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Immunoproteomic Discovery of Novel T Cell Antigens from the Obligate Intracellular Pathogen *Chlamydia*[1]

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***Chlamydia* infections cause substantial morbidity worldwide and effective prevention will depend on a vaccine. Since *Chlamydia* immunity is T cell-mediated, a major impediment to developing a molecular vaccine has been the difficulty in identifying relevant T cell Ags. In this study, we used a combination of affinity chromatography and tandem mass spectrometry to identify 13 *Chlamydia* peptides among 331 self-peptides presented by MHC class II (I-A^b) molecules from bone marrow-derived murine dendritic cells infected with *Chlamydia muridarum*. These MHC class II-bound peptides were recognized by *Chlamydia*-specific CD4 T cells harvested from immune mice and adoptive transfer of dendritic cells pulsed ex vivo with the peptides partially protected mice against intranasal and genital tract *Chlamydia* infection. The results provide evidence for lead vaccine candidates for a T cell-based subunit molecular vaccine against *Chlamydia* infection suitable for human study. *The Journal of Immunology,* 2008, 180: 2459–2465.**

World-wide *Chlamydia trachomatis* is annually responsible for >92 million sexually transmitted infections and 85 million ocular infections (1). Public health programs have targeted *C. trachomatis* as a major problem because the organism causes long-term sequelae such as infertility, ectopic pregnancy, and blindness. Sexually transmitted *C. trachomatis* is also a potent cofactor facilitating the transmission of HIV (2) and interacts with oncogenic human papilloma virus in the pathogenesis of cervical neoplasia (3). In developed countries, public health measures to control *Chlamydia* are failing as case rates continue to rise, perhaps due to early antibiotic treatment interfering with the acquisition of immunity (4); these approaches to control *Chlamydia* are not feasible for much of the developing world. Thus, a new approach, such as an effective vaccine, is needed if *Chlamydia* control is to be achieved in the developing and developed world.

Previous vaccine research using inactivated *Chlamydia* bacterial cells demonstrated partial short-term protection which may have been complicated by immunopathology during breakthrough infection (5). These vaccine trials yielded important lessons for the modern era of *Chlamydia* vaccinology, namely, that an effective *Chlamydia* vaccine will need to be molecularly defined and engender long-lived protective immune responses. Immunity to *C. trachomatis* is now known to depend on cell-mediated immune (CMI)[3] responses, especially Th1-polarized cytokine responses (6). Abs appear to play a secondary role (7). Experience has shown that developing vaccines for intracellular pathogens that require protective CMI is more difficult than for pathogens that simply require protective Ab (8). Part of the problem has been the identification of Ags that induce CMI responses because such Ags need to be presented to T cells by MHC molecules, and identifying MHC-bound microbial epitopes has been notoriously difficult (6). Since T cell responses mainly recognize protein Ags, protective vaccine candidates should be found within the proteome of an organism and, therefore, with the *Chlamydia* genome having been entirely deciphered, all of the potential vaccine Ags for this organism are now known, in principle (9, 10).

Dendritic cells (DCs) are at the center of the initiation of immune responses by naive T cells (11) and appear to be particularly important to the development of *Chlamydia* immunity (6). They capture Ag in the periphery and migrate to regional lymph nodes where they present processed Ag on MHC molecules to naive T cells. Su et al. (12) previously demonstrated that DCs pulsed ex vivo with *Chlamydia* protected mice against chlamydial infection. Our previous work has demonstrated that expression of GM-CSF (a cytokine known to mobilize DCs) in the mouse airway significantly enhanced systemic Th1 cellular immune responses following mucosal immunization with inactivated *Chlamydia muridarum* (13). Subsequent in vitro observations demonstrated that DCs exposed to live or to UV-irradiated *C. muridarum* develop distinct phenotypes, such that DCs exposed to live *Chlamydia* become mature and effectively present Ag to *Chlamydia*-specific CD4 T cells, while DCs exposed to UV-irradiated *Chlamydia* were less immunologically mature (14). Using *GeneChip* microarrays we observed that 33 genes were differentially expressed in response to live vs UV-irradiated *Chlamydia,* including three neutrophil chemoattractant chemokines, KC, MIP-2, and LIX (15). This correlated with the in vivo observation that *Chlamydia* induced

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[3] Abbreviations used in this paper: CMI, cell-mediated immune response; DC, dendritic cell; BMDC, bone marrow-derived DC; MS/MS, tandem mass spectrometry; IFU, inclusion-forming units; EB, elementary body; HK-EB, heat-killed EB.

significant DC and neutrophil infiltration during infection (15). Since *Chlamydia*-infected DCs are so effective at presenting *Chlamydia* Ags to CD4 T cells, we hypothesized that their surface MHC molecules should present *Chlamydia* peptides central to immunity.

Mass spectrometry (MS) provides a direct experimental means for identifying MHC-presented peptides through an approach termed immunoproteomics (16). These methods have been applied to several immunological problems (for review, see Ref 16) but instrument sensitivity has severely limited its applicability to infectious diseases. Recent advancements in tandem MS (MS/MS) technology now bring sensitivity limits near 1 femtomole and are able to measure peptide masses to within one part per million accuracy (17). This allows the detection technology to achieve a range compatible with the levels of microbial peptides that can reasonably be purified from MHC molecules presented on the surface of APCs such as DCs.

We used MS/MS to identify 13 MHC class II and 1 class I peptides derived from the *Chlamydia* proteome. Evaluation of class II peptides demonstrated that they were recognized by CD4 T cells from immune mice. When ex vivo peptide-pulsed DCs were used to immunize mice by adoptive transfer, mice were partially immune to pulmonary and genital tract infection with *Chlamydia*.

Materials and Methods

Chlamydia

C. muridarum strain Nigg was grown in HeLa 229 cells in Eagle's minimal essential medium (Invitrogen Life Technologies) supplemented with 10% FCS. Elementary bodies (EBs) were purified from HeLa cells on discontinuous density gradients of Renografin-76 (Squibb Canada) as described previously (13).

Mice

Female C57BL/6 mice were purchased from Charles River Laboratories and used at 8–10 wk of age. The mice were housed in a pathogen-free animal facility and all experiments were performed in accordance with University of British Columbia guidelines for animal care and use.

Generation of bone marrow-derived DCs (BMDCs)

BMDCs were prepared as previously described (12). Briefly, bone marrow cells were isolated from the femurs of C57BL/6 mice and cultured in Falcon petri dishes at 2×10^7 cells in 20 ml of IMDM supplemented with 10% FCS, 0.5 mM 2-ME, 4 mM L-glutamine, 50 μg/ml gentamicin, 10 ng/ml murine GM-CSF (R&D Systems), and 5% of IL-4 culture supernatants of Hybridoma X63 (provided by Dr. F. Melchers, Basilea Institute, Switzerland). On day 3, culture supernatants, including nonadherent cells, were removed and fresh medium containing GM-CSF was added. On day 5, nonadherent cells were transferred to new plates and cultured at 37°C in 5% CO_2 for 2 h. This was repeated once more to remove adherent macrophages. The nonadherent cells (purity of >50% $CD11c^+$) were harvested and used as DCs in adoptive transfer and immunoproteomics experiments. In some experiments, these cells were further purified using anti-CD11c beads (Miltenyi Biotec), resulting in a purity of >90% $CD11c^+$ cells as determined by FACS (BD Biosciences).

Purification of MHC-bound peptides

We grew 4×10^9 BMDCs (>50% $CD11c^+$) for each experiment to generate at least femtomole levels of MHC-presented peptides that might be present in as little as one copy per cell ($4 \times 10^9/6.02 \times 10^{23} \cdot mol^{-1} \approx 6.6$ fentomoles). Immature BMDCs were infected at a 1:3 multiplicity of infection with *C. muridarum* for 24 h. Infected BMDCs were lysed at 1×10^8 cells/ml in lysis buffer (1% CHAPS, 150 mM NaCl, 20 mM Tris-HCl (pH 8), 0.04% sodium azide, and protease inhibitors). MHC class II ($I\text{-}A^b$) and class I ($H2\text{-}K^b$ and D^b) molecules were isolated using the mAbs Y-3P (specific to $I\text{-}A^b$; ATCC HB183 (18)), AF6-88.5.3 (ATCC HB158 (19)), and 20-80-4S (ATCC HB-11 (20)) in immunoaffinity columns (protein A-Sepharose Fast Flow; GE Healthcare). Since 20-80-4S is specific to both $H\text{-}2K^b$ and $H\text{-}2D^b$, AF6-88.5.3 was used first to purify $H\text{-}2K^b$ alleles and 20-80-4S was then used to purify $H\text{-}2D^b$ in the subsequent step. MHC molecules bound to the affinity column were sequentially washed with 1) lysis buffer, 2) 20 mM Tris (pH 8.0)/0.15 M NaCl, 3) 20 mM Tris (pH 8.0)/1 M NaCl, and 4) 20 mM Tris (pH 8.0). Purified MHC molecules were separated from the peptides using 0.2 N acetic acid and subjected to ultrafiltration through a 5-kDa cutoff membrane to remove high molecular mass material (Ref. 21).

Identification of MHC-bound peptides

Purified MHC-bound peptides were analyzed using a linear-trapping quadrupole/Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FT; ThermoFisher Scientific) on-line coupled to an Agilent 1100 Series nanoflow HPLC using a nanospray ionization source (Proxeon Biosystems) as previously described (22). Fragment spectra were extracted using DTASuperCharge (http://msquant.sourceforge.net) and searched using the Mascot algorithm (v2.1, http://www.matrixscience.com) against a database comprised of the protein sequences from mouse (self) and *C. muridarum* using the following parameters: peptide mass accuracy, <5 parts per million; fragment mass accuracy, <0.6 Da; no enzyme specificity and ESI-TRAP fragment characteristics. Only those peptides with IonScores exceeding the individually calculated 99% confidence limit (as opposed to the average limit for the whole experiment) were considered as accurately identified.

Bioinformatics

Cellular location of each source protein was classified as membrane, soluble nonsecreted or secreted, according to the LOCATE database (23) using Entrez accession numbers or other aliases under the Entrez gene entry (including RIK codes).

Synthetic peptides

Chlamydia peptide sequences ($RplF_{51-59}$, $FabG_{157-165}$, $Aasf_{24-32}$, $PmpG\text{-}1_{303-311}$, $TC0420_{54-62}$, $ClpP\text{-}1_{36-44}$, $PmpE/F\text{-}1_{351-359}$ and $Gap_{148-156}$; shown in Table I) corresponding to the eight MHC class II epitopes were synthesized and purified (Sigma-Aldrich). Peptides were solubilized in DMSO at a concentration of 4 mg/ml and stored at −20°C. These *Chlamydia* peptides were pooled and used in all of the experiments reported here. The control peptides comprised one self -HC class II-binding peptide derived from Notch 2 (SPDQWSSSSPHSASDW) and one irrelevant sequence known to bind $I\text{-}A^b$ and derived from OVA (ISQAVHAAHAEINE).

ELISA

CD4 T cells were isolated from the spleens of mice immunized i.p. with *Chlamydia* (14) or naive mice using MACS CD4 T cell isolation kit (Miltenyi Biotec). CD4 T cells of >90% purity were obtained as measured by FACS (data not shown). Purified BMDCs were cultured in a 96-well plate at 2×10^5cells/well and matured with LPS (1 μg/ml) overnight, followed by treatment with 2 μg/ml *Chlamydia* peptides or control peptides for 4 h, at which point the cells were washed to remove unbound peptides. After a 48-h coculture with CD4 T cells (5×10^5/well), supernatants were collected and the production of IFN-γ in the supernatants was determined by ELISA as described previously (14).

ELISPOT assay

The IFN-γ ELISPOT assay was performed as described previously (24). Briefly, 96-well MultiScreen-HA filtration plates (Millipore) were coated overnight at 4°C with 50 μl (2 μg/ml) of murine IFN-γ mAbs (BD Pharmingen) per well. Splenocytes (1×10^6) (25) or purified CD4 T cells (2×10^5) were combined with DCs (4×10^4) in complete RPMI 1640 medium (Sigma-Aldrich) and added to each well of coated plates in the presence of *Chlamydia* peptides (2 μg/ml), control peptides (2 μg/ml), or heat-killed EBs (HK-EB; 5×10^5 inclusion-forming units (IFU)/ml). After a 20-h incubation at 37°C and 5% CO_2, the plates were washed extensively and incubated with biotinylated anti-murine IFN-γ mAbs (BD Pharmingen) at a concentration of 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 NBT and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich).

Adoptive transfer

BMDCs prepared as described above were mixed with live EBs at a ratio of 1:3 for 40 h or pulsed with *Chlamydia* peptides (2 μg/ml) for 4 h following maturation with LPS (1 μg/ml) overnight, and then the cells were collected and washed three times with PBS. BMDCs that had not been treated with peptides were used as a negative control. DCs (1×10^6 in 200 μl of PBS) were injected i.v. into the tail veins of naive C57/BL6. This process was repeated 2 wk later.

Table I. *MHC class I- and II-bound* C. muridarum *peptides identified using immunoproteomics*

Peptide Sequence	Source Protein	Abbreviation[a]	Entrez Gene Identification
MHC class I (H2-K^b and D^b)			
SSLFLVKL	Amino acid permease	$Aap_{379-386}$	1246014
MHC class II (I-A^b)			
GNEV**FVSPAAHIID**			
GNEV**FVSPAAHII**DRPG	Ribosomal protein L6	$RplF_{51-59}$	1246168
KGNEV**FVSPAAHII**DRPG			
EV**FVSPAAHII**DRPG			
SPGQTN**YAAAKAGII**GFS	3-oxoacyl-(acyl carrier protein) reductase	$FabG_{157-165}$	1245868
SPGQTN**YAAAKAGII**G			
KLDG**VSSPAVQES**ISE	Anti-anti-σ factor	$Aasf_{24-32}$	1246070
ASPI**YVDPAAAGG**QPPA			
SPI**YVDPAAAGG**QPPA	Polymorphic membrane protein G	PmpG-$1_{303-311}$	1246433
DLN**VTGPKIQTD**VD	Hypothetical protein TC0420	$TC0420_{54-62}$	1245773
IGQE**ITEPLANTV**IA	ATP-dependent Clp protease, proteolytic subunit	ClpP-1_{36-44}	1245608
AFHL**FASPAANYI**HTG	Polymorphic membrane protein F	PmpE/F-$2_{351-359}$	1246432
MTT**VHAATATQS**VVD	Glyceraldehyde 3-phosphate dehydrogenase	Gap_{148156}	1246159

[a] The range of numbers next to the protein abbreviation represent the nine core amino acid residues (in bold) for MHC class II peptides.

Pulmonary and genital tract infections and determination of Chlamydia *titer*

Two weeks following the final adoptive transfer of control or peptide-pulsed DCs, mice (5–10/group) were infected intranasally with *C. muridarum* (2000 IFU in 40 μl of PBS). Ten days following 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 of *Chlamydia* (26). For genital tract infections, 1 wk following the final adoptive transfer of control or peptide-pulsed DCs, mice (10–12/group) were injected s.c. with 2.5 mg of medroxyprogesterone acetate (Depo-Provera; Pharmacia and Upjohn). One week after Depo-Provera treatment, the mice were infected intravaginally with *C. muridarum* (1500 IFU in 10 μl of PBS). Cervicovaginal washes were taken at selected days postinfection and stored at −80°C before titration on HeLa cells as described previously (26).

Statistical analysis

Statistical analysis was performed by using the Student *t* test or two-sided χ^2 test. Differences between the means of experimental groups were analyzed at the level of $p < 0.05$.

Results

Identification of MHC class I- and class II-bound peptides

To mimic in vivo conditions and identify MHC-bound *Chlamydia* peptides presented on DCs, BMDCs were infected in vitro with *C. muridarum* for 24 h and then lysed. MHC molecules were isolated using MHC allele-specific mAb affinity columns. Peptide-protein and protein-protein interactions in the column were disrupted with low pH and peptides were separated from high molecular mass material by ultrafiltration. The purified MHC-bound peptides were then identified using nanoflow liquid chromatography/tandem mass spectrometry and a hybrid linear trapping quadrupole/Fourier transform-ion cyclotron resonance mass spectrometer as described in *Materials and Methods*.

A total of 523 MHC-binding peptides was identified from two separate experiments performed by infecting two different sets of BMDCs with *C. muridarum*. Among the 523 peptides identified, 344 were MHC class II (I-A^b)-binding and 179 were class I (H2-K^b or D^b)-binding peptides. The 344 MHC class II-bound peptides were identified with an estimated error rate of <1%; 331 peptides were derived from mouse proteins and 13 peptides were derived from *Chlamydia*. Because of varying degrees of proteolytic processing, the 344 MHC class II peptides mapped to 166 distinct epitopes derived from 153 unique source proteins. Each of these epitopes was represented by as many as 14 different sequence length variants (Table II; supplementary table[4]). Of most interest, the 13 *Chlamydia* class II-binding peptides were derived from 8 unique *Chlamydia* proteins (Table I). All eight MHC class II epitopes identified from *C. muridarum* were aligned with the corresponding protein sequences of human strains of *C. trachomatis* serovars D and L2 to see whether these epitopes are conserved in sequence between mouse and human strains of *Chlamydia*. All except PmpG and PmpF epitopes are highly conserved between the two species, indicating there may be a high chance of identifying the same epitopes if human serovars are used in this type of study. The *Chlamydia* peptides accounted for <4% of the total peptide diversity identified by MS as presented by MHC class II molecules on the surface of murine BMDCs infected by *C. muridarum*. Of 179 MHC class I-bound peptides isolated, 178 were self-peptides from 164 unique source proteins (supplementary table). The single MHC class I *C. muridarum* peptide identified originated from amino acid permease (Table I). The fraction of proteins that generated MHC-binding peptides that were chlamydial in origin was significantly higher for class II than class I (Table II; 8 of 153 class II vs 1 of 165 class I, $p < 0.05$).

Properties of MHC class I- and class II-bound peptides

As has been reported by others (27), class I-bound peptides varied between 8 and 13 aa, with most being 8 or 9 aa in length. The length of class II peptides was much more heterogenous and ranged between 9 and 24 aa (Table II and supplementary table). The analysis of the cellular location of the eight *Chlamydia* source proteins that generated the 13 MHC class II peptides identified in this study indicated that they originated from both cytosol and membrane compartments. PmpE/F-2 and PmpG-1 are two known outer membrane proteins that belong to the polymorphic membrane protein family (10).

[4] The online version of this article contains supplemental material.

Table II. *Summary of the number of* Chlamydia *and murine-derived MHC class I and II-bound peptides, epitopes, and source proteins identified in this study*

	Peptides		Epitopes		Proteins	
	MHC Class		MHC Class		MHC Class	
	I	II	I	II	I	II
Mouse	178	331	178	159	164	145
Chlamydia	1	13	1	8	1	8
Total	179	344	179	166	165	153
	(523)[a]		(345)		(318)	

[a] Numbers in parentheses, total number after combining MHC class I- and II-associated peptides, epitopes, and source proteins.

In vitro recognition of Chlamydia *MHC class II-binding peptides by immune T cells*

As a first step to validating the immunological relevance of the identified class II *Chlamydia* peptides, we determined whether class II *Chlamydia* peptides are recognized by CD4 T cells from immune mice. Since IFN-γ has been found to play a major role in mediating control of *Chlamydia* infection, we performed both ELISA and ELISPOT assay to measure IFN-γ production by *Chlamydia*-specific CD4 T cells in response to the identified *Chlamydia* peptides. The results from ELISA (Fig. 1*A*) showed that the IFN-γ level present in the supernatant of cocultures of immune CD4 T cells with *Chlamydia* peptide-pulsed DCs was significantly higher than that from cocultures in which either naive CD4 T cells or DCs pulsed with nonrelevant peptides were used.

To quantitate the fraction of IFN-γ-secreting cells, we next performed ELISPOT assays. Splenocytes (Fig. 1*B*) and purified CD4 T cells (Fig. 1*C*) from live EB-immunized mice or naive mice were stimulated in vitro with *Chlamydia* peptides, control peptides, or HK-EB and the number of IFN-γ-secreting cells was determined. The results showed that >1000 IFN-γ-secreting cells were detected among 10^6 immune splenocytes or among 2×10^5 immune CD4 T cells stimulated with HK-EB; substantial numbers of IFN-γ-secreting cells were also observed following stimulation of immune splenocytes and CD4 T cells with *Chlamydia* peptides (263 ± 37 and 198 ± 11, respectively); only low numbers of IFN-γ-secreting cells were detected following stimulation of immune splenocytes and CD4 T cells with control peptides (3.5 ± 1.5 and 7.5 ± 2.5, respectively). In addition, naive splenocytes stimulated with the *Chlamydia* peptide pool or HK-EB showed very low background levels. Taken together, these results show that the MHC class II *Chlamydia* peptides but not irrelevant peptides were recognized by *Chlamydia*-specific CD4 T cells as measured by Ag-specific IFN-γ production.

In vivo protection against Chlamydia *infection by adoptive transfer of DCs pulsed ex vivo with* Chlamydia *MHC II peptides*

To evaluate whether the *Chlamydia* MHC class II peptides were able to protect mice against either *Chlamydia* pulmonary or genital tract infection, we undertook adoptive transfer studies using LPS-matured DCs loaded ex vivo with *Chlamydia* peptides. The peptide-pulsed DCs were adoptively transferred i.v. to naive C57BL/6 mice which were subsequently challenged with *Chlamydia*. As controls, one group of mice received LPS-matured DCs that had not been treated with peptides and another group received DCs incubated with viable *C. muridarum*. Two weeks following the second adoptive transfer, both groups of mice were infected intranasally or vaginally with *C. muridarum*.

Ten days after the intranasal challenge, the lungs were harvested and *Chlamydia* titers were determined by plating serial dilutions of the homogenized lungs onto HeLa 229 cells. As shown in Fig. 2*A*, on day 10 postinfection, the yield of *Chlamydia* was $3 \pm 0.8 \times 10^6$ IFU from the lungs of the mice immunized with *Chlamydia* peptide-pulsed DCs while mice immunized with DCs alone had $15.6 \pm 1.7 \times 10^6$ IFU ($p = 0.0002$). Mice vaccinated with viable *C. muridarum*-pulsed DCs were well protected as shown by the yield of $0.08 \pm 0.01 \times 10^6$ IFU compared with the DC control group ($p < 0.0001$).

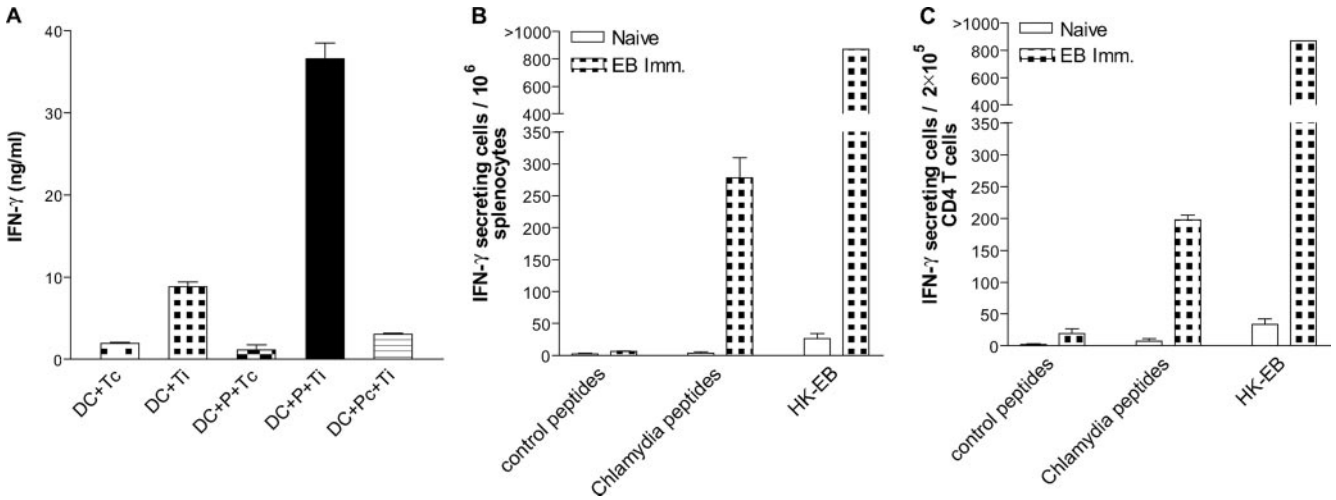

FIGURE 1. Recognition of *Chlamydia* peptides eluted from DCs by CD4 T cells. Splenocytes, purified CD4 T cells and BMDCs were generated as described in *Materials and Methods*. *A,* IFN-γ production by *Chlamydia*-specific CD4 T cells in response to BMDCs pulsed with *Chlamydia* MHC class II-binding peptides determined by ELISA. DC + Tc = DC + T cells purified from naive mice; DC + Ti = DC + T cells purified from immune mice recovered from *Chlamydia* infection; DC + P + Tc = *Chlamydia* peptide-pulsed DCs + T cells purified from naive mice; DC + P + Ti = *Chlamydia* peptide-pulsed DCs + T cells purified from immune mice recovered from *Chlamydia* infection; DC + Pc + Ti = control peptide-pulsed DCs + T cells purified from immune mice recovered from *Chlamydia* infection. Data are shown as the mean ± SEM of three mice. These data are representative of three similar experiments. *B* and *C,* Specific immune responses to *Chlamydia* MHC class II-binding peptides using spleen cells (*B*) and purified CD4 T cells (*C*) from live EB-immunized mice identified by IFN-γ ELISPOT assay. The results represent the average of duplicate wells and are expressed as the means ± SEM of peptide-induced IFN-γ-secreting cells per 10^6 splenocytes for groups of five mice (*B*) and per 2×10^5 CD4 T cells for groups of three mice (*C*). The data in *C* are representative of two similar experiments.

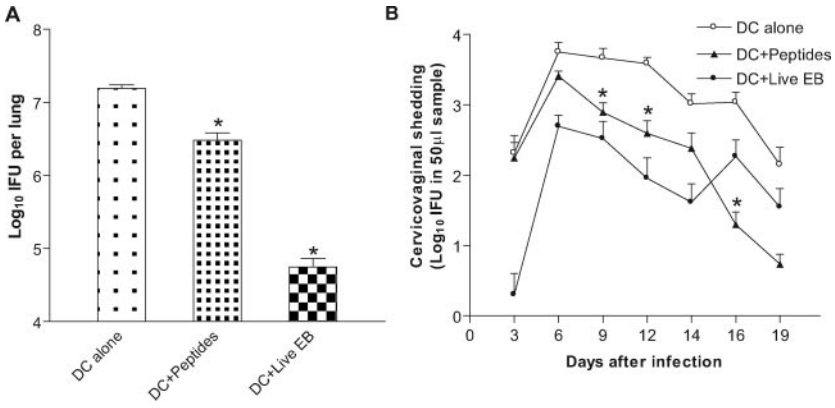

FIGURE 2. Resistance to *Chlamydia* infection following adoptive transfer of DCs pulsed with *Chlamydia* peptides. Synthetic peptides from PmpG-1, PmpE/F-2, Gap, TC0420, FabG, ClpP-1, RplF, and Aasf were pooled and used to pulse LPS-matured BMDCs for 4 h at 37°C. Lung bacterial titer (*A*) was measured at day 10 after intranasal challenge for groups of five mice and cervicovaginal shedding (*B*) was measured after genital challenge for groups of 12 mice. The mean *Chlamydia* IFU ± SEM is indicated. *, $p < 0.05$. These data are representative of three similar experiments.

Protection against intravaginal infection was assessed by isolation of *Chlamydia* from cervicovaginal washes and determination of the number of IFU recovered from each experimental group at the indicated time points (Fig. 2*B*). The results showed that the mice vaccinated with *Chlamydia* peptide-pulsed DCs exhibited resistance to challenge infection as indicated by reductions in the number of *Chlamydia* shed. At days 9, 12, and 16 the cervicovaginal shedding of *C. muridarum* among mice immunized with *Chlamydia* peptide-pulsed DCs was significantly lower than that of mice who received control DCs ($p < 0.05$). In aggregate, these in vivo data demonstrate that *Chlamydia* peptides administered via activated DCs elicit partial protective immunity.

Discussion

Chlamydia immunity appears to be predominantly T cell-mediated and the major problem in developing a vaccine against this intracellular pathogen has been difficulty in identifying relevant T cell Ags (6). DCs are professional APCs with the unique capacity to stimulate naive T cells. DCs capture Ag and following processing, the resulting peptides are loaded onto MHC molecules that are transported to the plasma membrane where peptide-MHC complexes are recognized by Ag-specific T cells (28). Because of high efficiency for peptide processing and presentation, Ag-pulsed DCs have been successfully used for *Chlamydia* vaccination (12). Since *Chlamydia*-pulsed DCs efficiently present Ag to *Chlamydia*-specific CD4 T cells in vitro (14) and induce immunity in vivo, we hypothesized that *Chlamydia* peptides presented on cell surface MHC of pulsed DCs could be identified by high sensitivity MS/MS.

In this study, we identified eight distinct class II and one class I *Chlamydia* peptides presented by MHC molecules on DCs infected with *C. muridarum*. The experiments show that *Chlamydia* peptides can be successfully isolated from MHC molecules of professional APCs and analyzed by MS/MS. The identified peptides were immunologically relevant because they were recognized by *Chlamydia*-specific CD4 T cells from immune mice and when delivered via adoptive transfer of matured DCs pulsed ex vivo conferred significant protection to *C. muridarum* pulmonary and cervicovaginal infection in mice. These results demonstrate that immunoproteomics using DCs along with high-resolution MS/MS is a productive combination that allows for the discovery of new T cell Ags.

Reverse vaccinology is a term coined by Rappuoli et al. (29) and is based on using genomic information to guide vaccine Ag discovery. Reverse vaccinology has been successfully used to identify B cell Ags suitable for vaccine development. Several recent studies have also used the approach to identify T cell epitopes derived from infectious disease agents including *Mycobacterium tuberculosis*, *Plasmodium falciparum*, HIV-1, and *Chlamydia pneumoniae* (30–33). The approach involves bioinformatic identification of candidate epitopes followed by testing each Ag's ability to elicit an immune response against the pathogen of interest. However, the analysis often yields many more candidate T cell epitopes than can be reasonably validated. For instance, a recent study of vaccinia virus-induced murine T cell responses using consensus epitope prediction identified 2256 potential epitopes that were eventually distilled down to 49 immunologically positive epitopes (34). Additionally, algorithmic epitope prediction is much more successful for MHC class I than for MHC class II peptides in part due to the greater variability in amino acid sequences that are loaded on MHC class II molecules, thereby limiting its use for pathogens that require CD4 T cells for protective immunity (35).

Similar to reverse vaccinology, immunoproteomics is also based on whole genome information. Our experience shows that immunoproteomics is able to readily identify class I and II peptides in the context of DC Ag processing and presentation of the whole organism. This reduces the number of false positives and identified epitopes appear to have significant immunological relevance in the context of natural infection. Thus, reverse vaccinology and immunoproteomics can be viewed as complementary techniques able to translate genomic information into viable vaccine Ag discovery.

Using empirical approaches, investigators have previously uncovered a limited number of *Chlamydia* proteins containing T cell Ags, some of which have been demonstrated to elicit partial protection in mice (6). Both cysteine-rich protein A (CrpA) and class I-accessible protein 1 (Cap1) contain MHC class I epitopes and are localized in the chlamydial inclusion membrane. The major outer membrane protein (MOMP) and the outer membrane protein 2 (OMP2) are quantitatively dominant *Chlamydia* membrane proteins and MOMP in particular is known to contain both class I and class II T cell epitopes. Other *Chlamydia* proteins containing T cell Ags comprise both cytoplasmic and membrane proteins and include: heat shock protein 60 (Hsp60), homolog of *Yersinia pseudotuberculosis* YopD (YopD), enolase, polymorphic membrane protein D (PmpD), and *Chlamydia* protease-like activity factor (CPAF) (6, 36).

Chlamydia proteins corresponding to MHC class II peptides found in this study were also composed of both cytosolic and membrane proteins (Table I). PmpE/F-2 and PmpG-1 belong to the polymorphic membrane protein family known to contain immunogenic amino terminal passenger domains associated with the outer membrane (37). In a recent comparative genome study, Carlson et al. (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. The authors concluded that *Chlamydia* PmpF could be a particularly important immune target of T cells and that variability in the MHC-binding epitopes of PmpF could help the pathogen evade host immune responses. This speculation is further supported by the finding that PmpF is the most polymorphic protein among all *C. trachomatis* Pmps (39). The identification of PmpE/F-2-derived peptide in this study could be explained by its possible unique role in MHC peptide binding and/or by its abundance because *pmpF* also has the highest mRNA expression level among tested *C. trachomatis* strains (40). PmpG has also been identified as recognized by CD4 T cells during *C. pneumoniae* infection in mice (41). Less is known regarding immune recognition of the *Chlamydia* proteins Gap, RplF, FabG, Aasf, TC0420, ClpP-1, and the class I protein Aap. Of interest, the protein TC0420 appears to be unique to *Chlamydia* species. Because these proteins appear relevant in terms of immune recognition, they are a high priority for further detailed immunobiological study.

Surprisingly, we did not identify T cell epitopes from *Chlamydia* MOMP or *Chlamydia* Hsp60 which other studies have shown to contain multiple CD4 T cell epitopes. *Chlamydia* Hsp60 is known to be a potent B cell Ag and anti-Hsp60 Abs are abundantly generated in patients with the late disease sequelae of *Chlamydia* infection (42). Additionally, IFN-γ T cell responses to *Chlamydia* Hsp60 are known to correlate with protection to cervical infection among commercial sex workers (43). It may be that in C57BL/6 mice, MOMP and Hsp60 generate low-affinity or low-frequency MHC class II peptides under the growth conditions we used and this may have limited detection of derived immunopeptides by MS/MS. Harvesting MHC molecules from DCs under different growth conditions or at different times in the developmental cycle may yield additional T cell peptide epitopes. Likewise, more starting material or more sensitive instrumentation may also identify more MHC-presented *Chlamydia* peptides.

Interestingly, we observed that few MHC class I-bound *Chlamydia* peptides were identified relative to class II-bound peptides. Furthermore, the single MHC class I peptide (Aap) that we identified was not recognized in the in vitro IFN-γ ELISPOT assay (data not shown). Even though this finding may support previous studies demonstrating that CD4 T cells are the main effectors for the control of *Chlamydia* infection (44), the rarity of class I peptides was somewhat unexpected as *Chlamydia* is known to secrete proteins into the vacuolar membrane and host cytosol (45). The low abundance of class I peptides might be due to the presence of recently identified *Chlamydia*-encoded deubiquitinating enzymes that are speculated to reduce proteasome generation of antigenic peptides for class I presentation (46). Other possible reasons might include the lack of consensus motif sequences or proteolytic cleavage sites in secreted *Chlamydia* proteins, low-affinity interactions with MHC, or synthesis at time points in the *Chlamydia* developmental cycle not assessed in this study.

Our in vitro results showed that MHC class II peptides derived from the *Chlamydia* proteome are recognized by *Chlamydia*-specific CD4 T cells from immune mice as determined by ELISA and the ELISPOT assay. Additional experiments using adoptive transfer of ex vivo-pulsed DCs demonstrated that *Chlamydia* MHC class II peptides are capable of engendering partial immunity following pulmonary and genital tract challenge with *C. muridarum*. Taken together, these results suggest that one or more of the identified MHC class II immunopeptides are presented to protective CD4 T cells in vivo. Current experiments are underway to determine which individual peptides are maximally antigenic.

To be useful as vaccine in outbred populations, it will be important to use the peptide information we have generated to identify proteins that are immunodominant T cell Ags in the context of different MHCs. In preliminary experiments, we have observed that proteins containing the MHC class II peptides that we identified are recognized by immune CD4 T cells from both C57BL/6 and BALB/c mice (data not shown). If it can be established that such proteins contain MHC class II peptides that are indeed recognized in outbred populations, then such proteins will be very useful for further evaluation as *Chlamydia* vaccine candidates. It also appears likely that the immunoproteomic approach used in this study will be useful to identify Ags for other problematic intracellular pathogens such as tuberculosis, malaria, and HIV for which vaccine solutions are needed.

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Disclosures

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