Administration of the stress protein gp96 prolongs rat cardiac allograft survival, modifies rejection-associated inflammatory events, and induces a state of peripheral T-cell hyporesponsiveness

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Abstract High-dose gp96 has been shown to inhibit experimental autoimmune disease by a mechanism that appears to involve immunoregulatory CD4⁺ T cells. This study tested the hypothesis that high-dose gp96 administration modifies allograft rejection and associated inflammatory events. Wistar cardiac allografts were transplanted into Lewis recipient rats and graft function was monitored daily by palpation. Intradermal administration of gp96 purified from Wistar rat livers (100 µg) at the time of transplantation and 3 days later significantly prolonged allograft survival (14 vs 8 days in phosphate-buffered saline [PBS]-treated recipients; $P = 0.009$). Rejected allografts from gp96-treated animals were significantly less enlarged than allografts from their PBS-treated counterparts (2.8 vs 4.3 g; $P < 0.004$). Gp96 was also effective when administered on days 1 and 8 (13 vs 7 days), but not if it was derived from recipient (Lewis) liver tissue or administered on days 0, 3, and 6. In parallel studies, CD3⁺ T cells from gp96-treated untransplanted animals secreted less interleukin (IL)-4, IL-10, and interferon (IFN)- γ after in vitro polyclonal stimulation than CD3⁺ T cells from PBS-treated animals. Gp96 administration might therefore influence the induction of immunity to coencountered antigenic challenges and inflammatory events by inducing what appears to be a state of peripheral T-cell hyporesponsiveness.

INTRODUCTION

Gp96 is a 94–96-kDa member of the Hsp90 family of molecular chaperones/stress proteins that resides within the lumen of the endoplasmic reticulum. In addition to its role as an intracellular chaperone (Gething and Sambrook 1992; Young et al 1993), the administration of tumor-derived gp96 induces tumor-specific cytolytic T cells and a

protective tumor-specific immunity that is defined by tumor-derived peptides with which the administered gp96 is associated (Udono and Srivastava 1994; Chandawarkar et al 1999; Binder and Srivastava 2005).

In contrast to its capacity to induce tumor-specific immunity, protective anti-tumor immunity is not apparent when high doses (2×10^6 µg intradermally) of tumor-derived gp96 are administered to mice (Chandawarkar et al 1999). Furthermore, work has shown that high-dose gp96 purified from normal liver (2×10^6 µg subcutaneously) can suppress the onset of diabetes in nonobese diabetic mice and myelin basic protein- or proteolipid protein-induced autoimmune encephalomyelitis (EAE) in

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SJL mice (Chandawarkar et al 2004), as well as prolong the survival of murine skin allografts exhibiting minor or major histocompatibility differences (Kovalchin et al 2006a).

Currently, the mechanism by which high-dose gp96 can influence inflammatory events is uncertain. Published data indicate that the anti-inflammatory properties of high-dose gp96 are not dependent on the tissue source of the administered gp96 (Chandawarkar et al 2004; Kovalchin et al 2006a) but do appear to involve the induction, activation, and/or recruitment of as yet unidentified immunoregulatory T-cell populations. Evidence for this comes from studies that have demonstrated that the adoptive transfer of CD4⁺ T cells from animals that have been treated with high-dose gp96 inhibits the induction of tumor immunity by low-dose gp96 and protects against diabetes and EAE (Chandawarkar et al 1999, 2004).

The immunoregulatory properties of high-dose gp96 appears to be dependent on the timing of its administration, in that, for this to be observed, gp96 must be administered at the time of, or shortly after, the antigenic stimulus has been encountered (Chandawarkar et al 2004). It might therefore be that the immunoregulatory effects of high-dose gp96 administration is targeted toward recently activated rather than memory T cells (Chandawarkar et al 2004). Targeting immunoregulatory effects toward recently induced immune responses to transplants might allow established immunity and immune potential to be retained and thereby limit the long-term morbidity and mortality problems that are inevitably associated with chronic, nonspecific immunosuppressive protocols in recipients. High-dose gp96-induced immunoregulatory activity might therefore be an effective and applicable approach by which to control allospecific immunity and transplant rejection responses.

This study used a rat cardiac transplantation model to determine whether gp96 can modify immunological events that are involved in the development and progression of transplant rejection.

MATERIALS AND METHODS

Animals

Adult Lewis rats (~300 g), Wistar rats (~300 g), and C57BL/6 mice (20–25 g) were obtained from Harlan UK Limited (Bicester, UK). All procedures were undertaken with UK Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986 and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Animals were killed by anesthetic overdose in a manner which is consistent with UK legislation and the

recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Purification of gp96

Gp96 was purified from Wistar rat livers essentially as described previously by others (Srivastava 1997) and ourselves (Fairburn et al 2006). Briefly, livers were homogenized in phosphate-buffered saline (PBS) containing the protease inhibitor phenylmethylsulphonylfluoride (0.2 mM; 60 mL buffer/30 g liver tissue) and unbroken cells, nuclei, and other debris from the homogenate by centrifugation for 10 minutes ($2,000 \times g$, 4°C). The supernatant was then subjected to ultracentrifugation at $100,000 \times g$ for 90 minutes. The supernatant was brought to 50% (w/v) saturation with ammonium sulfate, and the resultant precipitate was removed by centrifugation at $5,000 \times g$ for 20 minutes. The supernatant was diluted 1:1 with PBS containing 2 mM Ca²⁺ and 2 mM Mg²⁺ and applied to a concanavalin A (Con A)–Sephacrose column. The bound fraction was eluted with 10% (w/v) α -methylmannopyranoside. The eluted fraction was then passed through a PD10 size exclusion column, and the protein-containing eluate was applied to a diethylaminoethyl-Sephacrose column equilibrated with 5 mM sodium phosphate buffer (pH 7.0).

Seven 2-mL fractions were collected, and only those eluting between 450 and 500 mM NaCl (fractions 3 and 4) contained detectable levels of protein when measured by the Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and gp96 when assessed by ECL Western blot analysis (Fairburn et al 2006). The endotoxin content of the purified gp96 preparations was independently measured by the Cambrex Bioproducts LAL Testing Service (Vervier, Belgium), and this was less than 0.001 endotoxin units (0.1 pg) per microgram of gp96 for all preparations used in this study (Fairburn et al 2006). No contamination of the preparations with Con A was observed by two-dimensional gel electrophoresis and tandem mass spectrometry (Fairburn et al 2006).

Rat heterotopic cardiac transplantation and intradermal administration of gp96

Hearts were harvested from heparinized donor animals and stored in cold saline before transplantation. Cardiac grafts were anastomosed to the carotid artery and jugular vein in the neck of recipients with a combined cuff and suture technique, essentially as described by others (Xiu et al 2001). This model minimizes the surgical trauma to the recipient animal and markedly reduces the cold ischemic time (<60 minutes; Xiu et al 2001). Isografted animals (LEW → LEW) acted as surgical controls. The ex-

perimental groups used in the study are detailed in Table 1.

Purified gp96 in PBS or PBS alone was administered to Lewis recipient rats by intradermal injection at the doses and times indicated in the *Results* section. Intradermal administration was confirmed by the presence of a raised bleb. The concentrations of gp96 (w/w basis and taking into account that the intradermal route is 10-fold more efficient at inducing the biological effects of gp96 than the subcutaneous route [Chandawarkar et al 1999]) are similar to those that have previously been used to induce CD4⁺ T-cell-mediated immunoregulatory activity (Chandawarkar et al 1999, 2004) and to prolong skin allograft survival (Kovalchin et al 2006a) in mice. Cardiac graft function was monitored on a daily basis by palpating the neck of the recipient (Koizumi et al 1996; van Denderen et al 2001; Pêche et al 2003). Graft rejection was defined as the total cessation of cardiac muscle contraction, at which time, animals were killed and cardiac tissue was collected. Heart rates were also measured on a daily basis, the first measurement being taken approximately 2 hours after surgery to allow the recipient time to recover and the graft to stabilize. Palpation is a more appropriate approach for evaluating antirejection strategies than electrocardiography because it appears to provide a more reliable evaluation of the time of cardiac arrest (Koizumi et al 1996).

Histology and immunohistochemical analysis of graft cardiac tissue

Graft injury and cellular infiltration were assessed by histology and immunohistochemistry. Harvested tissue was fixed for 36 hours in formal saline, after which it was transferred into PBS and embedded in paraffin. The grading system of the International Society for Heart and Lung Transplantation was used to evaluate cardiac rejection, in which Grades 1 R, 2 R, and 3 R signify mild, moderate, and severe rejection, respectively (Stewart et al 2005). The histological evaluation was undertaken blindly by an experienced cardiac pathologist (S.K.S.). Immunohistochemistry was performed with monoclonal antibodies (mAbs) reactive with CD3, CD4, CD8, CD25, CD45, CD68 (ED1, macrophages), OX62 (dendritic cells, DCs), and major histocompatibility complex (MHC) class II antigens, all of which were obtained from Serotec Ltd (Kidlington, UK). Primary mAb binding was detected with a biotinylated anti-murine immunoglobulin secondary antibody, followed by a streptavidin-horseradish peroxidase (HRP) conjugate and diaminobenzidine substrate (BD Pharmingen anti-mouse Ig HRP detection kit, BD Biosciences, Oxford, UK). Sections were counterstained with hematoxylin, and the extent of myocardial damage, mononuclear and granulocyte cell infiltration, and vas-

culitis was evaluated blindly. It was not possible to evaluate CD4 and CD25 expression because these mAbs are not reactive with the antigens in fixed tissue.

Individual cell counts were not recorded; rather, an overall intensity of expression of the given antigen was evaluated. The range of staining intensities that were generated for a given antigen was blindly assessed, and this was then converted into a graded scale, essentially as described previously (Reid et al 1995; Uff et al 1995; Ogita et al 2000). CD45 and MHC class II expression was graded on a scale of 1 (no expression) to 4 (highest level of expression observed). The range of staining intensities that were observed dictated that CD3, CD8, and CD68 expression was graded on a scale of 1 to 3, and OX62 expression was graded on a scale of 1 to 2. All such analyses were performed blindly.

Effect of gp96 administration on the in vitro activities of splenic CD3⁺ T cells

The influence of intradermal administration of high-dose gp96 on the subsequent in vitro responsiveness of splenic CD3⁺ T cells to polyclonal activation via the T cell receptor in the form of immobilized (plate-bound) anti-CD3 mAb and soluble anti-CD28 mAb was evaluated in parallel studies. For these studies, gp96 purified from C57BL/6 mouse livers in PBS (100 µg) or PBS alone was administered to Lewis rats by intradermal injection into the abdomen on days 0 and 7. Ten days after the second injection, animals were killed and splenocytes were obtained by mechanical disruption for cell subset analysis by flow cytometry and in vitro functional studies. Splenic CD3⁺ T cells were isolated from PBS- and gp96-treated animals to a final purity of >92% (as assessed by flow cytometry; data not shown) according to the manufacturer's recommended protocol with a MagCollect Rat CD3⁺ T cell isolation kit (R&D Systems Europe Ltd, Abingdon, UK). Cells were washed twice in RPMI 1640 growth medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid), 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Invitrogen Ltd, Paisley, UK).

The secretion of IL-4, IL-10, and IFN-γ by CD3⁺ T cells from PBS-treated and gp96-treated animals (10⁶ cells/well) after 5 days incubation in 24-well tissue culture plates coated with anti-CD3 mAb (2 µg/mL; BD Biosciences) in the presence of costimulation with an anti-CD28 mAb (2 µg/mL; BD Biosciences) was evaluated. The optimal concentrations of the mAbs required to induce activation of responding CD3⁺ T cells were determined in our laboratory.

At the end of the 5-day culture period, CD3⁺ T cells were harvested gently with a pipette, and cell-free su-

Table 1 Experimental groups

Procedure	Treatment	Group size
Surgical and rejection controls		
Lewis recipients of Lewis cardiac transplants (isograft)	None	5
Lewis recipients of Wistar cardiac transplants (allograft)	None	5
Treatment on days 0 and 3		
Lewis recipients of Wistar cardiac transplants (allograft)	PBS: days 0, 3	5
	100 µg Wistar gp96: days 0, 3	5
	200 µg Wistar gp96: days 0, 3	5
Treatment on days 1 and 8		
Lewis recipients of Wistar cardiac transplants (allograft)	PBS: days 1, 8	3
	100 µg Wistar gp96: days 1, 8	3
Treatment on days 0, 3, and 6		
Lewis recipients of Wistar cardiac transplants (allograft)	100 µg Wistar gp96: days 0, 3, 6	3
Allospecificity of immunoregulatory effect?		
Lewis recipients of Wistar cardiac transplants (allograft)	100 µg Lewis gp96: days 0, 3	5

pernatants were collected for the analysis of cytokines that are characteristic of the proinflammatory Th1 (IFN- γ) and anti-inflammatory Th2 (IL-4, IL-10) phenotypes with the use of commercial enzyme immunoassays for IFN- γ and IL-4 (Diacclone Eli-pair assays via ImmunoDiagnostic Systems Ltd., Boldon, UK) and for IL-10 (BD OptEIA, BD Biosciences).

Effect of gp96 administration on splenic leukocyte populations

Leukocyte populations in the spleens of PBS- and gp96-treated animals were profiled by flow cytometry with fluorescein isothiocyanate (FITC)-conjugated mAbs reactive with CD3, CD4, CD8, and ED1 antigens and with PE-conjugated mAbs reactive with the OX62 antigen, CD45, and CD28. All mAbs were obtained from Serotec Ltd. The ED1 mAb recognizes a single-chain glycoprotein of 110 kDa that is expressed by the majority of tissue macrophages (Dijkstra et al 1985). It is the rat homolog of human CD68 that identifies cells of the monocyte/macrophage lineage. OX62 recognizes the 150-kDa integrin α_{E2} subunit, and this was originally described as being expressed on most, if not all, DCs in the rat (Brenan and Puklavec 1992). Subsequent work has shown that the majority (80%) of splenic DCs are OX62⁺, whereas only 50% of lymph node cells are OX62⁺ (Trinité et al 2000). Plasmacytoid DCs are OX62⁻ (Hubert et al 2004). Our own studies have demonstrated that rat bone marrow-derived DCs express the OX62 antigen (Muthana et al 2004).

Briefly, cells were incubated for 30 minutes at 4°C with 2 µL FITC- and PE-conjugated mAbs or appropriate isotype-matched, nonreactive negative control immunoglobulin (Serotec Ltd) in 12- × 75-mm polycarbonate tubes (Falcon, BD Biosciences). Cells were washed twice in PBS (CellWASH, BD Biosciences) containing 1% (w/v) bovine

serum albumin (Sigma-Aldrich Company Ltd, Gillingham, UK), and the cell pellet was resuspended in the residual volume. Cells were fixed in CellFIX (BD Biosciences; 100 µL/tube) and stored at 4°C until analysis no later than the following day. The proportion of cells expressing the given antigen and the intensity of antigen expression were determined with a FACSort flow cytometer and CELLQuest Pro data acquisition and analysis software (both from BD Biosciences).

Statistical analysis

All data are derived from the indicated number of animals and experiments. Differences in allograft survival times were compared by the Mann-Whitney *U*-test, as were all nonparametric data. Parametric data were compared by the Student's *t*-test. All statistical analyses were performed with SPSS v14.0 for Windows (SPSS UK Ltd, Woking, UK).

RESULTS

Gp96 administration prolongs rat cardiac allograft survival

Intradermal administration of gp96 purified from Wistar livers into Lewis rats at the time of transplantation (day 0) and again on day 3 significantly prolonged the survival of Wistar heterotopic cardiac allografts from a median of 8 days to a median of 14 days ($P = 0.009$, Mann-Whitney *U*-test; Fig 1). Long-term allograft survival was observed in one of the gp96-treated recipients.

Graft function (heart rate) was measured daily by palpation. On day 7 after transplantation, the graft heart rate in PBS-treated animals was only 29% of its immediate posttransplant level, whereas it was 92% of its immediate posttransplant level in gp96-treated animals ($P = 0.029$,

Fig 1. Gp96 administration prolongs cardiac allograft survival and preserves cardiac function. Upper panel: Effect of Wistar gp96 administration (100 μ g intradermally on days 0 and 3) on the survival of Wistar cardiac allografts transplanted into Lewis recipients. Lower panel: Cardiac graft function (heart rate) in isografted animals, untreated Lewis recipients of Wistar allografts, and Wistar gp96-treated (100 μ g, days 0 and 3) and PBS-treated (days 0 and 3) Lewis recipients of Wistar allografts in the first 8 days after transplantation. Data at later time points are not presented because of the much reduced number of animals in the untreated and PBS-treated groups as a result of rejection-mediated graft loss. Inset: Cardiac graft function (heart rate) in Wistar gp96-treated (100 μ g, days 0 and 3) Lewis recipients of Wistar allografts on days 9 to 14 after transplantation. Long-term allograft survival was observed in 1 gp96-treated recipient. The axes are the same as those indicated in the main figure. In all instances, data are means $\pm$ SEM from groups of 5 animals.

Fig 1). The administration of gp96 also appeared to markedly improve graft function in the immediate posttransplantation period (days 1–3; Fig 2). Although the mechanism underlying this effect is currently unknown, it might be that it reflects a nonspecific anti-inflammatory property of gp96. Indeed, although the administration of Lewis gp96 to Lewis recipients on days 0 and 3 had no effect on the survival of Wistar cardiac allografts (see lat-

er), it did appear to improve allograft function (as indicated by changes in heart rate) in the immediate posttransplantation period (265 beats/min on day 0, vs 324, 305, and 307 beats/min on days 1, 2, and 3, respectively). Alternatively, the effects of gp96 on immediate graft function might in some way reflect the potent wound-healing properties of this protein (Kovalchin et al 2006b).

In their comparison of methods to evaluate acute rejection of rat cardiac allografts, Koizumi et al (1996) used electrocardiography (ECG) to monitor changes in the graft heart rate as acute rejection developed. The intensity of the heart beat was evaluated by palpation and assigned a score (– to 3+). As might be expected (and as we have found), the heart rate progressively declines as rejection develops, and in the Koizumi et al (1996) study, this decline in heart rate was mirrored by a corresponding decrease in the beat intensity. Although the quantitative data are difficult to compare because of the use of different experimental models (Lewis $\times$ Brown Norway F1 donors into Lewis recipients vs Wistar donors into Lewis recipients), the pattern of heart rate changes determined by ECG in their study appears to be similar to those that have been observed in this study (297 ± 39 vs 206 ± 9 beats/min on day 0; 334 ± 24 vs 208 ± 5 beats/min on day 1; 340 ± 20 vs 190 ± 9 beats/min on day 3; 265 ± 43 vs 122 ± 46 beats/min on day 5 for ECG and palpation, respectively). To our knowledge, no other studies have reported heart rate data that have been obtained by palpation of the graft.

As would be expected, histological analysis revealed that the difference in the grading of isografts (median 0) and rejected allografts from untreated recipients (median 3 R) was of statistical significance ($P = 0.005$, Mann-Whitney U -test; data not shown). Isografts removed on day 35 displayed calcification as a sign of stress. Grafts from gp96- and PBS-treated recipients exhibited a mixture of both moderate and severe rejection, and no overall differences in the histological grading of cardiac allografts from these 2 groups of animals were observed (median grades of 3 R and 3 R, respectively; $P = 0.650$, Mann-Whitney U -test; data not shown).

CD3, CD45, and OX62 expression was increased in allografts from PBS-treated recipients, and a diffuse T-cell infiltrate was apparent (data not shown). Differences in CD3, CD8, CD45, CD68, OX62, and MHC class II expression in isografts and rejected allografts from untreated recipients were of statistical significance ($P = 0.017$, 0.015, 0.006, 0.003, 0.050, and 0.042, respectively; Mann-Whitney U -test; data not shown). Cardiac myocytes in isografts and untreated allografts unexpectedly stained positive with some of the mAbs tested.

The overall expression of CD8, CD45, CD68, OX62, and MHC class II in gp96- and PBS-treated recipients was similar and of no statistically significant difference. Al-

Fig 2. Change in cardiac graft function (heart rate) in PBS-treated (days 0 and 3) and Wistar gp96-treated (100 μ g, days 0 and 3) recipients of Wistar allografts in the first 8 days after transplantation. Data are expressed as a percentage of the heart rates that were recorded approximately 2 hours after surgery. Data at later time points are not presented because of the much reduced number of animals in the untreated and PBS-treated groups as a result of rejection-mediated graft loss. Inset: Change in cardiac graft function (heart rate) in Wistar gp96-treated (100 μ g, days 0 and 3) Lewis recipients of Wistar allografts on days 9 to 14 after transplantation. The axes are the same as those indicated in the main figure. In all instances, data are means $\pm$ SEM (as indicated) from groups of 5 animals.

though CD3⁺ T cells appeared to be more prevalent in gp96-treated recipients, the difference was not of statistical significance.

Intradermal administration of 100 μ g of Wistar gp96 on days 1 and 8 prolonged allograft survival to a similar degree to that achieved by the administration of the same dose of gp96 on days 0 and 3 (median survival 11, 13, 15 days vs 6, 7, 12 days in PBS-treated recipients; $P = 0.127$, Mann-Whitney U -test). Although the increase in allograft survival was not of statistical significance, this most likely results from the small group size (3 animals). Gp96 treatment also maintained graft function at its immediate posttransplantation level for the first 7 days after transplantation (98% vs 35% in PBS-treated recipients). Increasing the dose of gp96 to 200 μ g was no more effective at influencing allograft survival (median survival 8, 9, 11, 12, 16 vs 7, 8, 8, 9, 10 days in PBS-treated animals). Interestingly, the administration of 100 μ g of gp96 on days 0, 3, and 6 had no effect on allograft survival (median survival 8, 9, and 10 days), which suggests that gp96 administration on day 6 in some way inhibits induction of the immunoregulatory state.

In contrast to the previous proposition that the anti-inflammatory effect of gp96 is not tissue or source dependent (Chandawarkar et al 2004; Kovalchin et al 2006a), the capacity of gp96 to prolong allograft survival appeared to exhibit specificity because the administration of Lewis gp96 to Lewis recipients of Wistar grafts had no effect on allograft survival (7, 8, 9, 10, >35 vs 7, 8, 8, 9, 10 days in PBS-treated recipients). However, as stated earlier, it did appear to improve allograft function (as indi-

cated by changes in heart rate) in the immediate post-transplantation period.

Gp96 administration influences inflammatory events associated with rat cardiac allograft rejection responses

Cardiac allografts from untreated and PBS-treated recipients were more enlarged than day 35 isografts and native hearts, which reflects the inflammatory response induced during the rejection process (Fig 3). Cardiac allografts from gp96-treated animals (administered on days 0 and 3 and on days 1 and 8) were equivalent in size to isografts, suggesting that, although rejected, inflammatory events associated with the rejection process had been modified in some way (Fig 3 and data not shown, respectively).

The weights of rejected grafts from recipients that had been administered 100 μ g of gp96 on days 0 and 3 were also significantly lower than those from PBS-treated recipients ($P = 0.002$, Student's t -test) and were equivalent to those of isografts (Fig 4). Higher doses of Wistar gp96 (200 μ g) also had a significant effect on the size of rejected grafts ($P = 0.048$, Student's t -test; Fig 4). As reported earlier, the administration of Wistar gp96 on days 0, 3, and 6 had no effect on allograft survival, nor did it have an effect on the weight of cardiac allografts obtained from Lewis recipients (data not shown). However, allografts from these recipients were significantly smaller than their PBS-treated counterparts (3.1 ± 0.10 vs 4.3 ± 0.40 g; $P = 0.006$, Student's t -test).

Fig 3. Effect of gp96 treatment on the inflammatory events induced by allograft rejection. Upper panel: (A) Rejected allograft from untreated animal, (B) rejection allograft from PBS-treated animal, (C) rejected allograft from Wistar gp96-treated animal (100 μ g administered on days 0 and 3), (D) 35-day isograft from untreated recipient, (E) native heart. Lower panel: Internal architecture of rejected allograft from PBS-treated animal (left) and rejected allograft from Wistar gp96-treated animal (100 μ g administered on days 0 and 3; right).

Gp96 has no effect on circulating leukocyte populations but does induce a state of splenic CD3⁺ T-cell hyporesponsiveness

PBS and gp96 administration had no effect on the proportions of CD3⁺, CD4⁺, CD8⁺, ED1⁺, OX62⁺, and CD45⁺ leukocytes in the spleens of treated animals, nor did it influence the intensity of antigen expression (data not shown). Gp96 administration also had no effect on the constitutive secretion of IL-4, IL-10, and IFN- γ by splenic CD3⁺ T cells because levels of these cytokines in the supernatants of unstimulated cultures of CD3⁺ T cells from PBS- and gp96-treated animals were below the detection limits of the respective assays (data not shown). However, the secretion of IL-4, IL-10, and IFN- γ by CD3⁺ T cells from gp96-treated animals induced by stimulation with anti-CD3 and anti-CD28 mAbs was considerably lower than that by CD3⁺ T cells from PBS-treated animals (medians: 11 vs 52 pg/mL; 99 vs 2351 pg/mL, and 394 vs

Fig 4. Weights of Wistar cardiac isografts and rejected allografts. (A) Recipients received 100 μ g of gp96 intradermally on days 0 and 3 ($n = 5$); (B) recipients received 100 μ g of gp96 intradermally on days 1 and 8 ($n = 3$); and (C) recipients received 200 μ g of gp96 intradermally on days 0 and 3 ($n = 5$). Data are means + SEM from the indicated number of experiments.

6159 pg/mL for IL-4, IL-10, and IFN- γ , respectively; Fig 5).

Further analysis revealed that gp96 administration resulted in an increase in the IL-4/IFN- γ ratio secreted by polyclonally activated CD3⁺ T cells from these animals (median 0.027 vs 0.006), whereas the IL-10/IFN- γ ratio, which also reflects the balance between pro- and anti-inflammatory activities (Katsikis et al 1995), secreted by CD3⁺ T cells from gp96-treated animals was lower than that secreted by CD3⁺ T cells from their PBS-treated counterparts (median 0.01 vs 0.222). Although the quantitative nature of the shifts in cytokine secretion ratios was small and the biological relevance of these changes is therefore uncertain, these data do suggest that gp96 administration might induce a state of CD3⁺ T-cell hyporesponsiveness that could reflect changes in the functionality of T-cell populations in vivo and thereby influ-

Fig 5. IL-4, IL-10, and IFN- γ secretion by CD3⁺ T cells from PBS-treated and gp96-treated animals after 5 days of stimulation in vitro with immobilized anti-CD3 mAb and soluble anti-CD28 mAb. Each box and whisker plot shows the median (horizontal line), quartiles (box), and extreme values (lines). Data are generated from 5 independent experiments. Although the levels of cytokines generated in

ence the qualitative and quantitative nature of immune responses that are generated to coencountered antigens.

DISCUSSION

The "holy grail" of transplantation is the development of clinically applicable strategies that are capable of inducing long-term acceptance of transplanted organ allografts without the need for chronic immunosuppressive therapy. High doses of the stress protein gp96 have been shown to induce regulatory CD4⁺ T cells, which have the capacity to down-regulate tumor immunity and experimental autoimmune disease (Chandawarkar et al 1999, 2004). In this study, we have demonstrated that high-dose gp96 ($2 \times 100 \mu\text{g}$) administered in the peri- and post-transplant periods can significantly prolong the survival of Wistar rat cardiac allografts transplanted into Lewis rat recipients. Although of statistical significance, the magnitude of the prolongation was only modest, and it might be that a more marked effect would be observed had a less rigorous, low-responder PVG or DA rat strain been used. This possibility must be evaluated in future experiments. Notwithstanding this, these findings confirm those of others that have shown gp96 administration to prolong the survival of allogeneic skin grafts in mice (Kovalchin et al 2006a). An important feature of the high-dose gp96-induced immunoregulatory activity appears to be the timing of gp96 administration because it is equally apparent if the gp96 is administered at the time of, or 1 day after, the antigenic stimulus has been encountered. This confirms previous studies (Chandawarkar et al 2004) and adds support to the concept that the primary targets of the suppressive activity might be recently activated T-cell populations.

The mechanism via which high-dose gp96 induces immunoregulatory activity is currently unknown. One possibility is that it influences the phenotype of key antigen-presenting cells (APCs), such as DCs, and thereby the phenotype of responding T-cell populations. The capacity of low-dose gp96 administration to induce protective anti-tumor immunity has been proposed to involve the spontaneous internalization of gp96 by APCs such as DCs by a mechanism involving receptor-mediated endocytosis

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different experiments varied, the comparative responses of CD3⁺ T cells from PBS-treated and gp96-treated animals in individual experiments were consistent in all experiments. Outliers for IL-4 and IFN- γ levels secreted by CD3⁺ T cells from PBS-treated animals (117 and 60 191 pg/mL, respectively) are not presented in the plots but are included in the analyses. IL-4, IL-10, and IFN- γ levels in the culture supernatants of cells that were cultured in the absence of CD28 mAb were below the detection limit of the relevant assay. The differences in cytokine secretion by cells from PBS- and gp96-treated animals were not of statistical significance.

(Arnold-Schild et al 1999; Castellino et al 2000; Singh-Jasuja et al 2000) via the α_2 -macroglobulin receptor (CD91 molecule, Binder et al 2000; Binder and Srivastava 2004), a CD91-independent mechanism (Berwin et al 2002), or both. Endocytosis of gp96 leads to the transfer of chaperoned peptides into the intracellular pathways for MHC class I-restricted presentation to CD8⁺ T cells (Arnold-Schild et al 1999; Binder et al 2000; Castellino et al 2000; Singh-Jasuja et al 2000; Berwin et al 2002) and MHC class II-restricted presentation to CD4⁺ T cells (Doody et al 2004; SenGupta et al 2004). Although this sequence of events is commonly accepted, we have been unable to demonstrate that gp96 influences the phenotype, function, or activation status of rat bone marrow-derived DCs (Mirza et al, in press). We have also shown that it does not bind to these cells in a receptor-mediated manner; rather, it is internalized by pinocytosis (Mirza et al 2006).

Although these findings indicate that gp96 is not a universal activator of APCs, they do provide some insight into how it might elicit its anti-inflammatory effects. DCs can subsequently present antigenic peptides that are derived from pinocytosed exogenous material to T cells (reviewed in Norbury [2006]). Given that gp96 does not activate DCs (Mirza et al 2006), then the presentation of gp96-associated peptides to responding T cells by APCs that have endocytosed gp96 would occur in the absence of essential costimulatory signals—the consequence of which could be the induction of T-cell populations with the capacity to attenuate inflammatory disease. It is also possible that gp96 directly influences T-cell populations, and in this regard, we (Mirza et al 2006) and others (Banerjee et al 2002) have demonstrated that gp96 can bind to T cells and act as a costimulatory molecule that promotes the secretion of anti-inflammatory type 2 cytokines.

It might also be that gp96 influences the presence or activities, or both, of the naturally occurring CD4⁺CD25⁺ immunoregulatory T-cell subset (Shevach 2002; Gavin and Rudensky 2003; Wood and Sakaguchi 2003; Lee et al 2004; Waldmann et al 2004), especially given that these cells express Toll-like receptors (TLRs; Caramalho et al 2003; Zanin-Zhorov et al 2006) for which gp96 is a reported ligand (Vabulas et al 2002a, 2002b; Binder et al 2004). It is certainly known that the ligation of TLRs on naturally occurring CD4⁺CD25⁺ T cells by LPS (Caramalho et al 2003) or the stress protein Hsp60, which is also a reported ligand for TLRs (Ohashi et al 2000; Vabulas et al 2001; Zanin-Zhorov et al 2003, 2006; Cohen-Sfady et al 2005), can enhance the regulatory function of CD4⁺CD25⁺ T cells (Caramalho et al 2003; Zanin-Zhorov et al 2006). Although this is a potential mechanism, it does not necessarily explain the immunoregulatory properties of gp96 because the suppressive effect of high-dose gp96 has

been reported not to partition with the CD4⁺CD25⁺ or CD4⁺CD25[−] phenotypes (Chandawarkar et al 2004).

Our findings that CD3⁺ T cells from gp96-treated animals were hyporesponsive to polyclonal stimulation *in vitro* might immediately be perceived as inconsistent with the idea of allospecific immunoregulation, the concept that gp96 in some way influences the activity of regulatory T cell populations, or both. Were gp96 to influence regulatory T cell populations, then it might be expected that *in vitro* activated T cells should secrete more IL-10 and IL-4 and less IFN- γ . However, it is known that naturally occurring CD4⁺CD25⁺ regulatory T cells are unresponsive to stimulation via the T-cell receptor *in vitro* (Takahashi et al 1998; Thornton and Shevach 1998; Itoh et al 1999). It is also known that naturally occurring anergic T-cell clones can inhibit antigen-specific and allo-specific T-cell proliferation (Lombardi et al 1994; Vendetti et al 2000) and prolong skin allograft survival (Chai et al 1999). In addition, although anergic *in vitro*, CD4⁺CD25⁺ regulatory T cells are not anergic *in vivo*, and it therefore appears that the activities of antigen-specific regulatory T cells cannot be predicted from their *in vitro* behavior (Klein et al 2003). Taken together, our data support the concept that gp96 induces a population of regulatory T cells with the capacity to allospecifically influence allograft rejection responses rather than argue against it.

The observation that the immunoregulatory response induced by gp96 administration exhibits specificity provides some insight into why high-dose gp96 only prolongs rather than prevents allograft rejection. Given the proposed mechanisms via which gp96 induces tumor immunity, the specificity of the immunoregulatory response will be defined by alloantigenic peptides that are chaperoned by or associated with the administered gp96, in which case it will be to indirectly rather than directly presented alloantigen (alloantigenic peptides presented to recipient T cells by recipient APCs and alloantigenic peptides presented to recipient T cells by donor APCs, respectively). This would explain the inability of gp96 to have a more pronounced effect in the early stage of rejection because this is primarily driven by allogeneic responses to directly presented antigen. This might be an important finding because responses to indirectly presented alloantigen are more resistant to current antirejection therapies. Were this to be the case, then gp96 administration might have more profound effects on chronic allograft rejection, given that indirect alloantigen presentation and recognition is thought to play an important role in this type of rejection response (Ciubotariu et al 1998; Hornick et al 1998; Hornick et al 2000; Krasinskas et al 2000; Lee et al 2001).

The gp96 preparations used in this study contained approximately 6% calreticulin (Fairburn et al 2006), an endoplasmic reticulum protein, to which have also been at-

tributed tumor immunity-inducing immunoregulatory properties that are similar to those that have been reported for gp96 (Basu and Srivastava 1999; Nair et al 1999). To our knowledge, calreticulin has not been reported to possess anti-inflammatory properties; hence, were calreticulin to have any influence on cardiac allograft rejection responses, it is more likely that it would promote rather than attenuate rejection. For the induction of tumor-specific immunity in mice, calreticulin has been administered intravenously ($2 \times 10 \mu\text{g}$; Nair et al 1999) or subcutaneously ($11 \mu\text{g}$ twice weekly; Basu and Srivastava 1999). In the latter study, no anti-tumor immunity was induced when a dose of $5.5 \mu\text{g}$ was administered. Assuming a mouse body weight of approximately 20 g and that intradermal administration of calreticulin is no more effective at eliciting its biological effects than intravenous or subcutaneous administration (which is not known), then on a weight to weight basis, calreticulin doses of approximately $150 \mu\text{g}$ would be required to elicit similar immune responses in 300-g rats. This dose of calreticulin is greatly in excess of that which was administered in this study (6 and $12 \mu\text{g}$). Unless intradermal administration of calreticulin is more effective at eliciting biological effects than the intravenous or subcutaneous routes, then it seems unlikely that the calreticulin that was present in the gp96 preparation influenced its capacity to influence cardiac allograft rejection responses.

In this study, we demonstrated that gp96 can prolong allograft survival in a manner that appears to be source (ie, alloantigen) specific. More studies aimed at characterizing the specificity of the immunoregulatory response induced by high-dose gp96 administration are required, as are studies aimed at providing insight into the mechanism or mechanisms involved and elucidating its effects on allograft rejection responses that are generated in the absence of directly presented antigen.

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