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Glucose-Regulated Protein 94/Glycoprotein 96 Elicits Bystander Activation of CD4^+ T Cell Th1 Cytokine Production In Vivo

Julie C. Baker-LePain,* Marcella Sarzotti, † and Christopher V. Nicchitta 2 ♠

Glucose-regulated protein 94 (GRP94/gp96), the endoplasmic reticulum heat shock protein 90 paralog, elicits both innate and adaptive immune responses. Regarding the former, GRP94/gp96 stimulates APC cytokine expression and dendritic cell maturation. The adaptive component of GRP94/gp96 function reflects a proposed peptide-binding activity and, consequently, a role for native GRP94/gp96-peptide complexes in cross-presentation. It is by this mechanism that tumor-derived GRP94/gp96 is thought to suppress tumor growth and metastasis. Recent data have demonstrated that GRP94/gp96-elicited innate immune responses can be sufficient to suppress tumor growth and metastasis. However, the immunological processes activated in response to tumor Ag-negative sources of GRP94/gp96 are currently unknown. We have examined the in vivo immunological response to nontumor sources of GRP94/gp96 and report that administration of syngeneic GRP94/gp96- or GRP94/gp96-N-terminal domain-secreting KBALB fibroblasts to BALB/c mice stimulates CD11b^+ and CD11c^+ APC function and promotes bystander activation of CD4^+ T cell Th1 cytokine production. Only modest activation of CD8^+ T cell or NK cell cytolytic function was observed. The GRP94/gp96-dependent induction of CD4^+ T cell cytokine production was markedly inhibited by carrageenan, indicating an essential role for APC in this response. These results identify the bystander activation of CD4^+ T lymphocytes as a previously unappreciated immunological consequence of GRP94/gp96 administration and demonstrate that GRP94/gp96-elicited alterations in the in vivo cytokine environment influence the development of CD4^+ T cell effector functions, independently of its proposed function as a peptide chaperone. The Journal of Immunology, 2004, 172: 4195–4203.

Glucose-regulated protein 94 (GRP94/gp96), a member of the heat shock protein 90 (Hsp90) family of molecular chaperones, elicits both innate and adaptive immune responses. The latter activity has been proposed to reflect a peptide-binding function for GRP94/gp96 (1, 2). In this view, GRP94/gp96 forms stable complexes with antigenic peptides and thereby assumes the immunological identity of its tissue of origin (1, 2). When presented to professional APCs, GRP94/gp96-peptide complexes access the MHC class I Ag processing pathway, with the bound peptides being presented on APC class I molecules to yield the activation of CD8^+ T lymphocyte responses (3–5). Support for this model has been obtained in experiments wherein GRP94/gp96-peptide complexes, formed in vitro, were demonstrated to elicit peptide-directed CTL (4–6). In addition, studies in mice have demonstrated that immunization with GRP94/gp96 derived from virally transformed or viral protein-infected cells elicits viral peptide-restricted CTL (1, 4, 7).

In addition to its proposed role as a cross-presentation Ag, recent studies have established a role for GRP94/gp96 in the activation of innate immune responses (1, 2). For example, GRP94/gp96 stimulates dendritic cell maturation and elicits cytokine and chemokine secretion from macrophages and dendritic cells in vitro (8–10). These responses occur irrespective of the GRP94/gp96 tissue source and, by virtue of their Ag independence and APC target, identify a potential function for GRP94/gp96 in the regulation of innate immune responses. The identity of the signaling pathway(s) for GRP94/gp96-elicited innate immune responses is under continuing investigation, with recent studies identifying bacterial endotoxin as the stimulus for a number of the reported cellular responses to biochemically purified heat shock/chaperone proteins (11–13). Nonetheless, analyses using tissue culture media derived from GRP94/gp96-secreting cells, low endotoxin preparations of GRP94/gp96, or tissue culture cells expressing a cell surface form of GRP94/gp96 have clearly demonstrated that GRP94/gp96 can elicit the activation/maturation of APCs (8, 11, 14, 15).

Past studies of GRP94/gp96-elicited immune activation emphasized a role for adaptive immunity (16–18). Though such studies have proven instrumental in establishing a tumor suppressive function for GRP94/gp96, they did not explore the potential contribution of innate immune responses to the observed suppression of tumor growth. To distinguish between a GRP94/gp96 function in the activation of innate vs adaptive responses, we have used a cell-based GRP94/gp96 administration strategy wherein animals are administered syngeneic fibroblasts transfected with vectors containing secretory forms of GRP94/gp96 or GRP94/gp96 N-terminal domain (GRP94.NTD), a region lacking canonical peptide-binding motifs (15). Under these conditions, an in situ source of non-tumor-derived GRP94/gp96 is provided to the animal, thereby minimizing potential artifacts arising from antigenic and nonantigenic contaminants commonly observed in biochemically purified protein preparations (11, 19, 20). Using this experimental approach, we previously demonstrated that mice immunized with...
GRP94/gp96- or GRP94.NTD-secreting syngeneic fibroblasts displayed a significant suppression of the growth and metastasis of 4T1 tumor cells, a murine mammary carcinoma cell line. These results suggested that tumor Ag-independent sources of GRP94 possess immune stimulatory functions, though the immunological consequences of tumor Ag-free GRP94/gp96 administration remained unclear (15, 17, 19, 21). The current study investigates the immune responses produced through the in vivo administration of GRP94/gp96- or GRP94/gp96.NTD-secreting syngeneic fibroblasts. We report that GRP94/gp96-secreting fibroblasts elicit a marked increase in TNF-α and IFN-γ production in the peripheral lymphoid tissues. The production of TNF-α and IFN-γ occurred via the GRP94/gp96-dependent activation of CD11b+ and CD11c+ APC and, unexpectedly, through activation of Th1 cytokine production in CD4+ but not CD8+ T cells. These data identify a novel role for GRP94/gp96 in the bystander stimulation of Th1 cytokine expression by BALB/c CD4+ T cells.

Materials and Methods

Mice

Female BALB/c mice (5–6 wk old) were obtained from the National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). Animal experiments were performed in strict accordance with Institutional and Animal Care Use Committee guidelines.

Cell lines, construct preparation, and fibroblast injections

KBALB and YAC-1 cells were obtained from American Type Culture Collection (Manassas, VA) and were tested and found negative for mycoplasma DNA. 4T1 cells were obtained from Dr. C.-Y. Li (Duke University Medical Center, Durham, NC). GRP94.3KDEL and GRP94.NTD cDNAs were subcloned into the pEF/myc/cyto vector (Invitrogen, San Diego, CA) as described previously (15). For transfections, cells were transfected using Lipofectamine transfection reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Mock transfections were performed using empty pEF/myc/cyto vector and Lipofectamine. Twenty-four hours after transfection, KBALB cells were gamma-irradiated using a Mark I 137 Cs irradiator (J. L. Shepherd, San Fernando, CA). Irradiated cells were washed extensively with sterile 1× Dulbecco’s PBS without calcium and magnesium (Life Technologies) before s.c. injection into the left hind limb of animal recipients (1–3 × 106 cells/50 μl). In all experiments wherein animals received four injections, cells were administered on days −21, −18, −14, and −7 before sacrifice. In experiments wherein animals received two injections, cells were administered on days −5 and −2 before sacrifice. In experiments involving one immunization, cells were administered as indicated.

Cytotoxicity assays

Spleens were obtained aseptically and kept in ice-cold CMEM (MEM containing Earle’s salts and glutamine supplemented with 10% heat-inactivated FCS, 25 mM HEPES buffer, 5 × 10−5 M 2-ME, 1% nonessential amino acids, and 1% penicillin-streptomycin-glutamine; Life Technologies) before mechanical separation into single cell suspensions (22). RBCs were lysed with ammonium chloride potassium chloride (ACK) buffer (23) and cells were washed thoroughly into CMEM before further processing. For in vitro stimulations, splenocytes (3 × 106/well) and either irradiated 4T1 cells (2.5 × 106/well) or irradiated BALB/c splenocytes (4 × 106/well) were plated into 24-well plates as previously described (24). On day 5 of culture, effector cells were harvested from replicate wells, pooled, washed, and counted for use in a51Cr release assay. For in vivo stimulations, animals injected previously with irradiated KBALB cells received 1 × 106 viable 4T1 cells by i.p. injection. One week later, animals were sacrificed, and splenocytes were harvested as above. For 51Cr release assays, 4T1 or YAC-1 target cells were labeled with 51Cr (PerkinElmer/NEN Life Sciences, Boston, MA) for 1 h at 37°C and then washed extensively with CMEM. Effecter cells were plated into microtiter plates with labeled targets at various E:T ratios, with a 200-μl final volume per well. After incubation at 37°C for 8.5 h (4T1 targets) or 4 h (YAC-1 targets), 100 μl of cell-free supernatant were collected from each well and counted in a 1272 Cihingamma counter (LKB Wallac, Turku, Finland). Cytotoxicity values were determined by comparing 51Cr counts in wells containing both effectors and targets (experimental) with 51Cr counts in wells containing targets only (spontaneous). The percent-specific cytotoxicity was calculated according to the following formula: [(experimental – spontaneous)/(total – spontaneous)] × 100.

Cytokine ELISA

Lymph node drainage of the left flank area was established by injection of India ink (25). Subcutaneous injections in the left flank drained within 24 h to the ipsilateral popliteal, inguinal, and para-aortic lymph nodes but not to the mesenteric lymph nodes. Animals receiving KALB-GRP94.KDEL, KBALB-GRP94.NTD, or KBALB-Mock cells were sacrificed, and the spleen, draining lymph nodes (ipsilateral popliteal, inguinal, and para-aortic nodes) and nondraining lymph nodes (contralateral popliteal, inguinal, and para-aortic nodes) were harvested. Following mechanical tissue sepa-

Flow cytometric analysis and intracellular cytokine staining

For intracellular cytokine staining, ~2 × 106 cells per sample were incubated with Golgi Plug reagent (BD Biosciences, Mountain View, CA) for 5 h at 37°C, according to the manufacturer’s instructions. Cells were then washed into FACS buffer (PBS, 2% FCS, 0.2% NaN3) and blocked with purified anti-FcγRIII mAb (clone 2.4G2; BD Biosciences). Staining was performed using the appropriate fluorophore- or biotin-conjugated mAbs against cell surface markers: anti-CD11c (clone HL3), anti-CD11b (clone M1/70), anti-CD3 (clone 500A2), anti-CD4 (clone GK1.5), or anti-CD8 (clone 53-6.7), all purchased from BD Biosciences. Further incubation in allogeneic cytokin-in-conjugated streptavidin (BD Biosciences) was performed in some samples wherein a biotinylated mAb was used. Cells were subsequently processed using a Cytofix/Cytoperm kit (BD Biosciences), and intracellular cytokine staining was performed using PE-conjugated anti-TNF-α (clone MP6-XT22; BD Biosciences) or anti-IFN-γ (clone XMG1.2; BD Biosciences) mAbs. Samples were analyzed by the Duke Comprehensive Cancer Center Flow Cytometry Facility using FACSScan and CellQuest software (BD Biosciences). Single-color controls were used for electronic compensation, and 30,000 to 80,000 events were acquired for each sample. Samples were gated on forward and side scatter profiles, and then were gated on expression of the indicated cell surface marker. Percentage of cells present within the gates was determined using Floo software (TreeStar Software, San Carlos, CA).

Flow cytometric analysis of peripheral blood

Blood from animals immunized with KALB-GRP94.KDEL, KALB-GRP94.NTD, or KBALB-Mock cells was collected by cardiac puncture into heparinized syringes and kept on ice. Cells were obtained by mixing 10 μl of whole blood with 90 μl of 2% glacial acetic acid and counting cell nuclei. 51Cr-labeled samples (100 μl) were diluted with an equal volume of FACS buffer (PBS plus 1% BSA), and blocked with purified anti-FcγRIII mAb (clone 2.4G2; BD Biosciences). Staining was performed using FITC-conjugated pan-NK mAb (clone DX5; BD Biosciences). After washing, RBCs were lysed using a whole blood lysing reagent kit (Beckman Coulter, Fullerton, CA). Remaining cells were fixed in 1% paraformaldehyde/PBS and analyzed directly using single-color flow cytometry.

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In vivo depletion of phagocytes

Phagocytic cells were depleted by i.p. injection of 2 mg of iota type II carrageenan (Sigma-Aldrich, St. Louis, MO) on days −5 and −3 before injection of irradiated KBALB cells. Animals were sacrificed 1 wk later, and splenocytes were harvested and processed for two-color flow cytometry by intracellular cytokine staining, as above.

Results

In vivo administration of GRP94/gp96-secreting fibroblasts does not elicit sustained activation of NK activity

With most studies examining the effects of GRP94/gp96 on tumor-directed adaptive immunity, relatively little is known about GRP94/gp96-mediated activation of innate immunity in vivo, in the absence of tumor burden or tumor challenge. One recent study demonstrated that immunization of C57BL/6 mice with GRP94/gp96-secreting E.G7-OVA thymoma cells elicits a transient NK cell activation (26). As a role for NK cells in innate immunity is well established, we determined whether administration of GRP94/gp96-secreting syngeneic fibroblasts resulted in the activation of NK cell function in BALB/c mice. In these experiments, animals were immunized with fibroblasts secreting GRP94/gp96 (KBALB-GRP94KDEL), fibroblasts secreting the GRP94/gp96 N-terminal domain, a region known to lack peptide binding sites (KBALB-GRP94.NTD), or mock-transfected cells (KBALB-Mock, empty vector control). Splenocytes or draining lymph node cells were assayed as effectors against 51Cr-labeled YAC-1, an NK-sensitive lymphoma cell line (27). A modest induction of NK activity was observed in the total splenocyte population of animals immunized with KBALB-GRP94KDEL, KBALB-GRP94.NTD, or KBALB-Mock cells, as compared with naive animals (Fig. 1A). Although there was a trend toward increased NK activity in animals receiving KBALB-GRP94KDEL or KBALB-GRP94.NTD cells, as compared with animals receiving KBALB-Mock cells, these differences were not statistically significant (p = 0.20 for KBALB-GRP94KDEL vs KBALB-Mock; p = 0.16 for KBALB-GRP94.NTD vs KBALB-Mock). In the draining lymph nodes, the magnitude of the NK cell stimulation in immunized animals was less than that found in the spleen, consistent with the presence of fewer NK cells in this location compared with spleen (Fig. 1B). As with the spleen-derived cells, the induction of lymph node NK activity was observed in animals receiving KBALB-GRP94KDEL, KBALB-GRP94.NTD, and KBALB-Mock cells and thus was not a consequence of GRP94/gp96 secretion (Fig. 1B) (p = 0.14 for KBALB-GRP94KDEL vs KBALB-Mock; p = 0.70 for KBALB-GRP94.NTD vs KBALB-Mock). To rule out that a significant increase in NK activity was not missed due to the early kinetics of the response, we examined YAC-1 killing in BALB/c mice receiving KBALB-GRP94KDEL cells 0, 2, and 4 days following cell administration, and again we observed no significant increase (data not shown). As an internal control in these experiments, 51Cr-labeled YAC-1 targets were incubated with splenocytes from naive BALB/c mice stimulated in vitro with 1000 U/ml rIL-2, a potent stimulus for NK activity (28). Under these conditions, a pronounced activation of YAC-1-directed NK function was observed (Fig. 1). The effects of GRP94/gp96-secreting fibroblast administration on the circulating levels of BALB/c NK (DX5+) cells was also examined one week following the final injection; no statistically significant increases in DX5+ NK cell number were observed at this time point (percentage of cells in the large, granular lymphocyte gate was 8.95 ± 0.7 for KBALB-GRP94KDEL, 8.20 ± 0.4 for KBALB-GRP94.NTD, and 8.60 ± 0.12 for KBALB-Mock animals; p = 0.58 for KBALB-GRP94KDEL vs KBALB-Mock and p = 0.40 for KBALB-GRP94.NTD vs KBALB-Mock). In summary, under the described experimental conditions, the administration of syngeneic, GRP94/gp96-secreting fibroblasts to BALB/c mice does not yield a significant induction of NK cell activity. These observations, combined with parallel studies indicating that GRP94/gp96-secreting fibroblasts do not elicit (4T1-directed) CD8+ T cell activation (data not shown), indicate that in the absence of coadministered tumor or foreign Ag(s), GRP94/gp96 does not elicit a substantial activation of cytotoxic effector cell function.

GRP94/gp96 elicits TNF-α and IFN-γ production in peripheral lymphoid organs

As reported previously, suppression of 4T1 tumor growth and metastasis observed in mice receiving non-tumor-derived GRP94/gp96 occurs with minimal tumor lymphocyte infiltrate, suggesting a role for soluble mediators, rather than cytotoxic effector cells, in this phenomenon (15). Given the established role of cytokines in the regulation of tumor growth and metastasis, the effects of GRP94/gp96 administration on cytokine expression were therefore examined. In these experiments, KBALB-GRP94KDEL, KBALB-GRP94.NTD, or KBALB-Mock cells were injected s.c. into BALB/c mice (four administrations), and, one week after the final boost, single cell suspensions were cultured from three anatomical locations: 1) the spleen, 2) the ipsilateral popliteal, inguinal, and para-aortic lymph nodes draining the injection site, and 3) the contralateral popliteal, inguinal, and para-aortic lymph nodes. Culture supernatants were subsequently collected and assayed for the presence of the proinflammatory cytokines IL-12, TNF-α, and IFN-γ. As depicted in Fig. 2, a significant elevation in the levels of TNF-α and IFN-γ...
was observed in cells obtained from animals immunized with GRP94/KDEL- and GRP94.NTD-secreting cells, as compared with control (KBALB-Mock) cells. Splenocytes from animals administered KBALB-GRP94/KDEL or KBALB-GRP94.NTD cells displayed a 1.8- to 2.2-fold increase in TNF-α production and a 7.1- to 8.8-fold increase in IFN-γ production vs KBALB-Mock controls (Fig. 2). Similarly, cells of the draining lymph nodes from animals administered GRP94/KDEL or KBALB-GRP94.NTD cells displayed a 2.6- to 3.0-fold increase in TNF-α production and an 11.7- to 13.3-fold increase in IFN-γ production, as compared with KBALB-Mock controls (Fig. 2). This response was prominently evident near the site of injection; elevated cytokine levels were seen in cells obtained from the ipsilateral but not contralateral lymph nodes (Fig. 2). In contrast to TNF-α and IFN-γ, neither spleen- nor lymph node-derived cells displayed, at the indicated assay periods, enhanced synthesis of IL-12 (data not shown). These data identify the induction of proinflammatory cytokines (TNF-α, IFN-γ) in the peripheral lymphoid organs of BALB/c mice as a prominent in vivo immunological response to GRP94/gp96.

GRP94/gp96 elicits TNF-α and IFN-γ release from CD11b+ and CD11c+ cells

APCs, chiefly macrophages and dendritic cells, display cell surface receptors for GRP94/gp96 and undergo activation in response to GRP94/gp96 addition in vitro (1, 2, 4, 9, 29–31). Evidence for GRP94/gp96-elicited activation of APC in vivo has recently been provided in the findings that GRP94/gp96 elicits the transient expansion of CD11c+ cells in the peripheral blood (26) and the draining lymph nodes (32). To determine whether APC undergo activation in response to tumor Ag-negative preparations of GRP94/gp96 in vivo, BALB/c mice were administered GRP94/gp96-secreting KBALB cells (four injections), and single cell suspensions were prepared from the spleen one week after the final injection. The unfractionated splenocyte population was analyzed for the production of TNF-α or IFN-γ using intracellular cytokine staining. As shown in Fig. 3A, a marked increase in the production of both TNF-α and IFN-γ was observed in CD11b+ cells. Production of TNF-α and IFN-γ by CD11c+ cells was determined after metrizimide gradient enrichment of dendritic cells from the splenocyte population. In contrast to the CD11b+ cells, CD11c+ cells did not display significant production of TNF-α or IFN-γ after four administrations (data not shown). However, previous studies examining dendritic cell proliferation indicate that this cell type may become activated only transiently in response to GRP94/gp96 (26). Thus, we hypothesized that CD11c+ cells may produce proinflammatory cytokines in the immediate interval surrounding administration of GRP94/gp96-secreting cells. As shown in Fig. 3B, CD11c+ cells displayed a significant up-regulation of TNF-α but not IFN-γ production following two injections of GRP94/gp96-secreting fibroblasts. These observations identify CD11b+ and CD11c+ cells in the cytokine response to GRP94/gp96 in vivo and suggest that APC activation in response to tumor Ag-free sources of GRP94/gp96 may be transient (as in the case of CD11c+ cells) or sustained (as in the case of CD11b+ cells) through the course of the cell activation protocol.

**FIGURE 2.** Administration of GRP94/gp96-secreting KBALB fibroblasts elicits expression of TNF-α and IFN-γ in the spleen and draining lymph nodes. KBALB cells were transfected with constructs encoding either a secretory form of GRP94/gp96 (KBALB-GRP94/KDEL), a secretory form of the GRP94 N-terminal domain (KBALB-GRP94.NTD), or empty vector (KBALB-Mock) and administered to BALB/c mice by s.c. injection into the left flank. One week after the final injection, cells were harvested from the spleen, draining lymph nodes (left popliteal, inguinal, and para-aortic nodes), and the corresponding nondraining lymph nodes (right popliteal, inguinal, and para-aortic nodes). Supernatants from the cultured cells were harvested on day 4 and tested for the presence of the indicated cytokines by ELISA. Data represent mean ± SEM. Data are representative of three independent experiments (n = 2 for each experiment). *, A significant increase compared with cells from the same anatomical region harvested from KBALB-Mock-injected mice (Student’s t test, p ≤ 0.05).

GRP94/gp96 elicits the activation of the CD3+/CD4+ T cell subset

It has recently been reported that immunization with GRP94/gp96-secreting tumor cells elicits a dramatic activation and expansion of tumor-directed CD8+ T cells in vivo (26). T cells are abundant residents of both lymph node and spleen and secrete TNF-α and IFN-γ upon activation and so were examined as potential targets in the in vivo response to GRP94/gp96-secreting fibroblasts (33, 34). Splenic T cell cytokine production in response to GRP94/gp96-secreting fibroblasts was determined by two-color flow cytometry, staining for the cell surface T cell marker CD3 and intracellular TNF-α or IFN-γ. As shown in Fig. 4, a significant increase in the proportion of CD3+ cells producing both TNF-α and IFN-γ was observed in cells obtained from KBALB-GRP94/KDEL- and KBALB-GRP94.NTD-treated mice. In the CD3+ population, there was an ~3.0-fold increase in TNF-α-producing cells and an ~3.5-fold increase in IFN-γ-producing cells (Fig. 4). Having identified CD3+ T cell activation as a consequence of in vivo administration of GRP94/gp96-secreting cells, the effects of GRP94/gp96 on CD4+ and CD8+ T cell subsets were subsequently examined. Splenocytes from animals administered KBALB-GRP94/KDEL, KBALB-GRP94.NTD, or KBALB-Mock cells were obtained and analyzed by two-color flow cytometry for CD4 or CD8 expression and intracellular TNF-α or IFN-γ accumulation. The results of these analyses are
depicted in Fig. 5, where it is shown that administration of KBALB-GRP94.KDEL or KBALB-GRP94.NTD resulted in a 2.5- to 2.7-fold increase in intracellular TNF-α staining in the CD4⁺ population; no comparable increase in TNF-α staining was observed in the CD8⁺ population. Strikingly, the CD4⁺ T cell fraction obtained from KBALB-GRP94.KDEL or KBALB-GRP94.NTD treated animals displayed a 5.0- to 5.5-fold increase in intracellular IFN-γ staining (Fig. 5); again, no comparable increase was observed in the CD8⁺ population. These data indicate that GRP94/gp96 elicits the activation of CD4⁺ T cells to produce cytokines characteristic of the Th1 phenotype.

Kinetics of CD4⁺ T cell cytokine production following administration of GRP94/gp96

To further characterize the GRP94/gp96-elicited activation of the CD4⁺ T cell population, the kinetics of the response and the requirement for multiple administrations of GRP94/GRP94.NTD-secreting fibroblasts was determined. As detailed in Fig. 6, a
GRP94/gp96-dependent activation of the CD4+ T cell population was not observed at day 3 postadministration. However, by day 7, TNF-α and IFN-γ production had increased substantially (Fig. 6). To further examine this response, animals were given a second injection of KBALB-GRP94.NTD-producing cells on day 7, and CD4+ T cell production of TNF-α and IFN-γ was examined alongside the responses from animals receiving a single injection of KBALB-GRP94.NTD (Fig. 6). Under these conditions, further enhancement of IFN-γ but not TNF-α production was observed in the CD4+ T cell population (Fig. 6). Together, the data indicate that the activation of cytokine expression by the CD4+ T cell subset is evident following a single administration of GRP94.NTD-secreting cells. The absence of added Ag, combined with the large fraction of responding cells (>20%), suggests that the observed CD4+ T cell activation occurs via a bystander process.

Phagocytic cells are required for GRP94/gp96-elicited CD4+ T cell activation

As shown above, GRP94/gp96 administration elicits CD11b+ /CD11c+ APC and CD4+ T lymphocyte activation. For the CD4+ T cell population, activation is evident in a large fraction of the gated cells. Such responses are indicative of a cytokine-mediated, Ag-independent bystander phenomenon, perhaps mediated by the observed APC-derived cytokines. To test this hypothesis, animals were injected with either PBS (control) or carrageenan, a compound that abrogates phagocytic cell function in vivo (35, 36). Subsequently, animals received a single s.c. injection of KBALB-GRP94.NTD cells, and following a 7-day interval, splenocytes were harvested and stained using anti-CD4, anti-IFN-γ, and anti-TNF-α Abs. As shown in Fig. 7, carrageenan treatment markedly attenuated the magnitude of the CD4+ response to GRP94/gp96, suggesting an essential role for phagocytic cells in the phenomenon of GRP94/gp96-elicited CD4+ T cell activation.

Discussion

In the current study, the immunological consequences of in vivo administration of GRP94/gp96 were examined. In these experiments, nontumor, haplotype-matched cells, bearing expression vectors encoding secretory forms of GRP94/gp96 were used as Ag-negative sources of GRP94/gp96. Three primary findings are reported: 1) in vivo administration of GRP94/gp96 elicits the activation of CD11b+ and CD11c+ APCs; 2) in vivo administration of GRP94/gp96 is accompanied by a rapid and substantial induction of Th1 cytokine production by CD4+ T lymphocytes; and 3)

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**Figure 5.** GRP94/gp96 administration elicits TNF-α and IFN-γ production from CD4+ T cells. BALB/c mice were administered KBALB-GRP94ΔKDEL, KBALB-GRP94.NTD, or KBALB-Mock cells, and splenocytes were harvested and incubated for 5 h in Golgi Plug reagent before staining with anti-CD4 or anti-CD8 cell surface markers. Intracellular staining with anti-TNF-α or anti-IFN-γ was then performed as in Fig. 3. Numbers represent the percentage of CD4+ or CD8+ cells present within the TNF-α+ or IFN-γ+ gates. Results are representative of three independent experiments (n = 2 for each experiment).

**Figure 6.** Kinetics of CD4+ T cell cytokine production in response to GRP94/gp96 administration. BALB/c mice were given one s.c. injection of KBALB-GRP94.NTD cells, and splenocytes were harvested at various time points (days 0, 3, and 7) and tested for the production of TNF-α or IFN-γ using intracellular staining, as in the previous figures. A subset of animals was boosted with a second injection of KBALB-GRP94.NTD cells on day 7, and splenocytes were harvested 2 days later (day 9) and analyzed as above. Samples were analyzed using two color flow cytometry, as above. Numbers represent the percentage of CD4+ cells in the TNF-α+ or IFN-γ+ gate.
GRP94/gp96-dependent activation of CD4⁺ T lymphocyte cytokine production is dependent upon phagocytic cell function. These data demonstrate that GRP94/gp96 can stimulate APC function in vivo and identify the bystander activation of CD4⁺ T cells as a novel mechanism whereby GRP94/gp96 modulates cellular immune responses.

The observation that tumor Ag-negative sources of GRP94/gp96 elicit significant immune responses in vivo and that, as previously reported, such responses can be sufficient for tumor rejection is consistent with a prominent adjuvant, or immune response modifier, function for GRP94/gp96 (2, 15, 17, 19). It is important to note that distinguishing between immune responses to GRP94/gp96 alone (i.e., irrespective of predicted bound Ags) and immune responses to putative GRP94/gp96-Ag complexes is difficult when tumor tissue serves as the source of GRP94/gp96. In scenarios where GRP94/gp96 is derived from tumor tissue via biochemical purification, the absolute immunological purity of the protein is of concern (20). In experimental scenarios where secretory or cell surface forms of GRP94/gp96 are derived from engineered tumor cells, it is unclear whether the elicitation of a tumor-directed CD8⁺ T cell response is a direct consequence of the cross-presentation of predicted GRP94/gp96-associated tumor Ags or, alternatively, the processing of the engineered tumor cells themselves by APC (8, 26, 37). As it is well established that tumor cell-derived GRP94/gp96 can dramatically facilitate CD8⁺ T cell activation, the resolution of these questions will bear significantly on understanding regarding the mechanism of chaperone/heat shock protein-elicted tumor rejection (1, 8, 19, 21, 26, 38).

T cell activation is known to require three signals: 1) Ag, 2) costimulation, and 3) adjuvant or IL-12. Drawing from this paradigm, tumor cell-secreted GRP94/gp96 could serve either a "signal 1" role, through its proposed function as an in vivo peptide binding protein, and/or a "signal 3" role, via a biological adjuvant function (1, 10, 14, 19, 37, 39). As demonstrated in the current study, non-tumor-derived GRP94/gp96 elicits APC activation and the bystander activation of Th1 cytokine production from BALB/c CD4⁺ T cells, but not the activation of tumor-directed cytotoxic cell function. It would appear, then, that GRP94/gp96 can function as a "signal 3" in vivo. This conclusion is consistent with the view that heat shock/chaperone proteins, as constitutively intracellular proteins, signal immunological "danger" when present in the extracellular space (40). In support of this view, a recent study reported that transgenic mice bearing a cell surface form of gp96 (96tm-Tg) under control of a pan-specific promoter displayed chronic dendritic cell activation and systemic autoimmune disease, suggesting a GRP94/gp96-elicted loss of peripheral tolerance (41). The observation that the hyperstimulated DC phenotype of 96tm-Tg mice is accompanied by the activation of self-reactive T cells raises the possibility that such self-reactive cells, or other cytotoxic effectors, could function in the suppression of tumor growth and/or the provision of tumor Ags to dendritic cells (21). In all likelihood, the magnitude of such a response would reflect the GRP94/gp96-elicted dendritic cell activation state and its temporal persistence, both of which would be expected to correlate with GRP94/gp96 dose and availability. These and related questions are currently under investigation.

Adjuvants interact with pattern recognition receptors expressed on APCs to yield complex patterns of activation, maturation, and cytokine expression (42-44). GRP94/gp96 has been reported to elicit APC activation via Toll-like receptors 2 and 4, both important regulators of the innate immune response (14, 41). In addition, in vitro studies using endotoxin-free purified GRP94/gp96 or tissue culture media containing secreted GRP94/gp96 demonstrate that GRP94/gp96 elicits dendritic cell maturation (9, 10, 15, 32) and macrophage activation (11). Further evidence that GRP94/gp96 or GRP94.NTD functions in vivo as a biological adjuvant is evident in the data presented in Fig. 3, which demonstrates that cell-based administration of GRP94/gp96 or GRP94.NTD yielded the enhanced production of TNF-α and IFN-γ by APC. The observation that cell-based delivery of GRP94/gp96 or GRP94.NTD
elicits cytokine production by CD11b+ and CD11c+ APC suggests that these cells may serve as a source of cytokines that direct the subsequent activation of GRP94/gp96-elicited CD4+ T cell response. Such a proposal is consistent with the view that the local cytokine environment serves as the primary arbitrator of CD4+ T cell differentiation and is further supported by the observations that CD11b+ APC perform an important role in the regulation of Th1-polarized CD4+ T cell differentiation (45-48).

Cell-based administration of GRP94/gp96 or GRP94.NTD to BALB/c mice results in IFN-γ production in a relatively large fraction (>20%) of the CD4+ T cell population, suggesting that GRP94/gp96 can elicit an Ag-independent bystander activation of BALB/c CD4+ T cells. In support of this conclusion, we note that syngeneic fibroblasts were used as the source of GRP94/gp96 and GRP94.NTD in the current study; no defined Ag was concurrently provided. In addition, administration of irradiated, control (empty vector transfected) KBALB fibroblasts resulted in a far weaker CD4+ response. Lastly, cell-based secretion of the GRP94.NTD, which lacks a canonical peptide (Ag) binding site, is as effective in eliciting CD4+ cytokine production as the full-length protein. Interestingly, TCR-independent bystander activation of CD4+ IFN-γ production has previously been reported to occur in response to particular cytokine microenvironments (49, 50). For example, Chakir et al. (51) report that stimulation of naïve CD4+ cells with IL-2 and IL-12 or IL-18, in the absence of TCR ligation, yields the activation of IFN-γ secretion; Yang et al. (52) report that IL-12 and IL-18, combined, are sufficient to elicit TCR-independent activation of CD4+ IFN-γ production. In light of these findings, we favor a model in which GRP94/gp96 elicits APC activation and migration to peripheral lymphoid tissues, with the cohort of cytokines secreted by the GRP94/gp96-activated APC fraction functioning to direct the bystander activation of CD4+ T cells.

BALB/c mice, the animal model used in the current study, display an impaired IL-12/IFN-γ-mediated Th1 response, which is associated with a high susceptibility to infectious diseases such as cutaneous leishmaniasis (53). Recent studies suggest that BALB/c CD11b+ dendritic cells display altered cytokine profiles, as compared with those seen in Leishmania-resistant strains (i.e., C57BL/6), and such differences result in the relative inability of BALB/c mice to generate Th1 responses to Leishmania infection (46-48). As GRP94/gp96 elicits a (bystander) Th1 CD4+ phenotype in BALB/c mice, there must exist BALB/c APC receptor/signaling pathways whose activation is sufficient for the elicitation of Th1 responses. We hypothesize that the GRP94/gp96-mediated activation of such pathway(s) is sufficient to circumvent the inherent defect in the BALB/c response to parasitic infection.

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