SUPPLEMENTAL MATERIAL

Supplementary Methods

Cardiac Transplantation:

All animals were used between the age of eight to twelve weeks. Medetomidine (Domitor, Pfizer Animal Health, New York, USA; 1.6mg/Kg) and Ketamine (Vetalar, Zoetis UK Limited, Surrey, UK; 110mg/Kg) were used subcutaneously as general anaesthetics. Cardiac transplantation was performed as first described by Corry *et al.* (1). A large v-shaped incision was made in the donor, and the gut was removed to reveal the inferior vena cava (IVC) and the aorta. 500µl of heparinized saline (200units/ml) (Leo Laboratories, Buckinghamshire, UK) was injected into the IVC and allowed to circulate for 1 minute, then the aorta was cut to allow exsanguination of the animal. The rib cage was opened to reveal the heart; and the inferior vena cava, azygous veins, and the superior vena cava were tied with 7/0 silk suture (Pearsalls, Ltd., Taunton, UK) and cut. The aorta and the pulmonary artery were cut straight across using microscissors (Fine Science Tools, Heidlberg, Germany). A tie was then placed around the entire heart and it was removed from the animal, and placed in sterile saline on ice, whilst the recipient was prepared. The anaesthetized recipient was shaved, the abdomen swabbed with Povodine Iodine, as Vetasept (Animalcare, York, UK), and a sterile drape placed over the operating area. A midline incision was made, and the bowels and bladder retracted and covered in saline-soaked sterile gauze. The IVC and aorta were exposed, and the lumbar veins which drain into the IVC were tied with 7/0 silk suture. Microvessel clamps (Fine Science Tools) were placed at the top and bottom of the IVC and aorta. A venotomy and aortotomy were made using a 21-gauge needle (Terumo Ltd., Surrey, UK), and extended with microscissors. Anchor stitches were placed at the top and bottom of

the donor and recipient's aorta, and the left-hand walls were anastomosed with a running stitch using 10/0 nylon suture (Unik sutures, Taiwan). This was tied to the top anchor stitch; then the heart was flipped over to complete anastomosis of the right-hand wall. The pulmonary artery was anastomosed to the recipient IVC in the same way, except that the right wall was completed first. A small piece of Spongostan (Ferrosan Medical Devices, Soborg, Denmark) was placed over the anastomoses before removal of the clamps. The midline incision was closed with a 4/0 suture (Unik sutures). Cardiac grafts were monitored by palpation of the abdomen, and the strength of beating assessed according to the scale developed by Superina *et al.* (2). No detectable difference in the strength of beating was observed between gender matched and HY-mismatched grafts, as the palpation method is too crude and subjective for chronic rejection models. This has been previously reported in this model, as the Cardiac Allograft Vasculopathy (CAV) is not manifested as a decline in force of contract (3). To objectively determine the amount of injury to the donor graft, histological techniques, especially the assessment of vasculopathy as we have done here, is necessary.

Immunohistochemistry

Donor grafts were harvested and processed for immunohistochemistry; sections were stained with antibodies against CD4 (clone H129.19, BD Pharmingen, Oxford, UK), CD8 (clone 53-6.7, BD Pharmingen), CD68 (clone FA-11, Serotec, Oxford, UK), or LYVE1 (ab14917 Abcam plc, Cambridge, UK), as previously described (4). With the addition of a non-specific FC receptor block, before addition of the primary antibody (10% FCS, Sigma Aldridge, 1% BSA, Sigma Aldridge, in PBS for 30min at RT). Positive cells were counted in 20 random high-power fields (HPF, magnification 400x). For $CD68^{+}$ macrophage staining, a 63-point grid was

overlaid onto a 400x magnification of macrophage staining. The percentages of grid points positive for CD68 staining were counted. LYVE-1^+ vessels were counted in at least 10 medium power fields (MPF, magnification 200x). To determine the cross-sectional area of the lymphatic vessels. Images of MPF were analysed with Image J software. All analysis was conducted by two observers blinded to the experimental conditions and a mean was calculated. To determine if vessels were of donor or recipient in origin, the donor cardiac transplants were harvested and staining of lymphatic vessels was performed with the primary antibody anti-LYVE-1 (ab14917, Abcam plc) using a modified protocol. The recipient EYFP cell signal was amplified with FITC conjugated goat anti-YFP (ab6662, Abcam plc) as described below.

LYVE-1 staining method

5µm sections of tissue were fixed for 40s in 2% Paraformaldehyde (Sigma-Aldridge) in Acetone (BD Pharmingen) at 4^0C to preserve the EYFP expression. Endogenous peroxidase activity within the tissue was quenched with 3% hydrogen peroxide (Sigma-Aldridge) in PBS at RT for 10 mins. Non-specific FC receptor binding was blocked by incubation in 10% FCS, 1% BSA in PBS for 30min at RT. The primary antibodies rabbit anti-LYVE-1 and goat anti-YFP were incubated for one hour at RT. For LYVE-1 detection sections were incubated with a biotinylated goat, anti-rabbit (Vector Labs) at RT for 30 mins, subsequent to incubation with Streptavidin-PE for 30mins RT. Then sections were mounted in vectasheild with dapi (Vector Labs). Donor lymphatic vessels will be $EYFP^-$ and LYVE-1^+, recipient lymphatic vessels will be $EYFP^+$ and LYVE-1^+ and where the donor and recipient's vessels have grown into each other there will be $LYVE1^+$ vessels that are partially a mix of $EYFP^{+/-}$.

Elastic Van Gieson staining and calculation of percentage luminal occlusion

Graft vasculopathy was quantified by measuring luminal narrowing and intima to media ratio in donor arteries as described previously (5). Briefly, the harvested heart was fixed in 10% formalin, embed in paraffin and sectioned into 5µm sections. The sections were stained with Elastica van Gieson to highlight the IEL. elastin-stained cross-sections of the coronary arteries with a well-defined smooth muscle cell layer and IEL in the vascular wall were photographed using Slides were analyzed using a Diaplan microscope (Leitz) and a DXM1200DF digital camera (Nikon), using Lucia G software (Nikon). Digital images at 200x magnification were processed using Image-J software and the area encompassed by the lumen and the Internal elastic lamina IEL was traced (Figure 4g-i). The area of luminal stenosis in each section was calculated according to the following formula:

Luminal occlusion % = [IEL area (I) - luminal area (L)/ IEL area (I)] x 100

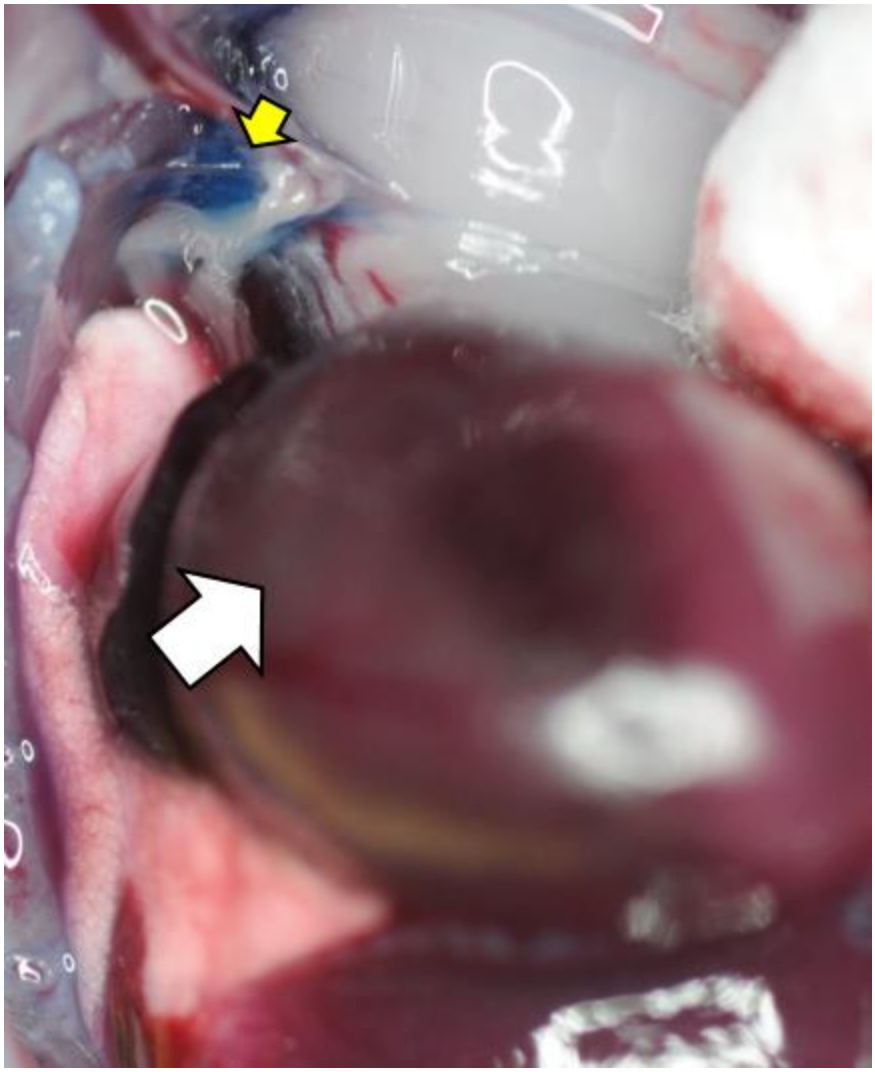
Two independent observers calculated the severity of CAV, these people were blinded to the sample type. The scores are shown as a mean ± SEM of the percentage luminal occlusion score of the two independent observers.

Quantitative PCR

To determine the number of donor cells that have migrated out of the grafts we isolated genomic DNA from the mediastinal dLN to quantify the number of male donor cells present in the female recipients after transplantation using a standard curve generated by real-time PCR. Zfy1 is a zinc finger gene within the testis determining region (6) and is located on the Y chromosome. Genomic DNA was chosen because the Y-chromosome specific gene, Zfy1, is

not expressed in the female recipients and each male donor cell carries four copies of this gene. As we are amplifying genomic DNA rather than mRNA, the level is not affected by the level of expression of the gene concerned, thus four copies represent one male cell. This technique is mouse strain independent and it has been reported that the gene expression level of Zfy1 corresponds well to the amount of male cell DNAs in a mouse transplantation model (7-8). This technique is highly sensitive and can detect donor cell engraftment at <1%. Furthermore, unlike conventional flow cytometry-based method samples can be stored for later analysis, which is a requisite when working with radioactive samples. The PCR reaction was performed using a Biorad C1000 real-time PCR machine and data was analysed using the BioRad CFX software. The standard curves were generated by plotting the mean of triplicate Ct values versus the log of the Y copy number and calculation of a regression line. The copy number of the unknown samples was determined by applying the mean Ct value of triplicates to the standard curve and corrected for the total amount of DNA in the sample. The percentage of male DNA in the entire dLN can be calculated, which in turn can be converted to the number of male donor cells that have trafficked to the mediastinal dLN. The primer sequences are as follows: Zfy1 5'-TGGAGAGCCACAAGCTAACCA and 5'-CCCAGCATGAGAAAGATTCTTC.

Supplemental Figure 1



Supplemental Figure 1. Visualisation of lymphatic Flow from the cardiac grafts to the mediastinal lymph nodes of a HY-mismatched recipient mouse. HY-mismatched or gender-matched donor hearts were transplanted into the abdomen of recipient mice. For direct visualisation of lymph drainage from donor heart grafts after transplantation Evan's Blue was injected into the apex of the donor heart grafts (n=3 in each group). Shown here is a representative of a HY-mismatched recipient donor graft showing the left mdLN (yellow arrow) above the native heart (white arrow) staining blue with Evan's Blue dye.

Supplemental References

(1) Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice – Role of H-2D, H-2K, and non-H-2 antigens in rejection. Transplantation. 1973; 16, 343-350.

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(4) Tapmeier TT, Fearn A, Brown K, Chowdhury P, Sacks SH, Sheerin NS, Wong W. Pivotal role of CD4+ T cells in renal fibrosis following ureteric obstruction. Kidney Int. 2010; 78: 351-62.

(5) Hasegawa T, Visovatti SH, Hyman MC, Hayasaki T, Pinsky DJ. Heterotopic vascularized murine cardiac transplantation to study graft arteriopathy. Nat Protoc. 2007; 2: 471-80.

(6) Nagamine CM, Chan K, Hake LE, Lau YF. The two candidate testis-determining Y genes (Zfy-1 and Zfy-2) are differentially expressed in fetal and adult mouse tissues. Genes & development. 1990; 4: 63–74.

(7) Byrne P, Huang W, Wallace VM, Shean MK, Zhang Z, Zhong Q, Theodossiou C, Blakesley H, Kolls JK, Schwarzenberger P. Chimerism analysis in sex-mismatched murine transplantation using quantitative real-time PCR. Biotechniques. 2002; 32: 279-4.

(8) An N, Kang Y. Using quantitative real-time PCR to determine donor cell engraftment in a competitive murine bone marrow transplantation model. J Vis Exp. 2013; 73: e50193.

Supplemental Video legends

Supplementary Video 1. A representative SPECT/CT 3D reconstruction of images of a recipient mouse transplanted with an HY mismatched donor heart in the abdomen five weeks previously. Four hours after injection, Tc-99m could be seen at the injection site within the transplanted heart in the mid abdominal cavity. Tc-99m activity migrated from the peritoneal cavity to a distinct hot spot in the thoracic cavity, suggesting that lymph from the donor organ has flowed towards the mediastinal lymph nodes. The identity of these lymph nodes was confirmed by dissection and biodistribution studies at post-mortem examination. Tc-99m activity was also seen in the liver after four hours. This was most likely to be due to lymph from the donor organ entering the blood stream *via* the lymphatic route. Radioactivity was also seen in the kidney and excreted into the urinary bladder.

Supplementary Video 2. A representative SPECT/CT 3D reconstruction of images of a recipient mouse transplanted with a gender-matched donor heart into the abdomen five weeks previously. Four hours after injection, Tc-99m could be seen at the injection site within the transplanted heart in the mid-abdominal cavity as described for the HY-mismatch graft. However, no distinct hot spots were detected in the location of the mediastinal lymph nodes. The mediastinal lymph nodes were dissected and confirmed to have only background radioactivity by biodistribution studies at post-mortem examination.