.3\\ 12.1\pm1.0\end{array}$	5.3 ± 0.4 3.8 ± 0.3				
P-value	0.009**	0.079	0.012*	0.0007***	0.019*				



0.07

11.90

Donor liver

Fig. 5. $CD4^+$ cell depletion but not iNKT cell depletion in the donor liver is protective against IRI following transplantation. (A) Dot plots representative of CD4 and CD8 staining of splenocytes from donor animal, 6 days after treatment with PBS or CD4-depleting antibody. (B) Serum ALT measured 6 hours after liver transplantation of WT (n = 6), CD4-depleted (n = 7), and iNKT KO (n = 8) donor liver into WT recipient. Data represent mean \pm SEM. **P* < 0.05; ns, not significant by Student *t* test.

resident leukocytes, appear to mediate much of the injury. CD39tg livers were more resistant to IRI and were deficient in both CD4⁺ T cells and iNKT cells. Reconstitution of these livers with a WT immune system (restoring resident T-cell number and function) abolished resistance. Furthermore, WT donor livers depleted of CD4⁺ T cell showed similar protection to CD39tg donor livers. Although CD4⁺ iNKT cells represent 20% to 40% of hepatic T cells,²⁷ conventional CD4⁺ T cells appeared to be the prime orchestrators of early hepatic injury, as livers from iNKT KO mice were not protected. The role of CD4⁺ T-cell subsets in warm hepatic IRI has been defined.²⁸ T-cell activation occurring through antigen-dependent and -independent mechanisms mediates liver injury through neutrophil recruitment and activation. Further, NKT cell activation can cause direct liver injury in partial hepatic warm IRI.²⁸ Our data are in accordance with

recent observations in a similar mouse liver transplantation model using CD1d KO donors and WT recipients,²⁹ but is at odds with data from a warm model of hepatic IRI where systemic blocking of NKT cells was protective and adoptive transfer of NKT cells in Tcell-deficient mice restored injury.¹⁴ This discrepancy suggests that warm and cold IRI have two distinct pathophysiologies and that the immune response against the transplanted organ differs from the response to local ischemia. Further, NK and NKT cells mediate phase-specific responses in IRI: depletion of NK1.1 cells, which encompass both NK and NKT cells, failed to moderate IRI at early timepoints¹⁷ but significantly reduced later hepatocellular damage.¹⁴ NK and NKT cells are a prime source of IFN-y, which becomes critically important at 24 hours of reperfusion. Our data confirm that CD4⁺ iNKT cells of donor origin have minimal effect during the early phase (within 6 hours) of IRI.

We have previously shown that the overexpression of CD39 on the renal parenchyma mitigates IRI up to 72 hours following transplantation.¹⁵ However, overexpression of CD39 within the hepatic parenchyma appears to play a minor role, if any, in this model of liver transplantation. Recipient circulating T cells, particularly CD4⁺ T cells, are recruited to the liver within hours of perfusion.¹⁶ It was anticipated that the adenosine-rich milieu created by CD39 overexpression would modify the inflammatory response. However, there was no significant difference in the susceptibility to IRI of WT or CD39tg donor livers following reconstitution with WT bone marrow, suggesting minimal if any effect of tissue restricted overexpression of CD39. This was unexpected, given the potent antiinflammatory effects of adenosine, but may be accounted for by the very short half-life of adenosine in the circulation.³⁰ Others employing the use of stable A2a receptor agonists have shown protection against IRI in models of hepatic warm IRI,¹⁴ CD4⁺ T-cell-dependent (Concanavalin A) liver injury,³¹ renal IRI,³² and myocardial infarction.³³ Adenosine signaling through the A2a receptor inhibits IFN-y production by both CD4⁺ T cells and iNKT cells.^{14,34} Injection of an A2a receptor agonist at the time of reperfusion resulted in a decrease of serum IFN- γ and was protective against hepatic IRI.¹⁴ In these models the response against ischemia reperfusion is modified by adenosine in the whole animal. The effect, if any, on the resident hepatic leukocytes has not been determined.

Prolonged ischemia generates a hypoxic environment within the liver. The main sensor of oxygen deprivation is the transcription factor HIF (hypoxiainducible factor), which regulates several genes involved in adaptation to hypoxia. Hypoxia and inflammation are interconnected because HIF regulates functions of both innate and adaptive immune cells and, conversely, inflammation triggers hypoxia.35 Sp1 activates the transcription of CD39 in response to hypoxia,³⁶ which may enhance the level of endogenous CD39. In addition, the promoters of the CD73, A2a, and A2b adenosine receptor genes contain an HIF-responsive element,37 and hypoxia might therefore contribute to the generation and signaling of adenosine. Recently, it has been shown that ATP signaling through P2X7 triggers NLRP3 inflammasome activation in hepatic immune cells in response to chemically induced liver toxicity; mice lacking CD39 were more sensitive to liver injury, whereas administration of apyrase reduced the immune infiltrate and injury.³⁸ It was thus surprising that the overexpression of CD39 did not confer protection in our transplant model; rather, the reduced number of hepatic resident CD4⁺ T cells was the most critical factor in protection. Mice overexpressing CD39 are deficient in CD4⁺ T cells due to a blockade in thymic maturation. A large proportion of developing thymocytes undergo apoptosis due to a failure to pass the selection criteria during Tcell development. Although present in very low concentrations in the extracellular space, ATP is present in millimolar concentrations within cells. When cells apoptose, ATP is released into the extracellular space and is hydrolyzed by ectoenzymes generating adenosine. Extracellular adenosine triggers apoptosis on thymocytes mainly through A2a receptor signaling, with DP thymocytes being the most sensitive population.³⁹ CD39 overexpressing mice have an enhanced capacity to generate adenosine due to increased NTPDase activity.²⁴ Although the catalytic activity was not directly measured within the thymus, strong CD39 expression was evident by flow cytometric and real-time PCR analysis (not shown). However, the observed thymocyte deficiency was not mediated through the A2a receptor, because the phenotype persisted in CD39tg

mice bred onto the A2aRKO background (not shown). The roles of the other adenosine receptors in this unanticipated effect of CD39 overexpression have not been investigated. The absolute number of iNKT cells was markedly

decreased in the CD39tg mice. The CD4-negative iNKT cell subset was spared, suggesting that the iNKT cell deficiency results mainly from a global CD4⁺ T-cell depletion. Intriguingly, the CD4⁺ Treg subset was not affected by CD39 overexpression. The sparing of this population may relate to the endogenous expression of CD39^{22,40} and CD73^{22,41} rendering these cells less susceptible to the toxic effects of adenosine possibly through the development of compensatory protective mechanisms.

In summary, we have shown that a reduction in the number of resident CD4⁺ T cells in donor livers, either as a consequence of overexpressing CD39 or pharmacological depletion, resulted in significant protection against IRI associated with cold storage and liver transplantation. Clinically, perfusion of the donor liver with an anti-CD4 depleting antibody may replicate these effects, reducing inflammation associated with transplantation and decreasing the risk of primary graft nonfunction and subsequent graft loss.

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