Archaeosomes Induce Enhanced Cytotoxic T Lymphocyte Responses to Entrapped Soluble Protein in the Absence of Interleukin 12 and Protect against Tumor Challenge

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ABSTRACT

Archaeosome adjuvants formulated as archaeal ether glycerolipid vesicles induce strong CD4+ as well as CD8+ CTL responses to entrapped soluble antigens. Immunization of mice with ovalbumin (OVA) entrapped in archaeosomes composed of the total polar lipids of Methanobre vibacter smithii resulted in a potent OVA-specific CD8+ T-cell response, and subsequently, the mice dramatically resisted solid tumor growth of OVA-expressing EG.7 cells and lung metastasis of B16OVA melanoma cells. Prophylactic protection was antigen-specific because tumor curtailment was not seen in mice injected with antigen-free archaeosomes. Similarly, there was no protection against B16 melanoma cells lacking OVA expression. Furthermore, in vivo depletion of CD8+ T cells abrogated the protective response, indicating that the antitumor immunity was mediated by CTLs. Depletion of CD4+ T cells also resulted in partial loss of tumor protection, suggesting a beneficial role for T-helper cells. Interestingly OVA-archaeosomes induced enhanced CTL response in the absence of interleukin 12 and IFN-γ. Furthermore, interleukin 12-deficient mice mounted strong tumor protection. However, IFN-γ-deficient mice, despite the strong CTL response, were only transiently protected, revealing a need for IFN-γ response in tumor protection. Archaeosomes also facilitated therapeutic protection when injected into mice concurrent with tumor cells. Interestingly, even archaeosomes lacking entrapped antigen mediated therapeutic protection, and this correlated to the activation of innate immunity as evident by the increased tumor-infiltrating natural killer and dendritic cells. Thus, archaeosomes represent effective tumor antigen delivery vehicles that can mediate protection by activating both innate as well as acquired immunity.

INTRODUCTION

Host CD8+ CTL responses are critical for long-term protection against tumors. In mice, spontaneous tumors develop in the absence of CD8+ T-cell cytotoxicity (1, 2). In humans, host CD8+ CTL responses to tumor-associated antigens have been shown to be highly beneficial to patients, particularly those with metastatic disease (3). Thus, there is considerable interest in the generation of cancer immunotherapies (4). The success of cancer vaccines depends on two key aspects: identification of specific antigenic targets and the ability to evoke a strong and appropriate immune response. In recent years, advances in the molecular characterization of tumors, coupled with a clearer understanding of tumor immunology, has lead to the identification of several tumor-associated antigens that could be used as effective vaccination targets (5). However, the use of highly purified antigens poses a challenge for delivering antigen to the appropriate antigen-processing pathway for the induction of CD8+ T-cell responses that are traditionally induced only by endogenous proteins.

Two main approaches are being investigated for induction of tumor antigen-specific response. In the first, successful vaccination in experimental models has been achieved by antigen-primed dendritic cells (6). The use of this approach, although efficient at targeting antigen to the appropriate cellular compartment for induction of CD8+ T-cell response, is cumbersome for human use because it involves use of patient cells, purification, and culture of dendritic cells. Thus, the second approach involving cell-free vaccination would be more suitable for clinical use. This, however, necessitates the availability of effective and safe adjuvants capable of inducing CD8+ CTL responses to exogenous antigens. Thus far, IFA is the most common adjuvant of T-cell responses used in cancer vaccination clinical trials. IFA not only provides strong immunostimulation but also protects peptide antigens from rapid degradation and has been effectively used in immunotherapy against melanoma (3). Nevertheless, IFA is not preferred for human vaccination because of its undesirable side effects such as erythema and induration at the injection site. Furthermore, tolerance rather than immunity with IFA has been reported previously (7).

Liposomes constitute particulate antigen carriers and are conventionally composed of natural or synthetic ester phospholipids. Liposomal adjuvants have been studied extensively (8), and a liposome-based vaccine against hepatitis A has been licensed for human use (9). However, often the antigenic depot provided by liposomes leads only to MHC class II presentation of the processed antigen. Furthermore, codelivery of immunostimulating agents such as Lipid A, Cholera toxin, or cytokines is required for effective costimulation and sustained immunity (10–12).

Archaea consist of organisms distinct from eubacterial and eukaryotic cells due, in part, to their unique, polar lipid structures (13). The TPLs of various archaea can be formulated into stable liposomal vesicles, termed archaeosomes (14). We have previously reported the ability of archaeosomes to induce long-lasting antibody, CD4+ T-helper cell and CD8+ CTL responses to entrapped proteins (15, 16). Furthermore, archaeosomes recruit and activate dendritic cells, making them attractive adjuvant candidates for tumor vaccination (17). We report here that archaeosomes induce prophylactic and therapeutic tumor-protective CD8+ CTL responses in two different experimental tumor models and even in the absence of IL-12. Furthermore, archaeosomes facilitate innate immunity by promoting the infiltration of dendritic and natural killer cells to the tumor site.

MATERIALS AND METHODS

Growth of Archaea and Extraction of Lipids. Methanobre vibacter smithii ALI (DSM 2375) was cultivated in a 75-liter fermenter as described earlier (18). Total lipids were extracted from frozen cell pastes, and the TPLs were collected as the acetone-insoluble fraction (18).

Antigens. OVA, grade V was purchased from Sigma Chemical Company (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). On SDS-PAGE gel analysis, it was noted to be homogeneous, lacking any fragments and/or peptides.

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3 The abbreviations used are: IFA, incomplete Freund’s adjuvant; APC, antigen-presenting cell; IL, interleukin; FBS, fetal bovine serum; OVA, ovalbumin; TPL, total polar lipid.
Preparation and Characterization of Archaeosomes and Conventional Liposomes. Archaeosomes were prepared by attaching L-dimyristoylphosphatidylcholine (DMPC), L-dimyristoylphosphatidylglycerol (DMPG), and cholesterol (CHOL) purchased from Sigma Chemical Company. Archaeosomes and conventional liposomes were prepared by hydrating (1 h at 35°C) 20 mg of dried lipid film in 2 ml of pyrogen-free deionized water. The multilamellar vesicles obtained by vortexing were passaged through an EmulsiFlex pressure extruder (Avestin, Inc., Ottawa, Ontario, Canada) to obtain unilamellar vesicles. The vesicles were lyophilized and then rehydrated in a small quantity of water, and the size of vesicles reduced by pressure extruding through polycarbonate membrane filters (serially through 1000- and 400-nm pore diameter filters) using a Liposofast (Avestin, Inc.) apparatus. The vesicles were annealed by incubation at 4°C for 18 h and then filter sterilized (0.45 µm; 25-mm Millex-HV, nonpyrogenic, low protein binding filter; Millipore Corporation, Bedford, MA). Where required, antigen was encapsulated into the archaeosomes by the dried-reconstituted vesicle method (19). Briefly, OVA (in aqueous solution at 1:5 ratio by weight of the lipid) was added to the antigen-free vesicles before the lyophilization step, and the formulation was mixed by vortexing. The lyophilized preparation was rehydrated in one-tenth the original volume of water. The average vesicle diameter was reduced by extruding through polycarbonate filters followed by annealing. OVA that was not associated with the vesicles was removed by ultracentrifugation (200,000 × g, for 30 min), followed by washing the vesicle pellet twice in water. The formulations were filter sterilized and diluted to achieve the final concentration in PBS (10 mM potassium phosphate (pH 7.14) containing 160 mM NaCl). The formulations were stored at 4°C until use. The mean vesicle diameters were in the range of 110–170 nm, determined by number-weighted Gaussian size distributions using a Nicomp Particle Sizer (model 370; Nicomp, Santa Barbara, CA). The amount of protein or peptide incorporated into the vesicles was estimated by the SDS Lowry method after lipid removal (20) and compared with standard curves constructed for OVA. The ratio of protein to lipid (µg/mg) was based on the salt-free dry weights of the vesicles. All glassware used in preparation of archaeosomes was prebaked (6 h at 180°C) to render it pyrogen-free and sterilized by autoclaving before use. Pyrogen-free water was used throughout.

Mice and Immunizations. Inbred, 6–8-week-old female C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). C57BL/6–IL-12-deficient (B6.129-IL12m1ts) and C57BL/6–IFN-γ-deficient (B6.129S7-Ifnγm1ts) mice and their controls were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the animal facility of the Institute for Biological Sciences, National Research Council, in accordance with guidelines from the Canadian Council on Animal Care. Mice were inoculated intraperitoneally with 1 × 10^7 cells of the tail with antigen-free archaeosomes or OVA in PBS (no adjuvant), or entrapped in archaeosomes (OVA-archaeosomes). Immunization volume was 100 µl, antigen dose 15 µg/injection, and lipid concentration 0.23 mg/injection. The immunization schedules were as described in figure legends.

Cell Lines. EL-4 (H-2b) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 2-mercaptoethanol, 8% FBS (HyClone, Logan, UT), and 10 µg/ml gentamicin (Life Technologies, Inc.). EG.7 cells, a subclone of EL-4 stably transfected with the gene encoding OVA (21), were obtained from Dr. D. Erber (University of Rochester, Rochester, NY). B16 cells were maintained in RPMI 1640 containing 8% FBS, whereas B16OVA cells were cultured in RPMI 1640 plus 8% FBS, additionally containing 400 µg/ml G418.

CTL Assays. Single-cell suspensions from pooled spleens (n = 2–4) of immunized mice were selectively lysed by erythrocytes with Tris-buffered ammonium chloride (pH 7.2; Sigma Chemical Company). After washing, 30 × 10^6 spleen cells were cultured with 5 × 10^5-irradiated (10,000 rads) EG.7 cells in 10 ml of RPMI 1640 plus 8% FBS containing 0.1 ng/ml IL-2 in 25-cm² tissue culture flasks (Falcon, Becton Dickinson, Franklin Lakes, NJ) kept upright. After 5 days (37°C, 8% CO₂), the cells were recovered from the flask and used as effectors in a standard 3¹Cr-release CTL assay. Briefly, for the CTL assay, EL-4 and EG.7 (OVA expressing) target cells were labeled with 51Cr (100 µCi) for 45 min, in 50 µl of RPMI 1640 plus 8% FBS. Targets were then washed, and various ratios of effectors and targets were cocultured for 4 h in 96-well round-bottomed tissue culture plates (Falcon). Supernatants were collected, and radioactivity detected by gamma counting. The percent-specific lysis was calculated using the formula: [(cpm experimental – cpm spontaneous)/cpm total-cpm spontaneous] × 100.

Enumeration of IFN-γ-secreting Cells. Enumeration of IFN-γ-secreting cells was done by ELISPOT assay (22). Briefly, spleen cells were incubated in anti-IFN-γ antibody-coated ELISPOT plates in various numbers (in a final cell density of 5 × 10^6/well) in the presence of IL-2 (0.1 and 1 ng/ml) and RPMI 1640 or OVA (257–264) (10 µg/ml) for 48 h at 37°C, 8% CO₂. The plates were subsequently blocked, incubated with the biotinylated secondary antibody (4°C, overnight) followed by avidin-peroxidase conjugate (room temperature for 2 h). Spots were revealed using 3,3-diaminobenzidine.

Tumor Models. Two murine tumor models (solid tumor and metastasis) were established to assess the relative protective potential of CD8+ T cells induced by archaeosomes. Both tumor models used cells expressing the gene for OVA. For the solid tumor model, 7 × 10⁶ OVA-expressing EG.7 cells (in PBS plus 0.5% normal mouse serum) were injected in the shaved lower dorsal region. From day 5 onwards, detectable solid tumor was measured using calipers. Tumor size, expressed in mm², was obtained by multiplication of diametrically perpendicular measurements. In the metastasis model, 5 × 10⁶ B16OVA or control B16 tumor cells were injected i.v., and 15 days later, lungs were removed and the number of black tumor foci counted visually under a dissection microscope.

In Vivo Depletion of T Cells. In vivo depletion of CD4+ or CD8+ T cells was achieved by injection of mice with control (rat-IgG), anti-CD4 (GK1.5) or anti-CD8 (243) antibody. Each mouse was injected twice a week, with 250 µg of antibody i.p. Antibody injections commenced a week before tumor challenge and continued until 2 weeks post-tumor challenge. This regimen of antibody injection consistently yielded >95% depletion of the appropriate T cell populations.

Flow Cytometric Analysis of Tumor-infiltrating Lymphocytes. Tumors were excised on day 15, and single-cell suspensions of the tumors were made by gently grinding between frosted ends of two glass slides in 5 ml of HBSS (Life Technologies, Inc.) containing collagenase (Boehringer Mannheim, La- val, Quebec, Canada) at a concentration of 0.28 mg/ml. This suspension was then transferred to a 50-ml Falcon tube and shaken in a 37°C water bath for 30 min. Enzyme reaction was stopped by addition of 40 ml of PBS containing 1% FBS. Cells were passed through a nylon strainer (Falcon) to remove any debris and washed twice before staining with selected antibodies for flow cytometric analysis. For flow cytometric analysis, cells were incubated on ice (10⁶ cells in 50 µl of RPMI 1640 plus 1% FBS) with antimouse CD3/CD16 (FcγRIII receptor). After 30 min, aliquots were washed and incubated in 50 µl of RPMI 1640 plus 1% FBS with phycoerythrin-labeled antinouse antibodies. The antibodies used included Mac1, DX5, CD4, and CD8 obtained from Pharmingen Canada, Inc. (Mississauga, Ontario, Canada). Antibody incubation was for 30 min on ice. Cells were washed and fixed in 1% formaldehyde in PBS and analyzed by flow cytometry (EPICS XL; Beckman Coulter Corp., Hialeah, FL) using the EXPO software.

Statistical Analyses. Student's t test was done to determine statistical significance between immunization groups for single point observations of solid tumors, whereas Mann-Whitney rank test was done for nonparametric data (e.g., metastatic tumor foci). For analysis of solid tumor progression trends over time, ANOVA was used. P < 0.05 was considered statistically significant.

RESULTS

Archaeosomes Induce CD8+ T-Cell Responses to Entrapped OVA. C57BL/6 mice were immunized (s.c.) on days 0 and 21 with OVA entrapped in archaeosomes composed of M. smithii TPL. On day 35, the spleens were removed, and the CTL response evaluated after restimulation with EG.7 cells for 5 days. In a standard 3¹Cr release assay, spleen cells from OVA-archaeosome-immunized mice
exhibited strong CTL activity toward EG.7 (specific targets expressing OVA) but not EL-4 cells (Fig. 1a).

To ascertain the T-cell frequencies, the number of antigen-specific IFN-γ-secreting CD8+ T cells was determined. The immunodominant CTL epitope in OVA for H-2Kb haplotype has been shown to be OVA257–264 (SIINFEKL; Ref. 23). Therefore, IFN-γ production in response to stimulation with this peptide was determined by ELISPOT. Fig. 1b shows the CD8+ T-cell frequency achieved after OVA-M. smithii immunization.

**OVA-Archaeosome-immunized Mice Are Protected against EG.7 Solid Tumor Challenge.** We determined whether the induction of a CD8+ T-cell response by OVA-archaeosomes would influence subsequent protection against OVA-expressing tumors. Fig. 2 illustrates the s.c. tumor progression in various groups of preimmunized mice upon challenge with OVA-expressing EG.7 cells. In naïve control mice, solid tumors grew steadily, being detectable as early as 5 days after challenge. By day 12–15, the tumors had reached a size of ~300 mm², and the mice were euthanized (Fig. 2a). Mice that were immunized with OVA in the absence of adjuvant also rapidly developed steadily progressing tumors (Fig. 2b). In contrast, mice that were immunized with OVA-M. smithii archaeosomes and subsequently challenged with EG.7 cells exhibited dramatic protection against tumor growth (P < 0.001), with mice remaining tumor free even at 20 days after challenge (Fig. 2c). To ascertain that the tumor-protective response noted was mediated by antigen-specific CTLs, we tested the effect of injecting mice with equivalent amounts of antigen-free archaeosomes. In the absence of antigen, archaeosomes did not afford prophylactic protection against tumor challenge (Fig. 2d). Fig. 2 is a representative of six such experiments conducted. Combining the data from all these experiments, of the 32 OVA-M. smithii mice tested, >50% remained tumor free in the long term (>60 days), whereas others developed small tumors beyond 4–6 weeks.

**The Antitumor Effect of Immunization with OVA-Archaeosomes Is Mediated by CD8+ T Cells.** To test whether the tumor-protective response observed after OVA-archaeosome vaccination was a result of the CTL response elicited, we depleted CD8+ or CD4+ T-cell subsets in vivo before tumor challenge. Subset depletion was ascertained by flow cytometry (data not shown), and consistently, we failed to detect the presence of the cells we were depleting in spleens. Fig. 3 indicates tumor progression in control and T-cell-depleted mice. In all groups, unimmunized mice showed rapid tumor progression, and these mice were euthanized by day 10–12. As seen previously, non-T-cell-depleted control mice immunized with OVA-archaeosomes demonstrated strong protection against tumor challenge (Fig. 3a). Fig. 3b clearly demonstrates that in the absence of CD8+ T cells, tumor protection afforded by archaeosomes is abrogated. Interestingly, in the absence of CD4+ T cells, OVA-archaeosome-immunized mice exhibited a delay in tumor progression (Fig. 3c) compared with the naïve CD4+ T-cell-depleted controls. For example, on day 7, only 2 of 5 mice in the archaeosome-immunized group had visible tumors (Fig. 3c), compared with >50 mm² tumors in all 5 mice in the naïve group.

![Fig. 1. Induction of CTL response to OVA entrapped in archaeosomes composed of M. smithii TPL. C57BL/6 mice were immunized s.c. on days 0 and 21 with 15 μg of OVA encapsulated in archaeosomes composed of the TPL of M. smithii. Splenocytes were obtained on day 35, and pooled spleen cells (n = 3/group) were stimulated with irradiated EG.7 cells for 5 days before assessing 4 h CTL activity against 51Cr-labeled targets (a). CTL data represent percentage of specific lysis of triplicate cultures ± SD at various E:T ratios on EL-4 (nonspecific target) and EG.7 (specific target expressing OVA peptides) cells. Various numbers of spleen cells were also stimulated with OVA257–264 (10 μg/ml) for 24 h in plates precoated with capture anti-IFN-γ antibody. The numbers of IFN-γ-secreting cells were then evaluated by ELISPOT assay (b). Number of spots/1 × 10⁶ spleen cells is indicated.](cancerres.aacrjournals.org)
OVA-Archaeosome-immunized Mice Resist Challenge by B16OVA-metastatic Melanoma Cells. We also measured protection against the tumor cells (B16OVA and B16) in the metastasis model where tumor cells proliferate in the lungs after i.v. injection. Mice were immunized with OVA-archaeosomes and then challenged with B16OVA or B16 tumor cells. Lung foci visible as black spots were enumerated 2 weeks post-tumor challenge. Naïve mice challenged with B16OVA tumor cells harbored between 250 and 500 tumor foci (Figs. 4 and 5). On the other hand, OVA-archaeosome-immunized mice were strongly protected (*P* < 0.01). Indeed, among the OVA-archaeosome-immunized group, most mice harbored no visible foci as clearly evident from the color illustration (Fig. 4). This prophylactic protection was antigen specific because mice that received injection of antigen-free *M. smithii* archaeosomes harbored substantial tumor foci (Fig. 5a). Furthermore, OVA-*M. smithii* immunized mice were not protected against challenge by B16 tumor cells, lacking OVA expression (Fig. 5b).

Archaeosomes Induce CTL Responses in the Absence of IL-12 and IFN-γ. As cytokines strongly influence T cell differentiation and expansion, we studied the effects of the two main inflammatory cytokines IFN-γ and IL-12, on archaeosome induced CTL responses. Fig. 6 shows the CTL response of effectors generated from the spleen cells of control and cytokine-deficient mice after OVA-*M. smithii* archaeosome immunization. Interestingly, in both IL-12- and IFN-γ-deficient mice the CTL response was substantially enhanced compared with the response observed in control mice (wild type). This response was specific as no killing was seen toward EL-4 targets lacking antigen. Fig. 6 is representative of four different experiments conducted in a total of 8 IL-12−/− and IFN-γ−/− mice each (cells from 2 mice/group were pooled for each experiment). In all experiments, the CTL response in cytokine-deficient mice was consistently 2–5-fold higher (based on lytic units) than controls. These results suggest that in the absence of IFN-γ and IL-12 expression, an even further CTL expansion can occur than that observed in control mice.

![Fig. 3. Role of CD8+ and CD4+ T cells in tumor protection mediated by OVA-archaeosomes. C57BL/6 mice were immunized on days 0 and 21 with 15 µg of OVA entrapped in 227 µg of *M. smithii* archaeosomes. Age-matched unimmunized mice were included as controls. Commencing on day 24, mice received injections of either control (rat IgG) or anti-CD4 (GK1.5) or anti-CD8 (2.43) antibody. Each mouse was injected twice a week until day 42, with 250 µg of antibody/injection. Mice were challenged with EG7 tumor cells on day 35. Tumor size was monitored using calipers, and tumor progression in individual mice among each group is indicated in the various panels. a, protection in OVA-*M. smithii*-immunized control mice is significantly different from naïve controls (*P* < 0.0001). b, tumor growth in naïve and OVA-*M. smithii*-immunized mice, after CD8+ T-cell depletion, is not significantly different (*P* = 0.41). c, CD4+ T-cell-depleted, OVA-*M. smithii*-immunized mice show significant (*P* < 0.001) delay in tumor progression in comparison to CD4+ T-cell-depleted naïve mice. Data analyzed by ANOVA over time.

![Fig. 4. Prophylactic vaccination against B16OVA metastatic melanoma using OVA-archaeosomes. C57BL/6 mice received injections of 15 µg of OVA entrapped in *M. smithii* archaeosomes on days 0 and 21. On day 35, groups of naïve and OVA-archaeosome-immunized mice were challenged i.v. with B16OVA melanoma cells. On day 49, mice were euthanized, lungs harvested, and the black metastatic foci enumerated under a dissection microscope. In 2 of the naïve control mice, because of the sheer high numbers, it was difficult to enumerate accurately the tumor foci, and therefore, they were arbitrarily scored as >500. The mean ± SD of the tumor foci is indicated in the figure. OVA-*M. smithii* values are significantly different (*P* = 0.0079) in comparison to naïve mice, as analyzed by Mann-Whitney rank test. These data are representative of three experiments.](cancerres.aacrjournals.org)
Archaeosomes Induce Tumor-protective Responses in the Absence of IL-12. We then tested whether the strong CTL response in cytokine-deficient mice correlated with tumor protection. Naïve and OVA-M._smithii_ archaeosome-immunized IL-12- and IFN-γ-deficient mice were challenged with EG.7 tumor cells. Fig. 7 indicates that tumors generally grew rapidly in IL-12- and IFN-γ-deficient naïve mice. Correlating with the enhanced CTL responses induced after OVA-archaeosome immunization, IL-12-deficient mice were strongly protected against tumor growth (Fig. 7a). Indeed, until day 20, only 1 of 4 mice showed visible tumors. In contrast, OVA-archaeosome immunized IFN-γ-deficient mice that also exhibited enhanced CTL responses displayed only a delay in the onset of tumors (Fig. 7b). Although no tumors were visible until day 10, subsequently, the tumors progressed rapidly. Thus, archaeosomes induce IL-12-independent tumor-protective CD8+ T-cell responses. In contrast, our results reveals a contrasting role for IFN-γ in tumor protection: although the expansion of tumor-protective CD8+ T-cell responses are enhanced in the absence of IFN-γ, both IFN-γ and CTL responses are required for tumor protection.

Therapeutic Tumor Protection Mediated by Archaeosomes. We then tested whether OVA-archaeosomes would facilitate protection when injected into mice concurrent to tumor challenge (Fig. 8). For this purpose, OVA entrapped in _M._smithii_ archaeosomes was injected into mice on days 1 and 7 post-tumor challenge. Fig. 8a shows the progression of tumors in naïve mice, and Fig. 8b indicates that OVA in PBS (no adjuvant) does not provide any therapeutic tumor regression. Figs. 8c shows that OVA-archaeosomes (15 μg of OVA entrapped in 227 μg of _M._smithii_TPL) facilitate substantial tumor regression. Surprisingly, considerable tumor regression was also seen with equivalent amounts of antigen-free archaeosomes (Fig. 8d), suggesting protection because of innate immunity. To study additionally the potential of archaeosomes to innately promote tumor regression, different doses of antigen-free archaeosomes were injected into mice. Fig. 9a shows the tumor sizes in control (PBS only, no archaeosomes), archaeosome-injected and conventional liposome-injected mice on day 10 post-tumor challenge. It is evident that even 50 μg of _M._smithii_ archaeosomes induce considerable tumor regression. More importantly, this effect is unique to archaeosome vesicles because even 1 mg of conventional liposomes has no effect on tumor regression. Fig. 9b demonstrates the rate of tumor progression in the various groups. A dose-dependent protective effect with various concentrations of antigen-free _M._smithii_ archaeosomes is clearly evident. Interestingly, an intermediate dose of archaeosome (150 μg) was more effective at mediating protection than the higher dose of 450 μg. Similar bell-shaped dose response curves have been observed previously by us for antibody response as well, using yet another archaeosome type (15).

Archaeosomes Facilitate Infiltration of Innate Immune Cells into the Tumors. We have previously reported that archaeosomes activate APCs such as dendritic cells and also facilitate their recruitment to the injection site in vivo (17). Thus, we tested whether the tumor regression mediated by archaeosomes lacking antigen is attributable to the recruitment of innate immune cells to the tumor site. Mice were challenged with EG.7 tumors and, on days 1 and 7, injected with 0.5 mg of antigen-free _M._smithii_TPL_ archaeosomes. Tumors were excised from the mice on day 15, single-cell suspensions made, and the cells stained and analyzed for the presence of various cell types. Fig. 10 shows that in comparison to tumors from naïve mice, tumors from mice treated with _M._smithii_ archaeosomes had a substantially higher percentage of DX5+ and Mac 1+ cells. Because DX5 is primarily expressed on natural killer cells and Mac 1 on dendritic cells and macrophages, infiltration of these host cell types into the tumor is indicated. There was also some increase in CD4+ and CD8+ cells, however, as no antigen was injected these may only represent nonspecific activation of T cells because of concurrent activation of other innate cells. None of these cellular markers were expressed on EG.7 cells (data not shown).

**Fig. 5.** Antigen-dependent protection against metastatic melanoma by archaeosomes. C57BL/6 mice received injections (days 0 and 21) of 15 μg of OVA entrapped in 0.29 mg of _M._smithii_ archaeosomes or equivalent amount (0.29 mg) of antigen-free _M._smithii_ archaeosomes. On day 43, groups of naïve and archaeosome-treated mice were challenged i.v. with B16OVA melanoma cells (a). In parallel, groups of naïve and OVA-M._smithii_ immunized mice were challenged i.v. with B16 melanoma cells, lacking OVA expression (b). On day 57, mice were euthanized, lungs harvested, and the black metastatic foci enumerated under a dissection microscope. The number of tumor foci in individual mice of every group is indicated. In the OVA-M._smithii_ group, all of the 5 mice that were challenged with B16OVA cells were sterile (P < 0.0001 in comparison to naïve controls by Mann-Whitney rank test) for tumor foci (a). In contrast, values for the antigen-free _M._smithii_ group are not significantly different from naïve control (P = 0.55 by Mann-Whitney rank test). Similarly, values for the naïve and OVA-M._smithii_ group of mice that were challenged with B16 cells (b) are not significantly different (P = 0.89 by Mann-Whitney rank test).

**Fig. 6.** Induction of CTL responses in IL-12- and IFN-γ-deficient mice after OVA-archaeosome immunization. C57BL/6 control and cytokine-deficient mice were immunized with 15 μg of OVA entrapped in _M._smithii_ archaeosomes on days 0 and 21. Spleens (n = 2/group) were obtained on day 35, and pooled spleen cells were stimulated with irradiated EG.7 cells for 5 days, and 4-h CTL activity against 51 Cr-labeled targets was assessed. CTL data represent percentage of specific lysis of triplicate cultures ± SD at various E:T ratios on EL-4 (nonspecific target) cells (left panel) and EG.7 (specific target expressing OVA peptides) cells (right panel). These data are representative of four independent experiments.
We have previously reported little attrition of antigen-specific CD8 T-cell frequencies that appear similar in magnitude to the memory responses induced by infections highlights the potent adjuvanticity of archaeosomes.

The potency of archaeosome adjuvants may be attributable to their unique lipid compositions. Archaeal lipids are composed of branched isoprenoid chains, which are often fully saturated and attached via ether bonds to the sn-2,3 carbon of the glycerol backbone. Two major classes of lipid moieties exist; namely, the archaeol lipid core ubiquitous to archaea and the membrane-spanning caldarchaeol core, found in only some archaean species. In contrast, conventional phospholipids found in other bacteria and eucarya have fatty acyl chains of variable length, which may be unsaturated, and which are attached via ester bonds to the sn-1,2 carbons of the glycerol backbone (13, 27). The TPLs of *M. smithii* are comprised of 40% caldarchaeol lipids and are dominated by archaetidyl serine and caldarchaetidylserine lipids (28). Because caldarchaeol lipids confer considerable stability to archaeosomes (18), it is possible that this feature bears strong implications for antigen delivery. However, stability of vesicles themselves cannot explain the ability of archaeosomes to provide immunostimulation. Because host immunity can be triggered through receptor-mediated signaling such as interaction of bacterial lipoproteins with Toll-like receptors on monocytes (29), it is possible that archaeal lipid head groups exposed on archaeosome surfaces interact with specific signaling receptors on APCs.

The induction and maintenance of CTL responses often requires help from CD4+ T-helper lymphocytes (30–33). We have previously reported that on a per cell basis, CD8+ T cells produce quantitatively lower cytokine levels than CD4+ T cells because of cytotoxicity of CD8+ T cells toward APCs (34). Production of reduced cytokine levels by CD8+ T cells can render them cytokine dependent, particularly at low dose immunizations (35). It is therefore understandable

**DISCUSSION**

Cell-free vaccination with the use of appropriate soluble tumor-associated antigens is an attractive approach for cancer immunotherapy, but it requires efficient and safe adjuvant formulations. Different adjuvants have been evaluated, including liposomal vesicles with capability to promote immune responses (8, 24). However, few can initiate effective CTL responses to soluble exogenous antigen in the absence of other coadjuvants. In this study, we show that vesicles composed of *M. smithii* archaeal ether lipids (archaeosomes) are potent immunostimulatory carriers for inducing tumor protective CTL responses even in the absence of IL-12. The dramatic protection observed after challenge with high numbers of tumor cells (7 × 10⁶ EG.7 cells) as used in the current study indicates the profound adjuvanticity of archaeosomes.

The key advantages of archaeosomes lies in their unique ability to deliver soluble antigen to both the MHC class I and II cellular compartments and to induce dendritic cell recruitment and activation for induction and maintenance of long-term memory T-cell responses (15–17). We determined the frequency of OVA<sub>257–264</sub>-specific, IFN-γ-secreting CD8<sup>+</sup> T cells and observed the frequencies in the range of 1:18,000 spleen cells. Although live pathogens such as *Listeria monocytogenes* and viral infections induce profound CD8<sup>+</sup> T-cell frequencies initially (22, 25), substantial attrition of T-cell responses occurs subsequently (25). T-cell frequencies in memory responses to infections are reported to be in the range of 1:10,000 spleen cells (22, 26). We have previously reported little attrition of antigen-specific CD8<sup>+</sup> T-cell responses after immunization with OVA-*M. smithii* archaeosomes (16). Thus the induction by archaeosomes (a particulate non-replicating antigen delivery vehicle) of CD8<sup>+</sup> T-cell frequencies that appear similar in magnitude to the memory responses induced by infections highlights the potent adjuvanticity of archaeosomes.

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In some tumor models, cytokine production by CD8+ T cells is indicated as a protective mechanism. CD4- T-cell-depleted mice showed a significant delay in tumor progression (36). We have also reported previously that M. smithii archaeosomes can induce CTL responses in CD4− T-cell-depleted mice (16). It is, however, apparent that a simple correlation between CTL response and tumor protection may not exist. In this context, although some adjuvants induce tumor protective responses, it is clear that depleting CD4+ T cells compromised OVA-archaeosome-induced protection against tumor development. However, CD4+ T-cell-depleted mice showed a significant delay in tumor progression (P < 0.001), indicating that T-helper cell-independent CD8+ responses were generated by archaeosomes, albeit conferring effective protection only for a short-term. We have also reported previously that M. smithii archaeosomes can induce CTL responses in CD4+ T-cell-deficient mice (36, 37). It is, however, apparent that a simple correlation between CTL response and tumor protection may not exist. In this context, although some adjuvants induce tumor protective responses in the absence of CD4+ T cells (36, 37), others such as coadministration of OVA with immunostimulating DNA induce high CTL activity without CD4+ T cell help but only intermediate tumor protection (37). Vaccination with an OVA-expressing plasmid DNA vaccine containing immunostimulating motifs stimulates low CTL activity in the absence of CD4+ T cells but provides tumor protection (37). In some tumor models, cytokine production by CD8+ T cells plays an important role in addition to cytotoxicity in facilitating tumor protection (38). Thus, CD4+ T cells may be required to maintain the functionality of CD8+ T cells besides preventing their deletion, and therefore, the mere induction of antigen-specific CD8+ T cells may be insufficient to guarantee effective protection. Interestingly, the activated state of T cells rather than frequency appears important for protection against some tumors (39). It is also possible that CD4+ NKT cells confer some innate protection, which is lost on depletion of CD4+ cells (40).

Cytokines have been shown to profoundly influence survival and responsiveness of T cells (41) and are often used as adjuvant vaccine responses (42, 43). Although IFN-γ correlates with cellular immunity against pathogens (44) and tumors (38), it can also have profound negative effects on T-cell survival. During an infection with Listeria monocytogenes (45) and Mycobacteria (46), expression of IFN-γ was shown to facilitate the erosion of T cells by enhancing apoptosis of effectors. In this context, the induction of enhanced CTL responses by archaeosomes in IFN-γ-deficient mice suggests that IFN-γ can restrict and/or regulate the expansion of CD8+ T cells even during immunization with a particulate immunogen. However, despite the enhanced CTL response and significant delay in the onset of tumors, all IFN-γ-deficient mice develop tumors. It has been shown that IFN-γ produced by CD8+ T cells enhances MHC class I expression of an otherwise low MHC class I-expressing tumor (38), thereby rendering the tumor more susceptible to CTL attack. In contrast, Winter et al. (47) showed that although IFN-γ aids tumor protection, IFN-γ produced by CD8+ T cells has little role. Thus, the role of IFN-γ in tumor protection appears to be complex and may depend on a variety of factors such as the nature of tumor model, recruitment of other inflammatory cells to the tumor site, and activation of cytotoxic intermediates like nitrate.

IL-12, a cytokine whose role is to primarily drive the differentiation of Th1 cells, is well known for its adjuvant abilities and is used in many experimental cancer immunotherapy regimens (48). We, however, show that M. smithii archaeosome adjuvants do not require IL-12 for induction of tumor-protective responses, and indeed, CTL responses are enhanced in IL-12-deficient mice. To our knowledge, this is the first demonstration that IL-12 may restrict CD8+ T-cell responses, similar to IFN-γ. Although, IL-12 has been shown to
adjuvant CD8+ T-cell responses (49–51), the effects of cytokines on priming versus maintenance of T-cell responses may not be the same. Because IL-12 mainly stimulates a Th1 response (52) associated with high levels of IFN-γ, it is reasonable to speculate that the reduced IFN-γ expression in IL-12-deficient mice favors enhanced expansion of CD8+ T cells. An alternative explanation for increased CD8+ T-cell response in IL-12-deficient mice is as follows. Recently, IL-4 produced by CD4+ helper T cells has been shown to immensely enhance the survival (53) and frequency (54) of CD8+ T cells. CTL-mediated tumor immunity is impaired in IL-4-deficient mice (55). Because archaeosomes induce both Th1 and Th2 responses to entrapped antigen (15), it is conceivable that the potential increase in T-cell IL-4, coupled with a decrease in IFN-γ production in OVA-archaeosome-immunized IL-12-deficient mice results in enhancement of the protective CTL responses. Compromised tumor protection observed in CD4+ T-cell-depleted mice by archaeosomes may then be explained by a reduction in the availability of IL-4 for promoting CTL expansion.

Another interesting observation of this study was the ability of archaeosomes lacking antigen to facilitate short-term therapeutic protection. In addition to the role of antigen-specific CD8+ T cells, innate immunity also exerts some protection against tumors (56). Indeed, live Mycobacterium bovis Bacillus Calmette-Guérin is used in immunotherapy against bladder carcinoma (57). Adjutants such as CpG DNA also augment tumor-protective transient innate immunity in the absence of antigen (58, 59). Our results also support this notion as increased numbers of innate immune effectors were noted in the tumor infiltrate even after injection with antigen-free archaeosomes. We have also previously demonstrated that M. smithii archaeosomes facilitate the recruitment and activation of dendritic cells in vivo (17). Dendritic cells can interact with natural killer cells and activate them to release IFN-γ and trigger antitumor cytolytic activity (60). This may explain the increased numbers of natural killer cells noted in the tumor infiltrate. Archaeosomes are unique particulate nonreplicating adjuvants because conventional liposomes composed of synthetic esters do not exhibit any effect on innate immunity. Thus, archaeal lipids appear to uniquely recruit innate immune cells and allow extravasation into the tumor site. Interestingly, antigen-free archaeosomes while mediating therapeutic protection failed to have any effect in the prophylactic model, probably reflecting the transient protective capability of innate immunity.

In all our studies thus far, archaeosomes have been found to have no adverse toxic effects, even after repeated daily injections at relatively high doses (61, 62). Overall, our results suggest a promising role for tumor antigen archaeosome-based cancer vaccines. We have used a model antigen (OVA) for our current studies, and similar efficacy with naturally occurring human tumor-associated antigens and/or peptides will need to be demonstrated. In a mouse model of Listeria monocytogenes infection, we have shown that a CTL peptide delivered in archaeosomes is efficient at inducing long-term protective immunity (63). In our preliminary experiments, vaccination of mice with CTL peptide from a melanoma differentiation antigen, tyrosinase-related protein, entrapped in archaeosomes confers protection against subsequent challenge with B16OVA melanoma cells (data not shown), indicating a positive trend for archaeosome-peptide-based cancer vaccines. A key advantage of archaeosomes may be their ability to augment IL-12-independent tumor-protective responses. To date, the most successful cancer immunotherapy regimens have been those that target the antigen-presenting capabilities of dendritic cells (6). Archaeosomes are a particulate, nonreplicating vaccine delivery vehicle that also effectively adjuvant dendritic cell responses.

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