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Novel *Chlamydia muridarum* T Cell Antigens Induce Protective Immunity against Lung and Genital Tract Infection in Murine Models[1]

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Using a combination of affinity chromatography and tandem mass spectrometry, we recently identified 8 MHC class II (I-A^b) -bound *Chlamydia* peptides eluted from dendritic cells (DCs) infected with *Chlamydia muridarum*. In this study we cloned and purified the source proteins that contained each of these peptides and determined that three of the eight peptide/protein Ags were immunodominant (PmpG-1, RplF, and PmpE/F-2) as identified by IFN-γ ELISPOT assay using splenocytes from C57BL/6 mice recovered from *C. muridarum* infection. To evaluate whether the three immunodominant *Chlamydia* protein Ags were also able to protect mice against *Chlamydia* infection in vivo, we adoptively transferred LPS-matured DCs transfected ex vivo with the cationic liposome DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate) and individual PmpG-1(25–500aa), RplF, or PmpE/F-2 (25–575 aa) proteins. The results showed that the transfected *Chlamydia* proteins were efficiently delivered intracellularly into DCs. Mice vaccinated with DCs transfected with individual *Chlamydia* protein PmpG-$1_{25–500}$, RplF, or PmpE/F-$2_{25–575}$ exhibited significant resistance to challenge infection as indicated by reduction in the median *Chlamydia* inclusion forming units in both the lung and genital tract models. The major outer membrane protein was used as a reference Ag but conferred significant protection only in the genital tract model. Overall, vaccination with DCs transfected with PmpG-$1_{25–500}$ exhibited the greatest degree of protective immunity among the four *Chlamydia* Ags tested. This study demonstrates that T cell peptide Ags identified by immunoproteomics can be successfully exploited as T cell protein-based subunit vaccines and that PmpG-$1_{25–500}$ protein may be a suitable vaccine candidate for further evaluation. *The Journal of Immunology,* 2009, 182: 1602–1608.

Chlamydia trachomatis is the leading cause of sexually transmitted bacterial infection, with more than 92 million new infections occurring annually worldwide (1). Although antibiotics are effective against *C. trachomatis*, most cases are initially asymptomatic and thus are often undetected and untreated. Untreated infection can spread throughout the reproductive tract and cause severe complications in women, such as pelvic inflammatory disease, ectopic pregnancy, and infertility. Additionally, infection with *C. trachomatis* facilitates the transmission of HIV (2) and might be a cofactor in human papillomavirus-induced cervical neoplasia (3). Public health measures to control *Chlamydia* appear to be failing, as case rates have risen during the past decade (4), perhaps due to early antibiotic treatment blunting the development of immunity to *C. trachomatis* (5). Therefore, the development of an effective vaccine remains an urgent public health priority.

Early vaccination trials in both human and nonhuman primates with whole inactivated *C. trachomatis* elementary bodies demonstrated that immunity to *Chlamydia* could be induced but that vaccine efficacy was incomplete and short-lived. Moreover, breakthrough *C. trachomatis* infection in some primate models resulted in more severe disease with worse inflammation postvaccination (6, 7). These observations were interpreted to suggest that *Chlamydia* contains both immunoprotective and immunopathological Ags and that an effective *Chlamydia* vaccine will need to be molecularly defined and delivered in such a manner as to engender long-term protective immune responses. Therefore, contemporary *C. trachomatis* vaccine research has focused on the production of subunit vaccines that are based on individual protective *C. trachomatis* proteins, which are administered with adjuvant or other delivery vehicles to enhance immunogenicity (8, 9).

Most subunit vaccine efforts have evaluated the *Chlamydia* major outer membrance protein (MOMP)[3] because this protein is abundant, highly conserved, and elicits T cell responses and neutralizing Abs (10–12). It has been both surprising and disappointing in primate models that while MOMP vaccines elicit strong immune responses, they confer only marginal protection even to a homologous strain (13). Various other candidate Ags that trigger T cell responses in humans and in mice have therefore been proposed (14–19); however, none has been yet evaluated in primate models, and thus the search for protective T cell Ags in *Chlamydia* remains a high research priority.

Studies in animal models and during human infection have established that *Chlamydia*-specific $CD4^+$ T cells producing γ IFN (IFN-γ) are critically involved in the clearance of a *Chlamydia* infection (20–22) and that Ab may play an important role in resistance to reinfection (22, 23); the role of $CD8^+$ T cells appears to be less important (22, 24). Therefore, selection of molecularly

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[3] Abbreviations used in this paper: MOMP, major outer membrance protein; CMI, cell-mediated immune response; DC, dendritic cell; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate; EB, elementary body; HK-EB, heat-killed EB; DAB, diaminobenzidine; IFU, inclusion-forming unit.

Table I. Chlamydia *peptides discovered by immunoproteomics and purified source proteins used in this study*

Peptide Sequence	Source Protein	Abbreviation	Purified Source Protein Format
KGNEVFVSPAAHIIDRPG	Ribosomal protein L6	RplF	Full length N-terminal GST-tag
SPGQTNYAAAKAGIIGFS	3-oxoacyl-(acyl carrier protein) reductase	FabG	Full length N-terminal GST-tag
KLDGVSSPAVQESISE	Anti-anti-factor	Aasf	Full length N-terminal GST-tag
ASPIYVDPAAAGGQPPA	Polymorphic membrane protein G	PmpG-1	25–500-aa N-terminal His-tag
DLNVTGPKIQTDVD	Hypothetical protein TC0420	TC0420	Full length N-terminal GST-tag
IGQEITEPLANTVIA	ATP-dependent Clp protease, proteolytic subunit	ClpP-1	Full length N-terminal GST-tag
AFHLFASPAANYIHTG	Polymorphic membrane protein F	PmpE/F-2	25–575-aa N-terminal His-tag
MTTVHAATATQSVVD	GAPDH	Gap	Full length N-terminal His-tag

defined Ags for a subunit vaccine that stimulate $CD4^+$ Th1 cells is central to the current design effort. While development of a vaccine for intracellular pathogens that require protective cell-mediated immunity (CMI) will be more difficult than for pathogens that simply require protective Abs, protective T cell Ag candidates can be chosen by identifying microbial peptides that bind to MHC molecules.

Using a combination of affinity chromatography and tandem mass spectrometry, we recently identified eight MHC class II ($I\text{-}A^b$)-bound *Chlamydia* peptides eluted from *C. muridarum*-infected dendritic cells (DCs) from C57BL/6 mice. Adoptive transfer of DCs pulsed with a pool of the eight peptides partially protected mice against challenge infection (25). In the present study, the parent protein containing each peptide sequence was cloned, expressed, and purified. Three of the eight *Chlamydia* Ags were immunodominant (PmpG-1, RplF, and PmpE/F-2), and vaccination with DCs transfected with individual *Chlamydia* proteins PmpG-$1_{25–500}$, RplF, or PmpE/F-$2_{25–575}$ induced significant protective immunity against lung and genital tract infections.

Materials and Methods

Chlamydia

C. muridarum strain Nigg (the mouse pneumonitis strain) was grown in HeLa 229 cells, and elementary bodies (EBs) were purified by discontinuous density gradient centrifugation and stored at −80°C as previously described (11). The infectivity of purified EBs was titrated by counting *Chlamydia* inclusion forming units (IFU) on HeLa cell monolayer with anti-EB mouse polyclonal Ab followed by biotinylated anti-mouse IgG (Jackson ImmunoResearch Laboratories) and a diaminobenzidine (DAB) substrate (Vector Laboratories) (26).

Chlamydia *peptides*

Eight MHC class II *Chlamydia* peptides (Table I) discovered by immunoproteomics (25) were synthesized and purified (Sigma-Aldrich). Peptides were solubilized in DMSO at a concentration of 4 mg/ml and stored at −20°C. One irrelevant sequence known to bind $I\text{-}A^b$ and derived from OVA (ISQAVHAAHAEINE) was used as a negative peptide control.

Chlamydia *peptide source proteins*

The source proteins containing the MHC II-binding *Chlamydia* peptides were cloned, expressed, and purified as follows: *rplF*, *fabG*, *aasf*, *pmpG-1*, *TC0420*, *clp-1*, *pmpE/F-2*, and *gap* DNA fragments were generated by PCR using genomic DNA isolated from *C. muridarum*. The PCR products were purified and cloned into either pGEX-6P-3 (GE Healthcare) for *rplF*, *fabG*, *aasf*, *TC0420*, and *clp-1* or pET32a (Novagen) for *pmpG-1, pmpE/F-2*, and *gap* after restriction enzyme digestion with *Bam*HI/*Not*I using standard molecular biology techniques. For *pmpG-1* and *pmpE/F-2*, only the first half of the gene (representing aa 25–500 and 25–575, respectively) was cloned into the vector for expression. The sequences of the subcloned genes were confirmed by sequencing with dye-labeled terminators using the ABI PRISM kit (Applied Biosystems). Plasmids containing the *rplF*, *fabG*, *aasf*, *pmpG-*$1_{25–500}$, *TC0420*, *clp-1*, *pmpE/F-*$2_{25–575}$, and *gap* genes were transformed into the *Escherichia coli* strain BL21(DE3) (Strategene) where protein expression was conducted by inducing the *lac* promoter for expression of T7 RNA polymerase using isopropyl-β-D-thiogalactopyranoside. The expressed RplF, FabG, Aasf, TC0420, and Clp-1 proteins with N-terminal GST-tag were purified from *E. coli* lysates by affinity chromatography using a glutathione Sepharose 4 fast flow purification system (GE Healthcare). PmpG-$1_{25–500}$, PmpE/F-$2_{25–575}$, and Gap proteins with N-terminal His-tag were purified by nickel column using the His bind purification system (Qiagen). LPS removal was conducted by adding 0.1% Triton 114 in the wash buffers during purification.

Mice

Female C57BL/6 mice (8 to 10 wk old) were purchased from Charles River Canada. The mice were maintained and used in strict accordance with University of British Columbia guidelines for animal care.

DC generation from bone marrow

DCs were generated following the protocol described by Lutz et al. (27). Briefly, bone marrow cells were prepared from the femora and tibiae of naive C57BL/6 mice and cultured in DC medium. DC medium is IMDM supplemented with 10% FCS, 0.5 mM 2-ME, 4 mM L-glutamine, 50 μg/ml gentamicin, and 5% of culture supernatant of murine GM-CSF-transfected plasmacytoma X63-Ag8 and 5% of culture supernatant of murine IL-4 transfected plasmacytoma X63-Ag8 that contained ~10 ng/ml GM-CSF and 10 ng/ml IL-4, respectively. The above two cell lines were kindly provided by Dr. F. Melchers (Basilea Institute, Switzerland). Culture medium was changed every 3 days. After 8-day culture, the cells were harvested for transfection with *Chlamydia* protein Ags. Approximately 65–70% of the cell preparations were DCs as judged by staining with anti-CD11c mAb.

DC transfection with Chlamydia *proteins*

DCs harvested on day 8 were washed twice in RPMI 1640. Sixty microliters of the liposomal transfection reagent *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate (DOTAP; Roche) and the *Chlamydia* proteins PmpG-$1_{25–500}$, RplF, PmpE/F-$2_{25–575}$, MOMP, or the negative control protein GST were mixed with 240 μl RPMI 1640 at room temperature in polystyrene tubes for 20 min (28, 29). The final concentration of PmpG-$1_{25–500}$, PmpE/F-$2_{25–575}$, MOMP, or GST protein in the DOTAP/protein mixtures is 0.2 mg/ml and RplF protein is 0.8 mg/ml. DCs ($2–3 \times 10^7$) in 3 ml RPMI 1640 were added to the DOTAP/protein mixtures. The protein-transfected DCs were incubated for 3 h at 37°C, washed twice, resuspended in DC medium, and then cultured overnight in the presence of 0.25 μg/ml LPS for maturation. DCs on day 8 pulsed with live EB (multiplicity of infection of 1) for 24 h were prepared as a positive control. Ag-loaded DCs were used for in vitro immunohistochemical analysis and in vivo immunization.

Immunohistochemistry

The protein-transfected DCs were deposited onto Micro Slides using Shandon Cytospin (Thermo Electron). The DCs on the slides were fixed for 20 min in 4% paraformaldehyde in PBS. Subsequently, they were permeabilized for 10 min in 0.5% Triton X-100 in PBS. The cells were blocked for 20 min with PBS containing 1% horse serum and incubated with corresponding Ag-specific polyclonal murine serum (1/200), respectively, for 2 h. All anti-*Chlamydia* protein polyclonal Abs (PmpG-$1_{25–500}$, RplF, PmpE/F-$2_{25–575}$, or MOMP) were made in our laboratory as follows: BALB/c mice were immunized three times s.c. with 10 μg of recombinant *Chlamydia* protein formulated with IFA (Sigma-Aldrich). Two weeks after the final immunization, sera from each group were collected and pooled. All anti-*Chlamydia* recombinant protein polyclonal Abs had titers ≥1/500,000 dilution as determined by ELISA. Biotinylated horse anti-mouse IgG (1/2000) (Vector Laboratories) was added and then the cells were incubated again for 1 h. Finally, the cells were incubated for 45 min with ABC reagent (Vector Laboratories) and incubated with peroxidase

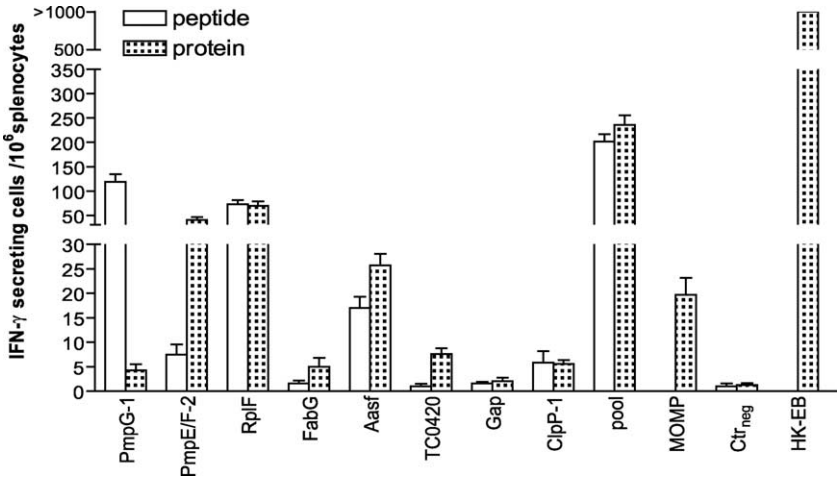

FIGURE 1. Recognition of individual *Chlamydia* peptides eluted from DCs and their source proteins in C57BL/6 mice recovered from live *C. muridarum* infection identified by IFN-γ ELISPOT assay. Mice were infected intranasally with 1000 IFU live *C. muridarum*. One month later, the splenocytes from recovered mice were harvested and stimulated in vitro for 20 h with 2 μg/ml individual peptide, 1 μg/ml individual protein, pooled peptides, or pooled proteins. One irrelevant OVA peptide and GST were used as peptide and protein negative controls (Ctr_{neg}), respectively, and HK-EB as positive control. MOMP protein stimulation was also set up as a reference. The results represent the average of duplicate wells and are expressed as the means ± SEM of *Chlamydia* Ag-induced IFN-γ-secreting cells per 10^6 splenocytes for groups of six mice. These data are representative of three similar experiments.

substrate solution (DAB substrate kit SK-4100; Vector Laboratories) until the desired stain intensity developed. The slides were rinsed in tap water, counterstained with 0.1% toluidine blue, and again rinsed in tap water. All incubations were performed at room temperature and the slides were washed in PBS three times between incubations.

ELISPOT assay

The IFN-γ ELISPOT assay was performed as described previously (30). Briefly, 96-well MultiScreen-HA filtration plates (Millipore) were coated overnight at 4°C with 2 μg/ml murine IFN-γ specific mAb (BD Pharmingen, clone R4-6A2). Splenocytes isolated from mice in AIM-V medium (Invitrogen) were added to the coated plates at 10^6 cells per well in presence of individual *Chlamydia* peptide at 2 μg/ml or individual *Chlamydia* protein at 1 μg/ml. After 20 h of incubation at 37°C and 5% CO_2, the plates were washed and then incubated with biotinylated murine IFN-γ specific mAbs (BD Pharmingen, clone XMG1.2) at 2 μg/ml. This was followed by incubation with streptavidin-alkaline phosphatase (BD Pharmingen) at a 1/1000 dilution. The spots were visualized with a substrate consisting of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma-Aldrich).

Adoptive transfer of DCs transfected with Chlamydia *protein Ags*

Mice were vaccinated i.v. three times with a 2-wk interval into the tail veins with 1×10^6 DCs transfected with *Chlamydia* protein PmpG-$1_{25–500}$, RplF, PmpE/F-$2_{25–575}$, or MOMP in 200 μl of PBS. DCs pulsed with live EB or GST protein were used as positive or negative controls, respectively. Two weeks after the last immunization, six mice of each group were euthanized to isolate splenocytes for IFN-γ ELISPOT assay. The remaining mice were used for *Chlamydia* infection challenge.

Pulmonary and cervicovaginal challenge and determination of Chlamydia *titer*

Two weeks after the final immunization, 5–10 mice from each group were intranasally challenged with 2000 IFU of *C. muridarum*. Weight loss was monitored each day or every 2 days. On day 10 after intranasal challenge, the mice were euthanized and the lungs were collected for *Chlamydia* titration. Single-cell suspensions were prepared by homogenizing the lungs with tissue grinders, and coarse tissue debris was removed by centrifugation at $1000 \times g$ for 10 min at 4°C. The clarified suspensions were serially diluted and immediately inoculated onto HeLa 229 monolayers for titration (31). For genital tract infections, 1 wk after the final immunization, 10 mice from each group were injected s.c. with 2.5 mg of medroxyprogesterone acetate (Depo-Provera; Pharmacia & Upjohn). One week after Depo-Provera treatment, the mice were challenged intravaginally with 1500 IFU of *C. muridarum*. Cervicovaginal washes were taken at day 6 after infection and stored at −80°C for titration on HeLa cells as described previously (31).

Statistical analysis

All data were analyzed with the aid of a software program (GraphPad Prism 3.0). Differences between the means of experimental groups were analyzed using an independent, two-tailed *t* test at the level of $p < 0.05$.

Results

Identification of immunodominant Chlamydia *Ags among the eight MHC class II-binding peptides*

Using an immunoproteomic approach, we previously identified eight MHC class II ($I\text{-}A^b$)-bound *Chlamydia* peptides eluted from DCs infected with *C. muridarum* (25). For this study we cloned and purified the eight source proteins that contained the corresponding peptides (Table I). To determine which individual peptides or proteins are immunodominant in the context of natural infection, we performed IFN-γ ELISPOT assay using splenocytes from C57BL/6 mice recovered from live *C. muridarum* infection.

Splenocytes from mice harvested 1 mo after *C. muridarum* infection were stimulated in vitro for 20 h with 2 μg/ml individual peptide, 1 μg/ml individual protein, pooled peptides, or pooled proteins. Irrelevant OVA peptide and GST were used as peptide and protein negative controls, respectively, and heat-killed EB (HK-EB) as positive control. Since MOMP has been a long-standing candidate in *Chlamydia* vaccine studies, MOMP was also set up as a reference Ag. As shown in Fig. 1, immune splenocytes exposed to HK-EB developed the highest numbers of IFN-γ-secreting cells where >1000 IFN-γ-secreting cells were detected among 10^6 splenocytes. In contrast, splenocytes stimulated with the OVA peptide or GST protein as negative controls showed nearly blank background levels, indicating that IFN-γ-secreting cells detected in the experimental system are *Chlamydia* Ag-specific. Stimulation by pooled peptides or pooled proteins induced significantly higher numbers of IFN-γ-secreting cells than did stimulation with individual *Chlamydia* Ags ($p < 0.05$).

Immune splenocytes stimulated with individual *Chlamydia* Ags exhibited markedly different levels of IFN-γ response (Fig. 1). The results demonstrated that IFN-γ responses in immune splenocytes to stimulation of PmpG-1 peptide, PmpE/F-$2_{25–575}$ protein, RplF peptide, and protein were strong; Aasf peptide, Aasf protein, or MOMP protein was moderate and others were weak. Thus, three of the eight Ags (PmpG-1, RplF, and PmpE/F-2) were determined as immunodominant based on their strong IFN-γ responses by ELISPOT assay to stimulation by either the peptide or parent protein. The above experiments were repeated three times and showed very consistent data.

Efficient intracellular uptake of Chlamydia *protein by DCs using DOTAP as a delivery system*

Because protein Ags require endocytotosis and lysosomal processing before peptide loading onto MHC class II molecules can take place, we used the cationic liposome DOTAP to deliver *Chlamydia*

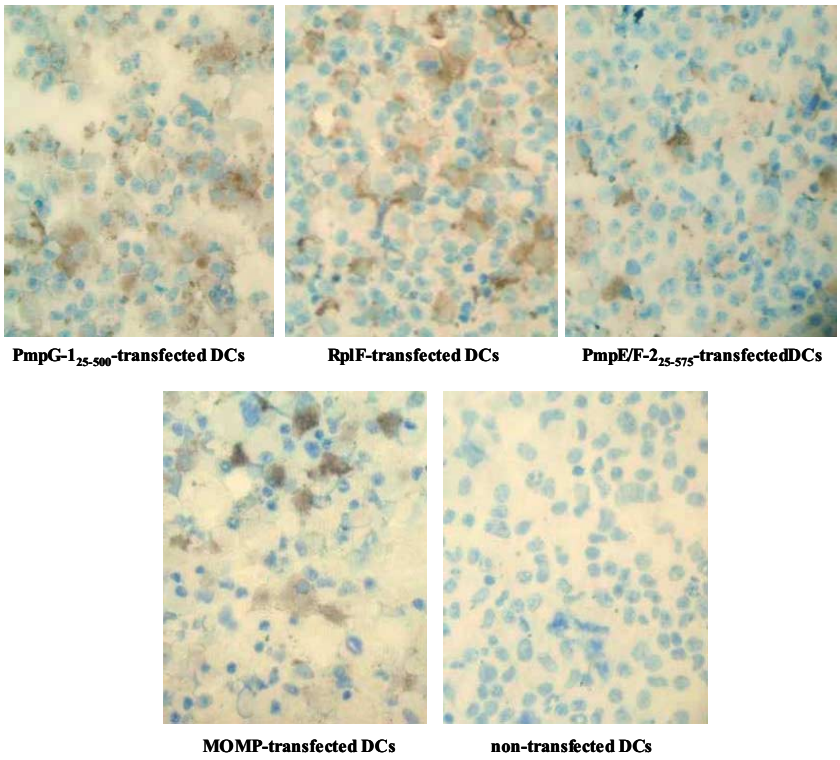

FIGURE 2. *Chlamydia* protein uptake by DCs detected by immunohistochemistry. *Chlamydia* protein uptake in DCs transfected with PmpG-$1_{25–500}$, Rplf, PmpE/F-$2_{25–575}$, MOMP, or without protein. The cationic liposome DOTAP was used to deliver *Chlamydia* protein into the DCs. The presence of *Chlamydia* protein after transfection was visualized with a protein corresponding polyclonal mouse Ab followed by a biotinylated horse anti-mouse IgG and a DAB substrate.

protein intracellularly into DCs. The intracellular uptake of PmpG-$1_{25–500}$, PmpE/F-$2_{25–575}$, RplF, or MOMP protein was visualized by immunohistochemistry following transfection. As shown in Fig. 2, strong expression of PmpG-$1_{25–500}$, RplF, PmpE/F-$2_{25–575}$, and MOMP was detected in the cytoplasm of the *Chlamydia* protein-transfected DCs, whereas no signal was detected in nontransfected DCs. Thus, the cationic liposome DOTAP efficiently delivered *Chlamydia* protein intracellularly into DCs.

After DC transfection with *Chlamydia* proteins, DCs were matured with LPS for 18 h. We evaulated the cell surface Ag expression of the DCs after LPS stimulation and found no phenotypic difference between DCs transfected with different *Chlamydia* Ags or GST-DCs stimulated with LPS-expressed enhanced levels of CD40, MHC class II, and CD86 compared with unstimulated DCs (data not shown).

Specific immune responses to Chlamydia *Ags following adoptive transfer of DCs transfected with* Chlamydia *proteins*

We next wanted to investigate whether adoptive transfer of transfected DCs induces Ag-specific immune responses. Again, a group of DCs transfected with MOMP was set up as a reference control Ag. One group of mice received DCs pulsed with GST protein as a negative control, and another group received DCs pulsed with viable C. *muridarum* EB as a positive control.

Two weeks following the final adoptive transfer, *Chlamydia*-specific immune responses in vaccinated mice were assessed by enumerating Ag-specific IFN-γ-producing cells in splenocytes from each group after exposure to *Chlamydia* Ags (Fig. 3). The results showed that the groups of DCs transfected with individual *Chlamydia* proteins developed significant Ag-specific IFN-γ responses to corresponding peptides and proteins but not to other unrelated *Chlamydia* Ags. Importantly, mice immunized with DCs transfected with individual *Chlamydia* proteins demonstrated strong specific immune responses to HK-EB ($p < 0.01$). As a positive control, mice that received DCs pulsed with live *C. muridarum* (EB) developed the strongest IFN-γ responses to HK-EB shown by >1000 IFN-γ-secreting cells detected among 10^6 splenocytes. This group also exhibited strong Ag-specific IFN-γ responses to PmpG-1 peptide/protein and RplF peptide/protein and moderate responses to PmpE/F-2 peptide/protein and MOMP. In contrast, naive and GST-DC-vaccinated splenocytes stimulated with the *Chlamydia* Ags or HK-EB showed low background levels except for the GST-DC group, which exhibited some responses to GST protein and the GST fusion protein RplF. IL-4 ELISPOT assays were also performed and showed no or very low *Chlamydia* Ag-specific IL-4 secretion in any groups immunized with DCs transfected with individual *Chlamydia* protein (data not shown).

Protection against Chlamydia *infection following adoptive transfer of DCs transfected ex vivo with the three immunodominant* Chlamydia *protein Ags*

To evaluate whether the *Chlamydia* protein Ags were able to protect mice against *Chlamydia* pulmonary and genital tract infection, we undertook adoptive transfer studies using LPS-matured DCs transfected ex vivo with PmpG-$1_{25–500}$, RplF, PmpE/F-$2_{25–575}$, or MOMP. Mice receiving DCs transfected with GST or pulsed with viable *C. muridarum* were set up as negative and positive controls, respectively. Two weeks following the final adoptive transfer, mice were challenged intranasally or vaginally with *C. muridarum*.

After the intranasal challenge, protection was measured by body weight loss and bacterial load in the lungs. As shown in Fig. 4*A*, mice adoptively immunized with live EB-pulsed DCs demonstrated excellent protection against infection, as indicated by no body weight loss. In contrast, mice immunized with GST-pulsed DCs exhibited the largest weight loss. The mean body weight loss on day 10 postinfection reached 24.4 ± 2.4% in the negative control group ($p < 0.001$ vs positive control). Mice vaccinated with individual *Chlamydia* Ag-transfected DCs showed varying levels of protection, as indicated by different degrees of body weight loss during the 10-day period. The mean relative body

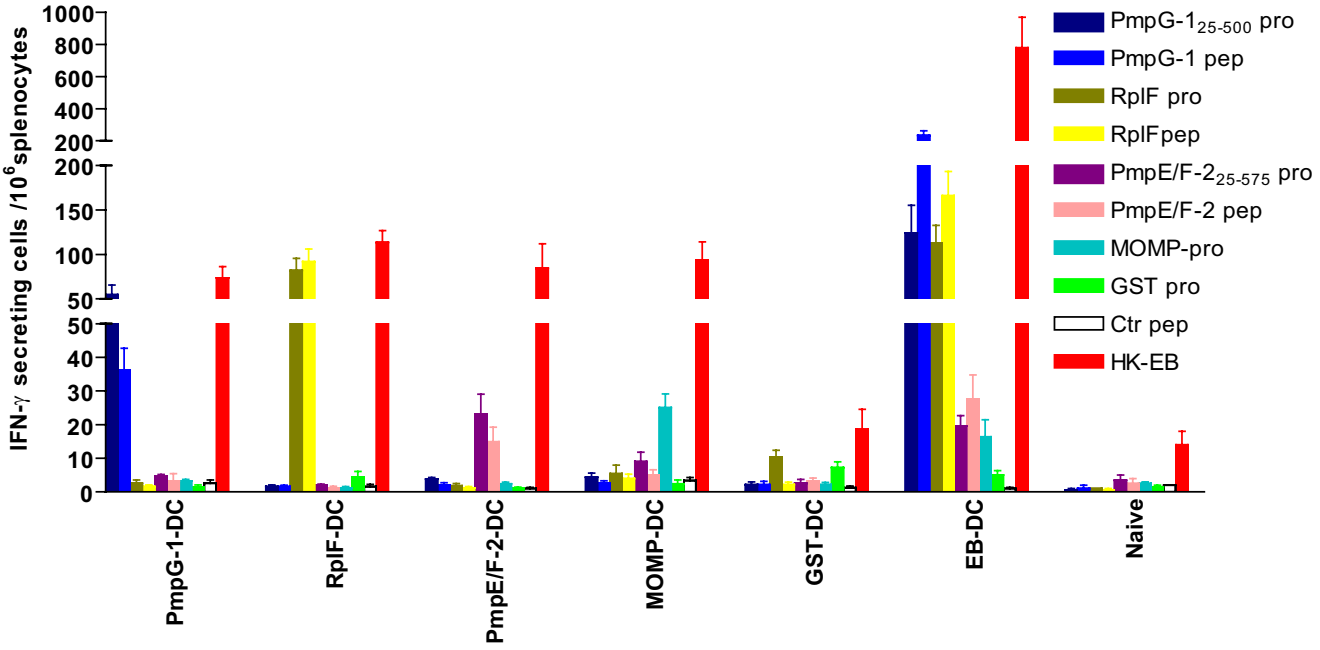

FIGURE 3. Specific immune responses to *Chlamydia* Ags following adoptive transfer of DCs transfected with *Chlamydia* proteins detected by IFN-γ ELISPOT assay. Mice were vaccinated three times with DCs transfected with *Chlamydia* protein PmpG-$1_{25–500}$, RplF, PmpE/F-$2_{25–575}$, or MOMP and matured overnight with LPS. DCs pulsed with live *C. muridarum* or GST protein were used as a positive or negative control, respectively. Two weeks after the last immunization, the splenocytes of each group were harvested for IFN-γ ELISPOT assay. The results are expressed as the means ± SEM of *Chlamydia* Ag-induced IFN-γ-secreting cells per 10^6 splenocytes for groups of six mice. These data are representative of two similar experiments.

weight loss at day 10 in groups of PmpE/F-2-DC, PmpG-1-DC, RplF-DC, or MOMP was 7.9 ± 3.1%, 8.1 ± 2.7%, 15.2 ± 3.4%, and 19.4 ± 2.8%, respectively. During the late period following infection, the recovery from lost body weight appeared most rapid in the PmpG-1-DC group among the four immunized *Chlamydia* Ag-transfected DC groups.

Ten days after the intranasal challenge, lungs were harvested and *Chlamydia* IFU were determined by plating serial dilutions of homogenized lungs onto HeLa 229 cells. When compared with the negative control group, the median *Chlamydia* titers decreased 1.8 orders of magnitude ($\log_{10}$) in mice vaccinated with PmpG-1-DC ($p < 0.01$) and decreased 1.2 and 1.1 orders of magnitude in mice vaccinated with RplF-DC ($p < 0.05$) and PmpE/F-2-DC ($p < 0.05$), respectively. There was no statistically significant difference in lung *Chlamydia* titers between mice vaccinated with MOMP-DC and the negative control group (Fig. 4*B*).

Protection against intravaginal infection was assessed by isolation of *Chlamydia* from cervicovaginal wash and determination of the number of IFU recovered from each experimental group at day 6 postinfection (Fig. 5). The results showed that the cervicovaginal shedding of *C. muridarum* in mice immunized with any of the four *Chlamydia* protein-transfected DCs was significantly lower than that of mice who received GST-transfected DCs ($p < 0.001$ in the PmpG-1 group, $p < 0.01$ in the RplF group, $p < 0.01$ in the PmpE/F-2 group, and $p < 0.01$ in the MOMP group).

Taken together, mice vaccinated with DCs transfected with *Chlamydia* protein PmpG-$1_{25–500}$, RplF, or PmpE/F-$2_{25–575}$ exhibited significant resistance to challenge infection as indicated by $\log_{10}$ reduction in the median *Chlamydia* titer in comparison with the negative control group in both the lung model and genital tract model. MOMP, as a reference Ag, conferred significant protection but only in the genital tract model. Overall, the data demonstrated that vaccination with DCs transfected with PmpG-$1_{25–500}$ developed the greatest degree of protective immunity among the four *Chlamydia* Ags evaluated.

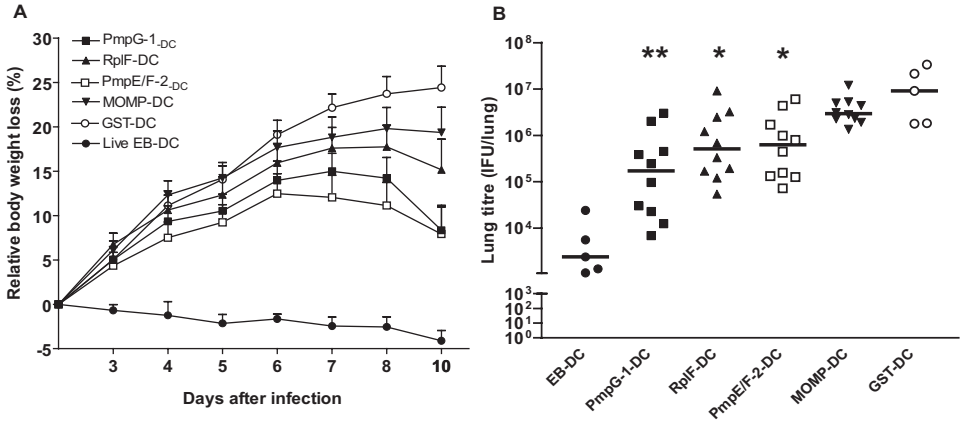

FIGURE 4. Resistance to *Chlamydia* pulmonary infection following adoptive transfer of DCs transfected with *Chlamydia* proteins. Adoptive transfer of DCs is described in *Materials and Methods.* Two weeks after the last immunization, mice were challenged intranasally with 2000 IFU live *C. muridarum*. Weight loss was monitored each or every 2 days after challenge (*A*). Ten days after intranasal challenge, the lungs were collected and bacterial titers were measured on HeLa 229 cells (*B*). *, $p < 0.05$; **, $p < 0.01$ vs GST-DC group. These data are representative of two similar experiments.

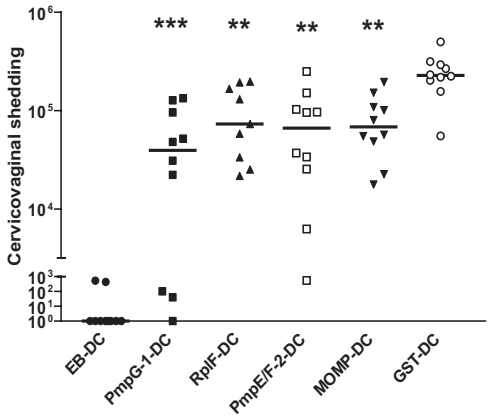

FIGURE 5. Resistance to *Chlamydia* genital tract infection following adoptive transfer of DCs transfected with *Chlamydia* proteins. Adoptive transfer of DCs is described in *Materials and Methods.* One week after the final immunization, the mice from each group were injected with Depo-Provera. One week after Depo-Provera treatment, the mice were infected intravaginally with 1500 IFU live *C. muridarum*. Cervicovaginal washes were taken at day 6 after infection and bacterial titers were measured on HeLa 229 cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs GST-DC group. These data are representative of two similar experiments.

Discussion

Chlamydia immunity is currently understood as T cell-mediated based on CD4 Th1 T cells producing IFN-γ. A major impediment to developing a vaccine against this intracellular pathogen lies in identifying relevant T cell Ags able to stimulate protective immunity (8). Our previous study using an immunoproteomic approach successfully identified eight MHC class II-bound *Chlamydia* peptides directly eluted from murine DCs infected with *Chlamydia*. A pool of the eight *Chlamydia* MHC II-binding peptides partially protected mice against *Chlamydia* infection when peptide-loaded DCs were adoptively transferred to mice before challenge infection (25). In this study we demonstrated that three of the eight Ags (PmpG-1, RplF, and PmpE/F-2) were immunodominant as identified by IFN-γ ELISPOT assay using splenocytes from mice recovered from natural infection. Furthermore, mice vaccinated with DCs transfected with individual *Chlamydia* protein PmpG-$1_{25–500}$, RplF, or PmpE/F-$2_{25–575}$ elicited IFN-γ immune responses and exhibited significant resistance to challenge infection as indicated by $\log_{10}$ reduction in the median *Chlamydia* titer in both the lung and genital tract models. Vaccination of DCs transfected with MOMP as a reference Ag demonstrated significant protective immunity in the genital tract model but not in the lung model. Among the four evaluated immunogens, PmpG-$1_{25–500}$ was the most promising vaccine candidate.

To our knowledge, this is the first published report to show that PmpG-$1_{25–500}$, PmpE/F-$2_{25–575}$, or RplF is able to engender protective immunity against challenge with *C. muridarum*. PmpG-1 and PmpE/F-2 belong to the polymorphic membrane protein (Pmp) family unique to Chlamydiales (32). There are nine *pmp* genes (*pmp*A to *pmp*I) identified for *C. trachomatis* (33, 34). The *pmp* genes are predicted to be localized in the outer membrane, and all Pmps have been detected by proteomics as membrane constituents (35). The *C. trachomatis* Pmps are abundant and account for 3.15% of the organism's protein coding capacity and are immunogenic for humans (18, 36). By protein structure analysis, they are predicted to be autotransporters that mediate the translocation of the N terminus to the bacterial surface (35). They contain multiple GGAI motifs, which have been associated in other organisms with adhesion to the host cell (37). It is likely that they play an important role in *Chlamydia* structural, functional, or antigenic polymorphism. *Chlamydia* genome analysis (38) showed that the PmpE/F-2 homolog from *C. trachomatis* (PmpF) contains a disproportionate number of single-nucleotide polymorphisms, many of which are located at predicted sites of T cell epitopes that bind to human MHC (HLA) class I and II alleles, indicating that *Chlamydia* PmpF could be a particularly important immune target of T cells. A recent study based on comparative expression profiling of the *C. trachomatis pmp* gene family demonstrated that *pmpF* also had the highest mRNA expression level among the nine *pmp* genes of tested *C. trachomatis* strains (39). There is less immunobiological information on PmpG of *C. trachomatis*; however, PmpG has been identified as recognized by CD4 T cells during *C. pneumoniae* infection in mice (40). RplF is the ribosomal protein L6 that has homology to the N terminus of a putative membrane protein adhesion of 18 kDa possessing HeLa-binding ability (41). Virtually nothing is known regarding immune recognition of RplF or the other *Chlamydia* peptides/proteins Gap, FabG, Aasf, TC0420, and ClpP-1 evaluated in this study. Since PmpG-$1_{25–500}$, RplF, and PmpE/F-$2_{25–575}$ induced significant levels of protection against *Chlamydia* challenge infection, they are a high priority for further detailed immunobiological investigation, including their potential role in human immunity to *Chlamydia* infection.

Since IFN-γ has been found to play a major role in mediating control of *Chlamydia* infection, we measured IFN-γ responses to *Chlamydia* Ags to evaluate antigenicity of the identified Ags in mice recovered from live *C. muridarum* infection and in mice vaccinated with the corresponding protein Ag. Following adoptive transfer of live EB-pulsed DCs, mice demonstrated strong IFN-γ responses to both peptides and proteins of PmpG-1 and RplF and moderate IFN-γ responses to both peptides and proteins of PmpE/F-2, whereas mice who recovered from live EB infection showed comparable levels of IFN-γ responses to both peptide and protein of RplF, PmpG-1 peptide, and PmpE/F-$2_{25–575}$ protein but none or low IFN-γ responses to PmpG-$1_{25–500}$ protein and PmpE/F-2 peptide (compare Fig. 1 with Fig. 3). These results may be explained in part by differential MHC class II loading from peptide vs protein sources. Additionally, it may be that PmpG-$1_{25–500}$ and PmpE/F-$2_{25–575}$ contain multiple immunoprotective peptides and that DCs pulsed ex vivo with live *C. muridarum* present a greater variety of *Chlamydia* peptides than do DCs in vivo which sample *Chlamydia* Ags via infected mucosal tissues during natural infection. Importantly, the results following vaccination with DCs transfected with PmpG-$1_{25–500}$ or PmpE/F-$2_{25–575}$ protein showed that both EBs and corresponding peptide and protein induced IFN-γ responses (Fig. 3).

Because an optimal Th1-polarized adjuvant that efficiently delivers *Chlamydia* Ags is as yet undefined, we used the cationic liposome DOTAP as a tool to deliver *Chlamydia* proteins directly into DCs and subsequently matured DCs with LPS overnight before vaccination. Our study demonstrated that liposome DOTAP remarkably increased the uptake of PmpG-$1_{25–500}$, RplF, Pmp E/F$2_{25–575}$, and MOMP protein by DCs compared with the uptake without DOTAP (data not shown). Although we did not evaluate the protective effect against *Chlamydia* challenge between groups of mice immunized with and without DOTAP in this study, our previous work showed that MOMP-pulsed DCs without DOTAP induced very poor protection (our unpublished data), whereas MOMP-pulsed DCs using DOTAP conferred significant protection against genital tract infection. One recent study reported that delivery of OVA to DCs using cationic liposomes resulted in a >100-fold increase in the efficiency of MHC class II Ag presentation (42). LPS-matured DCs exhibit enhanced levels of MHC

class II, CD86, and CD40 expression. The LPS treatment, in particular, dramatically increased the expression of CD40, which functions in the adaptive immune response as a trigger for efficient T cell activation (43).

Further studies will be conducted to determine whether the same *Chlamydia* protein Ags engender cross-species and cross-serovar immunity and provide protection in different MHC genetic backgrounds. A successful *Chlamydia* vaccine will likely need to be composed of multiple purified recombinant proteins and provide broad coverage in an outbred population and cross-protect against multiple variants of *C. trachomatis*. Additionally, immunogenicity for vaccine Ags will need to be optimized using compatible adjuvants to improve the partial protection that has been found with the current immunization protocol. This study provides a direct and useful strategy for discovery of T cell-based subunit molecular vaccines against *Chlamydia* as well potentially for other intracellular pathogens such as tuberculosis, malaria, and HIV.

Disclosures

The authors have no financial conflicts of interest.

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