CT043, a Protective Antigen That Induces a CD4⁺ Th1 Response during *Chlamydia trachomatis* Infection in Mice and Humans[▽]

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Despite several decades of intensive studies, no vaccines against *Chlamydia trachomatis*, an intracellular pathogen causing serious ocular and urogenital diseases, are available yet. Infection-induced immunity in both animal models and humans strongly supports the notion that for a vaccine to be effective a strong CD4⁺ Th1 immune response should be induced. In the course of our vaccine screening program based on the selection of chlamydial proteins eliciting cell-mediated immunity, we have found that CT043, a protein annotated as hypothetical, induces CD4⁺ Th1 cells both in chlamydia-infected mice and in human patients with diagnosed *C. trachomatis* genital infection. DNA priming/protein boost immunization with CT043 results in a 2.6-log inclusion-forming unit reduction in the murine lung infection model. Sequence analysis of CT043 from *C. trachomatis* human isolates belonging to the most representative genital serovars revealed a high degree of conservation, suggesting that this antigen could provide cross-serotype protection. Therefore, CT043 is a promising vaccine candidate against *C. trachomatis* infection.

Chlamydia trachomatis is an obligate intracellular human pathogen which exists in two highly specialized morphological forms: the infectious elementary body (EB) and the replicative reticulate body (RB). The pathogen has been classified into 19 different serotypes (serovars), on the basis of which variant of the major outer membrane protein (MOMP) is expressed on its surface. Worldwide, *C. trachomatis* is responsible for more than 92 million sexually transmitted infections and 85 million ocular infections per year (45). These infections cause several types of diseases (35, 44), including trachoma-induced blindness (serovars A to C), pelvic inflammatory disease, ectopic pregnancy and infertility (serovars D to K), and lymphogranuloma venereum (serovars L1 to L3). Furthermore, recent studies indicate that chlamydia infections facilitate the transmission of both human immunodeficiency virus (28) and human papillomavirus (16).

Although effective antibiotic treatments are available, they are often unsuccessful in halting the spread of the infection or inadequate to prevent the chlamydia-mediated long-term sequelae for a number of reasons. First, urogenital tract infections are frequently asymptomatic and therefore not properly treated in due time. Second, although *C. trachomatis* is generally sensitive to a panel of antibiotics, multiple-antibiotic-resistant strains of chlamydia have been reported (34). Third, there

are indications from in vitro studies that antibiotic treatment could lead to the formation of aberrant chlamydia forms that remain dormant within inclusions and may eventually turn into EBs under favorable environmental conditions (9, 47). Finally, epidemiological data in industrialized countries indicate that the rate of chlamydia reinfection is rising, and this has been attributed to the interference of early antibiotic treatment with the acquisition of immunity against chlamydia (2).

For these reasons, the development of an effective vaccine against *C. trachomatis* infection is urgently needed. Early trials with heat-killed preparations of whole EBs have been shown to elicit short-term protection, but a few cases of immunopathological reactions upon reexposure to chlamydia have also been reported (2). Based on these early findings, further efforts have been focused on the development of subunit vaccines (2, 13, 14). In particular, several studies have described the use of MOMP, the immunodominant chlamydial antigen accounting for 60% of the total mass of the chlamydia outer membrane (6). Immunization with MOMP purified from *C. trachomatis* elicited an immune response that fully protected mice against an intra-ovarian bursa chlamydia challenge (27). However, only correctly folded MOMP appears to provide protection, and no adequate, scalable processes for the production of soluble and properly folded recombinant MOMP have been developed yet. This has so far hampered the use of MOMP for vaccine development. Moreover, due to the sequence variability of MOMP, a broadly protective chlamydial vaccine will probably require the use of other immunogenic antigens in addition to, or in place of, MOMP.

The search for other chlamydial antigens has been driven by the elucidation of the mechanisms of immune responses to chlamydia infection and the demonstration of the importance

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of CD4⁺ T cells in natural immunity. Animal models have shown that protective immunity to *C. trachomatis* depends on the elicitation of a Th1-polarized cell-mediated immune response, in particular, gamma interferon (IFN- γ)-secreting CD4⁺ lymphocytes. In mice, the depletion of CD4⁺ T cells results in the loss of protective immunity and adoptive transfer of chlamydia-specific CD4⁺ T cells confers protection against *C. trachomatis* challenge (36). Studies in primates have also emphasized the role of Th1 cells in immunity to chlamydia (42, 43).

Here we report experimental evidence that CT043, a protein annotated as hypothetical, is a novel protective chlamydial antigen. This antigen appears to be associated with the bacterial cell surface and is expressed within the chlamydia inclusion in infected HeLa cells throughout the infection cycle. We show that CT043 is the target of CD4⁺ Th1 cells both in chlamydia-infected mice and in human patients with diagnosed *C. trachomatis* genital infection. Moreover, by using a DNA priming/protein boost immunization protocol, we show that the antigen significantly reduces the bacterial load in a mouse model of intranasal (i.n.) infection. Finally, sequence analysis of the CT043 gene in 22 *C. trachomatis* strains belonging to the most representative genital serovars revealed a high degree of conservation (99.4% amino acid identity), suggesting that the antigen could provide cross-serotype protection.

Altogether, our study demonstrates that this antigen is a promising candidate to be included in a subunit-based vaccine against *C. trachomatis*.

MATERIALS AND METHODS

Bacterial strains, cultures, and reagents. *Chlamydia muridarum* strain Nigg and *C. trachomatis* serovar D strain D/UW-3/CX were grown on confluent monolayers of LLCMK2 (ATCC CCL7) or HeLa 229 cells (ATCC CCL2.1) in Earle's minimal essential medium (EMEM) as described previously (3, 21). Purification of *C. trachomatis* and *C. muridarum* EBs was carried out by Renografin density gradient centrifugation as described previously (3). *Escherichia coli* DH5 α or BL21(DE3) was grown aerobically in Luria broth (LB) medium (Difco) at 37°C. When appropriate, ampicillin (100 μ g/ml) and isopropyl- β -D-galactopyranoside (IPTG; 0.5 mM) were added to the medium. Unless specified, all chemicals used in this study were purchased from Sigma. Restriction enzymes and DNA modification enzymes were from New England Biolabs. Fluorescence-activated cell sorter (FACS) reagents and antibodies were from BD Biosciences, Pharmingen. Confocal microscopy reagents were from Molecular Probes.

Gene cloning, protein purification, and serological analysis. To produce recombinant proteins CT043 and CT0681 (*C. trachomatis* MOMP [MOMP_{CT}]) and their *C. muridarum* homologs, TC0313 and TC0052 (MOMP_{CM}), genes were PCR amplified from *C. trachomatis* and *C. muridarum* chromosomal DNA using specific primers annealing at the 5' and 3' ends of either gene and cloned into plasmid pET21b⁺ (Invitrogen) so as to fuse a six-histidine tag sequence at the 3' end. Cloning and purification of His fusions were performed as already described (21). MOMP_{CT} and MOMP_{CM} expressed as His fusion proteins were purified from the insoluble protein fraction. MOMP_{CT} and MOMP_{CM} were also cloned into plasmid pMal c4 (New England Biolabs) fused to the *malE* gene of *Escherichia coli*, encoding a maltose-binding protein (MBP). MBP-MOMP_{CM} and MOMP_{CT} and MBP alone were affinity purified from the soluble fraction by amylose resin chromatography (New England Biolabs) according to the manufacturer's procedure.

For T-cell in vitro stimulation assays, lipopolysaccharide (LPS)-free proteins were prepared by washing column-immobilized proteins with buffer 10 mM Tris-HCl (pH 8), containing 1% Triton X-114 (35 ml) at 4°C. The amount of residual endotoxin was determined using a *Limulus* amoebocyte lysate analysis kit (QCL-100; BioWhittaker, Walkersville, MD).

For DNA immunization studies, the TC0313 and TC0052 genes were amplified from *C. muridarum* genomic DNA and cloned into plasmid pCMV KaSF2120 fused to the human tissue plasminogen activator signal sequence (12).

Large-scale preparations of the resulting plasmids pCMV-TC0313 and pCMV-MOMP were prepared at low endotoxin level by Aldevron LLC (Fargo, ND). Mouse antisera were generated and treated as described previously (21). Where specified, sera from mice immunized with 20 μ g of *E. coli* contaminant proteins (immobilized metal affinity chromatography-purified proteins from *E. coli* bacteria containing pET21b⁺ empty vector) were used as a negative control. Western blotting, enzyme-linked immunosorbent assay, and flow cytometry of *C. trachomatis* EBs were performed as described previously (10, 21).

Proteomic analysis of EB surface-exposed proteins. *C. trachomatis* EB surface digestion was carried out on 10⁸ IFU with 20 μ g of trypsin (Promega) in 500 μ l of sucrose-phosphate-glutamine buffer (SPG) (3) for 30 min at 37°C, and reactions were stopped with 0.1% formic acid. Peptides generated from the digestion were separated from the whole cell by filtration using 100-kDa pore-size filters (Millipore) and identified by nano-liquid chromatography tandem mass spectrometry, as described previously (30). The integrity of the EB preparation after trypsin digestion was >95%, as assessed by quantitative PCR detection of chromosomal DNA present in reaction tubes after protease treatment and in the peptide-containing fraction.

Analysis of antigen-specific CD4⁺ Th1 response in splenocytes and PBMCs. Splenocytes were isolated from BALB/c mice 10 days after *C. trachomatis* challenge with 10⁶ IFU, plated in duplicate on 96-well plates at 2 $\times$ 10⁶ cells/well, and stimulated with 20 μ g/ml of endotoxin-free specific antigen or with 4 μ g/ml of purified EBs, in the presence of 1 μ g/ml anti-CD28 antibody for 3 h at 37°C. Brefeldin A was added (2.5 μ g/ml) for an additional 16 h before intracellular staining.

Peripheral blood mononuclear cells (PBMCs) from mice were isolated from up to 2 ml of heparinized blood, diluted 1/4 in RPMI 1640 medium (Sigma), and separated by density gradient centrifugation over Lympholite-M (Cedarlane). PBMCs were further washed, resuspended in RPMI complete medium (containing penicillin-streptomycin solution, 200 mM L-glutamine, and 2.5% fetal calf serum), plated in duplicate (10⁶ PBMCs/well) together with 10⁶ mouse CD4-depleted splenocytes as antigen-presenting cells, and stimulated as described above. For intracellular cytokine assessment, cells were stained for viability with LIVE/DEAD (Molecular Probes) dye according to the manufacturer's instructions. Cells were then fixed and permeabilized using the Cytofix/Cytoperm kit, washed with Permwash, and stained with fluorochrome-labeled monoclonal antibodies for the detection of cells expressing CD3 and CD4 on the surface and intracellular IFN- γ or interleukin-4 (IL-4). Sample acquisition was done with a BD Canto flow cytometer, and data were analyzed using FlowJo software (Tree Star, Inc.). The intracellular expression of IFN- γ and IL-4 was analyzed on CD4⁺-expressing cells, previously gated for morphology, CD3 expression, and viability. For the study in humans, we used PBMC aliquots derived from samples from anonymous blood donors remaining after routine diagnosis of *C. trachomatis* infection. The samples were available at the S. Orsola Hospital—University of Bologna, which has authorization to treat such material for research purposes (4).

Analysis of CD4⁺ Th1 response was carried out as described above, with the exception that stimulation was done in the presence of anti-CD28 and anti-CD49 antibodies.

Mouse protection model. Groups of 6-week-old female BALB/c mice purchased from Charles River Laboratories (10 to 15 mice/group) were immunized intramuscularly (i.m.) at days 1, 15, and 28 with 3 doses (15 μ g) of purified recombinant TC0313 or *C. muridarum* MBP-MOMP formulated with 5 μ g of LTK63 as adjuvant (31) plus 10 μ g of CpG (ODN 1826) as Th1 immunoadjuvant (19). As a negative control, groups of mice that received the adjuvant alone or MBP were included. For the DNA priming/protein boost immunization regimen, 2 h before each DNA dose, mice were pretreated with an i.m. injection of 10 U of bovine hyaluronidase (20). pCMV-313 or pCMV-MOMP (50 μ g/dose) was injected at days 1, 21, and 42 into the tibial muscle, followed by electroporation with a BTX830 electroporator using a two-needle electrode array, as described previously (46). Three weeks after the third DNA dose, mice received one protein dose of TC0313 or MBP-MOMP (15 μ g) with LTK63-CpG adjuvant. As a negative control, mice received three i.m. injections of plasmid pCMV KaSF2120 and one adjuvant dose. Three weeks after the last immunization, mice were inoculated i.n. with 40 μ l of SPG buffer containing 10³ IFU of *C. muridarum*. The chlamydia challenge dose given to mice was confirmed by culturing in triplicate serial dilutions of the inoculating dose on LLCMK2 cell monolayers and, 24 h later, by counting chlamydial inclusions stained with FITC-conjugated antichlamydia antibody (Merifluor) using a UV microscope. Ten days postchallenge, lungs were harvested and homogenates were inoculated in LLCMK2 cell monolayers to count viable chlamydiae as described above. The detection limit of IFU recovered per lung was 500 IFU (2.7 log₁₀). All procedures were approved

by the National Health Institution and Novartis Vaccines Animal Care and Ethical Committee.

Confocal microscopy. To examine cellular localization of CT043 after infection, HeLa cells grown on glass coverslips were infected with *C. trachomatis*. At 6, 24, and 48 h postinfection, HeLa cells were fixed with 2% paraformaldehyde (PFA)-phosphate-buffered saline (PBS) followed by permeabilization with 1% saponin. After washing and blocking with 1% bovine serum albumin-PBS, the cell samples were stained with CT043 antiserum (1:5,000) and goat anti-mouse Alexa Fluor-conjugated antibody dye A₄₈₈. To reduce aspecific binding, CT043 antiserum was previously preadsorbed onto nitrocellulose strips containing *E. coli* BL21 and HeLa cell total proteins. DNA and actin were stained with propidium iodide and phalloidin conjugated with Alexa Fluor dye A₆₂₀, respectively. Antifade reagent was used to mount coverslips. Slides were scanned with a Bio-Rad confocal microscope with a 100× oil immersion objective lens.

Immunogold labeling and electron microscopy. Purified *C. trachomatis* EB or RB particles were charged on Formvar-carbon-coated nickel grids and saturated in blocking solution (1% bovine serum albumin-PBS) for 1 h at room temperature before immunogold labeling. Alternatively, monolayers of HeLa cells were grown onto polycarbonate Transwell membranes (0.4-μm pore size; Corning, Inc., Life Sciences) and then infected with *C. trachomatis*. At 24 h postinfection, cells were fixed in 2% PFA-PBS for 30 min, followed by permeabilization with 1% saponin-PBS buffer for 1 h at room temperature. Samples were then incubated with mouse anti-CT681 or anti-CT043 polyclonal antibodies and subsequently with goat anti-mouse gold-conjugated immunoglobulin G (IgG; 10-nm particle diameter). After washing, EBs or RBs were fixed in 2% PFA and stained with 1% phosphor-tungstic acid (pH 6.5), for 15 s. Infected HeLa cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer containing 1.8% sucrose (pH 7.2) and then rinsed in PBS buffer and postfixed in 1% osmium tetroxide. After dehydration in a graded series of ethanol, the membranes with infected cells were first slashed off from the wells and then embedded in an Epon-Araldite mixture. Ultrathin sections, obtained with a Reichert Ultracut II E, were routinely stained (1% uranyl acetate followed by 1% lead citrate). Samples were observed with a Philips CM 10 transmission electron microscope (Philips Electronic Instruments, Inc.) operating at 80 kV. Isolation and counting of gold particles of different sizes were done with the software EMAN (18).

Sequencing. Chromosomal DNA was extracted from EBs of ATCC strains and from EBs of clinical isolates by lysing EBs overnight at 37°C with 10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.6% sodium dodecyl sulfate, and 100 μg/ml proteinase K; sequential phenolic extraction; and alcohol precipitation. Fifty nanograms of each genomic DNA was used as template to amplify CT043 by PCR using Platinum *Taq* polymerase (Invitrogen). Amplicons were purified with a Qiagen PCR purification kit according to the manufacturer's instructions. PCR and sequencing primers were designed based on the nucleotide sequence of the CT043 gene from the serovar D strain D/UW-3/CX (GenBank accession no. AE001273.1). Both strands of the purified amplicons were sequenced using the Big-Dye 3.1 sequencing chemistry and the ABI 3730-xl 96-well capillary analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The CT043 sequences were edited, aligned, and analyzed using the software of the Wisconsin Package (GCG, Madison, WI).

Statistical analysis. Instat 3 (GraphPad software, Inc.) was used for statistical analysis. The Mann-Whitney U test was used to determine differences in CD4 Th1 frequencies and to evaluate differences in the lung bacterial load between experimental groups. Differences were considered statistically significant if *P* values were <0.05.

RESULTS

***C. trachomatis* mouse infection elicits CT043-specific CD4⁺ Th1 cells.** Our rationale to identify protective antigens is based on the observation that in mice *C. trachomatis* primary infection elicits IFN-γ-producing CD4⁺ T cells that protect mice from subsequent challenges (36). Therefore, we decided to systematically analyze the T-cell population of *C. trachomatis*-infected mice with the goal of defining their specificity toward the chlamydia antigens. Toward this aim, we initiated a high-throughput screening of *C. trachomatis* proteins selected on the basis of their being surface-predicted, virulent factors or hypothetical sharing sequence homologies with proteins from other pathogens known to be implicated in host interaction (Finco et al., unpublished observations). After their expression

FIG. 1. CT043- and MOMP-specific CD4⁺ IFN-γ⁺ response in BALB/c mice infected with a primary *C. trachomatis* infection. Groups of two or three BALB/c mice were infected intravaginally with 10⁶ *C. trachomatis* EBs. Ten days after infection, splenocytes were stimulated with 20 μg/ml of LPS-free recombinant CT043 (black bars) and MOMP (gray bars). The intracellular IFN-γ expression induced by each antigen was analyzed by flow cytometry on viable CD4⁺ T cells of infected mice and noninfected controls. Results represent the average values of 16 independent experiments. Error bars represent the standard error. Statistical difference among groups (*P*) was evaluated by the Mann-Whitney U test.

in *E. coli* and purification in a highly pure, LPS-free form, the proteins were ready to be tested for their capacity to stimulate IFN-γ production in splenocytes from chlamydia-infected mice. Positive antigens were subsequently screened in the *C. muridarum* mouse immunization model. Among the antigens analyzed so far, the hypothetical protein CT043 induced CD4⁺ T cells to produce IFN-γ with a frequency similar to the benchmark antigen MOMP. As shown in Fig. 1, the frequency of CT043-specific, IFN-γ-producing CD4⁺ T cells in splenocytes from chlamydia-infected mice was 86/10⁵ CD4⁺ T cells, a value comparable to that observed with MOMP. The frequency was significantly higher than the one observed in noninfected controls (*P* = 0.0088); indeed no appreciable levels of CT043-specific and MOMP-specific IFN-γ-producing CD4⁺ T cells were detected in the spleens from naive noninfected mice. Furthermore, the stimulation of splenocytes with both CT043 and MOMP did not reveal the presence of specific T cells producing IL-4. In the *C. muridarum* mouse infection model, the mouse strain largely used to study chlamydial biology and immunity carries a protein named "TC0313," which is almost identical to CT043, the two proteins sharing 97.6% of their amino acid sequence (corresponding only to a 4-amino-acid difference). Therefore, we verified whether *C. muridarum* mouse infection also induces a population of IFN-γ-producing, CD4⁺ T cells specific for this conserved antigen. Indeed, when splenocytes were stimulated with either CT043 or recombinant TC0313, specific CD4⁺ T cells were present at a frequency of

FIG. 2. Antigen specific CD4⁺ IFN-γ⁺ response in *C. trachomatis*-infected patients. PBMCs were isolated from patients infected by different *C. trachomatis* serotypes and healthy subjects and stimulated with heat-inactivated EBs, purified recombinant CT043, and MOMP. The left panel represents the number of specific CD4⁺ T cells producing IFN-γ, in both healthy subjects (white bars) and patients (filled bars), upon stimulation with whole EBs. The *C. trachomatis* serovar infecting each patient is represented by different bar patterns. The cutoff value of response to EBs was based on the upper 95% CI limit of the geometric mean for the control healthy subjects (dotted line). In the right panel, the frequencies of MOMP- and CT043-antigen specific CD4⁺ T cells that produce IFN-γ are reported for healthy subjects (white bars) and chlamydia-infected EB-responding patients (filled bars). Human subjects are reported in the same order in both graphs. Dotted lines within the graph represent the cutoff value of response to the individual recombinant proteins, defined as the upper 95% CI of the geometric mean for the healthy subjects.

168/10⁵, a frequency fivefold higher than what observed in the splenocytes from naïve mice (33/10⁵). Altogether, these data strongly suggest that upon infection, *C. trachomatis* and *C. muridarum* elicit a Th1 CD4⁺ T-cell response against the highly conserved CT043 and TC0313 antigens, respectively.

CT043 is a target of CD4⁺ Th1 cells in patients with genital *C. trachomatis* infection. We next investigated whether *C. trachomatis* infection in humans also elicits CT043-specific CD4⁺ T cells secreting IFN-γ. To this aim, we selected eight healthy volunteers (with no history of chlamydia-associated disease and serologically negative in the 5 years preceding the time of PBMC collection) and 18 patients divided into four groups on the basis of the chlamydia serovar they had been infected with: group Da (4 patients), group E (6 patients), group F (2 patients), and group G (6 patients). The pathogen isolated from patients was serotyped by DNA sequencing of the PCR-amplified MOMP gene (17). PBMCs from each individual were stimulated with serovar D heat-inactivated EBs, and the frequency of IFN-γ producing CD4⁺ T cells was assessed as described above. Twelve of the 18 patients showed a frequency of EB-specific CD4⁺ T cells producing IFN-γ of ≥11 out of 10⁵ CD4⁺ T cells (cutoff value based on the upper 95% confidence interval [CI] of the geometric mean for the control subjects) (Fig. 2, left panel), and 7 of them responded with a frequency higher than 50 positive cells on 10⁵ CD4⁺ cells. These “high responders” were all infected with serovar Da or E. No chlamydia-specific CD4⁺ T cells were found in PBMCs from the eight healthy volunteers. Subsequently, PBMCs from the 12 EB-responding patients and from the 8 healthy volunteers were stimulated with LPS-free recombinant CT043 and MOMP (Fig. 2, right panel). Eight out of 12 patients (67%) had a frequency of IFN-γ-producing CD4⁺ T cells specific for CT043 above the cutoff frequency of 14 positive cells/10⁵ CD4⁺ T cells. In particular, four patients had a frequency significantly higher than that of healthy subjects (>35 IFN-γ⁺ cells/10⁵ CD4⁺ T cells), while four patients were only margin-

ally positive. These CT043-responding patients did not predominantly belong to any of the four serovar groups. Among the healthy controls, only two subjects responded to CT043 (25%), with frequencies of 25 and 14 IFN-γ⁺ cells/10⁵ CD4⁺ T cells, respectively, and the mean frequency of IFN-γ-producing CD4⁺ T cells specific for CT043 was significantly higher in patients than in control healthy subjects ($P = 0.04$; Mann-Whitney rank sum test). The response to MOMP (serovar D) was also analyzed. Eight out of the 12 EB-responding patients (67%) had a measurable frequency of IFN-γ-producing CD4⁺ T cells in response to the recombinant serovar D MOMP, and 5 of them also responded to CT043 (Fig. 2, right panel). The serotyping of the infecting serovars highlighted serovars Da and E as predominant (67%) among the EB-responding patients, while four of the six patients not responding to serovar D EB stimulation belong to serovar G and F groups (67%). Finally, no IL-4-producing CD4⁺ T cells were found in both *C. trachomatis*-infected and healthy individuals in response to the tested recombinant antigens. Altogether, these data indicate that PBMCs from *C. trachomatis*-infected patients carry, as expected, CD4⁺ T cells which secrete IFN-γ upon stimulation with whole EBs. Among this population, MOMP-specific T cells and CT043-specific T cells are present. CT043 is likely to be well conserved and similarly expressed among different serovars, as judged by the capability of serovar D CT043 recombinant protein to stimulate CD4⁺ T cells from most of the patients, regardless of the *C. trachomatis* serovar with which they are infected.

The hypothetical protein CT043 is expressed by *C. trachomatis* at different developmental stages. Since CT043 is annotated as a hypothetical protein (<http://www.ncbi.nlm.nih.gov/>) and had never been characterized before, we studied its expression in *C. trachomatis* serovar D during its developmental cycle. As shown in Fig. 3A, Western blot analysis of total protein lysates of Renografin-purified EBs (corresponding to ~10⁷ EBs per lane) showed that CT043 antiserum recognized

FIG. 3. Analysis of CT043 expression. (A) Western blot analysis of CT043 expression in purified EBs and in *C. trachomatis*-infected HeLa cells. EBs and infected HeLa cells were lysed by sonication followed by 10' boiling, and total protein lysates (corresponding to $\sim 10^7$ EBs) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with CT043 and MOMP_C antisera (upper and lower panel, respectively). A Coomassie staining of the purified proteins is represented within each panel. Molecular mass standards are reported on the left side of each panel. α , anti-. (B) Confocal microscopy analysis of CT043 expression in infected HeLa cells. HeLa cells were grown on glass coverslips and infected with *C. trachomatis*. At times zero, 6, 24, and 48 h postinfection, cells were stained with CT043 antiserum followed by Alexa Fluor dye A₄₈₈-conjugated anti-mouse IgG (green). DNA was visualized with propidium iodide (red), and HeLa cell actin was stained with phalloidin-conjugated Alexa Fluor dye A₆₂₀ (blue). (C) Analysis of CT043 expression on the EB surface by FACS. Purified *C. trachomatis* EBs were incubated with either preimmune sera (empty peak) or with serum from mice immunized with recombinant CT043 (filled peak), and antibody binding was detected by R-phycoerythrin-conjugated anti-mouse IgG. (D) Transmission electron microscope analysis of CT043 expression on *C. trachomatis* RB and EB forms. Purified RBs and EBs (upper and middle panels) adsorbed to Formvar-carbon-coated nickel grids were incubated with anti-CT043 serum (panels 1 and 4), anti-MOMP serum (panels 2 and 5), or preimmune serum (panels 3 and 6) and detected with secondary gold-conjugated anti-IgG. Lower panels represent ultrathin sections of RBs and EBs within inclusions of HeLa cells at 24 h postinfection with *C. trachomatis*, stained with CT043 antiserum (panels 7 and 8) or preimmune serum (panel 9).

a single protein band of the expected molecular mass (18.4 kDa). We then investigated CT043 expression during *C. trachomatis* infection of human epithelial cells, assessing CT043 expression by Western blot analysis of total cell lysates after 24 and 48 h. The level of MOMP expression was used to normalize the amount of chlamydial proteins loaded on the gel as described previously (40). As shown in Fig. 3A, a single reactive band was observed at both time points, indicating that CT043 expression is substantially constant throughout the chlamydial biphasic developmental cycle. We also investigated CT043 cellular localization by confocal microscopy in infected HeLa cells at 6, 24, and 48 h postinfection. As shown in Fig. 3B, CT043 expression was detected within the inclusions as early as 6 h after infection and during the entire infection cycle.

CT043 is associated with the chlamydia surface. To assess CT043 cellular localization, we first investigated whether the protein is exposed on the chlamydia surface by FACS analysis, based on the capacity of anti-CT043 polyclonal antibodies to recognize purified EBs. As shown in Fig. 3C, anti-CT043 antibodies strongly recognized the entire population of EBs, giving a fluorescence shift of over 1 log with respect to the negative control serum. To confirm CT043 surface association, EBs were treated with trypsin to remove the protein domains protruding out of the bacterial surface (30). After digestion, the “shaved” EBs were removed by filtration and the proteolytic peptides present in the supernatant were analyzed by tandem mass spectrometry. The analysis revealed that the peptide mixture included one specific peptide matching the sequence LLEGSM LGGQMAGGGVGVATK present in CT043 from amino acids 75 to 95.

Finally, CT043 localization was also assessed via immunogold electron microscopy either on purified *C. trachomatis* EBs and RBs or on ultrathin sections of infected HeLa cells (at 24 h postinfection). As shown in Fig. 3D (panels 1 and 4), CT043 labeling occurred with a similar qualitative and quantitative surface distribution in both EBs and RBs (15 and 10 gold particles in RBs and EBs, respectively), while no colloidal gold was bound to the negative control samples (preimmune sera) (panels 3 and 6). MOMP antiserum showed similar recognition (panels 2 and 5), with 23 and 16 gold particles bound to the RB and EB surface, respectively. As shown in Fig. 3D (panels 7 and 8), CT043 antibodies mainly labeled the periphery of both RBs and EBs in fixed ultrathin sections of chlamydial inclusions in HeLa cells (8 and 5 gold particles, respectively), while no colloidal gold was bound to the negative control (panel 9). The observed lower counts of colloidal gold particles associated with the chlamydial EBs and RBs in ultrathin sections, as compared to the purified EBs and RBs, were probably due to the differences in sample preparation.

Altogether these data demonstrate that CT043 is associated with the chlamydia surface, differently from what was predicted by Psort (<http://www.psort.org/psortb>), which assigns it to the cytoplasmic compartment.

TC0313 confers protection against *C. muridarum* i.n. infection. After having demonstrated the capability of CT043 to induce a specific CD4⁺ Th1 response, we next evaluated the protective activity of this antigen using the *C. muridarum* mouse model of lung infection. The choice of this model has been mainly driven by the evidence that intravaginal challenge, the other model used in protection studies, requires treatment

with progesterone in order to be efficient (41), and progesterone has immunomodulatory properties (37) which might affect the immune responses elicited by the vaccine candidates under examination (24). The immunization was done by two different regimens, aimed at eliciting a potent CD4⁺ Th1 response and, possibly, high antibody titers. The first regimen was based on immunization with the recombinant protein formulated with the strong Th1 adjuvant LTK63-CpG (30). The second regimen was based on a DNA prime/protein boost schedule (5). In particular, for protein immunization, TC0313, the *C. muridarum* homolog of CT043 (97.7% identity at amino acid level) was used to immunize BALB/c mice formulated with LTK63 and CpG (ODN1826). As far as the DNA prime/protein boost immunization is concerned, the TC0313 gene was cloned generating the recombinant plasmid pCMV-TC0313. Mice received three i.m. injections of pCMV-TC0313 by electroporation followed, 3 weeks later, by a booster dose of TC0313 protein formulated with LTK63-CpG. Mice were challenged i.n. with 10³ *C. muridarum* EBs, and protection was measured by counting IFU in the lungs 10 days after challenge. As shown in Fig. 4, immunization with TC0313 protein induced an ~0.6-log reduction in IFU counts with respect to the animals immunized with the adjuvant alone. A slightly higher IFU reduction (about 1 log) was obtained when mice received a recombinant form of MOMP fused to MBP. Protection elicited by the pCMV-TC0313/TC0313 immunization was more pronounced, with an IFU reduction of 2.6 logs with respect to the negative control (empty plasmid immunization followed by LTK63-CpG injection). Interestingly, the observed protection was almost 1 log higher than that obtained in mice immunized with three doses of pCMV-MOMP DNA followed by a protein boost with MBP-MOMP fusion (Fig. 4). TC0313 protection appeared to correlate with the elicitation of IFN- γ -producing CD4⁺ T cells upon immunization, since the DNA prime/protein boost regimen induced a frequency of TC0313-specific CD4⁺ Th1 cells substantially higher than that obtained with protein immunization (Fig. 5). This was not the case for MOMP, which elicited a good CD4⁺ Th1 cell frequency with both regimens (Fig. 5). The reason why the prime boost immunization for MOMP did not improve with respect to protein alone might be related to the known inability of no native MOMP to confer full protection in the mouse model. The importance of cell-mediated immunity (CMI) over humoral response in protection against chlamydia infection was confirmed by analyzing the antibody responses to recombinant TC0313 in the pools of sera collected after the last immunization dose. As expected, TC0313-specific IgG titers were significantly higher in mice immunized with the protein immunization than in animals that received DNA priming/protein boost (titers of 1:146,000 and 1:20,000, respectively). Furthermore, antibodies elicited by both immunization regimens were not capable of neutralizing EB infectivity in vitro. In accordance with the Th1 type of immune response elicited by DNA immunization, a large proportion of antibodies elicited by DNA prime/protein boost immunization were of the IgG2a isotype (IgG2a titer, 1:32,000; IgG1 titer, 1:3,700).

CT043 is highly conserved among *C. trachomatis* serovars. The protection experiments described above suggest that CT043 could become an important component of a future chlamydia vaccine, given that the antigen is sufficiently con-

FIG. 4. Protective activity of TC0313 antigen. BALB/c mice were immunized i.m. with TC0313 or MOMP using either a protein immunization regimen or a DNA/protein regimen. For protein immunization, mice were immunized i.m. with 3 doses of TC0313 or MBP-MOMP, MBP alone, or adjuvant alone. For the DNA/protein immunization, mice received three doses of plasmid pCMV-TC0313 or pCMV-MOMP plus one booster dose of their respective recombinant *C. muridarum* proteins (designated as “pCMV-TC0313/TC0313” and “pCMV-MOMP/MBP-MOMP”) or were mock immunized with the pCMVKaSF2120 vector (pCMV). Three weeks after the last immunization, all groups were challenged i.n. with 10^3 *C. muridarum* EBs, and 10 days later, lungs were homogenized and used to measure viable chlamydiae (IFU/lung, expressed as $\log_{10}$). The graph represents the number of IFU/lung in each mouse. The numbers of infected versus total mice in each group are also reported within the graph. Black lines represent the geometric mean of $\log_{10}$ IFU/lung of each group. The dotted line marks the detection limit of $\log_{10}$ IFU recovered per lung ($2.7 \log_{10}$). Asterisks mark the statistically significant IFU/lung reductions in immunized groups as compared to their negative controls (mice receiving adjuvant alone or pCMV-immunized mice) determined by Mann-Whitney U test ($P < 0.001$).

served among the different chlamydial serovars. To analyze CT043 conservation, we PCR amplified the CT043 gene from the genomic DNA of 15 *C. trachomatis* clinical isolates (collected in Italy in the last 3 years) belonging to serovars D, Da, E, G, and F, as well as from ATCC strains representative of the urogenital biovars E, F, G, H, and J. The PCR products were sequenced and compared to the sequence of the CT043 gene from serovar D strain D/UW-3/CX (GenBank accession no. AE001273.1). CT043 was found to be highly conserved. Only two allelic variants were identified, differing by a single amino acid substitution at position 203 (R68K) (data not shown). Such a high conservation strongly suggests that CT043 should provide cross-protection among currently circulating isolates.

DISCUSSION

In the last few decades, substantial efforts have been dedicated to the elucidation of the mechanisms of natural immunity against *C. trachomatis* infections, with the aim of designing

FIG. 5. Antigen-specific CD4⁺ IFN- γ ⁺ responses in PBMCs from TC0313-immunized mice. PBMCs from BALB/c mice, immunized with protein regimen or DNA prime/protein boost regimen, were purified 10 days after the last immunization. Cells were stimulated with TC0313 (black bars), MOMP (gray bars), or an unrelated protein (white bars), and the frequency of specific CD4⁺ IFN- γ ⁺ cells detected every 10^5 CD4⁺ cells was measured by multiparametric FACS staining as previously described.

strategies for prophylactic intervention. Studies using nude mice and transgenic mice depleted in B cells and CD4⁺ and CD8⁺ T cells demonstrated that CD4⁺ T cells, and in particular those producing IFN- γ , play a major role in chlamydia immunity (23, 29). Antibodies are important in the resolution of reinfection (23) and synergize with CMI by augmenting the Th1 response via an Fc-mediated process (22).

Despite the importance of CD4⁺ T cells, few chlamydia antigens capable of eliciting a CD4⁺ T-cell response upon *Chlamydia* infection have been identified so far (2, 11). Therefore, we have recently undertaken a genome-based approach according to which a large panel of LPS-free recombinant chlamydia antigens are being used to stimulate splenocytes of chlamydia-infected mice and PBMCs of human patients, with the aim of identifying the proteins that during experimental and natural infections induce CD4⁺ T cells to produce IFN- γ (Finco et al., unpublished). While the screening is still in progress, one protein, the hypothetical protein CT043, was discovered to be promising from a vaccine development standpoint. This antigen was capable of eliciting CD4⁺ IFN- γ ⁺ T cells in BALB/c mice that received a *C. trachomatis* intravaginal infection or a *C. muridarum* i.n. infection. The frequency of CT043-specific CD4⁺ T cells was similar to that induced by MOMP, the *C. trachomatis* antigen highly immunogenic in both chlamydia-infected patients and mice (15). Moreover, CT043 was recognized by circulating CD4⁺ T cells from over 60% of the patients infected with genital serovars of *C. trachomatis*, suggesting that CT043 is a strong target of the natural T-cell response to chlamydia. This is in line with a recent report showing the stimulatory activity of the antigen on

PBMCs isolated from patients with *C. trachomatis* genital infection (25). Interestingly, patients with CT043-specific CD4⁺ T cells were infected by different genital serovars. This leads to two plausible conclusions. First, that CT043 is highly conserved in most if not all clinical isolates, and this conclusion is supported by our sequence analysis of 22 clinical isolates belonging to most chlamydia serovars showing the presence of only 1 amino acid variation among the analyzed isolates. Second, the protein should be sufficiently well expressed in most isolates/serovars to allow the elicitation of specific CD4⁺ T cells. In this context, it is interesting to observe that the frequency of CT043-responding patients was equal to the frequency of patients responding to the highly expressed and immunogenic MOMP and that five patients showed the capability to respond both to CT043 and MOMP. As far as MOMP-specific T-cell response is concerned, it appears that there is a preference for serovar D MOMP to stimulate PBMCs from patients infected with isolates belonging to serovar D or E (62%). This is in line with the fact that several CD4⁺ T-cell epitopes reside in the hypervariable region of MOMP which defines chlamydial serovars (15). However, not all of the patients infected with chlamydia serovar D responded to the homologous recombinant MOMP used to perform the assay. This might be due to the fact that patients were originally sensitized to native MOMP during infection and that T cells sensitized by native MOMP do not respond as well to recombinant MOMP. Moreover, it has to be pointed out that we do not have a well-characterized clinical and infection history of the selected patients, and it cannot be excluded that some of the patients might be exposed to serovar D for a period too short to elicit MOMP-specific CD4 T cells, while they might have been previously infected with other serovars.

The second important observation of this work is that CT043 not only elicits IFN- γ -producing CD4⁺ T cells during both natural and experimental infection but also reduces chlamydia infectivity in immunized mice. In the model we used, mice were immunized i.m. with TC0313, the *C. muridarum* homolog of CT043, using either a DNA prime/protein boost or a protein immunization regimen, and subsequently challenged i.n. with 10³ *C. muridarum* IFU. The DNA immunization approach was adopted, since conventional protein-based vaccines are usually limited in their ability to raise robust cell-mediated immune responses (33). Moreover, the DNA prime/protein boost regimen is known to elicit both cellular and humoral immunity and has been shown to be effective in vaccination experiments against different pathogens in small animals and nonhuman primates (5, 26, 39). Finally, DNA immunization could allow the elicitation of an immune response against conformational epitopes (1, 32, 38). Indeed, a previous study aimed at improving the protective property of a MOMP-based vaccine demonstrated that priming the immune response with MOMP-encoding plasmid, followed by boosting with MOMP, provided a higher protective efficacy in the mouse model than immunization with the recombinant MOMP (8).

In our study, the i.m. delivery of plasmid DNA encoding TC0313 (*C. muridarum* homolog of CT043) via electroporation, followed by a boost with the recombinant TC0313 protein, induced a robust protective cellular immune response against i.n. *C. muridarum* challenge, with a level of protection at least as potent as that achieved with MOMP. The TC0313

protein immunization regimen, although eliciting high antibody titers, was much less effective in inducing antigen-specific CD4⁺ Th1 cells and in protecting mice from chlamydia infection. This is in line with the notion that a strong humoral response, without the support of an efficient CD4⁺ T-cell response, does not provide protection against chlamydia infection.

Since CT043 has been so far uncharacterized, we investigated CT043 expression during the chlamydia infection cycle. This protein is constantly expressed by the bacterium during its biphasic developmental cycle and appears to remain confined within the chlamydial inclusion. Furthermore, differently from its topological prediction, the protein was found associated with the surface of both RBs and EBs. Although CT043 is annotated as a hypothetical protein, it has been assigned by the Conserved Domain Database (www.ncbi.nlm.nih.gov) to the CesT family, a family consisting of a number of bacterial proteins highly similar to the Tir (translocated intimin receptor) chaperone protein of *E. coli*. In enteropathogenic *Escherichia coli*, CesT serves as chaperone for the Tir protein, which is involved in enteropathogenic *E. coli* virulence by conferring the ability to alter host cell morphology following intimate bacterial attachment (7). Although further work is needed to understand the role of CT043 in the biology of *C. trachomatis*, its homology with CesT suggests that also in chlamydia this protein acts as a chaperone possibly involved in maintaining the conformation of a so-far-unknown substrate in a secretion-competent state.

In conclusion, our study demonstrates that this antigen should be considered for the development of a *C. trachomatis* subunit vaccine, it being able to confer protection in the mouse model of lung infection and to prime a CD4⁺ Th1 response in a heterogeneous human population. It would be interesting to evaluate whether CT043 is able to elicit a protective immunity in other models, such as lower and upper genital tract infection. Moreover, CT043 is particularly attractive since it is abundantly expressed during chlamydia growth and is highly conserved between different serovariants. The fact that a traditional protein immunization protocol does not appear to be as potent as the DNA-based approach in the elicitation of a protective cell-mediated response indicates that other efforts are deserved to optimize the formulation and delivery of this antigen in order to maximize its potential as a vaccine.

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