**Materials and Methods**

**Mice**

The mice were created using a transgene containing human Hsp-27 cDNA with a chicken beta-actin promoter and cytomegalus virus enhancer. Transgenic F1 hybrid mice (C57BL/10 x CBA/Ca) were bred with wild type B10.A mice for 9 generations to obtain heterozygous Hsp-27 transgenic (Hsp-27tg) B10.A. Henceforth, heterozygous female transgenic B10.A were bred with male wild type B10.A and transgenic male mice identified using PCR from tail clippings. Male Hsp-27tg mice and their male littermate controls (LCs) were used in our experiments. B10.A and C57BL/6 mice were purchased from Harlan Laboratory.

Mice were conventionally housed and fed and the experiments were performed under license (License number 70/6583) by the Animals and Scientific Procedures Act (1986).

**Heterotopic Heart Transplantation**

Mice were anaesthetized with 2-2.5% isofluorane inhalation anaesthesia (isofluorane-Vet, Merial) and every effort was made to minimise suffering. Donor aorta was anastomosed to the recipient aorta and donor pulmonary artery was anastomosed to the recipient vena cava. Hearts were palpated on a daily basis to determine day of rejection, they were palpated without knowledge of the experimental group to which they belonged.

**Western Blotting**

Tissues were snap-frozen in liquid nitrogen and proteins extracted directly by homogenisation in RIPA. The homogenate was incubated for 20 minutes on ice and centrifuged at 4°C for 10 minutes at 13K rpm. The supernatant was subjected to colorimetric BCA protein assay (Pierce). 40µg of total proteins solubilised in 1:3 loading buffer was loaded in each well. Proteins separated in 10-15% acrylamide Tris-Glycine gels were transferred onto PVDF membranes (GE Healthcare) for 1h at 100V. Membranes were then air dried. The efficiency of protein transfer was assessed by staining the membranes with Ponceau S. The house keeping gene, GAPDH was used as an internal control to check the equal loading of the proteins. Non-specific binding was blocked by incubating the membrane for 2h in 5% solution of dried milk powder in phosphate buffered saline (PBS) with 0.1% Tween-20 (PBST). Proteins were identified using an antibody against the HA tag (rabbit anti-HA from Santa Cruz, used at 1:200), rabbit anti-Hsp-25 (1:250, from Calbiochem) or rabbit anti-GAPDH (1:1000 from Cell-signalling) followed by blotting grade peroxidise conjugated anti-rabbit secondary antibody at 1:1000 (from Dako). Three 5 minutes washes with PBST were performed between each antibody step. The blots were then visualized with enhanced chemiluminescence (ECL, Amersham hyperfilm ECL, GE Healthcare). Band Intensities were quantified by scanning and processing with a Java-based image analysis program Image J (1.46r). Briefly, the area around each band was delimited by a rectangle and the program was used to quantify the intensity of each band. The intensities obtained for HA and Hsp-25 were normalised relative to those of loading control GAPDH.
**Immunocytochemistry**

Immunohistochemistry was used to analyse the presence of the HA in frozen sections of heart. Briefly, pieces of tissue were embedded in Oct and snap frozen in liquid nitrogen. They were stored in -80°C freezer until use. Cryostat sections (7μm thickness) were fixed in acetone for 6 minutes before staining with rabbit anti-HA primary antibody (at 1:50, from Roche) for 1hr 30 minutes at room temperature. Non-specific sites were blocked with 1%BSA/0.1%Tween 20 in PBS for 30 minutes before incubating the sections with the primary antibody. After washing in PBS, biotinylated rat anti-rabbit antibody (at 1:200, from Dako) in 2% mouse serum was added for 45 minutes followed by avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit, Vector laboratories). The sites of peroxidase binding were developed in PBS containing diaminobenzidine tetrachloride (0.3mg/ml) and hydrogen peroxide (0.01%) (Sigma fast, Sigma Aldrich). Slides were counterstained with Mayer's hematoxylin (Sigma).

**Measurement of alloantibodies**

Serum from recipient mice was collected at the time of sacrifice. 100,000 splenocytes were incubated with sera at 1:10, 1:20 and 1:40 dilutions for 30 minutes at 4°C. Samples were washed twice with PBS and incubated with secondary Goat anti-mouse Ig: FITC (AbD Serotec) at 1:30 dilution for 30 minutes at 4°C. Two further washes were performed and the splenocytes were then fixed in 0.5% formaldehyde and analyzed by flow cytometry.

**Enzyme linked immunoassay**

SDC 5µl of serum from Hsp-27 transgenic and littermate controls were diluted 1:10 and assayed according to manufacturer’s instructions. The absorbance was read using Spectrophotometer at 450nm. The quantity of Hsp-27 was determined by comparing the absorbance of the samples to the absorbance of standard (given by the manufacturer).

**Tunnel assay**

A piece of heart was frozen in liquid nitrogen and cut into 7μm section using a cryostat. The sections were fixed with 4% paraformaldehyde in PBS, pH7.4 and washed with PBS for 30 minutes. After permeabilisation (0.1% Triton X-100 and 0.1% sodium citrate for 2 minutes at 2-8°C), the sections were then incubated with 50μl per section of TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT), in the presence of fluorescently labelled nucleotides. Positive controls were obtained by incubating fixed permeabilised cells with DNAase I recombinant, grade I (2000U Tris-HCl, pH 7.5, 10mM MgCl2, 1mg/ml BSA). Sections incubated with nucleotides, in the absence of TdT, were used as negative controls. The slides were washed three times with PBS. These were then mounted using DAPI mounting media (Vectashield, Vector) and visualized under fluorescent microscope using an excitation wavelength in the range of 450-500nm.

**Caspase assay. SDC**

Hearts were homogenized in lysis buffer (125mM HEPES, 12.5mM CHAPS, 12.25mM DTT and protease inhibitor) and protein concentration was determined using Bradford protein assay reagents. 30µl of total protein was incubated at 37°C with 50µl of caspase assay buffer (200mM HEPES, 50mM DTT, 1%CHAPS, 20mM
EDTA) and the appropriate caspase substrate. The activity of caspase3, caspase 1 and caspase 9 were measured using specific substrates labelled with N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Sigma), N-acetyl-Tyr-Val-Ala-Asp-(7-amino-4-triflouroemethylcoumarin) and N-acetyl-Leu-Glu-His-Asp-(7-amino-4-triflouroemethylcoumarin) (Tocris) respectively and quantified by spectrofluorimetry (excitation wavelength 360nm and emission wavelength 460nm for caspase 3 and wavelength 360nm and emission wavelength 530nm for caspase 1 and caspase 9).

Quantitative PCR (qPCR) Analysis
100 ng of total RNA was used per 10 µl reaction volume with random hexamers and diluted to the RNA equivalent of 2 ng/µl with filter-sterilised water. qPCR was performed on Applied Biosystems’ 7500 Fast Real-time PCR system using Taqman reagents (Taqman Fast Advanced master mix, catalogue number 4444557) and 3 µl of diluted cDNA. Taqman probes were purchased for 18S ribosomal RNA (4310893E), and target CD4 (Mm00442754_m1), CD8 (Mm00438116_m1), CD3 (Mm00438095_m1), CD14 (Mm00438094_g1), IFNy (Mm00801778_m1), IL-1β (Mm01336189_m1), TGFβ (Mm01178820_m1), TNFα (Mm00443258_m1), IL-2 (Mm00434256_m1), IL-10 (Mm00439614_m1), IL-5 (Mm00439646_m1) and IL-4 (Mm00445260_m1). Target gene expression was normalised to 18S rRNA levels and expressed as fold induction over a control sample using the comparative cycle threshold (ΔΔCt) method. 18S expression was unaltered across the experimental groups and suitable for use as an endogenous control. All statistical analyses were performed using Graphpad Prism software. Data are given as mean±standard error of the mean except where stated. Non-parametric statistical tests were used throughout, and p≤0.05 was taken as significant.

Elispots. SDC
Single cell suspensions were prepared from spleens removed from transplanted animals. Red blood cell lysis was performed and CD3+ T cells isolated using the Dynal mouse T cell negative isolation kit (from Invitrogen). CD3+ T cells were stored frozen until use. Antigen Presenting cells (APC) were prepared by depletion of CD3+ T cells from spleen cell suspensions using magnetic beads (coated with antibody to CD3) and stored frozen until use. Ninety-six well PVDF-bottomed plates (Millipore) were coated overnight with mouse anti-rat IFNγ capture antibodies (BD Biosciences). The following day plates were washed and blocked for 1hour with 1% BSA in PBS, then washed again before the addition of cells. Responder cells were thawed, washed and resuspended in RPMI-1640 +10% FCS. Stimulator APC were thawed, washed, treated with mitomycin C (Sigma) (40µg/ml) for 20mins, washed 3x and resuspended in RPMI-1640 +10% FCS.

2x10^5 responder T cells and 2x10^5 APC were added to each well in a final volume of 200µl. Plates were incubated at 37°C, 5% CO₂ for 48hrs, after which they were washed with PBS + Tween and exposed to biotinylated anti-mouse IFNγ antibody (from BD Biosciences) for 2hrs at room temperature. After further washing, streptavidin HRP (Vector Laboratories Ltd.UK) was added for 1.5hrs before the addition of the substrate AEC (3-amino9-ethyl-carbazole). Spots were allowed to develop before the plates were washed with H₂O and left to dry overnight. Spots were enumerated using an AID elispot reader and software (Autoimmun Diagnostika GmbH)
SDC Figure S1. Increased expression of Hsp in transgenic hearts. Proteins present in the heart, lung, liver, kidney and spleen were analysed by western blot for the presence of HA-tagged Hsp-27 and the endogenous protein Hsp-25. GAPDH was used as control. Comparison of HA-tagged Hsp27 to HA+Hsp-25 showed a prominent increased expression of overall Hsp in transgenic heart (n=2).
SDC, Figure S2. Presence of alloantibodies was measured by flow cytometry. B10.A transgenic or LC or C57BL/6 heart was transplanted into C57BL/6 recipients. Sera from recipients were collected at 2 days (a), 5 days (b) or 12 days (c) post-transplant and incubated with B10.A splenocytes at 1/10, 1/20 and 1/40 dilutions. The intensity of staining was analyzed by flow cytometry and is shown as mean fluorescence.