The stress protein gp96 is not an activator of resting rat bone marrow–derived dendritic cells, but is a costimulator and activator of CD3⁺ T cells

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Abstract Although low doses of tumor-derived stress protein gp96 elicit protective immunity to the tumor from which it is isolated, protection is lost at high doses because of the induction of immunoregulatory CD4⁺ T cells. This study evaluated the influence of gp96 on resting rat bone marrow–derived dendritic cells (BMDCs) and purified CD3⁺ T cells. In contrast to previous reports, gp96 had no effect on adhesion and costimulatory molecule expression by BMDCs, nor did it influence interleukin (IL)-10 and IL-12 secretion or their allostimulatory capacity. Gp96 did not bind to BMDCs but dose-dependently bound to CD4⁺ and CD8⁺ T cells. At low concentrations (1 and 25 μ g/mL), gp96 acted as a costimulator of CD3⁺ T cells, inducing proliferation and the secretion of interferon (IFN)- γ and IL-10. Gp96 also increased the proliferation of CD28-costimulated CD3⁺ T cells and their secretion of IFN- γ , IL-4, and IL-10. Gp96 had no effect at higher concentrations (50 and 100 μ g/mL), despite the occurrence of cell surface binding at these concentrations. These findings indicate that gp96 can act as a costimulatory molecule for CD3⁺ T cells, and an observed increase in the IL-10:IFN- γ secretion ratio induced by gp96 suggests that it might, at appropriate concentrations, promote a regulatory T-helper 2 (Th2)–like phenotype.

INTRODUCTION

Gp96, also known as glucose-regulated protein (grp)94 (Lee 1981), endoplasmic (Koch et al 1986), and 99-kDa endoplasmic reticulum protein (ERp99; Lewis et al 1985) is a 94–96-kDa member of the HSP90 family of molecular chaperones/stress proteins that resides within the lumen of the endoplasmic reticulum. Research in the mid-1980s demonstrated that the administration to mice of a 96-kDa protein fractionated from a tumor cell lysate (subsequently shown to be gp96) induced resistance to the same tumor from which the protein had been originally isolated (Srivastava et al 1986). As intracellular chaperones, stress

proteins bind a large number of peptides derived from the cells from which they are isolated (Gething and Sambrook 1992; Young et al 1993)—the so-called “antigenic fingerprint” or repertoire of that cell (Srivastava and Udono 1994)—and it has now been proposed that the administration of gp96 isolated from murine tumor cells induces tumor-specific cytolytic T cells and anti-tumor immunity in mice, both of which are specific for tumor-derived peptides that are associated with gp96 rather than gp96 itself (Udono and Srivastava 1994; Chandawarkar et al 1999).

Although gp96 has been reported to induce peptide-specific protective immunity, at high doses, it can down-regulate anti-tumor immunity and attenuate autoimmune diseases. This observation was originally made in murine studies demonstrating that the induction of immunity to methylcholanthrene-induced Meth A fibrosarcoma by the

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administration of gp96 purified from Meth A displays a consistent dose restriction, in that 2 intradermal injections of 1 μ g protect, whereas 2 injections of 10 μ g do not (Chandawarkar et al 1999). It was proposed that the suppressive activity was source specific; that is, for the suppression of tumor immunity to occur, gp96 must be purified from that tumor (Chandawarkar et al 1999). However, subsequent work from the same investigators has shown that the administration of high-dose gp96 ($2 \times 100 \mu$ g subcutaneously) purified from normal liver tissue is equally able to suppress tumor immunity in mice, the onset of diabetes in nonobese diabetic mice, and the induction of autoimmune encephalomyelitis (EAE) by myelin basic protein and proteolipid protein in SJL mice (Chandawarkar et al 2004). For reasons that are currently unclear, administration of gp96 via the intradermal route is 10-fold more efficient at inducing its biological effects than administration via the subcutaneous route (Chandawarkar et al 1999).

The mechanism by which high-dose gp96 administration can down-regulate inflammatory events is unknown. The timing of the high-dose gp96 administration is important because the immunoregulatory activity is only apparent if gp96 is administered at the time of, or shortly after, an encounter with the antigenic stimulus (Chandawarkar et al 2004). This suggests that the primary targets of suppressive activity are recently activated rather than memory T cells (Chandawarkar et al 2004). In addition, high-dose gp96 administration appears to induce and/or activate immunoregulatory CD4⁺ T cell populations because the inhibition of tumor immunity and protection from diabetes and EAE can be achieved by the adoptive transfer of CD4⁺ T cells from animals that have been treated with high-dose gp96 (Chandawarkar et al 1999; Chandawarkar et al 2004).

Gp96 could influence the qualitative nature of an immune response at a number of different stages in the development of that response. First, gp96 might influence the phenotype (antigen expression, cytokine secretion profile) of antigen-presenting cells (APCs), particularly dendritic cells (DCs) and their capacity to activate and influence the functional phenotype of responding T cell populations. In this regard, a number of investigators have reported that gp96 induces the maturation of, and cytokine secretion from, these and other APCs and increases their capacity to stimulate the proliferation of T cells (Basu et al 2000; Singh-Jasuja et al 2000a; Zheng et al 2001; Baker-LePain et al 2002; Reed et al 2003). Gp96 might also have direct effects on T cells because a protein expressed on the surface of lipopolysaccharide (LPS)-activated B cells (B2), which has been identified as gp96, has been reported to act as a T-helper 2 (Th2)-specific costimulatory molecule for murine splenic T cells (Banerjee et al 2002).

We have recently reported a technique for generating resting immature rat bone marrow-derived DCs (BMDCs; Muthana et al 2004), and this study evaluated the influence of gp96 on the phenotype and activities of these cells. The study also investigated the direct influence of gp96 on the activities of purified CD3⁺ T cells in the absence and presence of costimulation via ligation of the CD28 molecule.

MATERIALS AND METHODS

Animals

Adult Wistar and Lewis rats (200–250 g) were obtained from Harlan UK Limited (Bicester, UK). All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986.

Purification of gp96

Gp96 was purified from Wistar rat livers essentially as described (Srivastava 1997; Fairburn et al 2006). Briefly, livers were homogenized in phosphate-buffered saline (PBS) containing the protease inhibitor phenylmethylsulfonylfluoride (0.2 mM, 60 mL of buffer per 30 g of tissue) with a Waring Laboratory Science laboratory blender and stainless steel minicontainer (Christison Particle Technologies Ltd, Gateshead, UK). The homogenate was centrifuged for 10 minutes at $2000 \times g$ at 4°C to remove unbroken cells, nuclei, and other debris, and the supernatant was ultracentrifuged at $100\,000 \times g$ for 90 minutes. The supernatant was brought to 50% (w/v) saturation with ammonium sulfate, and the resulting precipitate was removed by centrifugation at $5000 \times g$ for 20 minutes. The supernatant was diluted 1:1 with PBS containing 2 mM Ca²⁺ and 2 mM Mg⁺ and applied to a concanavalin A (Con A)-Sepharose column. The bound fraction was eluted with 10% (w/v) α -methyl mannopyranoside, and the eluate was passed through a PD10 size exclusion column. The protein-containing eluate was applied to a diethylaminoethanol-Sepharose column equilibrated with 5 mM sodium phosphate buffer (pH 7.0), and seven 2-mL fractions were collected. Only those eluting between 450 mM and 500 mM NaCl (fractions 3 and 4) contained detectable levels of protein when measured with the Bradford assay (Bio-Rad Protein Assay, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and gp96 when assessed with ECLTM Western blot analysis (data not shown). The endotoxin content of the purified gp96 preparations was measured by the Cambrex Bioproducts LAL Testing Service (Vervier, Belgium) and this was routinely found to be <0.001 EU (0.1 pg) per microgram gp96 (Fairburn et al 2006).

It has been reported that that gp96 purified by a Con A affinity purification step contains substoichiometric amounts of Con A, which can promote the formation of gp96–Con A oligomers, enhance the immunological activity of gp96 as a consequence (Monks et al 2005), and itself activate T cells because of its lectin-mediated mitogenic activity (Arnold-Schild et al 2000). However, no protein is present in the eluates from the Con A–Sephadex bead columns used for the purification of gp96 in this study, nor can bands that were observed in the Con A lanes of silver-stained gels and ECL Western blots (~28 kDa) be observed in lanes containing purified gp96 preparations (Fairburn et al 2006). In addition, no high-molecular mass gp96 oligomers are detectable, and none of the protein spots analyzed on 2-dimensional gels have been identified as being Con A by tandem mass spectrometry (Fairburn et al 2006). Taken together, these findings exclude the possibility that Con A is a contaminant of these preparations.

Generation of rat BMDCs

Rat BMDCs were generated as described previously (Muthana et al 2004). Briefly, bone marrow was flushed from the tibias and femurs of Wistar rats with a 25-gauge needle. 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, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Invitrogen Ltd, Paisley, UK; supplemented growth medium designated RPMI*) and were cultured (3×10^6 in 5 mL) in T25 Nunc[™] tissue culture flasks (Nalge Europe Ltd, Hereford, UK) in RPMI* containing recombinant rat granulocyte/macrophage colony-stimulating factors (GM-CSF; 1000 U/mL) and recombinant rat interleukin (IL)-4 (33 U/mL; both PeproTech EC Ltd, London, UK). On day three, 5 mL of GM-CSF- and IL-4-supplemented RPMI* were added. On day 7, nonadherent cells were removed, and the remaining adherent immature BMDCs were, without harvesting, used for all experiments.

This approach induces the differentiation of large numbers of a homogeneous population of adherent cells (typically 1.2×10^6 adherent cells/flask), all of which exhibit the morphological appearance of DCs and >90% of which bind the OX62 monoclonal antibody (mAb; Muthana et al 2006). The OX62 mAb recognizes the 150-kDa integrin α_{E2} subunit and was originally described as being expressed on most, if not all, DCs in the rat (Brenan and Puklavec 1992). Subsequent work has shown that most (80%) spleen DCs are OX62⁺, whereas only 50% of lymph node cells are OX62⁺ (Trinité et al 2000) and plasmacytoid DCs are OX62[−] (Hubert et al 2004). IL-4 inhibits the differentiation of monocytes/macrophages in bone marrow cell cultures, and only 2.12% of the resultant cell

population was positive for the myeloid cell (macrophage, neutrophil)-specific antigen CD68 (as measured by the ED1 mAb; unpublished observations). The proportions of contaminating CD3⁺ T cells and CD45⁺ B cells were also low (1.3% and 2.4%, respectively; unpublished observations). The OX62⁺ BMDCs generated under these conditions exhibit an immature phenotype, as defined by low levels of CD40, CD54, CD86, and major histocompatibility complex (MHC) class II expression and a pronounced capacity for antigen uptake (Muthana et al 2004). Cells were not harvested before use in any of the assays described below because the disruption and harvesting of DCs and DC clusters induces DC maturation (Pierre et al 1997; Talmor et al 1998; Gallucci et al 1999; Delamarre et al 2003) and might therefore influence the quantitative and qualitative responsiveness of BMDCs to exogenous stimuli.

Cytokine secretion by resting, LPS-treated, and gp96-treated BMDCs

Seven-day adherent BMDCs were incubated for 24 hours in RPMI* with LPS (1 µg/mL; Sigma-Aldrich Co Ltd, Poole, UK) or purified gp96 (0–100 µg/mL), after which supernatants were collected, centrifuged, and stored at −80°C. Levels of IL-10 and IL-12 were determined by enzyme immunoassay, with mAb pairs and recombinant proteins obtained from BD Biosciences (Pharmingen, Oxford, UK, BD OptEIA[™]) and Biosource International (Nivelles, Belgium, CytoSet[™]), respectively, in both instances, according to the manufacturers' recommended protocols. Two enzyme immunoassays for measuring IL-12 were used: one that detects only the bioactive IL-12 p70 heterodimer (IL-12_{p70}; Biosource, catalog no. CRC2374) and another that recognizes both the IL-12_{p70} heterodimer and the p40 subunit, IL-12_{p40} (IL-12_{p70+p40}; Biosource, catalog no. CRC0124). Samples were analyzed with both assays, and the levels of IL-12_{p70} heterodimer and the IL-12_{p40} subunit were calculated by subtraction. In all instances, cytokine concentrations were determined by reference to standard dose-response curves that were generated with Ascent data analysis software (Thermo Life Sciences, Basingstoke, UK).

Flow cytometric analysis

Surface antigen expression by resting, LPS-activated, and gp96-treated BMDCs was determined by 2-color flow cytometry with a phycoerythrin (PE)-conjugated murine mAb specific for OX62, in combination with fluorescein isothiocyanate (FITC)-conjugated murine mAbs specific for CD11b, CD54 (ICAM-1), CD80 (B7.1), and CD86 (B7.2) and monomorphic determinants on MHC class II (clone OX-6) expression, all of which were obtained from

Serotec Ltd (Kidlington, UK). An FITC-conjugated anti-CD40 mAb was purchased from BD Biosciences. Briefly, harvested cells were incubated for 30 minutes at 4°C with 2 µL of 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 (BSA, 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. Flow cytometric analysis was performed with a FACSort™ flow cytometer and the CELLQuest Pro™ data acquisition and analysis software (both from BD Biosciences).

Effect of gp96 on the activities of purified CD3⁺ T cells

The direct influence of gp96 on the proliferation of purified CD3⁺ and their secretion of interferon (IFN)-γ, IL-4, and IL-10 after incubation with solid-phase anti-CD3 mAb in the absence and presence of costimulation via CD28 ligation was evaluated. Lewis splenocytes were obtained by mechanical disruption, and CD3⁺ T cells were purified to a final purity of 92–95% (as assessed by flow cytometry; data not shown) according to the manufacturer's protocol for the MagCelect Rat CD3⁺ T cell isolation kit (R&D Systems Europe Ltd, Abingdon, UK).

The proliferation of Lewis CD3⁺ T cells was determined with the 5- (and 6-)carboxyfluorescein diacetate succinimidyl ester (CFSE)-based flow cytometric assay, which quantifies the progressive decline in the fluorescent intensity of CFSE-labeled cells as they divide (Lyons and Parish 1994). Freshly isolated Lewis CD3⁺ T cells (5 × 10⁶ cells/mL in PBS) were stained by gently mixing for 5 minutes at room temperature with CFSE (0.5 µM; Molecular Probes Europe BV, Leiden, The Netherlands). The fluorescence of unbound dye was quenched with an equal volume of FBS, after which cells were washed in PBS.

Freshly isolated CFSE-stained Lewis CD3⁺ T cells were cultured with or without gp96 (0–100 µg/mL) for 5 days in 24-well tissue culture plates coated with anti-CD3 mAb (2 µg/mL; BD Biosciences) in the presence or absence of costimulation with an anti-CD28 mAb (2 µg/mL; BD Biosciences). At the end of the culture period, cell-free supernatants were collected for the analysis of cytokines that are characteristic of the Th1 (IFN-γ) and Th2 (IL-4, IL-10) cytokine phenotype according to the manufacturers' recommended protocols with commercially available enzyme immunoassays (IL-10, BD Opt-EIA™, BD Biosciences; IFN-γ and IL-4, Diaclone Eli-pair assays from ImmunoDiagnostic Systems Ltd, Bordon,

UK). The proportion of cells that exhibited a reduced fluorescent intensity (ie, had proliferated) was determined by flow cytometry.

As controls, the influence of gp96, which had been boiled for 30 minutes or incubated for 3 hours at 37°C with proteinase K acrylic beads (Sigma-Aldrich Company Ltd), was also evaluated. Boiling resulted in a marked aggregation of gp96, as observed by silver staining of electrophoretic gels, and no protein bands were observed after proteinase K treatment (data not shown).

Binding of gp96 to BMDCs and CD3⁺ T cells

Purified gp96 and BSA were labeled with FITC according to the manufacturer's recommended protocol with a FluoroTag™ FITC conjugation kit (Sigma-Aldrich Company Ltd). Successful labeling was confirmed by observing an ~2-kDa shift in the apparent molecular masses of labeled proteins after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Resting or LPS-activated BMDCs were incubated with FITC-gp96 and FITC-BSA (0, 1, 50, 100 µg) for 30 minutes at 4°C (to determine specific, receptor-mediated interactions) or 37°C (to determine nonspecific uptake via pinocytosis). Cells were washed extensively with PBS/BSA at the end of the incubation period.

For the evaluation of FITC-labeled protein binding, freshly isolated and cultured (5 days in 24-well tissue culture plates coated with anti-CD3 mAb in the absence or presence of costimulation with an anti-CD28 mAb) CD3⁺ and CD4⁺ T cells were fixed in CellFIX™ (BD Biosciences), after which they were incubated with FITC-gp96 and FITC-BSA for 45 minutes at 4°C. CD4⁺ T cells were purified according to the manufacturer's protocol with a MagCelect Rat CD4⁺ T cell isolation kit (R&D Systems). Purified CD3⁺ T cells were also costained with a PE-conjugated mAb to CD4 to differentiate FITC-gp96 binding to CD4⁺ and CD8⁺ (ie, CD4⁻) T cells. In all instances, binding of FITC-labeled protein was analyzed by flow cytometry and visualized by fluorescent microscopy.

Statistical analysis

Where appropriate, data are expressed as mean ± SEM. The statistical significance of comparisons was calculated with the Student's *t*-test for paired data (SPSS UK Ltd, Woking, UK).

RESULTS

Effect of gp96 on immature rat BMDCs

In contrast to studies that have reported murine gp96 to induce the maturation of DCs and other APCs and their

secretion of cytokines (Basu et al 2000; Singh-Jasuja et al 2000a; Zheng et al 2001; Baker-LePain et al 2002; Vabulas et al 2002a; Reed et al 2003), yet in agreement with a report that human gp96 has no effect on the phenotype of human monocyte-derived DCs (Bethke et al 2002), we found a 24-hour incubation with rat gp96 to have no effect on the expression of CD54, CD40, CD80, CD86, and MHC class II by resting rat BMDCs (Fig 1). No effect was observed by increasing the incubation period with gp96 to 48 hours (data not shown).

The BMDCs that were used in this study predominantly secrete IL-12_{p40} and very little of the IL-12_{p70} heterodimer (Muthana et al 2006). Gp96 had no effect on the secretion of IL-12_{p40} and IL-10 (Fig 2). The BMDCs were responsive to maturation signals; LPS increased the intensity of CD40, CD54, and CD86 expression (Fig 1) and induced high levels of IL-12_{p40} and IL-10 secretion (Fig 2). Consistent with its lack of effect on cell surface antigen expression and cytokine secretion, gp96 pretreatment also had no effect on the capacity of Wistar BMDCs to stimulate the proliferation of allogeneic Lewis splenocytes or to induce their secretion of IFN- γ , IL-4, and IL-10 (data not shown).

Although it had no apparent effect on antigen expression, cytokine secretion, or the capacity of BMDCs to activate allogeneic splenocytes, gp96 did induce a dose-dependent progressive increase in the forward light scatter characteristics (size) of BMDCs when analyzed by flow cytometry, which, at 200 $\mu\text{g/mL}$, was of a similar magnitude to that induced by LPS (Fig 3). Immature DCs are rounded cells with small knoblike projections, whereas mature DCs exhibit a more irregular shape with large prominent ruffles or numerous long needlelike projections (Steinman 1991; Lagaraine et al 2005). Flow cytometers can detect differences in cell morphology (size vs granularity) on the basis of distinct light scatter characteristics, and the observed morphological change suggests that gp96 can, in some way at least, interact with and influence the morphology of these cells and possibly their biological status.

Effect of gp96 on purified CD3⁺ T cells

In contrast to its lack of effect on BMDCs, gp96 dose-dependently induced the proliferation of purified CD3⁺ T cells, as indicated by the proportion of cells that exhibited a decreased intensity of CFSE staining (Figs 4 and 5). Of particular interest was the observation that gp96 induced the proliferation of CD3⁺ T cells in the absence of concomitant costimulation via CD28 ligation, which indicates that it can act as a costimulatory molecule for T cells (Figs 4 and 5). For reasons that are currently unclear, at high concentrations (50 and 100 $\mu\text{g/mL}$), gp96 had no effect on the proliferation of CD3⁺ T cells. This was not

due to a cytotoxic effect, because gp96 treatment had no effect on the levels of lactate dehydrogenase, a stable cytosolic enzyme that is released into cell culture supernatants on cell lysis (data not shown).

Gp96 had no effect on IL-4 and IFN- γ secretion from CD3⁺ T cells in the absence of costimulation via CD28, nor did it have a statistically significant effect on IL-10 secretion (Fig 6). However, a trend toward low concentrations of gp96 inducing IL-10 and high concentrations inhibiting IL-10 secretion from these cells was suggested (Fig 6). In the presence of costimulation, a trend toward gp96 dose-dependently influencing the secretion of all 3 cytokines by CD3⁺ T cells was suggested (Fig 6). Boiled and proteinase K-treated gp96 had no influence on cytokine secretion (data not shown).

FITC-gp96 binding to BMDCs and T cells

In contrast to other studies that have reported FITC-gp96 binding to different DC populations (Arnold-Schild et al 1999; Vabulas et al 2002a), binding of FITC-gp96 to the resting and LPS-activated BMDCs used in this study was not observed (data not shown). This would appear to explain the inability of gp96 to influence the phenotype and activity of BMDCs; however, it is somewhat puzzling given that gp96 can influence the morphology of these cells (Fig 3). Although gp96 does not appear to bind to BMDCs via cell surface receptors, it is nonspecifically endocytosed by cells when these are incubated with gp96 at 37°C (Fig 7). The nonspecific nature of this uptake was confirmed by the observation that FITC-BSA was also taken up by BMDCs at 37°C (Fig 7). Such nonspecific endocytosis might provide an explanation for the capacity of gp96 to influence the morphology of BMDCs.

The ability of gp96 to act as a costimulator and activator of CD3⁺ T cells suggests that T cells express receptors for this protein. Although FITC-gp96 binding to freshly isolated CD3⁺ and CD4⁺ T cells was apparent (Fig 8), binding to CD3⁺, CD4⁺, and CD8⁺ T cells that had been cultured in the absence and presence of CD28 mAb, respectively, was much more pronounced (Figs 8 and 9). The specificity of FITC-gp96 binding was confirmed by its blocking with 4-fold excess unlabeled gp96 (data not shown).

DISCUSSION

The ability of tumor-derived gp96 to induce protective tumor-specific immunity has been suggested to arise from its capacity to form stable complexes with antigenic peptides (Srivastava 2002), and the mechanism via which gp96 can induce peptide-specific immunity has, at least in part, been elucidated. APCs such as DCs have been reported to spontaneously internalize gp96 by receptor-

Fig 1. Influence of gp96 on cell surface antigen expression by resting rat BMDCs. Adherent Wistar BMDCs were generated and cultured for an additional 24 hours with gp96 or LPS ($1 \mu\text{g/ml}$). The proportion (proportion, %; left panel) of OX62⁺ BMDCs expressing CD11b ($n = 12$ experiments), CD40 ($n = 4$), CD54 ($n = 13$), CD80 ($n = 4$), CD86 ($n = 12$), and MHC class II ($n = 12$) and its intensity of expression (median channel of fluorescent intensity, MFI; right panel) were determined by flow cytometry. Data are presented as mean $\pm$ SEM.

Fig 2. Influence of gp96 on IL-10 and IL-12 subunit (IL-12_{p40}) and heterodimer (IL-12_{p70}) secretion by resting rat BMDCs. Adherent Wistar BMDCs were generated and cultured with gp96 or LPS (1 µg/mL) without harvesting for an additional 24 hours. Levels of IL-10 ($n = 10$ independent experiments), IL-12_{p40} ($n = 10$), and IL-12_{p70} ($n = 5$) in cell-free culture supernatants were determined by enzyme immunoassay. Data are presented as mean $\pm$ SEM.

mediated endocytosis (Arnold-Schild et al 1999; Castellino et al 2000; Singh-Jasuja et al 2000b) 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. Chaperoned peptides are then transferred into the intracellular pathways for MHC class I-restricted presentation to CD8⁺ T cells (Ar-

nold-Schild et al 1999; Binder et al 2000; Castellino et al 2000; Singh-Jasuja et al 2000b; Berwin et al 2002) and MHC class II-restricted presentation to CD4⁺ T cells (Doody et al 2004; SenGupta et al 2004). Although it is widely thought that it is via this mechanism that gp96 induces tumor-specific immunity, the direct involvement of gp96-mediated chaperoning of tumor-derived peptides in inducing tumor-specific immunity has been questioned, and the primary involvement of innate (non-antigen-specific) immune activation has been proposed (Baker-LePain et al 2003; Nicchitta 2003).

DCs must be mature and express essential costimulatory molecules such as CD80 and CD86 to fully activate T cells, and gp96 has been reported to induce the maturation of, and cytokine secretion from, these and other APCs (Basu et al 2000; Singh-Jasuja et al 2000a; Zheng et al 2001; Baker-LePain et al 2002; Reed et al 2003). A commonly held view is that gp96 is a universal activator of DCs; however, this study demonstrates that this might not necessarily be the case because although gp96 influenced the morphology of BMDCs, it had no effect on their expression of surface antigens, their secretion of cytokines, or their capacity to activate allogeneic CD3⁺ T cells. The inability of gp96 to influence the phenotype of the BMDCs that were used in this study was explained by its lack of binding to these cells, and these data therefore question the widely held view that gp96 is a universal ligand for, and activator of, DCs.

Although our findings are supported, in part at least, by a previous study which has reported gp96 to have no effect on the maturation and functional status of human monocyte-derived DCs (Bethke et al 2002), the reasons for their discrepancy with other studies (Basu et al 2000; Singh-Jasuja et al 2000a; Zheng et al 2001; Baker-LePain et al 2002; Reed et al 2003) are currently unclear. It might be that receptors to which gp96 can bind are not expressed by rat BMDCs. Another possibility is that the BMDCs used in the study were more immature than those used by other investigators because they were not harvested before use, which can prime or induce DC activation and maturation (Pierre et al 1997; Talmor et al 1998; Gallucci et al 1999; Delamarre et al 2003). Although we could not detect gp96 binding to LPS-activated BMDCs (data not shown), this might not necessarily exclude the possibility that other non-LPS-inducible receptors are involved. More recent evidence demonstrating that low doses of gp96 (<50 µg/mL) do not activate murine BMDCs in the absence of Toll-like receptor 2 and 4 (TLR2 and TLR4) ligands (Warger et al 2006) supports, in part at least, our findings.

The mechanism underlying the capacity of high-dose gp96 to induce immunoregulatory activity is currently unknown. One possibility is that it influences the phenotype of key APCs such as DCs and thereby the phe-

Fig 3. Forward (FSC) and side (SSC) light scatter characteristics of resting gp96-treated and LPS-activated rat BMDCs. Adherent Wistar BMDCs were generated and cultured for an additional 24 hours with gp96 or LPS (1 $\mu\text{g}/\text{mL}$). The region in which the majority of cells were apparent in control cultures has been superimposed on FSC vs SSC scatterplots for cells cultured with gp96 and LPS for illustrative purposes. Plots are representative of at least 5 independent experiments.

notype of responding T cell populations. However, our findings argue against this being the mechanism. Alternatively, gp96 might directly or indirectly influence the activity and phenotype of T cell populations. The anti-inflammatory activity of CD4^+ T cells has been shown in a number of well-characterized model systems to partition, in part at least, into a naturally occurring CD25^+ subset (Shevach 2002; Gavin and Rudensky 2003; Wood and Sakaguchi 2003; Lee et al 2004; Waldmann et al 2004). However, although gp96 has the potential to influence the activity of $\text{CD4}^+\text{CD25}^+$ T cells because it is reported to be a ligand for TLRs (Vabulas et al 2002a, 2002b; Binder et al 2004), which are expressed on such regulatory T cells (Caramalho et al 2003), the suppressive effect of high-dose gp96 does not partition with the $\text{CD4}^+\text{CD25}^+$ or $\text{CD4}^+\text{CD25}^-$ phenotypes (Chandawarkar et al 2004).

Our finding that gp96 appears to influence the secretion of IL-10 by CD3^+ T cells is intriguing given that populations of regulatory CD4^+ T cells secreting large amounts of IL-10 (T regulatory type 1, Tr1) have also been described (Groux et al 1997; Cottrez et al 2000). Tr1 cells suppress bystander CD4^+ T cell proliferation in an IL-10- and TGF- β -dependent manner and prevent the development of Th1-mediated autoimmune disease and immune responses to alloantigens (Groux 2003).

Although gp96 induced the secretion of IFN- γ , the IL-

10:IFN- γ ratio, which is an important reflection of the balance between pro- and anti-inflammatory activities (Katsikis et al 1995) was increased from 1.5 to 2.1 for non-costimulated cells and from 0.52 to 1.1 for costimulated cells in the presence of 25 $\mu\text{g}/\text{mL}$ gp96. The effects of gp96 on CD3^+ T cells are highly dose dependent, and it is difficult at this stage to speculate on the in vivo consequences of high-dose gp96 administration on the basis of these in vitro data. In vivo studies aimed at providing insight into the in vivo consequences of high-dose gp96 administration on the survival of, and inflammatory events that are associated with, rat cardiac allografts are currently underway.

The dose-dependent effect of gp96 does not appear to result from inhibition of gp96 binding because of competition for a limited number of receptors because FITC-gp96 binding to T cell populations progressively increased at higher concentrations. Nor is it due to a cytotoxic effect. The dose-dependent effect of gp96 is similar to that observed for growth hormone, in that at high concentrations, its biological effects become dose-dependently diminished because of its influence on the capacity of its receptors to dimerize, the effect on the conformation of its dimerized receptors, which are members of the cytokine receptor superfamily, or both (Fuh et al 1992; Frank 2002).

Fig 4. Effect of gp96 on the proliferation of CD3⁺ T cells. CFSE-labeled Lewis CD3⁺ T cells were cultured for 5 days in anti-CD3 mAb-coated plates with different concentrations of gp96 in the absence (left panel) and presence (right panel) of costimulation with an anti-CD28 mAb. Proliferation (as indicated by a decrease in fluorescence intensity—relative proliferation) was determined by flow cytometry. Fluorescent histograms are representative of data that were obtained from a minimum of 7 independent experiments. The M1 (negative-staining) and M2 (positive-staining) regions were set with the use of the CFSE-stained unstimulated control. The proportion of cells in the M1 region (ie, exhibited a reduced fluorescence intensity compared with the unstimulated control) is indicated.

Fig 5. Effect of gp96 on the proliferation of CD3⁺ T cells. CFSE-labeled Lewis CD3⁺ T cells were cultured for 5 days in anti-CD3 mAb-coated plates with different concentrations of gp96 in the absence (left panel) and presence (right panel) of costimulation with an anti-CD28 mAb. Proliferation (as indicated by a decrease in fluorescence intensity) was determined by flow cytometry. Data (mean $\pm$ SEM) are presented as relative proliferation and were obtained from a minimum of 7 independent experiments.

Fig 6. Effect of gp96 on cytokine secretion by unstimulated (left panel) and costimulated (right panel) CD3⁺ T cells. CD3⁺ Lewis T cells were cultured for 5 days in anti-CD3 mAb-coated plates with different concentrations of gp96 in the absence of costimulation with an anti-CD28 mAb. Cell-free supernatants were harvested, and IFN- γ , IL-4, and IL-10 levels were determined by enzyme immunoassay. Data were obtained from 4 (for unstimulated cells) and 3 (for costimulated cells) independent experiments and are presented as mean $\pm$ SEM.

Fig 7. Uptake of FITC-gp96 and FITC-BSA into OX62⁺ BMDCs at 4°C (upper) and 37°C (lower). Propidium iodide (PI)-stained (nonviable) cells were excluded from the analysis. The M1 (negative-staining) and M2 (positive-staining) regions were set against the control (incubated in the absence of FITC-labeled protein). Although there is an apparent binding of FITC-gp96 to BMDCs at 4°C, this binding is nonspecific because FITC-BSA bound to a similar degree. Fluorescent histograms are representative of 2 independent experiments.

Further work aimed at identifying the receptors on T cells to which gp96 binds is required, and these studies are currently underway. Although possible candidates are TLRs, because TLR2 and TLR4 are expressed on immunoregulatory (Caramalho et al 2003; Zanin-Zhorov et al 2006) and activated (Liew et al 2004) T cell populations and TLR2 acts as a costimulatory receptor on activated (but not resting) and memory T cells (Komai-Koma et al 2004; Liew et al 2004), signaling via these receptors does not exhibit the type of dose dependency that has been observed in this study. An alternative explanation might be that higher doses of gp96 trigger immunoregulatory T cells that are present in the mixed CD3⁺ T cell population and thereby modify proliferative and cytokine responsiveness, as shown in a recent study, in which Hsp60 enhances CD4⁺CD25⁺ regulatory T cell function via TLR2 signaling (Zanin-Zhorov et al 2006).

Our study indicates that gp96-FITC does not bind to BMDCs at 4°C (a finding that argues against gp96 uptake occurring via specific receptor-mediated mechanism), but that it is taken up nonspecifically by pinocytosis when incubations are performed at 37°C. This suggests that gp96 might have an effect on BMDCs in a manner that does not involve surface receptor binding. It is known that immature DCs can take up antigens via pinocytosis in clathrin-coated pits and by fluid-phase pinocytosis (Lipscomb and Masten 2002). It is also known that receptors such as TLRs are located inside cells and can be activated intracellularly (Leifer et al 2004). This alternative route for entering BMDCs and the possibility that it con-

sequently activates intracellular receptors might explain why gp96 was able to influence the morphology of BMDCs, as determined on the basis of light scatter characteristics, in the absence of any apparent evidence that it binds to specific cell surface receptors.

The nonspecific uptake of gp96 by BMDCs might also, in part at least, explain the capacity of high-dose gp96 to delay the rejection of rat cardiac allografts in what appears to be an alloantigen-specific manner (LK Slack et al, personal communication). It is known that DCs can subsequently present antigenic peptides derived from pinocytosed exogenous material to T cells (reviewed in Norbury 2006), and our observations that gp96 does not activate DCs raise the possibility that antigen presentation to responding T cells occurs in the absence of essential costimulatory signals, in which case, the induction of T cell populations with the capacity to attenuate inflammatory disease might be the consequence.

In this study, we demonstrated that gp96 can act as a costimulatory molecule for CD3⁺ T cells and that it can, at appropriate concentrations, induce a cytokine secretion profile that is consistent with a Th2-like immunoregulatory phenotype (as indicated by an increased IL-10:IFN- γ ratio; Katsikis et al 1995). These findings, to some extent at least, confirm a previous study that reported gp96 acts as a Th2-specific costimulatory molecule (Banerjee et al 2002). The receptors and signaling events underlying these effects are currently under investigation, as are the *in vivo* immunoregulatory consequences of high-dose gp96 administration.

Fig 8. (a) Binding of 100 μ g of FITC-gp96 and 100 μ g of FITC-BSA to freshly isolated CD3⁺ (light shading) and CD4⁺ T cells (dark shading; data from 2 independent experiments). (b) Binding of FITC-gp96 (light shading) and FITC-BSA (dark shading) to cultured CD3⁺ and CD4⁺ T cells (data from 3 independent experiments). T cells were cultured for 5 days in anti-CD3 mAb-coated plates in the absence and presence of costimulation with an anti-CD28 mAb, at which time they were harvested, washed, and fixed. Differential binding of FITC-gp96 to CD4⁺ and CD8⁺ T cells was determined by costaining purified CD3⁺ T cells with PE-conjugated anti-CD4 mAb and analyzing FITC-gp96 and FITC-BSA binding to the CD4⁺ population (data from 2 independent experiments). In all instances, FITC-gp96 and FITC-BSA bindings were determined by flow cytometry, and data are presented as mean $\pm$ SEM.

Fig 9. Binding of FITC-gp96 and FITC-BSA to costimulated CD3⁺ T cells. Upper panel: concentration-dependent binding of FITC-gp96 to CD3⁺ T cells. Fixed CD3⁺ T cells were incubated with FITC-gp96 (left) or FITC-BSA (right) for 45 minutes at 4°C and washed twice, and binding was measured by flow cytometry. Concentrations of FITC-gp96 and FITC-BSA are indicated and fluorescence histograms are representative of 3 independent experiments. Lower panel: representative fluorescent micrographs of FITC-gp96 and FITC-BSA binding (both 50 µg) to activated CD3⁺ T cells (magnification 40×).

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