OPEN

Heat Shock Protein-27 Delays Acute Rejection After Cardiac Transplantation: An Experimental Model

Borggia Seemampillai,[1] Renee Germack,[2] Leanne E. Felkin,[2] Ann McCormack,[1] and Marlene L. Rose[1,3]

Background. Rejection is the major obstacle to survival after cardiac transplantation. We investigated whether overexpression of heat shock protein (Hsp)-27 in mouse hearts protects against acute rejection and the mechanisms of such protection.

Methods. Hearts from B10.A mice overexpressing human Hsp-27 (Hsp-27tg), or Hsp-27–negative hearts from littermate controls (LCs) were transplanted into allogeneic C57BL/6 mice. The immune response to B10.A hearts was investigated using quantitative polymerase chain reaction for CD3+, CD4+, CD8+ T cells, and CD14+ monocytes and cytokines (interferon-γ, interleukin [IL]-2, tumor necrosis factor-α, IL-1β, IL-4, IL-5, IL-10, transforming growth factor-β) in allografts at days 2, 5, and 12 after transplantation. The effect of Hsp-27 on ischemia-induced caspase activation and immune activation was investigated.

Results. Survival of Hsp-27tg hearts (35±10.37 days, n=10) was significantly prolonged compared with LCs (13.6±3.06 days, n=10, *P*=0.0004). Hsp-27tg hearts expressed significantly more messenger RNA (mRNA) markers of CD14+ monocytes at day 2 and less mRNA markers of CD3+ and CD8+T cells at day 5 compared with LCs. There was more IL-4 mRNA in Hsp-27tg hearts at day 2 and less interferon-γ mRNA at day 5 compared with LCs. Heat shock protein-27tg hearts subjected to ischemia or to 24 hr ischemia-reperfusion injury demonstrated significantly less apoptosis and activation of caspases 3, 9, and 1 than LCs. T cells removed from C57BL/6 recipients of Hsp-27tg hearts produced a vigorous memory response to B10.A antigens, suggesting immune activation was not inhibited by Hsp-27.

Conclusion. Heat shock protein-27 delays allograft rejection, by inhibiting tissue damage, through probably an antiapoptotic pathway. It may also promote an anti-inflammatory subset of monocytes.

Keywords: Hsp-27, Heat shock proteins, Transplantation, Cardiac transplantation, Transplant rejection

(*Transplantation* 2014;98: 29–38)

Rejection remains the major barrier to long-term graft survival after solid organ transplantation. Prevention of rejection requires life-long immunosuppression with agents such as steroids, antiproliferative agents, and calcineurin inhibitors (*1*). Unfortunately, life-long immunosuppression is associated with infections and malignancy. Identification of natural molecules that are cytoprotective may alleviate rejection and lessen the requirement for immunosuppression.

Heat shock protein (Hsp)-27 (in humans) and Hsp-25 (in rodents) are part of a family of heat shock proteins which bind denatured proteins in times of stress to prevent aggregation (*2*). The human Hsp-27 gene encodes a 199 amino acid polypeptide, which is induced in response to stress, such as heat shock, simulated ischemia, and oxidative stress (*3, 4*). The antioxidative effects, antiapoptotic ability, and ability to stabilize the cytoskeleton are well-known properties of Hsp-27 (*5, 6*), but these properties have been discovered almost entirely by cell-culture studies. Far less is known about the effects of Hsp-27 in whole animal models of disease.

Heat shock protein-27 is constitutively expressed in human cardiomyocytes (*7*), and ex vivo studies using mouse

This study was supported by the British Heart Foundation (RG/06/004) and B.S. was funded by the National Heart and Lung Institute Foundation.
The authors declare no conflicts of interest.

[1] Transplant Immunology Group, National Heart and Lung Institute, Imperial College, Harefield Heart Science Centre, Harefield, United Kingdom.
[2] Molecular Biology Group, National Heart and Lung Institute, Imperial College, Harefield Heart Science Centre, Harefield, United Kingdom.
[3] Address correspondence to: Marlene Rose, Ph.D., National Heart and Lung Institute, Imperial College, Heart Science Centre, Harefield Hospital, Harefield, Middlesex, UB9 6JH, UK.
E-mail: marlene.rose@imperial.ac.uk

B.S. participated in research design, writing of the article, performance of the research, and data analysis. R.G. participated in performance of research and data analysis. L.E.F. participated in performance of research and data analysis. A.M. participated in performance of the research and data analysis. M.L.R. participated in research design, writing of the article and data analysis.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

Received 17 December 2013. Revision requested 6 January 2014.
Accepted 13 March 2014.

ISSN: 0041-1337/14/9801-29
DOI: 10.1097/TP.0000000000000170

hearts overexpressing Hsp-27 have demonstrated that it protects the myocardium against ischemia and reperfusion injury (*8, 9*). Further studies have demonstrated that Hsp-27 is expressed within human blood vessels (*10*) and phosphorylated Hsp-27 is believed to be protective against atherosclerosis (*10–12*). Experimental studies have demonstrated that female Hsp-27 overexpressing mice crossed with the apoE knock out have less atherosclerosis than apoE knock out mice (*13*). Cardiac allograft vasculopathy (CAV) or occlusion of coronary arteries in donor hearts is a long-term complication after cardiac transplantation (*14*). It has many similarities to non–transplant atherosclerosis (*15*). The fact that significantly more Hsp-27 was found in cardiac biopsies from patients with long-term freedom from CAV compared with biopsies from patients who developed early CAV suggests a role for Hsp-27 in protection against transplant associated atherosclerosis (*16*). Whether Hsp-27 also has a role to play in protecting allografts from acute rejection has not been investigated.

We have used an experimental model of acute cardiac allograft rejection and demonstrate that overexpression of Hsp-27 in donor hearts delays acute rejection; we have investigated the mechanisms of delayed rejection.

RESULTS

Distribution of Hsp-27 in Transgenic Mice (Hsp-27tg)

To track expression of the transgene a hemagglutinin (HA) tag was placed contiguous with the Hsp-27 sequence (*17*). Hence, Hsp-27 could be tracked using rabbit antibody to HA. Organs were removed from Hsp-27tg mice and littermate controls (LCs) for analysis by Western blot and immunocytochemistry. Figure 1(a) shows representative data of Western blots probed with rabbit anti-HA and anti–Hsp-25 antibody. The HA protein was undetectable in heart, lung, liver, kidney, and spleen of LCs. However, prominent expression of HA was found in the hearts and lungs of Hsp-27tg mice, but no HA was observed in liver, kidneys, or spleen. In contrast, there was a wide-spread expression of endogenous Hsp-25 in hearts, lungs, kidneys, and spleen of LCs. In transgenic mice, there was no expression of Hsp-25 in kidneys. There was weak expression of Hsp-25 in the liver of Hsp-27tg and LCs. Immunocytochemistry of frozen sections of heart revealed a strong expression of HA-tagged Hsp-27 in the cardiomyocytes, but not endothelial cells, of hearts of transgenic mice (Fig. 1b). Quantification of Hsp-25 and HA-tagged Hsp-27 in all organs confirmed there to be considerably more Hsp-25/Hsp-27 in the hearts of transgenic mice compared with their LCs (**Figure S1, SDC**, http://links.lww.com/TP/A978). Enzyme-linked immunosorbent assay (ELISA) for Hsp-27 in the sera of Hsp-27tg and LCs demonstrated the presence of Hsp-27 in the sera of transgenic mice (Fig. 1c).

Acute Rejection

Hearts from B10.A transgenic or LCs (H-2^a) were transplanted into C57BL/6 recipients (H-2^b). This represents a complete major histocompatibility complex mismatch. Survival of hearts from B10.A Hsp-27tg mice (35±10.37 days, n=10) were significantly prolonged compared with that of hearts from LCs (13.6±3.06 days, n=10, P=0.0004). Syngeneic grafts from C57BL/6 mice showed no signs of rejection when recipients were killed at 8 weeks after transplantation (Fig. 1d).

Analysis of Acute Rejection by Quantitative Polymerase Chain Reaction

To investigate the mechanisms of delayed graft rejection, infiltration of inflammatory cells (CD3+ T cells, CD8+ T cells, CD4+ T cells, and CD14+ monocytes) in allografts was investigated by quantitative polymerase chain reaction (qPCR) at 2, 5, and 12 days after transplantation (Fig. 2). At 2 days, posttransplant allografts expressing Hsp-27 showed significantly increased expression of CD14 transcripts, reflecting monocytes, compared with control mice. At 5 days after transplantation, expression of CD3 and CD8 messenger RNA (mRNA) was induced in both Hsp-27 transgenic and LC allografts, but abundance of both transcripts was significantly lower in Hsp-27 transgenic allografts compared with controls. At day 12, there was no difference in CD3 and CD8 expression. Heat shock protein-27 seems not to have demonstrated an effect on CD4 expression; however, the quantity of mRNA for the CD4 gene is low compared with CD3 or CD8 mRNA.

The expression of proinflammatory (interferon [IFN]-γ, interleukin [IL]-2, tumor necrosis factor-α, and IL-1β) and anti-inflammatory cytokines (IL-4, IL-5, IL-10, and transforming growth factor-β) was investigated in the same hearts (Fig. 3). For the proinflammatory cytokines, Hsp-27tg allografts had significantly reduced expression of IFN-γ transcripts at day 5 after transplantation but significantly increased expression at day 12 compared with LC mice (Fig. 3a). Interleukin-2 transcript expression was induced at 5 days after transplantation in both transgenic and control mice by comparable amounts. However, by 12 days after transplantation, the abundance of IL-2 mRNA had dropped, although levels in Hsp-27tg allografts were significantly higher than controls (Fig. 3b). There was no significant difference in quantity of mRNA for tumor necrosis factor-α or IL-1β between transgenic hearts and control hearts at various times (Fig. 3c and d). For the anti-inflammatory cytokines, there was a substantial induction of IL-4 transcripts at day 2 in Hsp-27tg allografts compared with controls (Fig. 3e) at days 5 and 12 mRNA for IL-4 was barely detectable in any group. In contrast, mRNA for IL-10 and transforming growth factor-β (Fig. 3g and h) increased in levels between days 2 and 5, plateaued at day 12, and there was no difference between Hsp-27tg and LC hearts. There was also no difference in mRNA levels of IL-5 expression between Hsp-27tg and LCs (Fig. 3f). Taken together, qPCR analysis of cells and cytokines suggest the presence of the transgene Hsp-27 has promoted an early anti-inflammatory Th-2 response (characterized by mRNA for CD14 and IL-4) and delayed the Th-1 response (characterized by CD8+T cells and IFN-γ) in this particular model.

Alloantibody Responses

The presence of alloantibody to B10.A antigens was investigated in the sera of C57BL/6 mice at days 2, 5, and 12 after transplantation (**Figure S2, SDC,** http://links.lww.com/TP/A978). Alloantibody was detected at day 12, and there was no difference in the titre of antibody produced by recipients of Hsp-27tg or LC hearts.

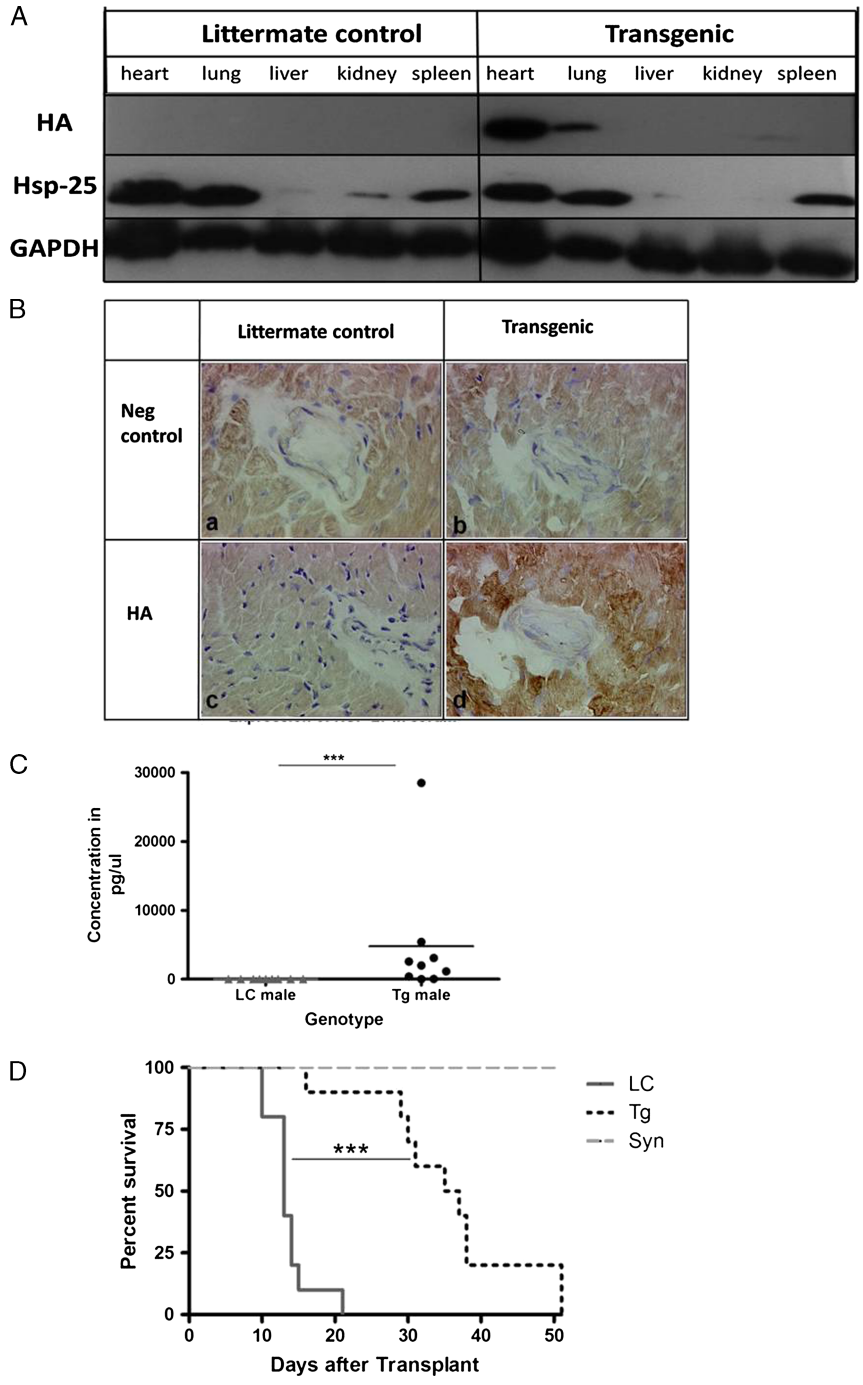

FIGURE 1. Distribution of HA-tagged Hsp-27 in transgenic mice and prolonged survival of transgenic allograft. Protein from hearts, lungs, liver, kidney and spleen from LC and Hsp-27tg mice were separated by SDS-PAGE and transferred to nitrocellulose membranes by Western blotting (a). They were probed with antibodies to HA, Hsp-25 or GAPDH followed by HRP-conjugated antibody. In b, frozen sections of hearts from Hsp-27tg and LCs were treated with peroxidase labeled irrelevant antibody (neg control) or antibody to HA, the peroxidase was visualized as described in the *Materials and Methods*. Hemagglutinin is predominantly expressed within cardiomyocytes of Hsp-27tg mice. In c, sera from LCs (n=8) and Hsp-27tg mice (n=8) were tested for soluble Hsp-27 by ELISA. The concentration of Hsp-27 was determined using the standard curves provided by the manufacture. Results are shown as mean±SEM, P=<0.001. In d, Hearts from Hsp-27tg (n=10) or their LCs (n=10) were heterotopically transplanted in C57BL/6 recipients. Survival of hearts from Hsp-27tg mice were significantly prolonged compared with hearts from LC (P<0.001, by Kaplan-Meier analysis). Syngeneic hearts were still beating at day 50 when all surviving mice were killed. HA, hemagglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LCs, littermate controls; Hsp, heat shock protein. ELISA, enzyme-linked immunosorbent assay.

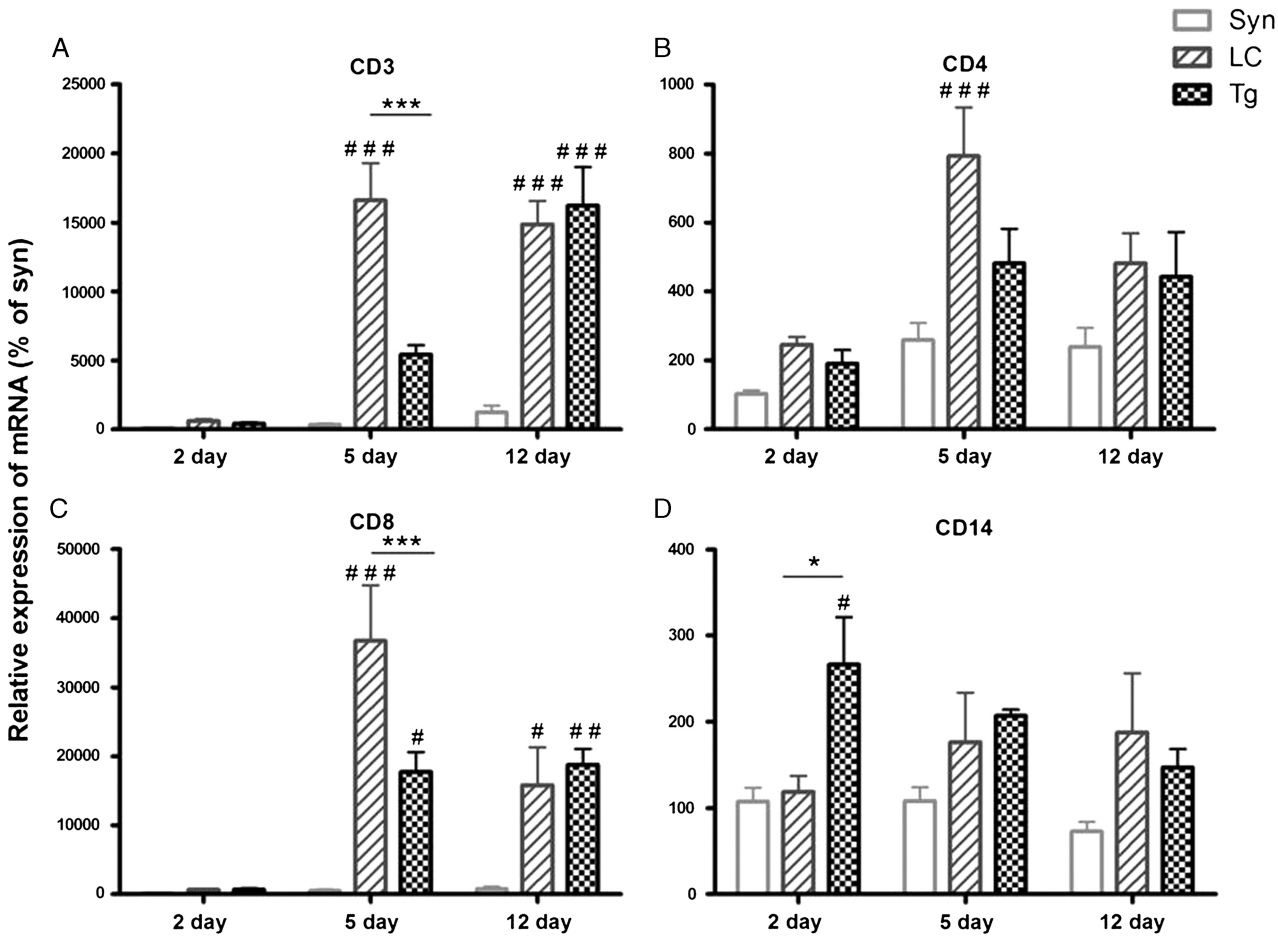

FIGURE 2. Effect of Hsp-27 on infiltrating cells. Recipients of transgenic and LC allografts were killed at days 2, 5, and 12 after transplantation and donor hearts were assessed for infiltration of CD3+ (A), CD4+ (B), and CD8+ (C) T cells as well as CD14+ (D) cells by RT-PCR. The y-axis shows relative expression of mRNA as a percentage of that present in syngrafts. It can be seen that transgenic allografts have less CD3+ and CD8+ T cells at day 5 compared with LC allografts and more CD14 cells at day 2 (* or #$P \leq 0.05$; ** or ##$P < 0.01$ and *** or ###$P < 0.001$, *transgenic vs. LC, #syngeneic vs. LC or syngeneic vs. transgenic, n=3–6). LC, littermate control; mRNA, messenger RNA; RT-PCR, reverse-transcriptase polymerase chain reaction; Hsp, heat shock protein.

Response of Hsp-27tg Hearts to Ischemia

Apoptosis

The observation that prolongation of heart survival was associated with early changes in the graft (i.e., presence of CD14 and IL-4 at day 2) suggested the possibility that early damage caused by ischemia or ischemic reperfusion may be modulated by Hsp-27. Hearts from Hsp-27tg and LC mice were subjected to 10 min cold and 40 min warm ischemia. Cryostat sections of hearts were analyzed for the presence of apoptotic cells using the terminal deoxynucleotide transferase-mediated 2′-deoxyuridine-5′-triphosphate nick-end labeling (TUNEL) assay (Fig. 4a and b). The increase in the numbers of apoptotic cells caused by ischemia in Hsp-27tg hearts (153% increase) was significantly attenuated compared with the increase in apoptotic cells in LC hearts (250%, P=0.0013, Fig. 4a and b).

Caspase Activity

Apoptosis is triggered through two main signaling pathways: intrinsic and extrinsic pathways (*18, 19*). Both of these pathways lead to the activation of caspase 3. The intrinsic pathway activates caspase 9, whereas the extrinsic pathway leads to the activation of caspase 8. Figure 4(c) shows similar levels of activity of caspase 3 in Hsp-27tg and control normoxic heart. Ischemia leads to increased activity of caspase 3 both in LC and Hsp-27tg hearts. However, the activity of caspase 3 was significantly higher in LC ischemic heart compared with transgenic ischemic heart (223% increase and 153% increase, respectively), suggesting that Hsp-27 may protect from ischemia-induced apoptosis by decreasing the activation of caspase 3.

To understand whether Hsp-27 acts through the intrinsic pathway or extrinsic pathway, the activity of caspase 9 was studied. The activity of caspase 9 was comparable in nonischemic, normoxic hearts of LC and Hsp-27tg (Fig. 4d). Ischemia induced approximately 184% increase in caspase 9 activity in LC group. No such increase of caspase 9 activity was seen in the Hsp-27tg group after ischemia (Fig. 4d). Hence, in this model, ischemia leads to the activation of the intrinsic pathway through activation of caspase 9.

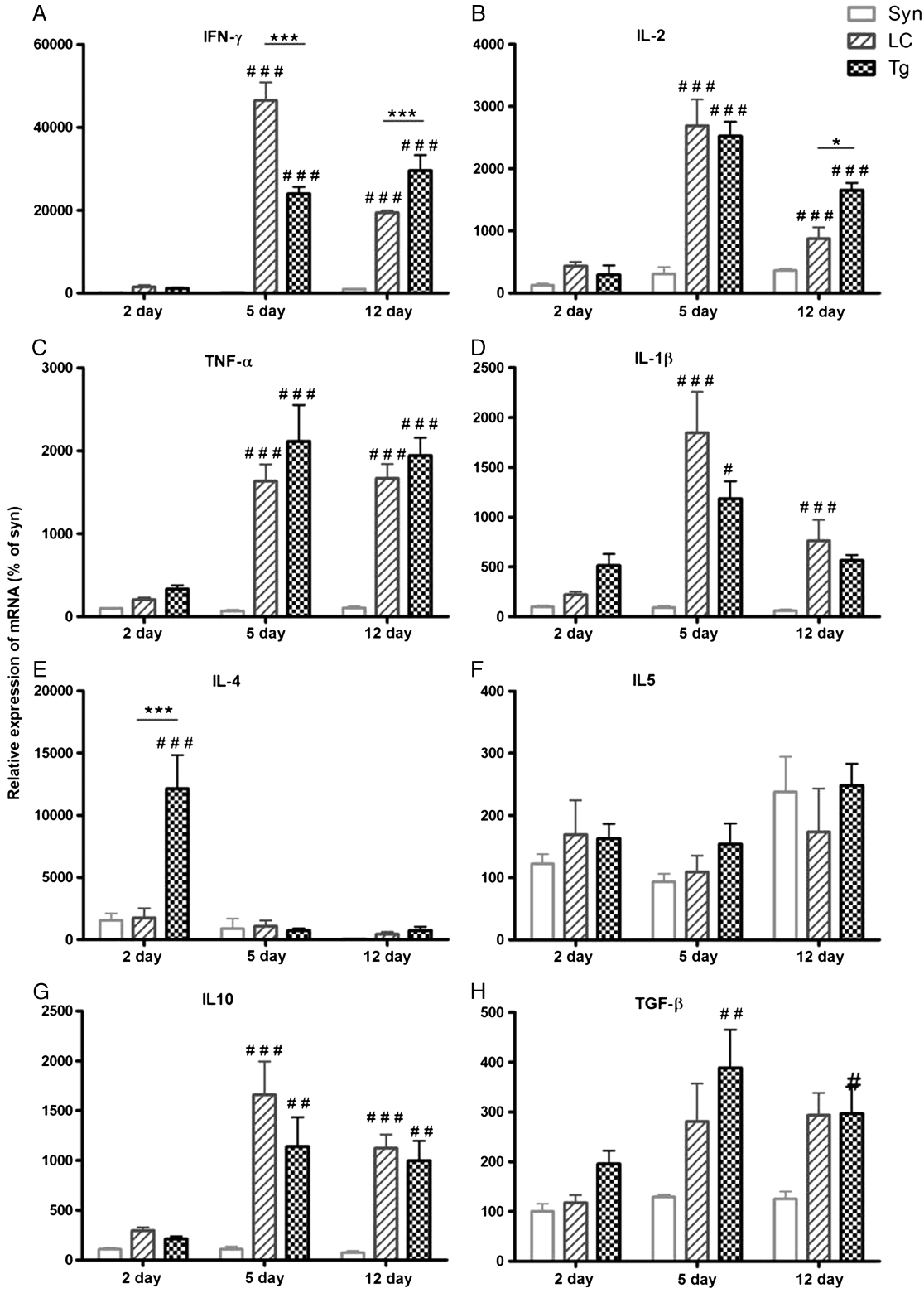

FIGURE 3. Effect of Hsp-27 on cytokines. Recipients of transgenic and LC allografts were killed at days 2, 5, and 12 after transplantation and donor hearts assessed for presence of IFN-γ (A), IL-2 (B), TNF-α (C), IL-1β (D), IL-4 (E), IL-5 (F), IL-10 (g) and TGF-β (H). The y-axis shows relative expression of mRNA as a percentage of that present in syngrafts. It can be seen that transgenic allografts express less IFN-γ at day 5 than LC allografts and more IL-4 at day 2 (*or #$P \leq 0.05$; ** or ##$P < 0.01$ and *** or ###$P < 0.001$, *transgenic vs. LC, #syngeneic vs. LC or syngeneic versus transgenic, n=3–6). IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; mRNA, messenger RNA; LC, littermate control; IFN, interferon; Hsp, heat shock protein.

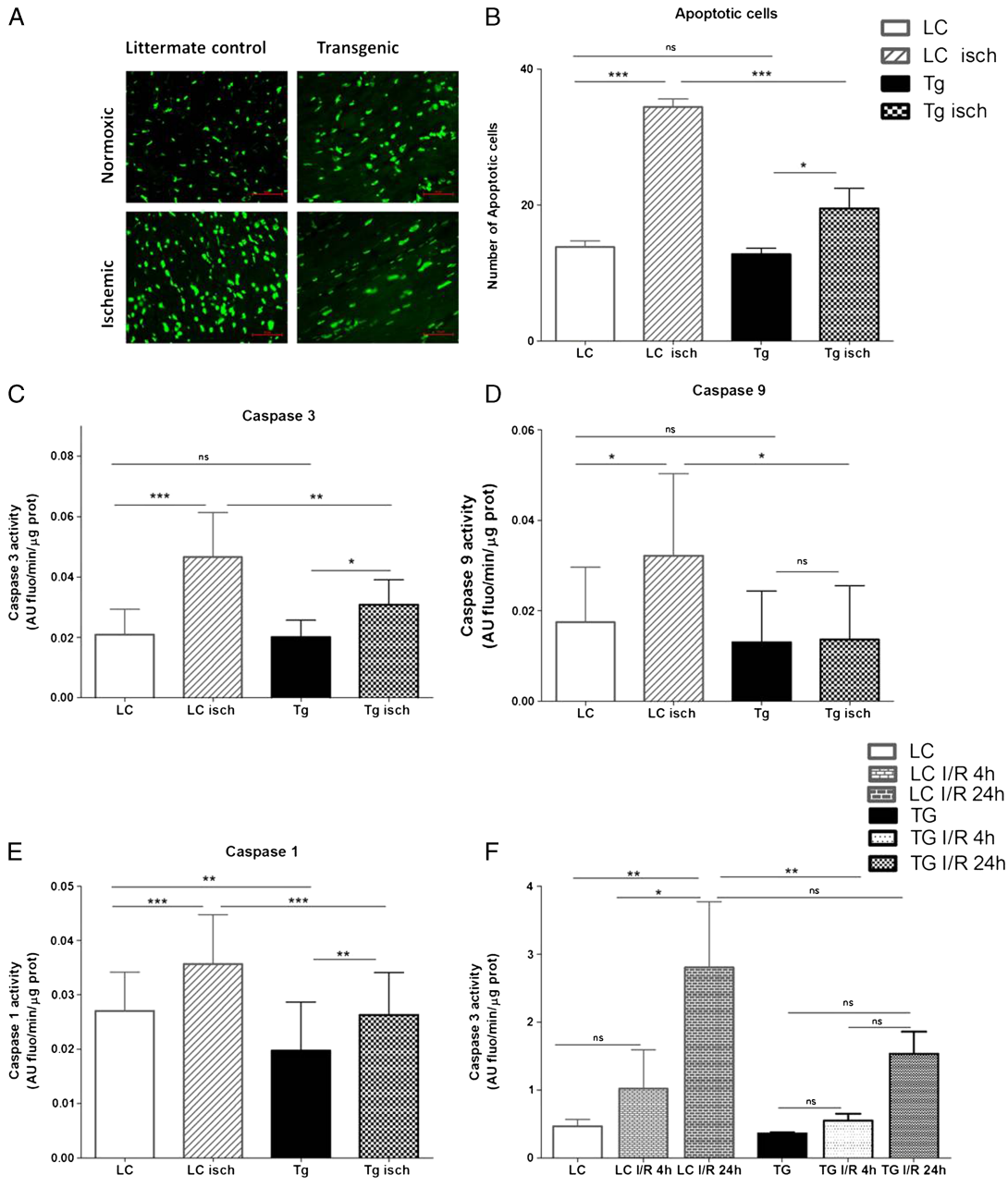

FIGURE 4. Comparison of apoptotic cells and caspase activity induced by ischemia-reperfusion injury in Hsp-27tg and LC hearts. Hearts from Hsp-27tg (n=3–6) and LC (n=3–6) mice were subjected to 10 min cold ischemia and 40 min warm ischemia ex vivo (A–E) or ischemia-reperfusion injury in vivo for 4 hr or 24 hr (F). The hearts from these mice were subjected to TUNEL assay (a and b) to reveal presence of apoptotic cells or examined for presence of caspases. A, A photomicrograph of cryostat sections of hearts from Hsp-27tg and LC hearts (normoxic and ischemic) showing green fluorescent TUNEL-positive apoptotic cells. B, Quantitation of apoptotic cells (y-axis — no of apoptotic cells) in hearts from nonischemic LC, LC ischemic, nonischemic Hsp-27tg and ischemic Hsp-27tg. Data derived from mean of 50 fields/heart. Heat shock protein-27tg hearts subjected to ischemia had less apoptotic cells than LC hearts subjected to ischemia. (C–E) Activity of caspase 3 (n=6 per group), caspase 9 (n=5 per group), and caspase 1 (n=4 per group), respectively, after ischemia measured as OD460/min/μg. It can be seen that ischemia-induced expression of caspase 3 and caspase 9 is significantly reduced in Hsp-27tg compared with LC hearts (C and D). The x-axis of f (n=3) shows the groups (LC normoxic, LC after 4 or 24 hr ischemia-reperfusion, Hsp-27tg normoxic, Hsp-27tg after 4 or 24 hr ischemia-reperfusion). It can be seen that there was no significant increase in caspase 3 at 4 hr in any group, but at 24 hr, there was significantly more caspase activity in LC hearts compared with normoxic hearts. One-way ANOVA with Bonferroni correction (*P<0.05, **P<0.01, and ***P<0.001). LC, littermate control; TUNEL, terminal deoxynucleotide transferase-mediated 2'-deoxyuridine-5'-triphosphate nick-end labeling; ANOVA, analysis of variance; Hsp, heat shock protein.

Caspase1 displays both proapoptotic and proinflammatory properties (*20*). Its activation results in maturation of pro–IL-1β and pro–IL-18 into mature forms. The effect of Hsp-27 on caspase 1 activation was investigated (Fig. 4e). There was no significant difference in the increase in caspase 1 activity between LC (132%) and Hsp-27tg (133%) ischemic hearts. However, in normoxic conditions, the activity of caspase 1 was significantly lower by 27% in Hsp-27tg compared with LC hearts. This result indicates that Hsp-27 regulates the expression of caspase 1 and not its activity and by doing so exhibits both antiapoptotic and anti-inflammatory effects.

Ischemia-Reperfusion Injury

Hearts from Hsp-27tg and LCs were transplanted into the abdomen of syngeneic B10.A. The animals were killed at 4 hr or 24 hr after transplantation, and their hearts were examined for activity of caspase 3 (Fig. 4f). There was a small but insignificant increase in caspase 3 activity at 4 hr in control mice, reaching significantly higher levels at 24 hr ($P<0.001$). In contrast, there was no significant increase in caspase 3 activity in hearts from Hsp-27tg mice after 4 or 24 hr of ischemic reperfusion. These results confirm the ex vivo results above that Hsp-27 may protect from ischemia-reperfusion injury by an antiapoptotic effect through the suppression of caspase 3 activity.

Effect of Hsp-27 on T-Cell Activation

The possibility that antigen-presenting cell (APC) from Hsp-27tg hearts are deficient in their ability to induce T-cell activation was investigated. Splenocytes were removed from C57BL/6 mice 5 days after they had received a syngeneic heart transplant or hearts from B10A mice (Hsp-27tg or LC mice). Splenocytes were incubated with APC from spleens of Hsp-27tg, LC, or third party controls (FVB mice) for 48 hr and numbers of T cells producing IFN-γ estimated by ELISA spot (Fig. 5a). Production of IFN-γ represents activation of memory T cells (*21*). It can be seen that splenocytes from mice transplanted with syngeneic hearts did not show IFN-γ responses to B10.A hearts from Hsp-27tg or LC mice. In contrast, splenocytes from mice that received an allogeneic LC B10.A heart showed memory IFN-γ responses to APC from both LC and Hsp-27tg mice, there being no difference between these responses. These mice had not been primed to third party FVB cells. Similarly, mice that had been exposed to allogeneic Hsp-27tg hearts became primed to B10.A antigens, it can be seen that their splenocytes produced IFN-γ to APC from both Hsp-27tg and LC mice, but not to third party controls. There was no significant difference in the magnitude of the response to LC APC from the mice that had been exposed to LC allogeneic hearts or Hsp-27tg allogenic hearts (Fig. 5a). Spleens were removed after 12 days, and the results were the same (Fig. 5b), although in all cases, the IFN-γ response was diminished compared with that at 5 days. These results demonstrate that exposure of splenic T cells to Hsp-27tg hearts for 5 or 12 days does not inhibit T-cell priming.

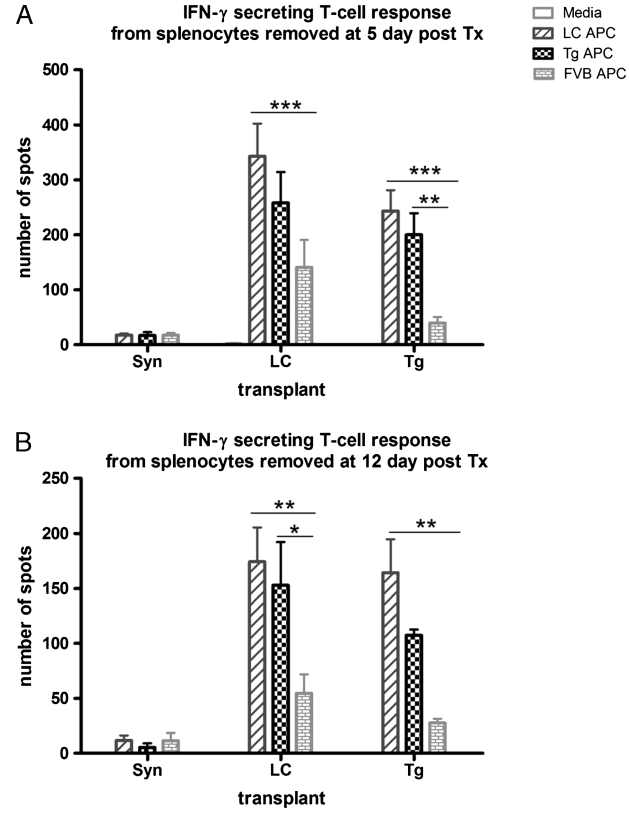

FIGURE 5. Frequency of alloreactive T cells from mice receiving Hsp-27tg and LC hearts grafts. Splenic T cells obtained from C57BL/6 recipients of syngeneic, LC, or Hsp-27tg allografts were removed at 5 (A) or 12 days (B) after transplantation and mixed with antigen-presenting cell (APC) isolated from LC, Hsp-27tg, third party (FVB) controls or medium alone. The cultures were tested for production of IFN-γ by ELISPOT. The frequency of IFN-γ–producing cells is shown on the y-axis as number of spots per million splenocytes. Splenocytes from recipients of LC or Hsp-27tg allografts demonstrated a vigorous memory response when cultured in the presence of APC from LC or Hsp-27tg mice, but exhibited a poor response to APC from third party controls. There is no evidence that the presence of the Hsp-27tg allograft for 5 or 12 days diminishes the ability of C57BL/6 splenocytes to become primed to B10.A antigens, *$P<0.05$, **$P<0.01$, ***$P<0.001$, n=3–8 per group. LC, littermate control; APC, antigen-presenting cell; IFN, interferon; ELISPOT, enzyme-linked immunosorbent spot; Hsp, heat shock protein.

DISCUSSION

These results demonstrate for the first time that overexpression of Hsp-27 in donor hearts significantly prolongs allograft survival. In this article, we have investigated the possible mechanisms. The properties of Hsp-27 that contribute to cytoprotection, such as antiapoptotic and antioxidative properties, have been discovered almost entirely using cell culture assays; in vivo effects are likely to be more complex because they involve interaction between different organ systems. Western blotting demonstrated the presence of HA-tagged Hsp-27 in the heart of Hsp-27tg mice, and immunocytochemistry strongly suggested it to be confined to cardiac myocytes and not present in endothelial or interstitial cells. The absence in interstitial cells is important because other studies have shown that macrophages contain and secrete Hsp-27 (*13, 22*). Overexpression of Hsp-27 in cells of monocytic or dendritic origin would suggest modulation

of antigen presentation as a possible mechanism of allograft prolongation by Hsp-27. However, although the control and Hsp-27tg mice demonstrated the presence of endogenous Hsp-25 in their spleens (Fig. 1a), the HA tag was not detectable, suggesting that in our mice, the transgene was not incorporated into the cells of monocytic or dendritic lineage. Immunocytochemistry of cytospins of bone marrow and spleen confirmed the absence of HA-tag in hematological precursor cells of Hsp-27tg mice (data not shown).

Analysis of donor hearts by qPCR demonstrated significantly reduced expression of CD3 and CD8 transcripts in Hsp-27tg hearts compared with hearts from LCs at day 5; by day 12, there was no difference. This suggests that Hsp-27 reduced the magnitude of the proinflammatory T_H1 response at early times after transplantation. Expression of CD4 was low compared with CD3 and CD8 (Fig. 2), suggesting acute rejection is mediated mainly by CD8+ T cells in this strain combination. However, qPCR data do not provide information about numbers of cells, and although visual analysis of sections by immunocytochemistry broadly confirmed qPCR results, the absence of quantitative data of numbers of cells or heart section is a weakness of this study. The possibility that the innate immune system was modulated by Hsp-27 over-expression in the myocardium was suggested by presence of more CD14 transcripts in the hearts of Hsp-27tg donors at 2 days (Fig. 2) and significantly more expression of the anti-inflammatory cytokine IL-4, which is produced by monocytes (Fig. 3). There are at least two subpopulations of monocytes which are distinguished by the expression of chemokine receptors and specific surface markers. The $Ly6C^{hi}$ (also known as Gr1+) monocytes express Ly6C and CD11b+ together with high levels of chemokine receptor CCR2 but low level of CX_3CR1. $Ly6C^{lo}$ (Gr1−) express low level of Ly6C and CCR2 but high level of CX_3CR1 (*23, 24*). These subsets of monocytes also differ functionally, $Ly6C^{hi}$ are proinflammatory, they are preferentially recruited to the site of inflammation, whereas $Ly6C^{lo}$ has been shown to be involved in inducing tolerance and wound healing but are weak phagocytes (*23, 25, 26*). In 2007, Nahrendorf et al. demonstrated that in a murine model of myocardial infarction (induced by coronary ligation), there is a biphasic recruitment of monocytes to the damaged heart; the first phase consists of $Ly6C^{hi}$ monocytes, and the second phase are $Ly6C^{lo}$ cells (*24*). Depletion of the second phase of $Ly6C^{lo}$ cells resulted in decreased remodeling and less granulation tissue in the heart, suggesting these cells are involved in the healing response. As far as we know, a biphasic monocyte response has not been sought in models of myocardial ischemic damage induced by organ transplantation. Further studies are required to explore whether the overexpression of Hsp-27 in cardiac myocytes alters the balance of proinflammatory versus anti-inflammatory or healing monocytes in the damaged myocardium.

In view of the predominance of CD14 in Hsp-27tg hearts early after transplantation, the possibility that Hsp-27tg modulates early tissue damage caused by ischemia was investigated. Myocardial ischemia-reperfusion injury induces cellular damage including membrane peroxidation, increase in reactive oxygen species, disruption of the cytoskeleton, and deterioration of mitochondrial function (*27–29*). Nontransplanted hearts were subjected to ischemia and investigated for presence of apoptotic cells and activation of caspases 3, 9, and 1 (Fig. 4a–e). Heat shock protein-27tg hearts demonstrated significantly less apoptotic cells than those in LC hearts, and there was significantly less activation of caspase 3 and caspase 9 in Hsp-27tg hearts compared with LCs. Similarly, caspase 3 activity increased significantly in littermate control hearts, but not Tg hearts, after transplantation in syngeneic recipients for a period of 24 hr (Fig. 4f). Hollander et al. demonstrated lower levels of the oxidative product malodialdehyde in Hsp-27tg hearts subjected to ischemia on Langendorff preparations compared with control hearts—and suggested less oxidative stress was associated with smaller infarct size (*9*). These results suggest that Hsp-27 overexpression inhibits activation of the intrinsic pathway which leads to apoptosis and cell death. In addition, the lower activity of caspase 1 in Hsp-27tg heart versus control (Fig. 4e) indicates that Hsp-27 regulates the antiapoptotic and anti-inflammatory effects mediated by caspase 1.

The results suggest therefore that early damage to cardiac myocytes is inhibited in Hsp-27tg hearts through an antiapoptotic pathway and probably an anti-inflammatory effect. Although there was no evidence that Hsp-27 was overexpressed in APCs in our mice (as said above), it is possible that Hsp-27 is released from overexpressing hearts and may inhibit T-cell priming in the spleen and lymphoid organs. Heat shock protein-27 is secreted from macrophages and can be found in the blood stream (*13*). Enzyme-linked immunosorbent assay detected the presence of Hsp-27 in the blood of our transgenic animals (Fig. 1c). Hence, experiments were performed to assess T-cell priming in recipients of allogeneic Hsp-27tg hearts (Fig. 5). Splenic T cells removed from allogeneic recipients of LC hearts demonstrated a primed IFN-γ response when challenged in vitro with splenic APC from Hsp-27tg mice or their LCs, demonstrating no deficiency in the ability of splenic APC to activate memory cells (Fig. 5, column 2) in this particular system. Similarly, spleens from mice which had received Hsp-27tg hearts 5 or 12 days previously also showed a memory response to APC from Hsp-27tg and control mice. (Fig. 5, 3rd column). The fact that the alloantibody response was not diminished in mice bearing Hsp-27tg allografts also suggests that T-cell activation has not been inhibited. These results strongly suggest that protection from early myocardial injury is the major mechanism whereby Hsp-27 enhances survival of Hsp-27tg hearts. Whether this is achieved by direct recruitment of anti-inflammatory CD14+ monocytes or simply by reducing numbers of apoptotic cells has yet to be determined. The results add support to current efforts to limit tissue damage during procurement and reperfusion (*30, 31*) and suggest that the use of natural inducers of Hsp-27 may protect hearts from ischemia-reperfusion injury and be of clinical relevance in heart transplantation.

MATERIALS AND METHODS

There follows a brief description of all *Materials and Methods*. More details can be found in the **SDC** (http://links.lww.com/TP/A978).

Mice

B10.A mice overexpressing the human transgene Hsp-27 (Hsp-27tg) were provided by Dominic Wells (Imperial College London). The generation and characterization of these mice has been described previously (*17*).

Heart Transplantation

B10.A (H-2^a) hearts from Hsp-27tg or littermate controls were heterotopically transplanted into C57BL/6 (H-2^b) recipients by the technique as described by Corry et al. in 1973 (*32*). Syngeneic controls consisted of transplanting C57BL/6 into C57BL/6.

Western Blots

Tissues were snap-frozen in liquid nitrogen and proteins extracted by homogenization in radioimmunoprecipitation assay buffer. Proteins were separated by gel electrophoresis and transferred onto polyvinylidene fluroride membranes (GE Healthcare, Amersham, UK). The house keeping gene, GAPDH was used as an internal control. Proteins were identified using an antibody against the HA tag (rabbit anti-HA from Santa Cruz [Wembly Middlesex, UK], used at 1:200), rabbit anti–Hsp-25 (1:250, from Calbiochem, Nottingham, UK) or rabbit anti-GAPDH (1:1000, from Cell Signalling, Hitchin, UK) followed by peroxidase conjugated anti-rabbit secondary antibody at 1:1000 (from Dako, Ely, UK). After visualization using enhanced chemiluminescence (Amersham hyperfilm *ECL*; GE Healthcare), band intensities were quantified by densitometry. The intensities obtained for HA and Hsp-25 were normalized relative to those of loading control GAPDH.

Immunohistochemistry

Cryostat sections were stained with rabbit anti-HA primary antibody (at 1:50, from Roche, Burgess Hill, UK) for 1 hr 30 min at room temperature. After washing in phosphate-buffered saline, biotinylated rat anti–rabbit antibody (at 1:200, from Dako) in 2% mouse serum was added for 45 min followed by avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories, Peterborough, UK). The sites of peroxidase binding were developed in phosphate-buffered saline containing diaminobenzidine tetrachloride (0.3 mg/mL) and hydrogen peroxide (0.01%) (Sigma fast; Sigma Aldrich, Poole, UK). Slides were counterstained with Mayer's hematoxylin (Sigma Aldrich).

Detection of Alloantibody

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 min at 4°C. Cells were washed and incubated with secondary goat anti-mouse Ig-fluorescein isothiocyanate (AbD Serotec, Kidlington, UK) at 1:30 dilution for 30 min 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

Plasma levels of Hsp-27 were assessed using an ELISA kit for Hsp-27 (Q1A 119 from Calbiochem). The assay has been found to have no cross-reactivity with Hsp-25 (*22*).

Induction of Ischemia

To mimic the effect of ischemia encountered during heterotopic heart transplantation, hearts were harvested from Hsp-27tg and LCs and subjected to 10 min cold ischemia (in saline at 4°C) followed by 40 min of warm ischemia (in saline at 25°C). Hearts where no ischemia was induced were used as controls.

TUNEL Assay

Apoptotic cells from the hearts of Hsp-27tg mice and their LCs were detected by TUNEL labeling detection of free 3′-OH groups in fragmented DNA in situ (In Situ Cell Death Detection Kit, POD; Roche). The assay was performed as described by Sgnoc et al. (*33*).

Caspase Assay

Thirty microliters of protein extracted from mouse hearts was incubated at 37°C with 50 μL of caspase assay buffer (200 mM HEPES, 50 mM dithiothreitol, 1% 3-[(3-cholamidopropyl) dimethyl-ammonio]-α-propanesulphonate, 20 mM ethylenediaminetetraacetic acid) and the appropriate caspase substrate. The activity of caspase3, caspase 1, and caspase 9 was measured using specific substrates labeled with N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Sigma), N-acetyl-Tyr-Val-Ala-Asp-(7-amino-4-triflouromethylcoumarin), and N-acetyl-Leu-Glu-His-Asp-(7-amino-4-triflouromethylcoumarin) (Tocris), respectively, and quantified by spectrofluorimetry

qPCR Analysis

For qPCR analysis, myocardium samples were snap-frozen in liquid nitrogen immediately after collection and stored at −80°C. Total RNA was extracted from frozen tissue using the RNeasy fibrotic tissue mini kit with DNAase treatment (catalogue number 74704; Qiagen) and quantified by ultraviolet (UV) spectrophotometry. complementary DNA was prepared using Life Technologies' Taqman reverse transcription reagents (catalogue number N8080234).

Enzyme-Linked Immunosorbent Spot Assays

T cells and APC were separated from mouse spleens using antibody coated magnetic beads. Ninety-six well PVDF-bottomed plates (Millipore, Watford, UK) were coated with mouse anti-rat IFN-γ capture antibodies (BD Biosciences, Oxford, UK). Responder and stimulator cells (pretreated with Mitomycin C) were resuspended in RPMI-1640 +10% fetal calf serum. A total of 2×10^5 responder T cells and 2×10^5 APCs were added to each well, and plates were incubated at 37°C, 5% CO_2 for 48 hr. After washing, they were exposed to biotinylated anti-mouse IFN-γ antibody (from BD Biosciences) for 2 hr at room temperature. Streptavidin HRP (Vector Laboratories Ltd., UK) was added for 1.5 hr before the addition of the substrate AEC (3-amino9-ethyl-carbazole). Spots were allowed to develop before the plates were washed with H_2O and left to dry overnight.

Spots were enumerated using an AID ELISPOT reader and software (Autoimmun Diagnostika GmbH, Straβberg, Germany).

Statistics

Statistical calculations were performed using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA). Comparison of two groups was performed with unpaired *t*-test. To compare three or more groups analysis of variance (one-way analysis of variance) and Bonferroni posttesting (compare all pairs of columns) was performed, whereas two-way analysis of variance Bonferroni posttesting was used to compare two factors. *P* less than 0.05 was considered statistically significant with **P* less than 0.05, ***P* less than 0.01, ****P* less than 0.001.

ACKNOWLEDGMENT

The authors thank Dr. Padmini Sarathchandra for the technical assistance.

REFERENCES

1. Sulemanjee NZ, Merla R, Lick SD, et al. The first year post-heart transplantation: use of immunosuppressive drugs and early complications. *J Cardiovasc Pharmacol Ther* 2008; 13: 13.
2. Fink AL. Chaperone-mediated protein folding. *Physiol Rev* 1999; 79: 425.
3. Arrigo AP, Welch WJ. Characterization and purification of the small 28,000-dalton mammalian heat shock protein. *J Biol Chem* 1987; 262: 15359.
4. Landry J, Lambert H, Zhou M, et al. Human HSP27 is phosphorylated at serines 78 and 82 by heat shock and mitogen-activated kinases that recognize the same amino acid motif as S6 kinase II. *J Biol Chem* 1992; 267: 794.
5. Arrigo AP. The cellular "networking" of mammalian Hsp27 and its functions in the control of protein folding, redox state and apoptosis. *Adv Exp Med Biol* 2007; 594: 14.
6. Concannon CG, Gorman AM, Samali A. On the role of Hsp27 in regulating apoptosis. *Apoptosis* 2003; 8: 61.
7. Schimke I, Lutsch G, Schernes U, et al. Increased level of HSP27 but not of HSP72 in human heart allografts in relation to acute rejection. *Transplantation* 2000; 70: 1694.
8. Efthymiou CA, Mocanu MM, de Belleroche J, et al. Heat shock protein 27 protects the heart against myocardial infarction. *Basic Res Cardiol* 2004; 99: 392.
9. Hollander JM, Martin JL, Belke DD, et al. Overexpression of wild-type heat shock protein 27 and a nonphosphorylatable heat shock protein 27 mutant protects against ischemia/reperfusion injury in a transgenic mouse model. *Circulation* 2004; 110: 3544.

10. Robinson AA, Dunn MJ, McCormack A, et al. Protective effect of phosphorylated Hsp27 in coronary arteries through actin stabilization. *J Mol Cell Cardiol* 2010; 49: 370.
11. Martin-Ventura JL, Duran MC, Blanco-Colio LM, et al. Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. *Circulation* 2004; 110: 2216.
12. Park HK, Park EC, Bae SW, et al. Expression of heat shock protein 27 in human atherosclerotic plaques and increased plasma level of heat shock protein 27 in patients with acute coronary syndrome. *Circulation* 2006; 114: 886.
13. Rayner K, Chen YX, McNulty M, et al. Extracellular release of the atheroprotective heat shock protein 27 is mediated by estrogen and competitively inhibits acLDL binding to scavenger receptor-A. *Circ Res* 2008; 103: 133.
14. Taylor DO, Stehlik J, Edwards LB, et al. Registry of the International Society for Heart and Lung Transplantation: Twenty-sixth Official Adult Heart Transplant Report—2009. *J Heart Lung Transplant* 2009; 28: 1007.
15. Rahmani M, Cruz RP, Granville DJ, et al. Allograft vasculopathy versus atherosclerosis. *Circ Res* 2006; 99: 801.
16. De Souza AI, Wait R, Mitchell AG, et al. Heat shock protein 27 is associated with freedom from graft vasculopathy after human cardiac transplantation. *Circ Res* 2005; 97: 192.
17. Akbar MT, Lundberg AM, Liu K, et al. The neuroprotective effects of heat shock protein 27 overexpression in transgenic animals against kainate-induced seizures and hippocampal cell death. *J Biol Chem* 2003; 278: 19956.
18. Crow MT, Mani K, Nam YJ, et al. The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ Res* 2004; 95: 957.
19. Czerski L, Nunez G. Apoptosome formation and caspase activation: is it different in the heart? *J Mol Cell Cardiol* 2004; 37: 643.
20. Martinon F, Tschopp J. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 2004; 117: 561.
21. Fitzpatrick DR, Kelso A. Nature versus nurture in T cell cytokine production. *J Leukoc Biol* 1999; 66: 869.
22. Rayner K, Sun J, Chen YX, et al. Heat shock protein 27 protects against atherogenesis via an estrogen-dependent mechanism: role of selective estrogen receptor beta modulation. *Arterioscler Thromb Vasc Biol* 2009; 29: 1751.
23. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* 2011; 11: 762.
24. Nahrendorf M, Swirski FK, Aikawa E, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. *J Exp Med* 2007; 204: 3037.
25. Swirski FK, Weissleder R, Pittet MJ. Heterogeneous in vivo behavior of monocyte subsets in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2009; 29: 1424.
26. Libby P, Nahrendorf M, Swirski FK. Monocyte heterogeneity in cardiovascular disease. *Semin Immunopathol* 2013; 35: 553.
27. Hoffman JW Jr, Gilbert TB, Poston RS, et al. Myocardial reperfusion injury: etiology, mechanisms, and therapies. *J Extra Corpor Technol* 2004; 36: 391.
28. Park Y, Kanekal S, Kehrer JP. Oxidative changes in hypoxic rat heart tissue. *Am J Physiol* 1991; 260: H1395.
29. Simpson PJ, Lucchesi BR. Free radicals and myocardial ischemia and reperfusion injury. *J Lab Clin Med* 1987; 110: 13.
30. Anaya-Prado R, Delgado-Vazquez JA. Scientific basis of organ preservation. *Curr Opin Organ Transplant* 2008; 13: 129.
31. Zaouali MA, Ben Abdennebi H, Padrissa-Altes S, et al. Pharmacological strategies against cold ischemia reperfusion injury. *Expert Opin Pharmacother* 2010; 11: 537.
32. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation* 1973; 16: 343.
33. Sgonc R, Wick G. Methods for the detection of apoptosis. *Int Arch Allergy Immunol* 1994; 105: 327.