SUPPLEMENTAL DIGITAL CONTENT 2

Materials and Methods

Mice

C57BL/6 (B6, H-2K^b) and BALB/c (Balb/c, H-2K^d) mice (10 to 12 weeks old) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), and C57BL/6J-RAG2KO (B6.Rag2KO, H-2K^b) mice were kindly provided by Dr. S. Nakae, National Research Institute for Child Health and Development, Tokyo, Japan. All mice were maintained under standard conditions and fed rodent food and water, in accordance with the guidelines of the Animal Use and Care Committee of the National Research Institute for Child Health and Development, Tokyo, Japan.

Generation and genotyping of conditional B6.CD98hc deficient mice

CD98 heavy chain genomic clones were isolated from a 129Sv/J mouse genomic library in the Lambda FIX 2 vector (Stratagene) using CD98hc cDNA as a probe. The targeting vector consisted of a 3.6-kb 5' homologous region and a 2.6-kb 3' homologous region. The region of exon 3 was flanked with a loxP site. A neomycin selection cassette flanked by Flpe site (provided by Dr. Okabe, University of Osaka, Osaka, Japan) was inserted in intron 3. The linearized targeting vector was electroporated into RW4 ES cells. Selection with G418 and ganciclovir was applied at 24h after plating and continued for 7 days. The targeted clones were confirmed by Southern blotting analysis using 3' and 5' probes. These clones were injected into B6 blastocyst-stage embryos to generate chimeric mice, which were crossed to B6 mice

for the production of targeted offspring. CD98hc neo mice were bred onto the Flpe deleted strain (BRC, RIKEN, Tsukuba, JAPAN) to excise the neomycin selection cassette. B6.CD4-cre mice (1) were supplied from Dr. Chris Wilson (University of Washington, WA, USA). For all experiments, the offspring of $CD98hc^{f/-}$CD4-Cre^{+} X $CD98hc^{f/f}$ matings were used. The genotypes of the offspring were identified by PCR. The specific PCR primer pairs were: lox, 5'-GTT ACC TCC ACT ATG AAT GC (primer49) and 5'-CAG ATT GTC AGT AAC AGA CA (primer43); null, 5'-TCA TGC GTG AGC GTA ATT TT (primer41) and primer43; and Cre, 5'-CCC AAG AAG AAG AGG AAG GTG TCC (primer86) and 5'-CCC AGA AAT GCC AGA TTA CG (primer87). PCR conditions were 94°C /1 min, 55°C/30 s, and 72°C /45 s for 35 cycles.

FACS analysis

Splenocytes were obtained from B6.wild or B6.CD98hc-/- mice, and suspended in PBS and then incubated at 4°C for 30 min with an optimal concentration of FITC- and/or PE-conjugated anti-mouse CD3, CD11a, CD11b, CD29, CD62L, CD98hc, CD103, and CD147 mAb (BD Pharmingen) diluted with PBS containing 0.5% fetal calf serum. Splenocytes were also obtained from Balb/c heart grafted B6.wild or B6.CD98hc-/- recipients on POD3, 7, and 14, suspended in PBS, and then incubated at 4°C for 30 min with an optimal concentration of anti-mouse CD4-PE/Cy5 mAb (BD Pharmingen) diluted with PBS containing 0.5% fetal calf serum. For intracellular Foxp3 and PS6 staining, followed by membrane staining for CD4, a BD

Cytofix/Cytoperm Kit was used according to the manufacturer's instructions. The PE anti-Foxp3 antibody (FJK-16s, eBioscience, San Diego, CA) or Alexa Fluor 488 phospho-S6 ribosomal protein (PS6, Ser235/236) rabbit antibody was used. The stained cells were analyzed by flow cytometry and the data were analyzed using the CellQuest software package.

A FACS analysis was also performed before the anti-CD98hc mAb therapy. Splenocytes were obtained from B6 naive mice or B6 mice who had received one intraperitoneal injection of 1mg anti-CD98hc mAb (clone 26-24) 3 days before, then cells were stained with anti-CD98hc mAb followed by anti-rat IgG2a-FITC and anti-mouse CD4-PE/Cy5, CD98hc-PE and CD4-PE/Cy5 (RM4-5), after incubation at 4ºC for 30min, cytometry was performed using a FACS Caliber Cytometer.

Lymphocyte proliferation assays

The mixed lymphocyte reaction (MLR) was performed with B6.wild or B6.CD98hc-/- mouse nylon-wool column (Wako)-enriched T cells ($1x10^5$/well) as responders, and 20-Gy irradiated Balb/c splenocytes ($1x10^5$/well) as stimulators. The cells were incubated in a flat-bottom 96-well white plate (Costar; Corning, NY) at a final volume of 200 µl/well of the GIT medium containing 50µM 2-mercaptoethanol (Wako) in a humidified atmosphere at 37˚C for four days. The proliferation of T cells was measured using cell-proliferation ELISA kits (Roche Diagnostics Gmbh, Penzberg, Germany). Briefly, the cells were labeled with 5-bromo-2-deoxyuridine (BrdU) solutions at 10µl/well and incubated for an additional 2hr at 37˚C. After centrifugation,

the supernatant was removed, 200µl/well of fixation and DNA denaturation (FixDenat) solution was added to the cells, and they were re-incubated for 30 min at 15 to 25˚C. The cells were cultured for 90min with anti-BrdU-peroxidase solution and subsequently washed three times. After adding substrate solution at 100µl/well, the BrdU incorporation was measured with a chemiluminescence reader (Wallac ARVO^{TM} SX; PerkinElmer, Inc., Wellesley, MA) and the data was processed using the Wallac1420 manager software package (PerkinElmer).

Heterotopic cardiac transplantation

The heart transplantation was performed from the sex-matched Balb/c donor to B6.wild or B6.CD98hc-/- recipients by using microsurgical techniques. Intra-abdominal vascularized heterotopic mouse cardiac transplantation was performed as described previously (2, 3). In brief, donor hearts were harvested and placed in chilled physiologic saline, during which time the recipient mice were prepared. The donor hearts were heterotopically transplanted into recipient mice by end-to-side anastomosis of the donor's aorta and pulmonary artery to the recipient's abdominal aorta and inferior vena cava, respectively. Cardiac graft survival was determined by the daily palpation of the recipient abdomen. Rejection was considered complete at the time of cessation of a palpable heartbeat, and confirmed visually by laparotomy.

Immunohistochemical analysis

Triple immunostaining of cardiac allografts was performed to detect intragraft cell infiltration. Four μm thick frozen sections of heart grafts were cut, stained with rat-anti-mouse CD4 or CD8α mAb, then incubated with alkaline phosphatase (ALP)-conjugated anti-rat Ig (A9316; Sigma-Aldrich, St. Louis, MO) and developed with Vector Blue (Vector Laboratories, Burlingame, CA). Thereafter, cryo-sections were incubated with rabbit-anti-mouse type IV collagen polyclonal Ab (LB1403; Cosmo Bio, Tokyo, Japan), then incubated with peroxidase-conjugated anti-rabbit Ig (55693; Mitsubishi Chemical, Tokyo, Japan) and developed with diaminobenzidine (Vector Laboratories). Subsequently, samples were treated with 4N HCl for 30 min and neutralized with borate buffer (0.1 M; pH 8.5) for 4 min. Bromodeoxyuridine (BrdU) was then detected with an anti-BrdU mAb, followed by an ALP-labeled sheep $F(ab')_2$ to mouse Ig, and developed with a Vector Red substrate kit (Vector Laboratories). Another part of the tissue was used for double immunostaining of Foxp3 and type IV collagen. The infiltration of CD4+, CD8+, BrdU+ cells and Foxp3+ cells were analyzed quantitatively using the WINROOF software package (4, 5). At least three fields of each section of the cardiac allograft were selected randomly and analyzed by an observer who was blinded to the research.

Histological analysis

Cardiac graft specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections were cut (1μm-thick) and stained with hematoxylin and eosin. Light microscopic analysis was performed to assess the overall cellular and myocardial

damage (6).

Isolation of graft infiltrating lymphocytes (GIL) and FACS analysis

To prepare GIL, cardiac grafts were cut into small pieces and digested with collagenase IV (0.5mg/ml, Sigma-Aldrich) and DNase I (0.1mg/ml, Sigma-Aldrich) for 30 min at 37˚C. GIL and splenocytes were stained with FITC anti-mouse CD4 and PE/Cy5 anti-mouse CD8α mAb. After incubation at 4ºC for 30min, cytofluorimetry was performed using a FACS Caliber Cytometer.

RNA isolation and quantitative real-time RT-PCR

The total RNA was extracted from the cardiac grafts and spleen using an RNeasy Mini Kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's protocol. The concentration of total RNA was determined by measuring the optical density at 260 nm. Each 600 ng RNA was reverse transcribed to cDNA using oligo (dT) primers and Super Script^{TM} reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Quantitative RT-PCR was performed using the TaqMan program on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers amplifying the mouse mRNA regions and a specific Taqman probe were designed using the Primer Express software package (Applied Biosystems). Data were expressed as the comparative cycle threshold (Ct). The normalized Ct value of each gene was obtained by the subtracting the Ct value of

18S rRNA. The fold change versus one sample of the control group was calculated as described previously (7).

In vitro migration assays and adoptive transfer study

Splenocytes were prepared from Balb/c heart grafted B6.wild or B6.CD98hc-/- recipients on POD7, and incubated with 5μM 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE). Then, $5x10^5$ B6.wild or B6.CD98hc-/- CFSE labeled lymphocytes in 100μl medium were loaded in Transwell filters (5μM PC; Costar, Corning, Inc,), which were placed in 24-well plates containing 600μl medium with or without chemokines CCL2, CCL5 (R&D Systems, Minneapolis, MN) at different concentrations (0, 0.1, 1, 10, 100 ng/ml). After incubation for 1h at 37°C, cells in the bottom well were collected and counted by flow cytometry. Chemotactic indices were calculated as the number of cells that had migrated to wells containing chemokines, divided by the number of cells that had migrated to wells containing medium alone.

For the adaptive transfer study, lymphocytes were obtained from the spleens of B6.wild or B6.CD98hc-/- naive mice, and incubated with CFSE. Labeled cells (50×10^6) were injected through the tail vein into B6.Rag2KO recipients immediately after transplantation of Balb/c heart grafts. On day 5 after transfer, spleen and cardiac grafts of B6.Rag2KO recipients were harvested and subjected to FACS and immunohistochemical analyses.

Antibodies and antibody therapy

An anti-CD98hc neutralizing antibody (clone 26-24) (8) and anti-CD25 depleting antibody (clone PC61) were purified by ammonium sulfate precipitation of a GIT medium cultured using a CL1000 flask (INTEGRA Biosciences AG, Switzerland) and added to the above culture system at various concentrations. To assess the effect of Treg cells on cardiac allograft tolerance in B6.CD98hc-/- mice, B6.CD98hc-/- mice were given an intraperitoneal injection of 1mg anti-CD25 mAb twice (Days -7 and -3), and then received a cardiac allograft from a Balb/c donor on Day 0. The effects of the CD98hc neutralizing antibody were also investigated. On the day the B6.wild mice received the Balb/c cardiac allograft, the recipient was given one intraperitoneal injection of 500μg anti-CD98hc mAb (clone 26-24), and then 250μg twice a week for 2 weeks. The graft survival was determined by daily palpation of the recipient abdomen.

Statistical Analysis

Student's *t*-tests were used to compare the paired and unpaired analyses. A statistical evaluation of mouse survival was performed using the Kaplan-Meier test. *P* values less than 0.05 were considered statistically significant. All *in vitro* experimental data were representative of three independent experiments and represented the mean ratio of triplicate results in each experiment.

Supplementary Figure legends:

Supplementary figure 1. *Generation and characterization of CD98hc conditional knockout mice.* (A) Restriction maps showing the endogenous 129/SVJ CD98hc gene and the targeted alleles. The position of PCR genotyping primers 41, 43 and 49 are shown. (B) Identification of the different CD98hc alleles. The specific PCR reactions were used to identify the CD98hc alleles. (C) Splenocytes obtained from B6.wild and B6.CD98hc-/- naive mice were stained with anti-CD3-FITC and anti-CD98hc-PE, and showed that the population of the CD98 molecule on T cells was significantly reduced in the cells from deficient mice. (D) Histograms demonstrating that the CD3-positive T cells from wild type (dotted line) and deficient mice (solid line) expressed the same level of the CD11a, CD11b, CD29, CD62L, CD103, and CD147. Data are representative of at least three separate experiments.

Supplementary figure 2. *Cell number of spleen in the B6.wild and B6.CD98hc-/- mice.* The results of the total spleen cells obtained from B6.wild (white bar) and B6.CD98hc-/- (gray bar) naive mice, and showed no difference between the wild type and deficient mice. Data are representative of three independent experiments and indicate the mean ratio of triplicate results from each experiment.

Supplementary figure 3. *Cell subsets characterization of B6.wild and B6.CD98hc-/- mice.* Bone marrow, thymus, and spleen obtained from B6.wild and B6.CD98hc-/- naive mice, were stained with anti-CD3, CD4, CD8, CD11b-FITC, anti-B200-PE

(gray line represent isotypic control, blue line represent B6. wild, and red line represent B6.D98hc-/-). The proportion of CD3+, CD4+ T cells, CD11b+ macrophage cells and B220+ B cells showed no difference between the wild type and deficient mice. The expression of the CD8 is down-regulation on the spleen cells in deficient mice. Data are representative of at least three separate experiments.

Supplementary figure 4. The response of B6.wild and B6.CD98hc-/- naïve mice T cells to anti-CD3 and anti-CD28. Splenic T cells were prepared from B6.wild (white bar) and B6.CD98hc-/- (gray bar) naive mice, and were cultured with anti-CD3 and anti-CD28. The results are shown no difference between the B6.wild and B6.CD98hc-/- recipients. Data are representative of at least three separate experiments.

Supplementary figure 5. Cell survival of B6.wild and B6.CD98hc-/- mice. (A) Naïve splenic T cells were prepared from B6.wild and B6.CD98hc-/-mice. (B) Splenic T cells were prepared from B6.wild and B6.CD98hc-/- mice, and were cultured with irradiated Balb/c spleen cells for 4 days. (C) Splenocytes were obtained from B6.wild and B6.CD98hc-/- recipients on POD7. The proportion of PI and AnnexinV+ cells showed no difference between the B6.wild and B6.CD98hc-/- recipients. Data are representative of at least three separate experiments.

Supplementary figure 6. mTOR and PS6 significantly decreased in B6.CD98hc-/-

recipients on POD7. (A) Quantitative RT-PCR for the mTOR mRNA expression in the spleens and cardiac grafts obtained from the B6.wild isograft recipients (light gray bar), B6.wild allograft recipients (white bar) and B6.CD98hc-/- allograft recipients (dark gray bar) on POD7. The expression of mTOR mRNA significantly decreased in the spleens and cardiac allografts harvested from B6.CD98hc-/- (dark gray bar) recipients on POD7, compared with the B6.wild (white bar) recipients (p=0.0110 and 0.0403). (B) Splenocytes were obtained from B6.wild naïve mice, B6.wild (dotted line) recipients and B6.CD98hc-/- (solid line) recipients on POD7, then were stained with anti-mouse CD4 and anti-PS6 (Ser235/236). The filled gray histogram represents the fluorescence of B6.wild naïve mouse cells. The expression of PS6 on the CD4+ T cells was decreased in the B6.CD98hc-/- (solid line) recipients on POD7 compared with the B6.wild (dotted line) recipients. The data are representative of three independent experiments.

Supplementary figure 7. *The expression of CTL-related mRNAs in cardiac grafts from B6.wild and B6.CD98hc-/- recipients.* Balb/c heart allografts harvested from B6.wild (white bar) and B6.CD98hc-/- recipients (gray bar) on POD7 were used for measurement of mRNA expression by quantitative real time RT-PCR. The changes in the mRNA expression *vs.* one sample of the control group were calculated. p=0.004, <0.001, 0.028, 0.004, 0.006, and 0.005, for the differences between B6.wild and B6.CD98hc-/- mice. The relative quantity is presented as the ratio of the comparative cycle threshold (Ct) of the target genes against those of the housekeeping gene 18s.

Data are representative of three independent experiments and indicate the mean ratio of triplicate results from each experiment.

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