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Title

Liver grafts from CD39-overexpressing mice are protected from ischemia reperfusion injury due to reduced numbers of resident CD4$^+$ T cells

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Abstract

Ischemia-reperfusion injury (IRI) is a major limiting event for successful liver transplantation, and $CD4^+$ T cells and invariant NKT (iNKT) cells have been implicated in promoting IRI. We hypothesized that hepatic over-expression of CD39, an ecto-nucleotidase with anti-inflammatory functions, will protect liver grafts after prolonged cold ischemia. CD39-transgenic (CD39tg) and wild type (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 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.

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Introduction

Ischemia reperfusion injury (IRI) is inherent to the transplant process. The duration of warm ischemia is relatively short for brain dead heart-beating 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 re-established due to ongoing inflammation and coagulation, which initiates a self-perpetuating cycle. In healthy tissue, 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 ADP to AMP, which is converted into adenosine by CD73 (4, 5). CD39 has both thromboregulatory and immunoregulatory functions through modulating 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

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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). Natural killer (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 via diminished IFNγ production (10). CD1d 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 anti-coagulant and anti-inflammatory milieu that could alleviate renal IRI (15). Herein we extend these observations using a clinically relevant model of orthotopic liver transplantation characterised by prolonged cold storage of the donor organ. Further we explore putative mechanisms of protection through the immune phenotyping of these mice.

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Experimental procedures

Animals:

C57BL/6 wild type (WT) mice were purchased from the Walter and Eliza Hall Institute (WEHI, Melbourne, Australia). CD39tg mice over-expressing CD39 under the control of the mouse $H2K^b$ promoter (C57BL/6 background) (24) were backcrossed at least 10 generations. Invariant NKT knock-out 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 edition 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 PBS with 2% FCS and counted before flow cytometric analysis.

The livers of anaesthetised mice were perfused with phosphate-buffered saline (PBS) via 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, England) gradient and centrifuged. The cell pellet contained liver lymphocytes.

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Flow Cytometry

Freshly obtained leukocytes were stained with the following antibodies: 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, USA), CD4-APC, CD8a-APC (eBioscience). Intracellular staining for IFNγ-FITC and IL4-FITC (eBioscience) was performed using Cytofix/Cytoperm (BD Biosciences, San Jose, CA) reagents containing 1μg/mL of Brefeldin A (Sigma, St Louis, MO) and following manufacturer's instructions. Data acquired by FACSCalibur (BD Biosciences) were analysed using FlowJo software (Tree Star, Asland, OR).

In vivo iNKT cell functional assay

Mice were injected IP with 5 μg of α-Galactosylceramide (αGalCer) (Alexis Biochemicals, Lausen, Switzerland) diluted in PBS/0.05% Tween (BDH, Radnor, PA) or vehicle only. 2h post-injection, organs were harvested for flow cytometric analysis and serum was collected for IL-4 determination by ELISA using the Mouse IL-4 ELISA Ready-Set-Go kit (eBioscience) following manufacturer's instructions.

Cell culture and proliferation assay

RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT, USA), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen) and 50 μM 2-mercaptoethanol (Sigma) was used for T cell culture. Splenocytes were labelled with 5 μM

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CFSE (Invitrogen) and stimulated with 5 μg/mL plate-bound anti-CD3 (clone 145-2C11) and 5 μg/mL anti-CD28 (clone 37.51) in suspension (WEHI, Melbourne). After 48 hrs, proliferation was assessed by flow cytometry. Viable CFSE-labelled 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 injected IP 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

4-week-old recipient mice were irradiated twice for 20 min 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 previously (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.

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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. 6 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 manufacturer's instructions.

Histological procedures

Liver grafts were immersed in 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). Whole sections were analysed and scored following Suzuki's method (26) by a histopathologist blinded to treatment groups. Apoptosis was assessed using rabbit anti-cleaved caspase-3 antibody [Asp175] (Cell Signalling Technology, Danvers, MA). Sections were analysed and apoptosis scored according to percentage of central vein involvement by a histopathologist blinded to treatment groups.

Statistical analysis

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The computer software GraphPad Prism (version 5) (GraphPad Software, La Jolla, CA, USA) 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's t-test was used to give the stated 'p values'. A 'p value' of <0.05 was considered significant.

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Results

Donor livers from mice over-expressing CD39 are protected against IRI

To assess the impact of CD39 over-expression 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 10018 U/L (± 653) and 15638 U/L (±1513), respectively (Fig. 1A). The level of systemic pro-inflammatory cytokine IL-6 was also significantly reduced in recipients of CD39tg donor livers (2396 pg/mL ±438) compared to WT livers (4336 pg/mL ±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 non-confluent necrotic areas. Hepatocellular damage was graded according to the Suzuki's histological score (26) (Suppl. 1A): 5 out of 6 WT donor livers showed mild to severe congestion and vacuolization associated with more than 30% hepatocyte necrosis (score 2 or greater) compared to only 2 out of 6 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 and Suppl. 1B).

CD39 transgene expression in donor livers was confirmed by real-time PCR (Suppl. 2A) and flow cytometric analysis on donor liver lymphocytes (Suppl. 2B). To 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 sub lethal irradiation, WT mice were reconstituted with bone marrow

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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 over-expression to the liver parenchyma. Prior to liver transplantation experiments, the 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 15215 (±3894) and 13505 (±1167) U/L respectively for ALT (Fig. 1F) and 6709 (±2296) and 9725 (±4020) pg/mL respectively for IL-6 (data not shown). These results suggest that over-expression 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 analysed 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 analysed. Only CD4-

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expressing iNKT cells were deficient with $0.03x10^6$ (±0.01) CD39tg compared to $0.33x10^6$ (±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 (Treg), which also express the CD4 surface marker, were not deficient in the spleens from CD39 transgenic mice (Suppl. 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 hypo-proliferative compared to WT cells with 64% (±3) dividing cells versus 85% (±2) (Fig. 3A-B). The function of iNKT cells was determined *in vivo* 2 hours post-stimulation with αGalCer. 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 αGalCer (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 (Suppl. 3C-D).

CD39 transgenic mice have a T cell thymic development deficiency

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The peripheral lymphopenia, hypofunction and poor reconstituting capacities 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). TCRβ rearrangement and expression of CD69 are hallmarks of T cell development, but expression of both was virtually absent in CD39tg DP thymocytes (Fig. 4B). TCRβ 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 suggesting that an A2a-receptor-independent mechanism was responsible for the thymic maturation blockade.

$CD4^+$ 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 10296 ±1376) but not iNKT KO donor livers (ALT 26271 ±2231) (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.

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Discussion

Herein, we have shown that the over-expression 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 pan-lymphopenia 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 over-expression 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 inter-related coagulation system. There are several putative mechanisms by which CD39 over-expression may protect given its role in modulating both of these systems. In this model, passenger leukocytes which are donor-derived hepatic 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

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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 T-cell 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 time points (17) but significantly reduced later hepatocellular damage (14). NK and NKT cells are a prime source of IFNγ, 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 over-expression of CD39 on the renal parenchyma mitigates IRI up to 72 hours following transplantation (15). However, over-expression 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 over-expression 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 over-expression of CD39. This was unexpected given the potent anti-inflammatory

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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 signalling through the A2a receptor inhibits IFN-γ 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 (hypoxia-inducible factor), which regulates several genes involved in adaptation to hypoxia. Hypoxia and inflammation are interconnected since 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 signalling 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 over-expression 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 over-

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expressing 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 T-cell 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 hydrolysed by ectoenzymes generating adenosine. Extracellular adenosine triggers apoptosis on thymocytes mainly through A2a receptor signalling, with DP thymocytes being the most sensitive population (39). CD39 over-expressing 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 over-expression 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 over-expression. 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 over-expressing CD39 or pharmacological depletion,

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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 non-function and subsequent graft loss.

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Other manuscript elements

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Figure legends

Fig. 1. CD39tg donor livers are protected against IRI following orthotopic liver transplantation. Serum ALT (A) and IL-6 (B) measured 6h after transplantation of CD39tg or WT donor livers into WT recipients. n=6 per group, mean ± SEM. **, $p<0.01$; *, $p<0.05$ by Student's 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's 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 ± SEM, n=6 per group. ***, $p<0.001$ by Student's t-test. (F) Serum ALT measured 6h 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 ± SEM. ns, not significant by Student's t-test.

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 ± SEM, n=9 per group. ***, $p<0.001$; ns, not

significant by Student's 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.

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 ± 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's 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 ± SEM of serum IL-4 measured by ELISA 2 hours after stimulation, n=4 per group. ***, $p<0.001$ by Student's 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 ±SEM of the proportion of cells among total thymocytes, n=12 per group. ***, $p<0.001$; *, $p<0.05$; ns, not significant by Student's t-test. (B) Dot plots representative of CD69 and TCRβ 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 TCRβ expression on CD4 SP and CD8 SP thymocytes from CD39tg and WT mice. Data are representative of n=6 per group (B-C).

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 6h after liver transplantation of WT (n=6), CD4-depleted (n=7) and iNKT KO (n=8) donor liver into WT recipient. Data represent mean ± SEM. *, $p<0.05$; ns, not significant by Student's t test.

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 ±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

Thymocytes					
Mice	**Total**	**DN**	**DP**	**CD4 SP**	**CD8 SP**
WT (n=12)	150 ±11.1	3.6 ±0.2	155 ±8.2	19.8 ±1.3	5.3 ±0.4
CD39tg (n=12)	113 ±6.4	4.5 ±0.4	118.9 ±8.2	12.1 ±1.0	3.8 ±0.3
p-value	0.009 **	0.079	0.012 *	0.0007 ***	0.019 *

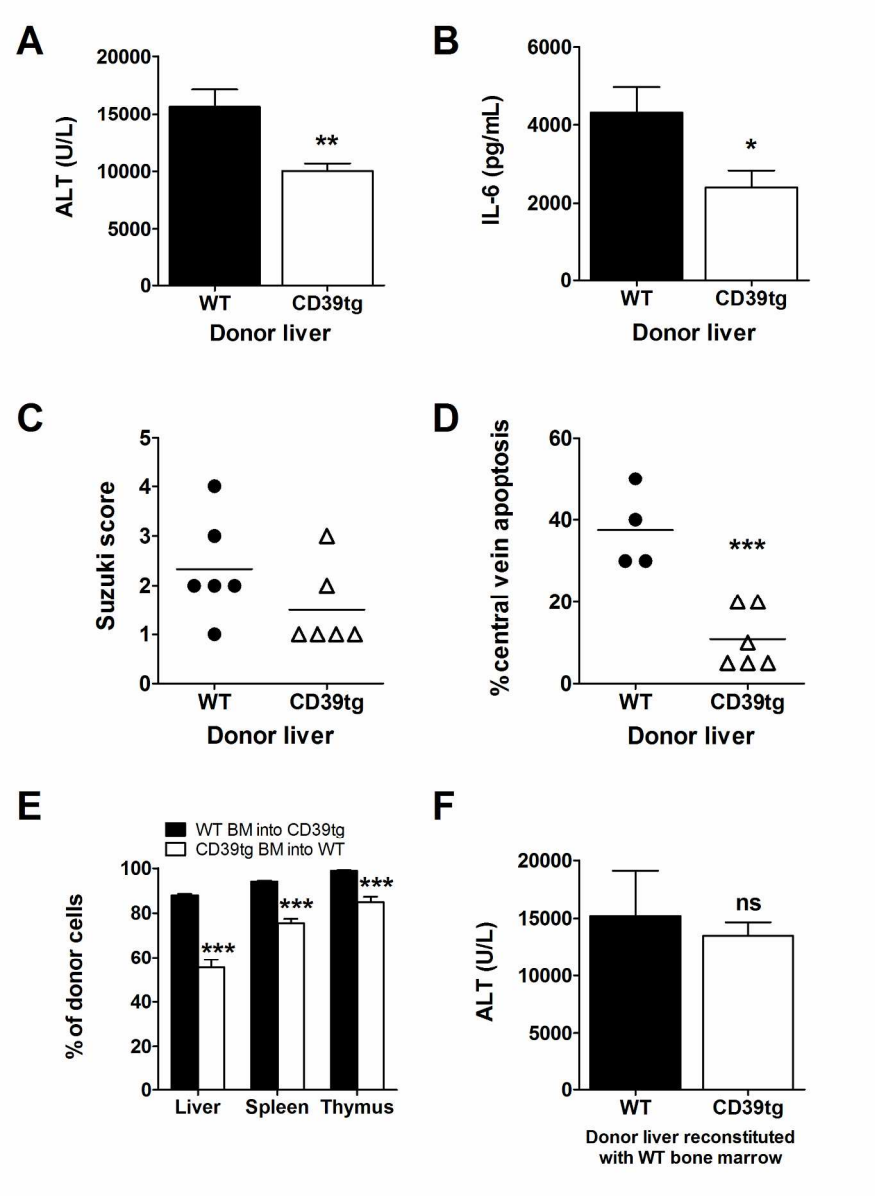

Figure 1
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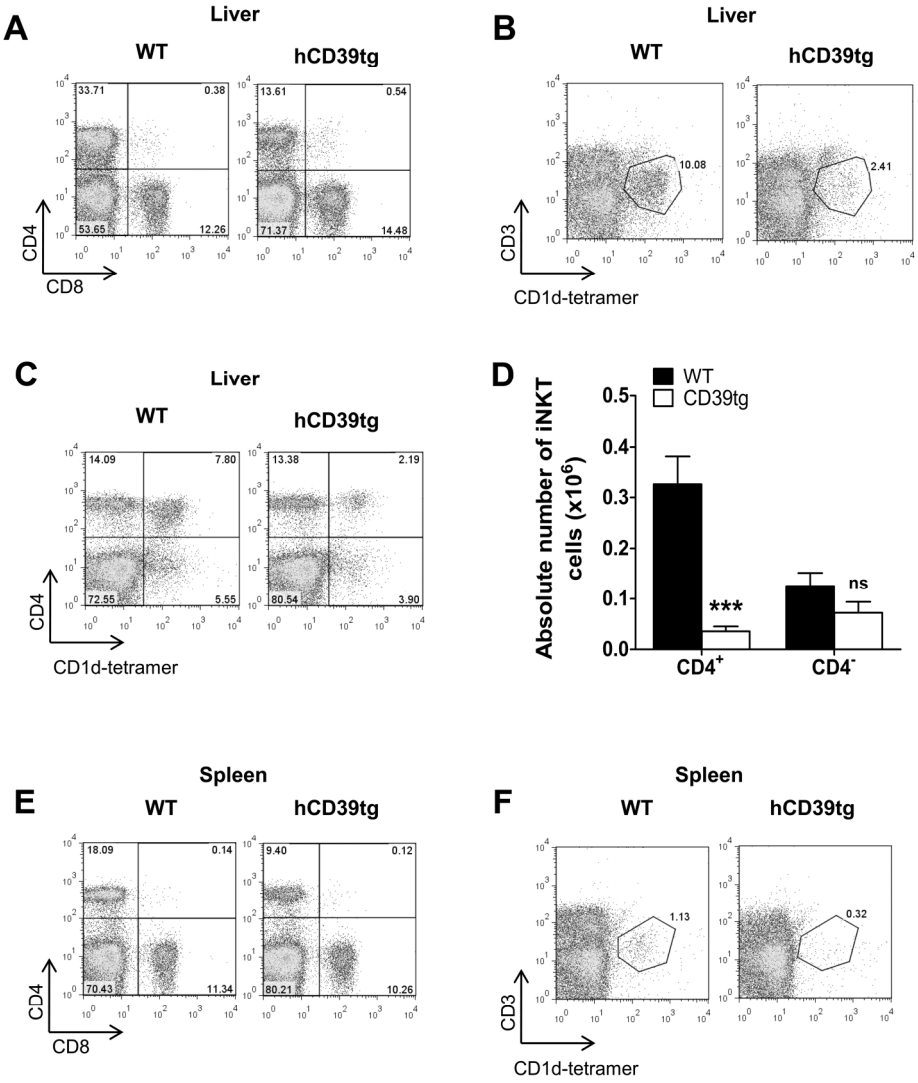

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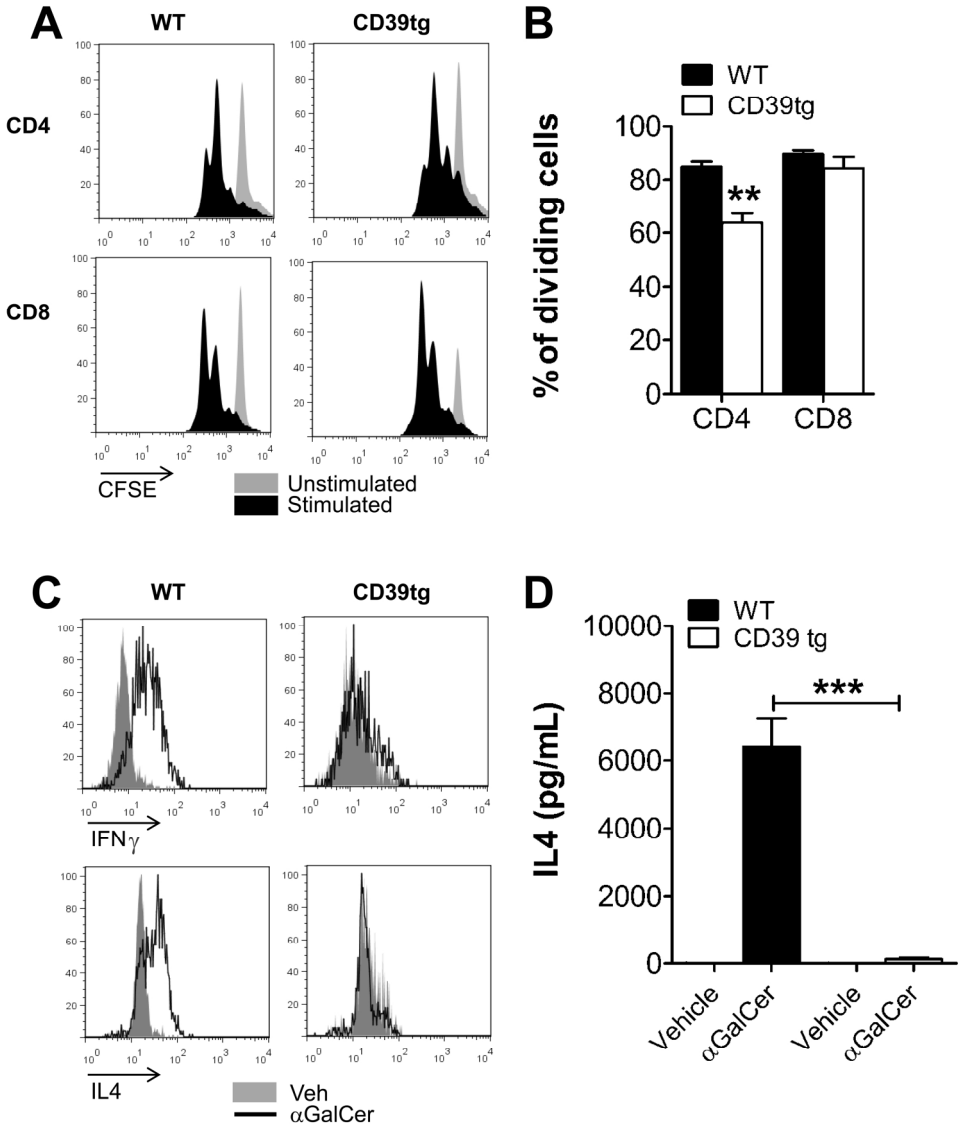

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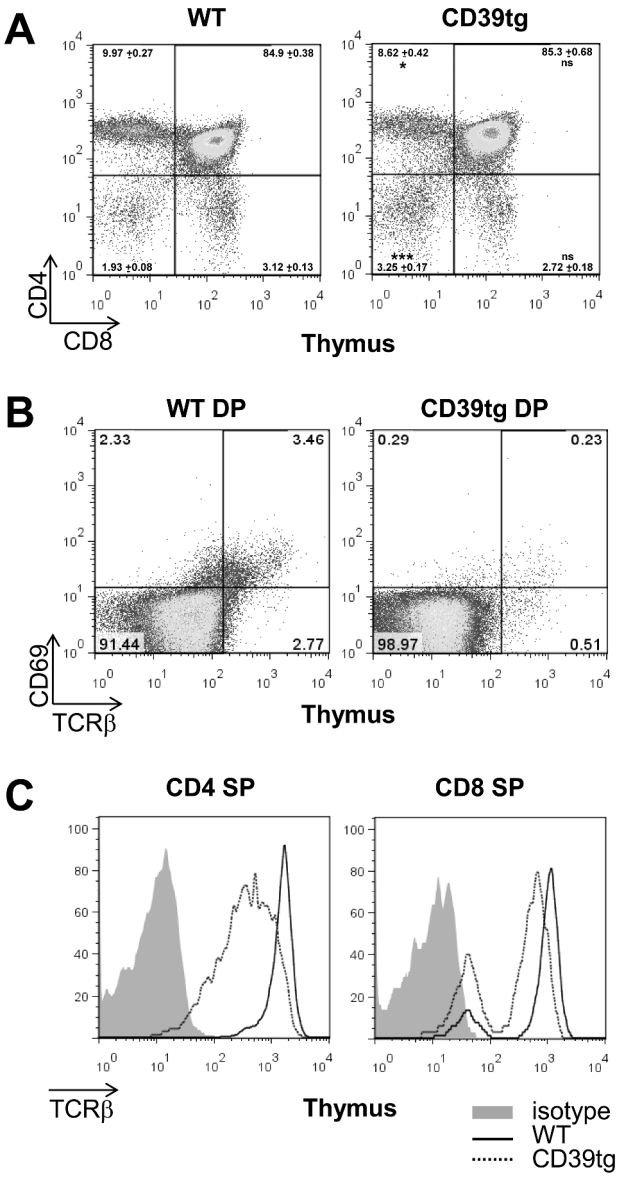

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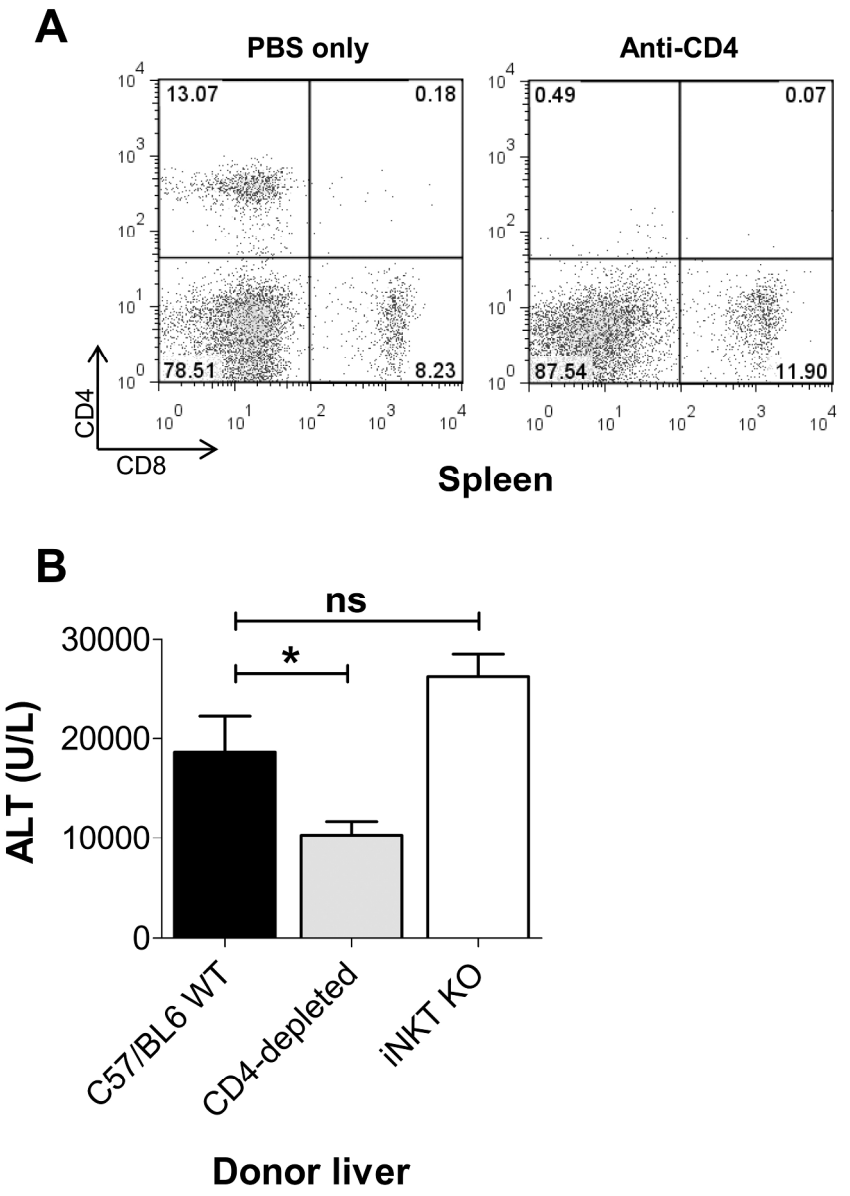

Figure 5
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Supplementary experimental procedures

Real-Time PCR

RNA extractions from transplanted livers were performed using RNeasy Midi Kit (Qiagen) following manufacturer's instructions and quantified on a Nanodrop spectrometer (Thermo Scientific). 5 µg of RNA was reverse transcribed at 50ºC with Superscript III (Invitrogen). Real-time PCR were performed using Taqman Master Mix and Gene Expression Assays primers and probes (human CD39: Hs00169946_m1 and mouse GAPDH: Mm99999915_g1) (Applied Biosystems) on the 7500 instrument and analysed with SDS software (Applied Biosystems). The relative expression of each gene was normalised to GAPDH and calculated as: $2^{-(\Delta Ctgene-\Delta CtGAPDH)}*1000$, where ΔCt represents the average of triplicates of the threshold cycle for each transcript.

Flow cytometry for T regulatory cells

FoxP3 intracellular staining was conducted using the anti-mouse/rat FoxP3 Staining Set (eBioscience) following manufacturer's instructions. Data acquired by FACSCalibur (BD Biosciences) were analysed using FlowJo software (Tree Star).

T regulatory cell suppression assay

Accessory cells ($CD4^-$), responder cells ($CD4^+CD25^-$) and T regulatory cells (Treg) ($CD4^+CD25^+$) were sorted on an Automacs cell sorter using Regulatory T Cell Isolation Kit (Miltenyi Biotec). Accessory cells were treated with 50µg/mL Mitomycin C (Sigma) (30 min at 37ºC) and responder cells were stained with CFSE (Invitrogen) (5 min at 37ºC). 5 x 10^4

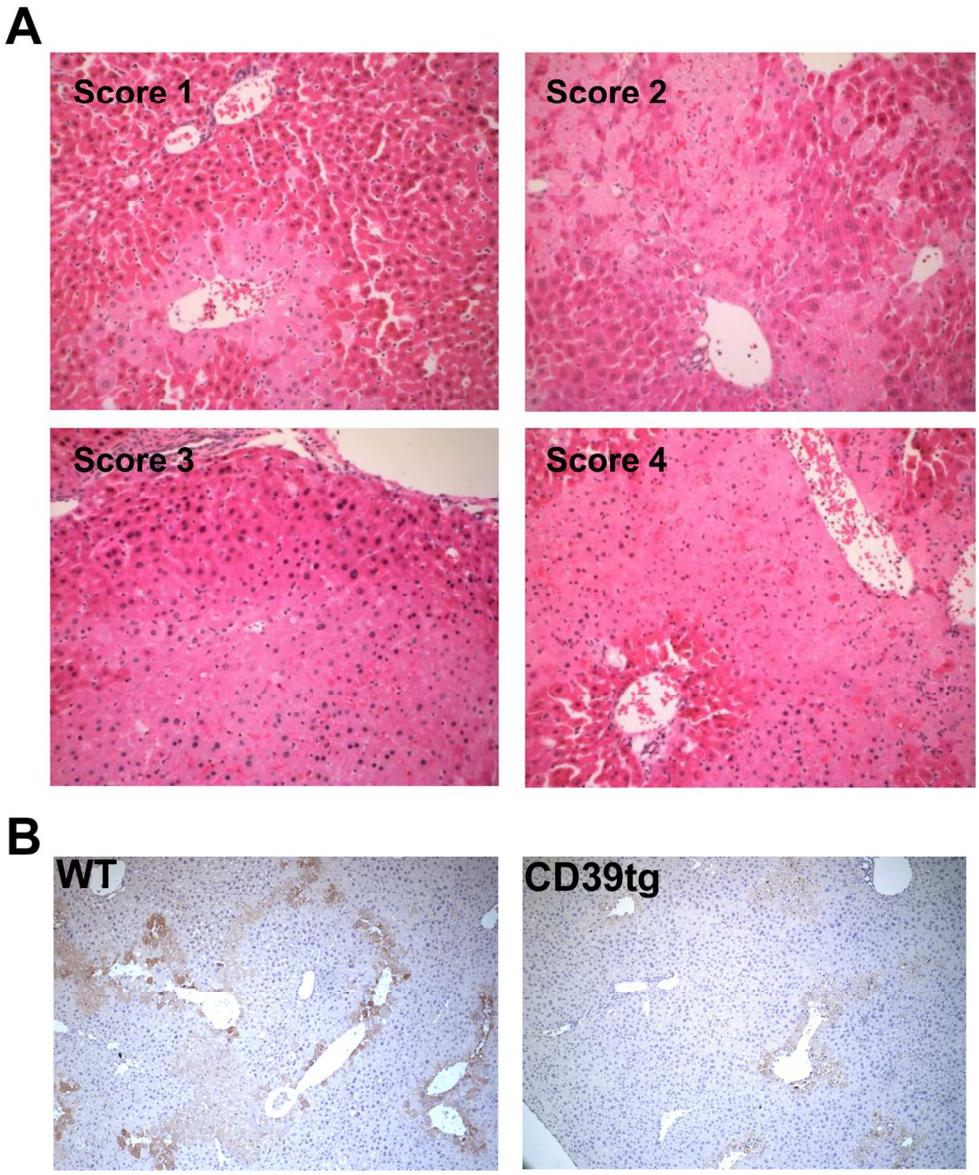
CFSE-labelled responders were cocultured with 5 x 10^4 accessory cells and unlabeled Treg at different ratios and stimulated with 1μg/mL anti-CD3 (clone 145-2C11) (WEHI, Melbourne). After 72 hrs of culture, responder cell proliferation was assessed by flow cytometry on $CD4^+$/7-AAD^- (BD Biosciences) gated CFSE-labelled cells. Percent suppression was calculated by dividing the number of proliferating cells determined using the FlowJo proliferation platform in the presence of Treg by the number of proliferating cells in the absence of Treg and multiplied by 100.

Supplementary figure legends

Suppl. 1. (A) Suzuki's score showing representative picture of scoring 1, 2, 3 and 4 on H&E staining at an original magnification of x200. (B) Representative picture of WT and CD39tg donor liver stained for cleaved caspase-3 at an original magnification of x100. Sections were scored based on the percentage of central veins with surrounding cleaved caspase-3 positive cells.

Suppl. 2. CD39 transgene expression on donor liver tissue and lymphocytes. (A) CD39 expression analysed by real-time PCR on WT and CD39tg donor livers. Bars represent means ± SEM of human CD39 gene expression relative to GAPDH (2^{-dCt}), n=6 per group. **, $p<0.01$ by Student's t-test. (B) Flow cytometric analysis of CD39 expression on donor lymphocytes from WT and CD39tg livers. Data represent MFI ± SEM, n=9 per group.

Suppl. 3. T regulatory cell number and function. Treg cell proportion (A) and absolute numbers (B) were determined in the spleen of WT and CD39tg mice using $CD4^+/FoxP3^+$ gating. (B) Histograms represent mean ± SEM, n=8 per group. ns, not significant by Student's t-test. (C) Histograms represent CFSE stained WT T effector cells after 72hrs coculture with WT accessory cells and WT (solid grey) or CD39tg (black line) Treg at different ratios Treg/Teff. (D) Percentage of WT and CD39tg Treg suppression on Teff proliferation at different ratios Treg/Teff. Each dot represents the percentage of suppression calculated with means of triplicates. Data are representative of three independent experiments.



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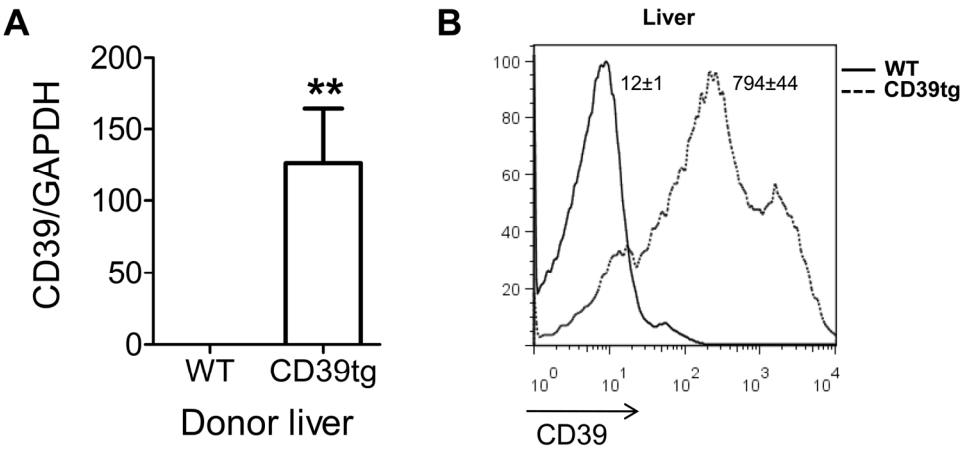

Supplementary figure 2
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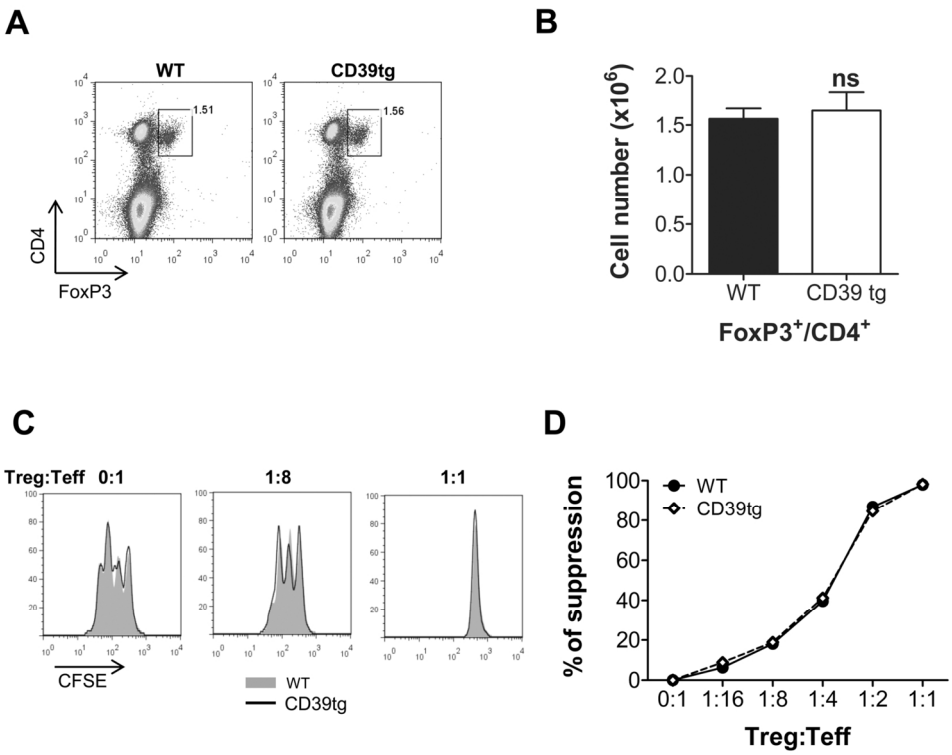

Supplementary figure 3
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