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GM-CSF Transgene-Based Adjuvant Allows the Establishment of Protective Mucosal Immunity Following Vaccination with Inactivated *Chlamydia trachomatis*

Hang Lu,* Zhou Xing,‡ and Robert C. Brunham2*†

Cellular and humoral immune responses induced following murine *Chlamydia trachomatis* infection confer almost sterile protection against homologous reinfection. On the other hand, immunization with inactivated organism induces little protective immunity in this model system. The underlying mechanism(s) that determines such divergent outcome remains unclear, but elucidating the mechanism will probably be important for chlamydial vaccine development. One of the distinct differences between the two forms of immunization is that chlamydia replication in epithelial cells causes the secretion of a variety of proinflammatory cytokines and chemokines, such as GM-CSF, that may mobilize and mature dendritic cells and thereby enhance the induction of protective immunity. Using a murine model of *C. trachomatis* mouse pneumonitis lung infection and intrapulmonary adenoviral GM-CSF transfection, we demonstrate that the expression of GM-CSF in the airway compartment significantly enhanced systemic Th1 cellular and local IgA immune responses following immunization with inactivated organisms. Importantly, immunized mice had significantly reduced growth of chlamydia and exhibited less severe pulmonary inflammation following challenge infection. The site of GM-CSF transfection proved important, since mice immunized with inactivated organisms after GM-CSF gene transfer by the i.p. route exhibited little protection against pulmonary challenge, although i.p. immunization generated significant levels of systemic Th1 immune responses. The obvious difference between i.p. and intrapulmonary immunization was the absence of lung IgA responses following i.p. vaccination. In aggregate, the findings demonstrate that the local cytokine environment is critical to the induction of protective immunity following chlamydial vaccination and that GM-CSF may be a useful adjuvant for a chlamydial vaccine. *The Journal of Immunology*, 2002, 169: 6324–6331.

*Chlamydia trachomatis* is an obligate intracellular bacterial pathogen that has tropism for mucosal epithelia and causes many important human diseases, including trachoma, urethritis, cervicitis, and salpingitis. Repeated or persistent infection appears to underlay much of the tissue-damaging effects of infection and can lead to infertility and blindness (1–4). Because of the public health importance of chlamydial diseases, there has been long-standing interest in developing an effective vaccine (5–7). However, progress in this area has been limited in part because of incomplete understanding of the mechanisms for protective immunity and immunopathology. Early human and primate chlamydia vaccine trials involved the use of an inactivated whole cell vaccine that only induced partial short-term protection (8). Lack of safety was a prominent concern for this type of vaccine and ultimately resulted in halting chlamydia vaccine research. Current vaccine research has centered on identification of purified subcomponents or the development of live attenuated strains that exhibit enhanced immunogenicity with reduced reactogenicity (9–12).

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3 Abbreviations used in this paper: DC, dendritic cell; BAL, bronchoalveolar lavage; EB, elementary body; IFU, inclusion-forming unit; i.n., intranasally; MIP, macrophage inflammatory protein; MoPn, mouse pneumonitis; UVEB, UV-inactivated EB; vEB, viable EB.
Th1-biased immunity and was similar in magnitude to that observed following infection (6, 16). The results indicate that the underlying mechanism in the initiation and maintenance of protective immunity induced by viable chlamydia may at least partly depend on the mobilization and maturation of DCs. Presumably immunization with inactivated organisms does not directly result in the mobilization of mature DCs. Indeed, several studies have provided evidence for the accumulation of MHC class II⁺ cells or dendritic-like cells at local sites following C. trachomatis infection of the genital tract (20) or peritoneal cavity (19). Studies have also shown that in vitro infection of epithelial cells with C. trachomatis results in the secretion of a variety of proinflammatory cytokines and chemokines, such as IL-1α, IL-1β, IL-6, IL-8, IL-18, GRO-α, and GM-CSF, that could result in the mobilization and maturation of DCs. Secretion was induced only by viable, but not by inactivated, organisms and was inhibited by antibiotics (21–23). These observations suggest that chlamydia-induced secretion of cytokines and chemokines from host cells may play a critical role in the activation of DC-mediated immunity. Inactivated organisms fail to do so and thereby may fail to induce protective immune responses.

This study was directed toward evaluating the usefulness of adenoviral GM-CSF transfection in enhancing protective immunity following the intrapulmonary delivery of inactivated chlamydia organisms. GM-CSF is a potent proinflammatory cytokine known to mobilize and mature DCs (15, 24, 25). The adenovirus vector carrying the murine GM-CSF gene is known to increase the numbers of DCs at local sites and enhance the development of TH1-mediated immunity (24). In the present study we found that adenoviral GM-CSF gene transfer significantly enhanced protective immunity elicited by inactivated chlamydia organisms, while similarly administrated inactivated organisms without prior GM-CSF transfection was unable to induce significant immune protection. Protection was correlated with systemic chlamydia-specific CD4⁺ TH1 and mucosal IgA responses. Our findings have implications for the development of a safe and effective vaccine against chlamydial infection.

Materials and Methods

Chlamydia organisms and animals

The C. trachomatis MoPn strain Nigg was grown in HeLa 229 cells. Chlamydia elementary bodies (EBs) were purified on discontinuous density gradients of Renografin-76 (Squibb Canada, Quebec, Canada) as described previously (23). Purified EBs were aliquoted and stored in a sucrose-phosphate-glutamic acid buffer at −80°C. The same stock of EBs was used for all experiments. The infectivity of purified EBs was titrated by infection of HeLa cell monolayers for 24–36 h, followed by the fixation of cells with methanol and enumeration of inclusions. Cells containing chlamydial inclusions were detected by staining with anti-chlamydial LPS mAb (ViroStat, Portland, ME) as described previously (23). Portions of purified EBs were inactivated by UV light (G15T8 UV lamp; D. William Fuller, Inc., Chicago, IL) at a distance of 5 cm for 1 h at room temperature. No chlamydial growth was detected when UV-inactivated EBs (UVEBs) were inoculated onto HeLa monolayers at a dose equivalent to 10⁵ inclusion-forming units (IFU)/10⁶ HeLa cells. For convenience, the number of UVEBs used in all experiments was calculated based on the number of IFU of the corresponding viable EBs preparations before UV light exposure.

Female BALB/c mice, 6–8 wk old, were purchased from Charles River Canada (St. Constant, Canada). Mice were housed under specific pathogen-free conditions. All animal procedures used in this study were approved by the animal care committee of University of British Columbia.

Gene transfer and immunization

A replication-deficient human type 5 adenoviral construct (adenovector) carrying murine GM-CSF cDNA in the E1 region of the viral genome (AdGM) was constructed as described previously (26). An adenoviral vector add70-3 without the GM-CSF transgene (Ad) was used as a control.

Mice were anesthetized, and AdGM or Ad was intranasally (i.n.) delivered to lungs as described previously (25). Briefly, a dose of 3 × 10⁵ PFU of viral vector in 50 μl PBS was delivered to mouse lungs. The same amount of PBS was delivered i.n. to control mice. On day 7 after gene transfer, some of the mice were sacrificed for measurement of GM-CSF levels in bronchoalveolar lavage (BAL); other groups of mice were immunized i.n. with 1 × 10⁵ IFU of UVEBs twice on consecutive days on two occasions at 3-wk intervals.

BAL analysis

BAL was performed at the indicated time points. Mice were usually sacrificed on day 7 postinfiltration of adenovector or at indicated times. A total of 450 μl PBS was used to lavage the lung on two occasions. BAL fluids were centrifuged in a microcentrifuge at 2000 rpm for 5 min, and supernatants were stored at −80°C until cytokine assays.

Challenge infection and quantification of MoPn

For a comparison of immune responses between groups immunized with viable or UVEBs without GM-CSF gene transfer, mice were immunized i.n. with 5000 IFU viable EBs or 1 × 10⁵ IFU UVEBs, respectively. On days 3, 7, 9, 10, 14, and 21, mice were sacrificed for assays of sera Abs and Ag-driven cytokine production by spleen cells.

On day 21 after immunization with UVEBs post-GM-CSF gene transfection, mice were i.n. challenged with 5000 IFU of viable MoPn and monitored daily for body weight changes. Mice were sacrificed at 10 days following inoculation, during the time of peak chlamydial growth in immunologically naive mice (27). Lungs were aseptically removed and homogenized with a cell grinder in 3 ml cold sucrose-phosphate-glutamic acid buffer. Tissue suspensions were centrifuged at 3000 × g for 15 min at 4°C to remove coarse tissue debris and were frozen at −80°C until tested.

For quantification of MoPn in lung homogenates, HeLa 229 monolayers were inoculated with serially diluted tissue supernatants for 2 h, followed by culture for 30 h. Cells with chlamydial inclusions were detected using an anti-LPS mAb as previously described (28).

Determination of MoPn-specific Ab levels

Sera were collected at the indicated time points for the determination of MoPn-specific Ab responses using an ELISA as previously described (28). Cell-ELISA plate (Cornell Glass Works, Corning, NY) was coated with 10⁵ IFU of MoPn EBs in 100 μl of 0.1 M NaHCO₃ buffer at 4°C overnight. Abs bound to the coated Ags were detected using biotin-conjugated, isotype-specific anti-mouse IgG2a and IgG1 (Caltag, Burlingame, CA) and IgA (BD Pharmingen, San Diego, CA), followed by the addition of streptavidin and substrate (Sigma, St. Louis, MO). ELISA Ab titers in sera were expressed as the geometric mean titer ± SEM and represented the inverse dilution of sera using the end point (cutoff at OD 405, 0.5) of the titration curves. Chlamydial MoPn-specific IgA in the supernatants of lung homogenates or BALs was also measured by ELISA. The lungs of mice were homogenized as described for the quantitation of MoPn EBs, but were centrifuged at 10,000 rpm. The clarified supernatant was diluted in 1/10 PBS for anti-MoPn IgA ELISA. The lung MoPn-specific IgA titer was expressed as an OD value at 405 nm.

Spleen cell culture and cytokine determination

Spleens were collected at the same time points as sera for Ab measurement. Spleen cells were cultured at 5 × 10⁶ cells/ml in the presence or the absence of UVEBs (1 × 10⁵ IFU/ml) for 3 days (28). Culture supernatants were harvested for IFN-γ, TNF-α, IL-10, and IL-4 analysis by sandwich ELISA. Cytokines in the lung homogenate supernatants or BAL were also tested using ELISA. Abs pairs for determination of mouse IFN-γ, TNF-α, IL-10, and IL-4, and GM-CSF, and matched standard murine cytokines were purchased from BD Pharmingen. To determine whether CD4 T cells were responsible for IFN-γ production, a CD4 mAb or isotype control Ig (BD Pharmingen) was added to spleen cell culture.

Histopathological analysis

The lungs from mice were isolated at indicated time points for fixation in 10% buffered formalin and embedded in paraffin as described previously (28). Tissue sections (7 μm) were stained with H&E and examined under light microscopy by a person blinded to the identification of mouse groups.

Immunohistochemical staining

Lung tissues were lyophilized with OCT embedding compound (Sakura Finetek, Torrance, CA) in liquid nitrogen and stored at −80°C. Five-micrometer sections were fixed with cold acetone and incubated with 0.5% H₂O₂ in methanol to block endogenous peroxidase activity. Slides were stained with rat anti-mouse DC mAb (HR-227; American Type Culture
Collection, Manassas, VA) or control Ig (BD PharMingen) in 2% goat serum/PBS, respectively. Following incubation with a 1/250 dilution of biotin-strepaavidi conjugated F(ab')$_2$, mouse anti-rat IgG and a 1/500 dilution of peroxidase-conjugated strepavidin (Jackson ImmunoResearch Laboratories, West Grove, PA), the slides were visualized through the addition of diamobenzidine substrate (Roche, Laval, Canada) and counterstained with hematoxylin. Images were acquired and processed with a computer equipped with a digital camera (CoolSNAP; Media Cybernetics, Silver Spring, MD).

**Statistical analysis**

Data are shown as the mean ± SEM unless otherwise indicated. Results were analyzed by Student’s t test and were considered statistically significant at $p < 0.05$.

**Results**

**Immunity elicited by infection is necessary and sufficient for protection against MoPn reinfection**

Our previous data and other studies have demonstrated that infection immunity is necessary and sufficient for eliciting protective immunity against *C. trachomatis* MoPn challenge infection (6, 16–19). Understanding the mechanism for this is important for the development of an effective chlamydial vaccine. We initially compared differences in immune responses between infection-elicited immunity and i.n. immunization with UVEBs by characterizing Ag-specific Ab and cytokine responses. Striking differences in systemic and pulmonary immunities were observed between two groups as shown in Fig. 1. By day 10–14 after primary infection, mice produced significant systemic MoPn-specific IgG2a and IgG1 Abs (Fig. 1, a and b), spleen cell IFN-γ response (Fig. 1c), and lung IgA Ab response (Fig. 1e). After primary infection the lung IFN-γ level peaked around day 10 and declined to minimal levels at day 21 postinfection (Fig. 1d). In contrast, i.n. immunization with UVEBs did not induce significant immune responses.

Mice who recovered from primary infection exhibited almost sterile protection against homologous reinfection, whereas mice who received UVEBs were fully susceptible to challenge infection (Fig. 1f). These data confirm the inability of mice to generate anti-MoPn immunity following i.n. immunization with UVEBs.

DCs appear to be a key APC for the development of chlamydia-specific immune responses and in previous experiments appeared in the peritoneum after i.p. chlamydia infection, but not after immunization with UVEBs (19). In the lung model we also found that increased numbers of DCs were present at the pulmonary site by day 5 postinfection. On the other hand, intrapulmonary immunization with UVEBs failed to increase the number of DCs (Fig. 1, g–i; infection group, 7.1 ± 3.1 DCs/field; UVEB group, 2.1 ± 0.7 DCs/field; naive mice, 1.5 ± 0.6 DCs/field; mean ± SEM number of DCs; three to five mice per group; $p < 0.05$). Thus, infection with viable EBs preferentially increased the number of DCs at the infection site compared with immunization with inactivated EBs. Accumulation of DCs preceded Ag-specific cellular and humoral immune responses (Fig. 1). The results suggest the immunization with UVEBs fails to induce protective immune responses at least partly because of its inability to increase the number of DCs at the local immunization site.

**Intranasal delivery of a transgene for GM-CSF via an adenovector increased the numbers of DCs at local tissue sites and induced Ag-specific cellular and humoral immune responses following UVEB mucosal immunization**

We next explored the importance of DCs in the initiation of Ag-specific immune responses using a transgene strategy. Similar to the report by Stampfl et al. (25), mice that received an i.n. administration of AdGM had significantly higher levels of GM-CSF...
in the BAL on day 7 after GM-CSF gene transfer than mice infestigated with Ad (AdGM group, 12 ± 1.9 pg/ml; Ad group, 0.5 ± 0.1 pg/ml of GM-CSF in BAL; mean ± SEM of four mice; p < 0.01). The levels of GM-CSF in the lungs decreased to background levels by day 28 after GM-CSF transfer (data not shown). As previously reported using the same strategy to study allergy induction (24, 25), increased GM-CSF expression was correlated with the appearance of large numbers of DCs in the lungs of mice who received AdGM, as demonstrated by immunohistochemistry with anti-DC Ab (TIB-229; American Type Culture Collection) staining. In contrast, few DCs were found in the lungs of mice who received Ad, indicating that the appearance of DCs was not due to the nonspecific effect of adenoviral vector effect. These findings confirm that i.n. delivery of the GM-CSF transgene causes transient local GM-CSF expression and increased the number of DCs in the lung. We next tested our hypothesis that UVEBs should be able to induce protective immunity when sufficient DCs are present at the local immunization site using the GM-CSF gene transfer strategy.

We initially determined whether UVEB immunization post-GM-CSF gene transfer selectively induces a Th1-like, IFN-γ-producing, T cell-dominant response. As shown in Fig. 2a, spleen cells from intranasal UVEB-imunized mice post-GM-CSF gene transfer had significantly elevated levels of IFN-γ, TNF-α, and IL-10 upon in vitro stimulation with MoPn EBs (p < 0.05 compared with mice immunized with UVEB after administration of adenovector). Naive spleen cells or spleen cells from control mice (Ad/UVEB) failed to produce significant amounts of IFN-γ, TNF-α, and IL-10 regardless of Ag stimulation, indicating that immunization with UVEB following GM-CSF gene transfer primed chlamydia Ag-specific T cells in vivo. There was only marginal IL-4 production, with no significant differences among the groups of mice (Fig. 2a). Increased production of IFN-γ from spleen cells was dependent on CD4 T cells, since anti-CD4 Ab abolished IFN-γ production (data not shown). We measured serum Abs against chlamydia, which provides further information on the biological activity of Th responses in vivo. Mice immunized with UVEB post-GM-CSF gene transfer had significantly higher titers of Ag-specific serum IgG2a and IgG1, but no detectable Ag-specific serum IgA (Fig. 2b and data not shown).

Mucosal IgA is a major effector molecule of the mucosal immune system against many microbial pathogens, including C. trachomatis. We measured local IgA levels to MoPn EBs in lung homogenates and BAL on day 10 postchallenge. As shown in Fig. 2b, IgA levels in the lungs of the mice immunized with UVEB after GM-CSF transfection were significantly higher than those in control groups and similar to the levels observed in mice infected with viable EB (vEB). Thus, the data indicate that the immunization of UVEB post-GM-CSF gene transfer was able to induce not only systemic Ag-specific immune responses, but also mucosal Ag-specific IgA responses.

*Immunity induced by UVEBs following transgene GM-CSF provides protection against MoPn infection*

To address the potential role of enhanced mucosal and systemic immune responses in protecting against infection, we next assessed protective immunity by measuring chlamydia growth and body weight changes following intranasal challenge with 5000 IFU of MoPn. Mice immunized with UVEB following GM-CSF gene transfer displayed milder systemic illness and significantly less body weight reduction and began to regain body weight earlier compared with control mice (Fig. 3a). We further evaluated the effect of UVEB immunization after GM-CSF gene transfer by quantitating infectious chlamydia recovery from mouse lung tissues. As shown in Fig. 3b mice immunized with UVEB post-GM-CSF gene transfer had >1000-fold less chlamydia recovered from lung tissue compared with other control groups (p < 0.05). Mice immunized with UVEB following Ad transfer were not protected.

Collectively, these observations show that the transfer of GM-CSF...
To investigate the potential role of enhanced immune responses in protecting against C. trachomatis lung infection, we next characterized the pathological features following challenge infection. We processed and examined lung tissues on day 10 postinfection (Fig. 4, e–h) using the morphology of lung before challenge among each group of mice as control (Fig. 4, a–d). There was much less severe peribronchial and perivascular infiltration of mononuclear cells in the lungs of mice immunized with UVEB post-GM-CSF gene transfer (Fig. 4g). The inflammatory infiltrate was comparable to that observed in challenged mice who had recovered from previous infection (vEB; Fig. 4b). In contrast, diffuse mononuclear cell infiltration involving between 60 and 90% of the lung parenchyma was present in infected control mice (Fig. 4e) and in mice immunized with UVEB after delivery of adenovector (Ad/vEB; Fig. 4f).

**Lung IgA correlates with protective immunity post-GM-CSF gene transfer following immunization with UVEB**

To determine whether local GM-CSF expression is required for the induction of protection, we compared the effects of alternate routes of GM-CSF gene delivery on cellular and humoral immune responses and on protective immunity. As expected, mice i.p. immunized with UVEB after i.n. delivery of AdGM (i.n. AdGM/i.p. UVEB) did not have significant Ab responses or spleen cell cytokine production upon Ag stimulation in vitro (Fig. 5, a–c). Mice immunized by i.p. injection of UVEB after i.p. delivery of AdGM or i.n. administration of UVEB after i.n. delivery of AdGM had strong Ab (IgG2a and IgG1) responses in sera and spleen cell Ag-driven cytokine (IFN-γ and TNF-α) responses (Fig. 5, a–c). Thus, the effect of AdGM as an adjuvant appears to be limited to the local site where the immunogen is delivered. Despite developing systemic immune responses, however, the i.p. immunized mice did not demonstrate protective immunity against challenge infection (Fig. 5d). Since both IFN-γ and mucosal IgA appear to be critical components of protective immunity against C. trachomatis infection, we measured lung IFN-γ and IgA levels among groups of mice. The results showed that i.p. immunized mice (i.p. AdGM/i.p. UVEB) had little local IgA response, whereas they exhibited high levels of IFN-γ in lung homogenates (Fig. 6, a and b). The high IFN-γ in lung homogenates correlated with the extensive mononuclear cell infiltration seen histopathologically (Fig. 6, c, e, and f) and the higher growth of chlamydia in lung tissue (Fig. 6d). Intranasally immunized mice (i.n. AdGM/i.n. UVEB) had higher lung IgA responses, lower lung IFN-γ levels

**Transgene GM-CSF-amplified immunity results in less severe lung inflammation in response to MoPn infection**

To investigate the potential role of enhanced immune responses in protecting against the tissue-damaging effects of chlamydial infection, we next characterized the pathological features following gene indeed enhances protective immunity following UVEB immunization. The degree of the protective immunity was similar to that observed among previously infected mice (Fig. 3b). Protection was unlikely to be due to the direct effect of GM-CSF alone, since mice that similarly received AdGM without UVEBs were not protected (Fig. 3, a and b).

**FIGURE 3.** Mice i.n. immunized with UVEB following GM-CSF gene transfer were protected against C. trachomatis MoPn lung infection. Groups of mice (eight mice per group) were immunized twice every 3 wk as described in Fig. 2. Three weeks after the last immunization mice were i.n. challenged with 5000 IFU of MoPn EB and monitored daily for body weight reduction (a). Day 10 postchallenge lungs were homogenized for titration of chlamydial growth in vitro (b). The data are the mean ± SEM IFU (log_{10}) per lung or body weight reduction (percentage). One of three separate experiments with similar results is shown. *p < 0.01; **p < 0.005 (compared with the AdGM-, Ad/UVEB-, or mock-immunized group).

**FIGURE 4.** Less severe inflammatory response to MoPn infection among mice immunized with UVEB post-GM-CSF gene transfer. Groups of mice (five or six mice per group) were immunized twice with a 3-wk interval as described in Fig. 2. Formalin-fixed mouse lungs before challenge (a–d) or on day 10 postchallenge with 5000 IFU of MoPn (e–h) among each group were stained with H&E. Magnification, ×200. a and e, Naive mice; b and f, mice immunized with UVEB following delivery of adenovector; c and g, mice immunized with UVEB post-GM-CSF gene transfer by adenovector; d and h, mice recovered from previous infection as positive controls. Mice immunized with UVEB post-GM-CSF gene transfer (g) had reduced inflammatory infiltration, as did reinjected mice who recovered from primary infection (h). Infected control mice (e and f) showed severe mononuclear inflammation.
Local GM-CSF expression was required for the induction of protective immunity. Groups of mice (eight mice per group) were i.p. immunized with UVEB after i.p. administration of AdGM (i.p. AdGM/i.p. UVEB) or i.p. immunized with UVEB following i.n. administration of AdGM (i.n. AdGM/i.p. UVEB) or i.n. instillation of UVEB following i.n. administration of AdGM (i.n. AdGM/i.n. UVEB), respectively, as described in Materials and Methods. Mock control mice were administrated PBS i.n. Mice previously i.n. infected with MoPn, but recovered, were used as positive controls (i.n.vEB). Before challenge, sera and spleens were collected for Ab and cytokine determinations by ELISAs. Spleen cells were cultured for 72 h in the presence of UVEB (1 × 10^7 IFU/ml) for IFN-γ, TNF-α, and IL-10 assays (a). Sera MoPn-specific IgG1 (b) and IgG2a (c) titers were expressed as the geometric mean titer ± SEM and represent the inverse dilution of the samples. Three weeks after the last immunization, mice were i.n. challenged with 5000 IFU of MoPn. On day 10 postchallenge, mice were sacrificed for lung homogenization, and the clarified supernatant was directly tested for lung chlamydia growth in vitro (d) as described in Fig. 1. Data represent one of the three independent experiments with similar results. *, p < 0.01; **, p < 0.05 (compared with mock control).

**Discussion**

In this study we used adenovirus-mediated gene transfer technology to demonstrate that local GM-CSF transfection significantly enhanced protective immunity against *C. trachomatis* infection following immunization with whole inactivated organism. Protection was correlated with Ag-specific CD4 Th1 immunity and local IgA responses and was accompanied by much reduced inflammatory pathology in the lung. These are important findings, as previous immunization studies have shown that optimal protective immunity against chlamydia infection could only be achieved using viable organisms or DCs pulsed ex vivo with inactivated organisms. Our studies indicate that an inactivated whole cell chlamydia vaccine or possibly one composed of subcomponents may be efficacious when adjuvancy is enhanced through increasing the numbers of DCs and facilitating the maturation of DCs at the immunizing site such as with an adenovirus vector containing GM-CSF. The data demonstrate that local delivery of GM-CSF and the immunogen to the mucosal site of challenge infection are essential to the induction of protective immunity.

Recently Shaw et al. (29) reported that DCs pulsed with chlamydial major outer membrane protein or UVEB induced Ag-specific CD4 T cell proliferation in vitro, but that only immunization with DCs pulsed with UVEB induced Th1-mediated protective immunity in vivo. Immunization with DCs pulsed with major outer membrane protein elicited Th2 immune responses without significant protection against challenge infection. The authors suggested that the nature of the Ag used to pulse DCs may influence the maturation of DCs and thereby skew the Th1-Th2 balance of the immune response in vivo (29). In the absence of maturation, Ag-loaded DCs may induce Ag-specific tolerance or polarize the immune response to a type 2 pattern (30). Whole EBs, as used in the current study, not only contain protective epitopes, but also appear to promote the maturation of DCs, perhaps through interaction with Toll-like receptors.

Epithelial cells maintain an effective antimicrobial barrier to mucosal infection in part through the direct induction of innate antimicrobial factors in response to infection, which results in the initiation of an inflammatory response followed by recruitment of
immune cells to the local infection site. The initial site of *C. trachomatis* infection is at mucosal surfaces such as the genital, respiratory, or ocular surfaces. In vitro infection of epithelial cells by *C. trachomatis* causes the secretion of many chemokines, proinflammatory cytokines, and other mediators through mechanisms dependent on chlamydia-derived protein synthesis. Inactivated chlamydia are unable to induce chlamydia-dependent cytokine secretion by epithelial cells (22, 23), suggesting that cytokine secretion by epithelial cells requires in vivo metabolism by the organism. For the present study we hypothesized that mediators secreted by epithelial cells following chlamydial infection recruit inflammatory and immune cells to the local site, which are critical to the induction of protective immunity. During this process, DCs may be mobilized and matured by molecules, such as GM-CSF, IL-1β, TNF-α, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MIP-3α, RANTES, and macrophage-derived chemokine. The relative in vivo importance of each of the molecules in DC mobilization and maturation has not yet been determined (31–37). Gene transfer technology provides a novel way to evaluate the importance of local cytokine expression in this process. Previous studies have demonstrated that adenovirus vectors are in many ways well suited for gene transfer to epithelial cells and have been informatively used for experimental study in tumor, allergy, and infection models (24, 26, 28, 39). The strategy has advantages over the use of recombinant cytokines, in that the transgene can be expressed in a dose-dependent manner in a specific tissue for a limited period of time. In the present study we used a low dose of the adenoviral construct (26) delivered via the airway to target epithelial cells and to certain extent alveolar macrophages (40), since these cell types are probably major targets for MoPh infection in vivo. We observed that the potent effect of GM-CSF was closely associated with the appearance of increased numbers of DCs, enhanced Ag-specific CD4-Th1 immune responses, and increased lung Ag-specific IgA levels and resulted in protection against challenge infection. In the absence of adenovirus-mediated GM-CSF transfection, UVEB immunization did not induce significant immune responses or protection (Figs. 2 and 3).

Local production of GM-CSF at the site of immunization appeared to be particularly important, since mice delivered GM-CSF and UVEB by separate routes had undetectable Ag-specific immune responses and no protection against challenge infection. Protection induced by immunization with UVEB post-GM-CSF transfection was unlikely to be due to the direct effect of GM-CSF, since mice similarly immunized with adenovector encoding GM-CSF alone were as susceptible to challenge infection as mock control mice (Fig. 3b).

Interestingly, mice i.p. immunized with UVEB following GM-CSF gene transfer generated a strong chlamydia-specific Th1 immune response and systemic Ab response. However, mice were not protected against lung challenge infection. This observation is similar to a previous report by Su et al. (6), who reported that i.p. immunization with heat-killed EB plus exogenous IL-12 elicited strong Th1-dominated immune responses, but no protective immunity against chlamydia genital tract infection. The authors suggested that Ag-specific T cell populations may not be appropriately sensitized after systemic immunization with inactivated EBs plus IL-12 to enable their homing to the genital mucosa (6). In our system we observed that mice i.p. immunized with UVEB after delivery of AdGM lacked local lung IgA responses despite having high serum Abs (IgG2a and IgG1) responses and spleen cell cytokine responses, including IFN-γ production upon Ag restimulation in vitro (Figs. 5 and 6). Lung homogenate IFN-γ on day 10 postchallenge did not correlate with protection, since mice recovered from previous infection or immunized locally with UVEB post-GM-CSF gene transfer showed significant protective immunity and yet exhibited very low levels of IFN-γ in the lung, whereas infected naive mice or i.p. immunized mice had poor protection, extensive pulmonary inflammation, and high lung levels of IFN-γ (Fig. 6). Previous studies have consistently shown that IFN-γ is central to immunity against *C. trachomatis* genital tract or lung infection (41–44). The experiments reported here demonstrate that local IgA is required for optimal protective immunity to chlamydia, and it may be that the protective effect of mucosal IgA is most apparent in the presence of Th1-related cytokines, such as IFN-γ. Mucosal immunity can be most efficiently induced by the mucosal immunization (45, 46). Mucosal IgA may neutralize chlamydia organisms when encountering the pathogen in the mucosal tract and thereby limit the need to mount a vigorous inflammatory response to cope with a large infection load, a concept that is probably important for vaccine development.

In conclusion, the differing efficacies of in vivo immunization with dead vs live chlamydia are probably due to the inability of dead chlamydia to induce the secretion of proinflammatory mediators such as GM-CSF and thereby mobilize DCs at the site of immunization. Information from our studies indicates that the design of a vaccine to prevent chlamydia infection should not only target the activation of the CD4+ Th1 cells and IgA-producing B cells with mucosal homing properties, but also enhance the mobilization and maturation of APCs such as DCs for Ag processing and presentation through the use of transient transgene expression such as GM-CSF or other key cytokine adjuvants.

**References**