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The Potent Adjuvant Activity of Archaeosomes Correlates to the Recruitment and Activation of Macrophages and Dendritic Cells In Vivo

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Modern vaccines are often based on protective soluble Ags of microbes or viruses that need to be suitably adjuvanted for induction of strong and sustained immune responses (1). Although adjuvants have been used empirically for many years, the mechanism(s) by which they function are less well understood. Understanding the mechanism(s) of adjuvant action should aid the design and improvement of vaccines and is critical from a regulatory perspective for ensuring the safety of adjuvant use in humans. Indeed, the only adjuvant currently approved universally for use in humans is alum (aluminum hydroxide), which however, is a poor stimulator of cell-mediated immune responses (2).

Efficient immune stimulation occurs when Ags are presented by potent APC populations. Primarily dendritic cells (DCs) and macrophages serve this function (3–5). These professional APCs effectively take up foreign Ags, and process and present antigenic peptides in the context of MHC molecules. Recognition of the MHC-Ag complex by the TCR triggers T cell activation. However, besides recognition of foreign Ags, T cells require additional signals to become fully active (6). These key signals are augmented by non-Ag-specific costimulatory molecules on APC surfaces interacting with their receptors on T cells (e.g., B7 with CD28). T cell activation results in cytokine production and subsequent clonal expansion of Ag-specific effectors. Thus, to induce strong adaptive T cell responses, adjuvants that effectively activate professional APCs may be most suitable for vaccines.

Liposomes primarily composed of natural or synthetic ester phospholipids (conventional liposomes) have been explored as possible Ag carriers, and a liposome-based vaccine against hepatitis A has been licensed for human use (7). Although liposomes are phagocytosed by macrophages and are used as carrier systems for targeting Ags to APCs (8), codelivery of other immunostimulating agents such as lipid A, cholera toxin, or cytokines is required for potentiation of strong adjuvant activity (9–11).

Archaea consist of organisms distinct from eubacterial and eukaryotic cells due in part to their unique, polar lipid structures (12, 13). The distinct structures of archaeal lipids confer considerable stability to liposomes (archaeosomes) formulated from the total polar lipids (TPL) of the different archaea (14) or from purified lipid subfractions (15). In earlier studies we reported archaeosomes to be superior adjuvants, capable of facilitating strong, long-lasting Ab, CD4+ T helper and CD8+ CTL responses to entrapped proteins (16–18). In this study we elucidate the mechanism(s) for the strong adjuvant activity of archaeosomes by evaluating their immunostimulating effects on APCs.

Materials and Methods
Preparation and characterization of archaeosomes and conventional liposomes

Methanobrevibacter smithii ALI (DSM 2375), was cultivated in a 75-L fermenter as described previously (19). Total lipids were extracted from frozen cell pastes, and the TPL were collected as the acetone-insoluble fraction (19). Archaeosomes were prepared from the TPL obtained from

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3 Abbreviations used in this paper: DCs, dendritic cells; TPL, total polar lipids.
the human gut archaeon isolate, *M. smithii*, t-α-dimyristoylphosphatidylcholine, t-α-dimyristoylphosphatidylglycerol, and cholesterol were purchased from Sigma (St. Louis, MO) for the preparation of conventional liposomes. *M. smithii* containing t-α-dimyristoylphosphatidylcholine:t-α-dimyristoylphosphatidylglycerol:cholesterol (1.8/0.2/1.5 molar ratio) vesicles. Vesicles (archaeosomes and conventional liposomes) were prepared by pressure extrusion at 23°C (20). Briefly, 20 mg of dried lipid was hydrated in 1 ml of PBS (10 mM potassium phosphate, pH 7.14, containing 160 mM NaCl) in either the presence or the absence of the protein Ag (10 mg/ml). After 18-h hydration, the multilamellar vesicles obtained were pressure extruded several times using a Liposofast apparatus containing two stacked 400-nm polycarbonate filters (Avestin, Ottawa, Canada). Ag that was not associated with the vesicles was removed by ultracentrifugation (200,000 × g for 30 min, three times) from 7-ml volumes of PBS, followed by washing pellets twice. Mean vesicle diameters were determined by number-weighted Gaussian size distributions using a particle sizer (model 370; Niconmp, Santa Barbara, CA). The vesicle sizes ranged from 96–263 nm. The amount of protein incorporated into the vesicles was estimated by the SDS Lowry method, after lipid removal (21) and comparison with standard curves constructed for the relevant protein. The ratio of protein to lipid (micrograms per milligram) is based on the salt-free dry weights of the vesicles.

**Cell lines**

The J774.A.1 (macrophage) cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 8% FBS (HyClone, Logan, UT) and 10 μg/ml gentamicin (Life Technologies). EG.7, a subclone of EL-4 stably transfected with the gene encoding OVA (22), was also obtained from American Type Culture Collection and maintained in RPMI plus 8% FBS additionally containing 400 μg/ml G418 (Rose Scientific, Edmonton, Canada). Wehi 164–13 cells were obtained from Dr. Tim Mosmann (University of Rochester, Rochester, NY) and maintained in RPMI 1640 medium supplemented with 8% FBS. All cells were cultured at 37°C under 8% CO2 in a humidified atmosphere.

**Generation of bone marrow-derived DCs**

Bone marrow was flushed from the femurs and tibias of one to three naive BALB/c mice, and single-cell suspensions were made by passing them through Falcon 2360 cell strainers (Becton Dickinson, Franklin Lakes, NJ) using a sterile 1-ml syringe plunger. Cells were counted, resuspended at 1 × 10⁶ cells/ml in RPMI medium supplemented with 8% FBS and 5 μg/ml of recombinant murine GM-CSF (ID Laboratories, London, Canada) in a Falcon tissue culture flask (Becton Dickinson), and cultured for 6–8 days at 37°C in 8% CO2. Nonadherent cells were removed on days 2 and 4 of culture, and fresh RPMI plus 8% FBS containing GM-CSF was added. On the day of harvest (days 6–8), nonadherent cells were harvested, washed, counted, and used. The DC preparations were consistently >80% CD11c⁺ by flow cytometry.

**In vitro activation of APCs**

J774.A1 macrophage cells or bone marrow-derived DCs were incubated in vitro with 25 μg/ml of empty archaeosomes or conventional liposomes in 24-well tissue culture plates (Falcon) at the appropriate cell densities stated in the figure legends. Alternatively, cells were activated with 10 μg/ml LPS (Escherichia coli). After 24 h at 37°C in 8% CO2 in a humidified atmosphere, the cells were recovered and stained for various cell surface markers or were used as APCs (after irradiation at 2500 rad) in T cell proliferation assays. For measurement of cytokine production by APCs, J774.A1 or bone marrow-derived DCs (1 × 10⁶) were cultured in vitro with empty archaeosomes or conventional liposomes, in 96-well tissue culture plates. Supernatants were collected at 48 h, and TNF production was measured by a bioassay using Wehi 164–13 cells (23).

**Mice**

Inbred, pathogen-free, 6- to 8-wk-old female BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (St. Constant, Canada). C3H/HeJ mice 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.

**Archaeosome injections and peritoneal exudate preparations**

BALB/c or C3H/HeJ mice were injected i.p. with empty archaeosomes (1 mg of lipid/injection). At various time points after injection, mice were euthanized, and peritoneal exudate cells were recovered by performing peritoneal lavage with 10 ml of warm RPMI plus 8% FBS medium. Erythrocytes were then selectively lysed with Tris-buffered ammonium chloride, pH 7.2 (Sigma). The cells were washed, resuspended in RPMI plus 8% FBS, counted, and analyzed for cell surface marker expression, used for cell sorting, or used as APCs for T cell proliferation assays.

**Flow cytometric analysis and sorting**

For flow cytometric analysis, cells were incubated on ice (10⁶ cells in 50 μl of RPMI plus 1% FBS) with anti-mouse CD32/CD16 (FcγRIII receptor). After 30 min, aliquots were washed and incubated in 50 μl of RPMI plus 1% FBS with the different FITC, PE, or biotinylated anti-mouse Abs as stated in the figure legends. The Abs used for the various experiments included Mac1, F4/80, CD80 (B7.1), CD86 (B7.2), H-2Kd (MHC class I), MHC class II, Bi2, CD4, and CD8. All Abs were obtained from PharMingen Canada (Mississauga, Canada), except for F4/80, which was obtained from Cedarlane (Hornby, Canada). Ab incubation was performed for 30 min on ice. Cells stained with biotinylated Abs were subsequently incubated with streptavidin-FITC or streptavidin-PE (50 μg/ml) in 1% FBS medium. Cells were fixed in 1% formaldehyde in PBS and analyzed by flow cytometry (EPICS XL; Beckman Coulter, Hialeah, FL) using EXPO software.

For flow cytometric cell sorting, peritoneal exudate cells (10 × 10⁶/ml) were stained (for 30 min on ice) with 5 μl of the anti-mouse CD32/CD16 Ab, followed by 5 μl of PE-labeled, anti-mouse Mac1, or B220 Ab. Cells were then washed and resuspended in 1 ml of RPMI 1640 supplemented with 1% FBS. Cells were sorted on an EPICS Elite ESP (Beckman Coulter) and collected in RPMI plus 8% FBS medium. Subsequently an aliquot of the sorted cells was analyzed on an EPICS XL to confirm purity.

**T cell stimulation assays**

C57BL/6 (H-2Kk) splenocytes obtained from naive control mice were enriched for CD4⁺ or CD8⁺ T cells by passing through appropriate T cell columns (Cytovax, Edmonton, Canada) according to the manufacturer’s instructions. Briefly, splenocytes (100 × 10⁶) were incubated with a rat anti-mouse CD4 or CD8 Ab, loaded onto glass-bead columns coated with goat anti-rat Ig and sheep anti-mouse Ig, and eluted. After passage through the column, cells were washed and resuspended in 1 ml of RPMI 1640 supplemented with 1% FBS. Cells were stained on an EPICS Elite ESP (Beckman Coulter) and collected in RPMI plus 8% FBS medium. Subsequently an aliquot of the sorted cells was analyzed on an EPICS XL to confirm purity.

**Ag-specific immune responses**

BALB/c mice were immunized i.p. on days 0 and 21, with OVA (grade V, Sigma) entrapped in archaeosomes or conventional liposomes (15 μg of Ag entrapped in vesicles consisting of ~1 mg lipid). Mice were euthanized on day 28 for evaluation of Ag-specific immune responses. For evaluation of humoral responses, mice were bled by cardiac puncture before euthanizing, the blood was collected in serum separator tubes (Becton Dickin- son) and serum was separated and stored at −20°C until assayed. The Ab levels in serum were determined by indirect Ag-specific ELISA (17). Ab titers are represented as endpoint dilutions exhibiting 0.3 OD unit above background. For evaluation of Ag-specific proliferation, splenocytes were selectively lysed for erythrocytes using Tris-buffered ammonium chloride and then were cultured for 72 h in triplicate with varying Ag concentra-tions, and IFN-γ production in the supernatant was determined by cytokine specific ELISA (24). For CTL assays, 30 × 10⁶ spleen cells were cultured with 5 × 10³ irradiated (10,000 rad) EG.7 cells in 10 ml of RPMI plus 8% FBS containing 0.1 ng/ml IL-2, in 25-cm² tissue culture flasks (Falcon), kept upright. After 5 days (37°C, 8% CO2), the cells recovered from the flask were used as effectors in a standard 51Cr release CTL assay, and the percent specific lysis against EG.7 targets was determined (18).

**Statistical analyses**

Student’s t test and ANOVA were performed to determine statistical sig-nificance between different groups. p < 0.05 was considered statistically significant.
Results
Archaeosomes up-regulate cell surface expression of MHC class II and costimulatory molecules on APCs

J774A.1 macrophages or bone marrow-derived DCs were incubated with M. smithii archaeosomes for 24 h, and the expression of cell surface molecules was analyzed by flow cytometry. Nonactivated J774A.1 cells constitutively show strong expression of MHC class I molecules and moderate expression of costimulatory marker CD86 (B7.2), but fail to express MHC class II and CD80 (B7.1). Exposure of J774A.1 cells to archaeosomes (Fig. 1) led to strong up-regulation of MHC class II, CD80 (B7.1), and CD86 (B7.2). An increase in the expression of various markers also occurred after treating cells with LPS, a known activator of macrophages. In contrast, comparable amounts of conventional liposomes failed to enhance the expression of these activation markers on J774A.1 cells. Similar results were obtained when bone marrow-derived DCs were exposed to archaeosomes for 24 h. Nonactivated DCs constitutively expressed high levels of MHC class II molecules. However, incubation of these DCs with archaeosomes led to an even further up-regulation of MHC class II expression (Fig. 2). Furthermore, there was also a strong up-regulation of B7.2 expression after archaeosome treatment. Thus, archaeosomes induced a rapid and potent up-regulation of MHC class II and costimulatory molecules on APCs.

Archaeosomes induce TNF production by APCs

Activated APCs often exhibit an enhanced capacity to secrete inflammatory cytokines, particularly TNF. Therefore, we tested whether treatment of APCs with archaeosomes led to enhanced secretion of cytokines. Macrophages (J774A.1) or DCs incubated with M. smithii archaeosomes for 48 h produced substantial amounts of TNF (Fig. 3) relative to nonactivated APCs. TNF production showed a dose-dependent correlation to increasing archaeosome concentrations. In contrast, incubation of APCs with conventional liposomes failed to invoke significant increases in TNF production.

Enhanced stimulation of T cells by archaeosome-activated APCs

As experiments in Figs. 1–3 suggest that archaeosomes activate APCs, we tested whether this translated into an increased functional ability of the APCs to stimulate T cell proliferation. Macrophages (J774A.1, H-2K\(^d\)) pre-exposed to archaeosomes strongly stimulated the proliferation of purified allo-specific (H-2K\(^b\)) CD8\(^+\) T cells (Fig. 4a). This proliferation was comparable to that achieved with LPS-activated macrophages. In the absence of any activation, J774A.1 APCs induced only modest CD8\(^+\) T cell proliferation. Similarly, nonactivated bone marrow-derived DCs (H-2K\(^d\)) showed low ability to induce the proliferation of purified naive allo-specific (H-2K\(^b\)) CD4\(^+\) T cells. However, prior activation of DCs with archaeosomes led to strong proliferation of the T cells (Fig. 4b). In contrast, DCs exposed to conventional liposomes failed to enhance T cell stimulation.

Archaeosomes facilitate recruitment of Mac1\(^+\)-positive cells in vivo

To decipher the effects of archaeosomes on cell populations in vivo, we analyzed the recruitment of APC populations into the peritoneal cavities of mice after i.p. injection of a dose of 1 mg of empty archaeosomes. Representative data in Fig. 5a shows the Mac1 expression profile of peritoneal exudate cells from control (PBS-treated) and archaeosome-treated mice. Three distinct populations can be identified based on Mac1 staining, Mac1\(^{low}\), Mac1\(^{high}\), and Mac1\(^{high}\), particularly in the archaeosome-treated mice. Fig. 5b summarizes the percentage of these three populations in the peritoneum at various time points after archaeosome injection (as deduced from several experiments for each time point). From these two figures it is evident that in control mice (injected with PBS alone), the primary cell population is Mac1\(^{low}\). In contrast, archaeosome-treated mice show a decrease in Mac1\(^{low}\) cells as early as day 2 and a concomitant increase in Mac1\(^{high}\) cells. By day 14 of archaeosome treatment, Mac1\(^{high}\) cells become the predominant population, accounting for >60% of the cells in the peritoneum. As Mac1\(^+\) expression is primarily seen on cells of the monocytic lineage (macrophages and DCs), these results suggest that archaeosomes facilitate the sustained recruitment of these cell populations into the injection site.

Archaeosome treatment leads to an increase in CD11c- and F4/80-expressing cells in vivo

DCs are known to be potent APCs and express high levels of CD11c (25, 26). Therefore, we evaluated the expression of CD11c on peritoneal exudate cells after archaeosome treatment. On day 5 after archaeosome treatment, there was a substantial increase in the percentage of CD11c-expressing cells, from 1.7% in the control mice (injected with PBS) to 5.2% in the archaeosome-treated group (Fig. 6). Similarly, there was a significant enhancement of F4/80\(^+\) cells in the peritoneum of archaeosome-treated mice (from 35% in the controls to 58% with archaeosome treatment). F4/80 is a myeloid Ag expressed mainly by macrophages (26). The peritoneum is normally rich in macrophages, and this number appears to further increase with archaeosome treatment (Fig. 6).

Archaeosomes up-regulate MHC class II expression of cells in the peritoneum

Next we wanted to determine whether the increased numbers of APCs in the peritoneum of archaeosome-injected mice were also in an activated state. Therefore, the peritoneal exudate cells were stained for MHC class II molecules as a representative activation marker. The profile in Fig. 7 indicates the MHC class II expression of cells in the peritoneum 5 days after archaeosome treatment. It highlights the strong up-regulation of MHC class II expression by...
archaeosomes compared with controls (peritoneal exudate cells from PBS-treated mice).

APCs induced in vivo by archaeosomes strongly stimulate T cell proliferation and cytokine production

Having demonstrated the ability of archaeosomes to recruit potent APCs in vivo, we evaluated the ability of these APCs to stimulate T cells. Peritoneal exudate cells were recovered from archaeosome-treated mice and used as APCs to stimulate allo-specific purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Peritoneal APCs from archaeosome-treated mice induced strong proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 8a). Furthermore, the T cells stimulated by archaeosome-activated APCs produced substantial IFN-γ (Fig. 8b). In contrast, peritoneal exudate cells from control mice (treated with PBS alone) were unable to induce T cell proliferation or cytokine production.

APCs activated in vivo by archaeosomes are Mac1<sub>high</sub>

Next we focussed on identifying the specific APC population that was being activated in vivo by archaeosomes and was capable of T cell activation. For this purpose peritoneal exudate cells recovered from archaeosome-treated mice were sorted by flow cytometry for various cell populations based on cell surface marker expression and then used as APCs for stimulating T cells. T cell proliferation was monitored as a measure of APC activation. In the first set of experiments, peritoneal cells obtained on day 5 after archaeosome treatment were sorted based on B220 expression. Peritoneal cells from both control and archaeosome-treated mice had ~40% B220<sup>+</sup> cells. After sorting, the purity of B220<sup>−</sup> and B220<sup>+</sup> populations obtained from peritoneal exudate of archaeosome-injected mice was > 90% (data not shown). Fig. 9a indicates that the sorted B220<sup>−</sup> cells were unable to stimulate the proliferation of T cells. In contrast, B220<sup>+</sup> cells stimulated the proliferation of T cells to a similar extent as unsorted cells. As B220 is

FIGURE 2. Up-regulation of cell surface molecules on bone marrow-derived DCs treated with archaeosomes in vitro. Bone marrow–derived DCs (10<sup>6</sup>/ml) were cultured for 24 h in the absence (a) or the presence (b) of <i>M. smithii</i> archaeosomes (25 μg lipid/ml). Cells were then washed, recovered, and single stained with anti-mouse Ia<sup>+</sup> (MHC class II), B7.1, B7.2, or appropriate isotype-specific Abs, and analyzed by flow cytometry. Data profiles were obtained after analysis of 20,000 events. a, Staining of normal bone marrow DCs for the various markers. b, Staining obtained after archaeosome treatment. The hashed line profile indicates negative staining (with the isotype-specific Ab), and the solid line indicates positive staining. The vertical dotted line represents the enhanced staining obtained with archaeosomes. The percentages within each panel indicate the number of cells staining strongly for each marker in the two groups.

FIGURE 3. Production of TNF by archaeosome-activated APCs. J774A.1 cells (a) or bone marrow DCs (b) were treated with varying concentrations of conventional liposomes or <i>M. smithii</i> archaeosomes for 48 h, and TNF production in the supernatant was assessed by a bioassay. The sensitivity of the TNF assay was <0.1 pg/ml. TNF production after archaeosome treatment is significantly different from that in conventional liposomes as determined by ANOVA.
primarily expressed on B cells, these results suggest that the APC population activated by archaeosomes in vivo excluded B cells.

Next, peritoneal exudate cells were sorted based on their expression of Mac1α. As described above, archaeosomes injected into the peritoneum of mice result in three distinct populations, Mac1αlow, Mac1αhigh, and Mac1αhigh. Because the best separation of these populations was evident between days 4–5 after archaeosome injection, this time point was chosen for cell sorting experiments. Mac1αlow and Mac1αhigh cells accounted for 34 and 26% of the peritoneal cells, respectively. On sorting, the purity of the two populations was >80% (data not shown). Fig. 9b shows that the stimulation shown previously for both CD4+ and CD8+ T cells was exclusively mediated by Mac1αhigh cells. Mac1αhigh cells are strongly expressed on cells of the monocytic lineage, including macrophages and DCs (26). Thus, the T cell proliferation results also suggest that these APC populations are being specifically activated by archaeosomes in vivo.

Mac1αhigh cells induced by archaeosomes in vivo include both macrophage and DC populations

To further characterize the Mac1αhigh cell population that was recruited and activated by archaeosomes in vivo, Mac1αlow and Mac1αhigh cells were sorted and cultured with GM-CSF in vitro for 48 h, and the characteristics of the cell population were analyzed. In Fig. 10a, the data indicate Mac1α staining of peritoneal exudate cells from archaeosome-treated mice. The cells were then sorted for Mac1αlow and Mac1αhigh expression. The data in Fig. 10b show the profile of the purified populations. These cells were then incubated with GM-CSF for 48 h, and the expressions of F4/80, CD11c, and B220 were analyzed. Interestingly, the Mac1αhigh cells gave rise to two populations expressing F4/80 or CD11c. As strong CD11c expression is often associated with mature DCs, and F4/80 expression with macrophages (26), it appears that the Mac1αhigh cells recruited and activated by archaeosomes in vivo included precursors of both these potent APC populations. Mac1αlow cells, in contrast, stained negatively for F4/80, and CD11c and were primarily B220+ cells.

Entrapment of Ag in archaeosomes facilitate strong Ag-specific immune responses

The potential of archaeosomes to evoke Ag-specific immune responses was tested after immunization of mice with OVA entrapped in these vesicles. The data in Fig. 11 demonstrate the potent adjuvant action of archaeosomes to entrapped soluble Ag and its superiority to conventional liposomes. Entrapment of OVA in archaeosomes facilitated strong Ag-specific Ab responses (Fig. 11a). Furthermore, the spleen cells of OVA-archaeosome immunized mice responded strongly to soluble OVA stimulation in vitro by producing IFN-γ, indicating induction of CD4+ Th helper responses as well (Fig. 11b). More interestingly, archaeosomes induced a strong CD8+ CTL response to the entrapped exogenous...
Thus, the ability of archaeosomes to activate professional APCs in vivo translates into their potent ability to evoke humoral and cell-mediated immune responses to entrapped Ag.

**Discussion**

Optimal T cell activation requires two signals, one initiated by TCR-MHC Ag peptide complex and the other facilitated through costimulatory molecules, particularly involving the interaction between CD28 on T cells and B7 on APC (6). CD28 recognizes two different ligands, B7.1 and B7.2, which are related, but distinct, costimulatory molecules (6). The ability of professional APCs such as DCs and macrophages to efficiently augment T cell activation is at least in part related to their strong expression of MHC and costimulatory molecules (25). We have previously shown that archaeosome adjuvants efficiently prime T cell responses to entrapped Ags (17, 18). The data in this study indicate that this may relate to the ability of archaeosomes to recruit professional APCs and up-regulate the expression of activation and costimulatory markers on their surface.

It is noteworthy that archaeosomes enhanced the expression of MHC class II on J774A.1 cells, as these cells constitutively fail to express MHC class II. In contrast, mature DCs constitutively express high levels of MHC class II (4), and archaeosome treatment leads to an even further up-regulation of expression. Some studies suggest that costimulation through either B7.1 or B7.2 differentially allows the development of Th1 or Th2 cells, respectively (27). Indeed, adjuvants such as cholera toxin that prime predominantly Th2 cells have been shown to preferentially activate B7.2 (28). In comparison, the ability of archaeosomes to up-regulate the expression of both B7.1 and B7.2 on J774A.1 macrophages conforms to their ability to prime both Th1 and Th2 responses to entrapped Ag (17). However, archaeosomes up-regulated the expression of B7.2, but not B7.1, on the surface of DCs. Recent evidence suggests that following bacterial ingestion, up-regulation of costimulatory molecules on DCs may occur in a sequential manner, with B7.1 being up-regulated much later than B7.2 (29). Thus, it is possible that the lack of B7.1 up-regulation observed on DCs relates to the kinetics of expression. Overall, the functionality of

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**FIGURE 6.** Recruitment of CD11c<sup>+</sup> cells in the peritoneum of archaeosome-treated mice. BALB/c mice (n = 3) were injected i.p. with 1 mg of *M. smithii* archaeosomes in 200 μl of PBS or with 200 μl of PBS alone. Peritoneal cells were recovered 5 days later, stained with anti-mouse CD11c-PE or F4/80-PE Ab, and analyzed by flow cytometry. Data from 20,000 events are indicated in the dot plots. The boxed area in each plot indicates the percentage of CD11c<sup>+</sup> or F4/80<sup>+</sup> cells.

**FIGURE 7.** Up-regulation of MHC class II expression on peritoneal exudate cells after archaeosome treatment. BALB/c mice (n = 3) were injected i.p. with *M. smithii* archaeosome or PBS as detailed in Fig. 5. Peritoneal cells were recovered 5 days later, stained with anti-mouse Ia<sup>+</sup>-FITC labeled Ab (MHC class II), and analyzed by flow cytometry. The MHC class II staining of peritoneal exudate cells as deduced from 20,000 events is indicated by the profiles. The hashed line indicates the expression profile of cells from PBS control mice, and the solid line indicates the expression profile of cells from *M. smithii* archaeosome-treated mice.

**FIGURE 8.** Stimulation of allo-specific T cells by peritoneal exudate cells from archaeosome-treated mice. BALB/c mice (n = 3) were treated with archaeosomes or PBS as detailed in Fig. 5, and 5 days later the peritoneal exudate cells were recovered. Cells (10<sup>6</sup>) were washed, counted, irradiated, and used as APCs to stimulate allo-specific (H-2K<sup>b</sup>) purified CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The proliferation of the T cells was monitored by <sup>3</sup>H incorporation at 72 h (a). IFN-γ production in the supernatant at 72 h was assayed by ELISA (b). Data represent the mean ± SD of values from triplicate cultures. **, Values statistically significant by ANOVA (p < 0.0001) for archaeosomes compared with PBS control. *, Values statistically significant by Student’s t test (p < 0.001) for archaeosomes compared with PBS control.
archaeosome activation of APCs is demonstrated by the potent ability of archaeosome-treated macrophages and DCs to efficiently stimulate T cells.

To correlate the ability of archaeosomes to activate APCs with the ability to adjuvant immune responses to entrapped Ag, we elucidated the effects of archaeosomes on various cell populations in vivo after an i.p. injection of empty archaeosomes. This approach was chosen because, firstly, the peritoneum offers an anatomically defined site that is readily accessible to most cell types. Secondly, in our earlier studies we had observed that i.p. injection of Ag entrapped in archaeosomes evoked a strong systemic immune response (16, 17). We also chose to use empty archaeosomes to address the immunostimulatory effects of archaeosomes independently of Ag-mediated stimulation.

Several microbial products are known to stimulate B cells, including LPS and bacterial porins (30, 31). Many of these microbial products also function as potent adjuvants for coadministered Ag (30). Although a typical Gram-negative, outer membrane structure containing LPS- and porin-like components is not encountered among archaeobacteria, we examined whether archaeobacterial lipids affected the Ag-presenting ability of B cells in the peritoneum. Firstly, we observed that the overall numbers of B220$^+$ cells (B cells) in the peritoneum of archaeosome-injected mice were not significantly different from those in controls. Furthermore, the inability of sorted B220$^+$ cells from peritoneal exudates of archaeosome-treated mice to induce T cell stimulation clearly ruled out direct effects of archaeosomes on Ag presentation by B cells.

The most dramatic effects of archaeosome injection were a sustained increase in Mac1$^+$ cells in the peritoneum, the up-regulation of MHC class II, and the ability of APCs to enhance T cell proliferation. One point of interest is that although there was an increase in CD11c$^+$ and F4/80$^+$ cells in the peritoneum of archaeosome-treated mice, these increases were less dramatic than that observed for Mac1$^{\alpha high}$ cells. CD11c and F4/80 are prominently expressed on mature DCs and macrophages, respectively (26). In contrast, Mac1$^{\alpha}$ appears to be more ubiquitously expressed on all cells of the monocytic lineage. Furthermore, macrophages and DCs are considered to arise from a common precursor (32). Thus, one may speculate that the Mac1$^{\alpha high}$ precursors recruited by archaeosomes to the site of injection, upon acquiring expression of CD11c and F4/80, migrate to other lymphoid organs for Ag presentation.

Macrophages and DCs are potent APCs for induction of Ag-specific immune responses. More interestingly, they appear to possess abilities to cross-prime exogenous Ag for MHC class I presentation and CTL induction (33, 34). Furthermore, effective
costimulation provided by DCs has been shown to bypass CD4+ T cell help for CTL induction (35–37). Archaeosomes are efficiently phagocytosed by macrophages (38), and we have also previously demonstrated the ability of archaeosomes to facilitate strong CTL responses to entrapped exogenous Ag even in the absence of CD4+ T cell help (18). The data of this study indicate that archaeosomes not only effectively target Ag to DCs and/or macrophages, but also sufficiently enhance costimulatory activity of these APCs for potentiation of strong cell-mediated immunity. In contrast, conventional liposomes that are also phagocytosed by macrophages, albeit to a lesser extent than archaeosomes (38), appear incompetent to sufficiently activate APCs. Consequently, they fail to induce a strong T cell response to entrapped Ag in vivo (17, 18), a conclusion consistent with our previous data demonstrating that conventional liposomes per se do not facilitate MHC class I processing of entrapped soluble Ags (18).

Many adjuvants appear to potenti ate different components of the innate immune system. ISCOMs have been shown to promote neutrophil, macrophage, and DC recruitment after i.p. injection into mice (39). CpG-DNA motifs are potent activators of NK and DCs (40). Adjuvants such as cholera toxin and porins modulate the expression of costimulatory molecules on APCs (28, 31, 41). Our observations indicating that the activation of APCs by archaeosomes conforms to this general scheme of innate immune activation promoting subsequent adaptive T cell responses. Indeed, our recent studies indicate that protection in mice against infection by Listeria monocytogenes develops rapidly within 7 days of immunization with archaeosomes and requires that a protective Ag be entrapped. Whether generalized inflammatory responses involving other cell types, such as neutrophils, also ensues with archaeosome injection needs further study. However, we have not observed increases in the overall numbers of peritoneal exudate cells after archaeosome injection (data not shown). Furthermore, archaeosomes do not exhibit direct mitogenic effects on spleen cells in vitro (L. Krishnan et al., unpublished observations). Thus, it appears probable that archaeosome effects are restricted to the APC component of the innate immune system.

Bacterial products are a major source of adjuvants. Peptidoglycans, monophosphoryl lipid A or lipid A components of LPS, and mycobacterial lipoproteins are examples of such products (30). Bacterial products may be immunomodulatory because they mimic the microbial structures that provide the danger signal of infection to the host. The presence of CD14, the LPS receptor on macrophages and neutrophils, is one such example (42). More recently, the induction of host immunity through interactions between bacterial lipoproteins and Toll-like receptors on monocytes has become evident (43). Archaeal polar lipids are composed of fully saturated, branched phytanyl chains of defined length that are attached via ether bonds to the sn-2,3 glycerol backbone carbon atoms. In contrast, conventional phospholipids found in other bacteria and eukarya have fatty acyl chains, often unsaturated and of variable length, that are attached via ester bonds to the sn-1,2 glycerol backbone carbon atoms (12, 13). The interaction of the unique ether lipid structures of archaea with specific receptors on APCs leading to immune activation may be a possibility. Furthermore, unique head-group structures are also seen among archaeal lipids. These may, again, facilitate specific interactions with cells. Interestingly, M. smithii lipids (from which archaeosomes were prepared) exhibit an abundance of arcaetidylserine and caldarcaethidylserine (44) that are analogues of phosphatidylserine present in eu bacteria that is recognized by CD36 on macrophages (45).

Many of the approaches for induction of strong vaccine-specific immunity aim to mimic the ability of pathogens to activate APCs and evoke strong immunity. Indeed, the use of Ag-pulsed DCs is extremely effective for induction of cell-mediated immunity (46).
The use of archaeosomes represents a relatively simple approach for activating DCs and adjuvanting immune responses. Furthermore, archaeosomes facilitate Ag-specific protective immunity even in the long term (17, 18). Additionally, M. smithii is a natural nonpathogenic inhabitant of the human colon (47), suggesting that lipids from such archaea may be more readily acceptable from a regulatory perspective in vaccine formulations. It has yet to be determined whether any of the lipids from the archaea normally resident in the human gut enter the systemic sites of the host. However, from our currently ongoing toxicology evaluations (A. Omri et al., unpublished observations) as well as from our animal studies (16–18), we can conclude that archaeosome vaccine formulations are safe. Elucidating the mechanism(s) by which archaeosomes signal APC activation may further aid the optimization and development of archaeosomes for vaccines.

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References