**Bordetella pertussis** Proteins Dominating the Major Histocompatibility Complex Class II-Presented Epitope Repertoire in Human Monocyte-Derived Dendritic Cells

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Knowledge of naturally processed *Bordetella pertussis*-specific T cell epitopes may help to increase our understanding of the basis of cell-mediated immune mechanisms to control this reemerging pathogen. Here, we elucidate for the first time the dominant major histocompatibility complex (MHC) class II-presented *B. pertussis* CD4⁺ T cell epitopes, expressed on human monocyte-derived dendritic cells (MDDC) after the processing of whole bacterial cells by use of a platform of immunoproteomics technology. Pertussis epitopes identified in the context of HLA-DR molecules were derived from two envelope proteins, i.e., putative periplasmic protein (PPP) and putative peptidoglycan-associated lipoprotein (PAL), and from two cytosolic proteins, i.e., 10-kDa chaperonin groES protein (groES) and adenylosuccinate synthetase (ASS). No epitopes were detectable from known virulence factors. CD4⁺ T cell responsiveness in healthy adults against peptide pools representing epitope regions or full proteins confirmed the immunogenicity of PAL, PPP, groES, and ASS. Elevated lymphoproliferative activity to PPP, groES, and ASS in subjects within a year after the diagnosis of symptomatic pertussis suggested immunogenic exposure to these proteins during clinical infection. The PAL-, PPP-, groES-, and ASS-specific responses were associated with secretion of functional Th1 (tumor necrosis factor alpha [TNF-α] and gamma interferon [IFN-γ]) and Th2 (interleukin 5 [IL-5] and IL-13) cytokines. Relative paucity in the natural *B. pertussis* epitope display of MDDC, not dominated by epitopes from known protective antigens, can interfere with the effectiveness of immune recognition of *B. pertussis*. A more complete understanding of hallmarks in *B. pertussis*-specific immunity may advance the design of novel immunological assays and prevention strategies.

Prevention of morbidity and mortality caused by the human pathogen *B. pertussis* has effectively relied on national vaccination programs since these were introduced in the 1940s and 1950s (1, 2). However, during the last decade an increase of whooping cough has been reported (3–9). Pertussis, mostly feared for affecting infants too young to be fully vaccinated, is more and more notified among adolescents and adults, who apparently gradually lose their vaccine-induced protection to current *B. pertussis* strains (2, 10–13). Understanding of protective adaptive immunity and its weaknesses is essential for us to be able to improve pertussis vaccination. Despite being implicated in protection against severe pertussis (14, 15), levels of preexposure human serum antibodies to the major vaccine antigens have never been exclusively correlated with the efficacy of pertussis vaccines (16–18). In addition to antibodies, CD4⁺ T cells contribute to immunological resistance against *B. pertussis* infection. First, CD4⁺ T cell responses are essential for avidity maturation of specific B cell responses. Second, murine (19–25) and human (26–33) *B. pertussis*-specific CD4⁺ T cell immunity has been associated with the secretion of Th1-, Th2-, and Th17-type cytokines capable of arm-
pertussis CD4+ T cell epitope repertoire on MDDC, such as strong epitope domination, may help to elucidate weaknesses in the human cellular immune response to pertussis and may provide leads on how to design new generations of pertussis assays and vaccines.

MATERIALS AND METHODS

Subjects and ethics statement. Blood from volunteers was obtained in accordance with the Declaration of Helsinki and with Dutch regulations, following approval, respectively, from the Sanquin Ethical Advisory Board (for citrated buffy coat donation from 20 HLA-typed healthy blood bank donors and for one leukapheresis donation from an HLA-typed healthy blood bank donor [trial BS05.0015-s]) and from the accredited Review Board STEG and the review boards of collaborating hospitals for heparinized blood sampling of participants from a clinical study (age range, 8 to 77 years [median, 40 years]) (30 pertussis patients within 12 months after laboratory-confirmed diagnosis of B. pertussis infection and 10 healthy household contacts negative for B. pertussis infection based on diagnostic serology [trial NVI-243, CCMO number NL1.6334.040.07]). All participants provided written informed consent for the collection of samples and subsequent analysis.

Isolation of PBMC. Peripheral blood mononuclear cells (PBMC) from citrated blood samples were separated by centrifugation on a Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala Sweden) or on a gel in heparinized CPT tubes (BD Biosciences). After washing and counting, cells were used directly or after cryopreservation.

Bacterial strains and growth conditions. The B. pertussis vaccine strain 509 was grown on Bordet-Gengou (BG) agar plates containing 15% defibrinated sheep blood. Liquid B. pertussis cultures were grown in either natural 14N-containing minimal Bioexpress cell growth medium or in 98-atm%-enriched 15N stable isotope containing minimal Bioexpress cell growth medium (Cambridge Isotope Laboratories) (both containing 0.15% lactic acid [Fluka, Switzerland] adjusted to pH 7.2 with NaOH). Cell growth medium (Cambridge Isotope Laboratories) (both containing 0.15% lactic acid [Fluka, Switzerland] adjusted to pH 7.2 with NaOH) and 14N- and 15N-concentrated bacterial biomass at an optimized final concentration of 3×109/ml in 150-mm tissue culture dishes (Corning Costar) in Iscove’s modified Dulbecco’s medium (GibcoBRL) supplemented with 1% fetal bovine serum (HyClone) and penicillin-streptomycin-glutamine (GibcoBRL) at 37°C and 5% CO2 in a humidified incubator for 2 h. After removal of the nonadherent fraction, adherent cells were further cultured for 6 days in medium containing 500 U/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech) and 250 U/ml recombinant human IL-4 (Strathmann Biotech GmbH, Germany). Culture medium and growth factors were refreshed on day 3. On day 6, 1.2×106 MDDC, still immature, were pulsed with a 1:1 wt/wt mixture of 14N- and 15N-concentrated bacterial biomass at an optimized final concentration/Aeq of 0.028 and incubated for 6 h. Thereafter, cells were further cultured and matured in the continuous presence of antigen, growth factors, and 20 ng/ml lipopolysaccharide (Salmonella abortus equi; Sigma). On day 8, B. pertussis-pulsed and matured MDDC were harvested and washed four times in PBS, counted, pelleted, and snap frozen before cell lysis and peptide isolation (Fig. 1B). Maturation of the MDDC was verified using flow cytometry by comparing the expression of CD83, CD40, CD80, and CD86 cell surface markers on day 6 and day 8 MDDC (data not shown).

Proteins and synthetic peptides. Recombinant P.69 pertactin (P.69 Prn) was expressed and purified from an Escherichia coli construct as previously described (39). Pertussis toxin (Ptx), filamentous hemagglutinin (FHA), and fimbriae subtypes 2 and 3 (Fim2/3) were purified in-house according to procedures described in the literature (40–42). Maturation of the MDDC was verified using flow cytometry by comparing the expression of CD83, CD40, CD80, and CD86 cell surface markers on day 6 and day 8 MDDC (data not shown).

Isolation and NanoLC-ESI-MS analysis of HLA-DR-associated peptides. B. pertussis-pulsed MDDC were lysed and HLA-DR-peptide complexes were isolated essentially as described previously (38), using the HLA-DR-specific monoclonal antibody B8.11.2 bound to CNBr-activated Sepharose 4B beads. Peptides were eluted using 10% acetic acid and spun through a 10-kDa cutoff Spinfilter (Millipore, USA) (Fig. 1C). The filtrate was concentrated to ~10 μl using a freeze dryer and prefractionated using strong cation exchange (SCX) chromatography. Peptide fractions were reconstituted in a 5% formic acid and 5% dimethyl sulfoxide solution in water containing angiogenin-III and oxytocin (each at a concentration of 250 amol/ml) as internal standard peptides and analyzed using NanoLC-ESI-MS/MS, as described earlier (45) (Fig. 1D). Characteristic heavy and light mass spectral doublets, representing MHC class II-associated peptide epitopes originating from corresponding 14N- and 15N-B. pertussis protein homologues, were allocated in all mass spectra using the MS-Xelerator mass spectral interpretation software (MsMetrix, the Netherlands) (Fig. 1E). Candidate epitopes were identified by targeted NanoLC-ESI-MS/MS analysis, using identical chromatographic conditions on the same quadrupole time of flight (Q-TOF Ultima API) mass spectrometer operated at optimized collision energy (Fig. 1F) and database searching for a sequence match. The expressed levels of epitopes were quantified based on the relative response factor (RFR) of each naturally processed and presented epitope relative to the two additional standard peptides in an SCX fraction and the RRF of the corresponding synthetic.
analogue compared to these two standard peptides when acquired under identical NanoLC-ESI-MS conditions.

**Functional assays to analyze immunogenicity of epitopes.** Immunogenicity of epitopes was tested by incubating PBMC in the absence or presence of the indicated synthetic peptide or peptide pool (Fig. 1G) (at 1 μM per peptide) in complete AIM-V medium (AIM-V medium containing streptomycin, gentamicin, and l-glutamine [GibcoBRL] supplemented with 2% human AB serum [Harlan]) at 10^5 cells per well in 150 μl in 96-well round-bottom plates (Greiner) for 6 or 10 days in 3-, 10-, or 40-fold replicated wells per condition, as indicated, at 37°C in a humidified 5% CO2 atmosphere. Plates were visually inspected and either supernatants were harvested for cytokine measurement, 0.5 μCi (18.5 kBq) [3H]thymidine (Amersham, USA) was added overnight to measure direct proliferation, or cultures were expanded on IL-2 and restimulated, as described below, before the addition of [3H]thymidine. Eighteen hours after labeling, cells were harvested and the [3H]thymidine incorporation

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**FIG 1** Immunoproteomics strategy to identify pathogen derived MHC class II-associated peptides. (A) Separate *B. pertussis* (Rp) cultures are prepared in 14N-containing or in 98 atom% 15N-enriched prokaryotic medium. This allows all bacterial proteins synthesized to incorporate heavy or light nitrogen residues. (B) Immature MDDC are loaded with a 1:1 (A600 ratio) mixture of 14N- and 15N-labeled heat-inactivated *B. pertussis* whole-cell suspensions and MDDC are left for 48 h to process and present bacterial antigens while cultured in normal (14N) eukaryotic medium. (C) HLA-DR-epitope complexes are affinity purified using monoclonal antibody B.8.11.2 and epitopes are acid eluted and separated from the HLA-DR molecules by size exclusion. (D) After the peptide sample is highly fractionated by SCX chromatography, a portion of each SCX fraction is subjected to NanoLC-ESI-MS analysis. (E) Pathogen-derived epitopes are easily allocated by searching mass spectra for 14N- and 15N-ion doublets with similar intensities and retention times, and an average mass difference of 1.2%. Notably, self-epitopes will form only a 14N-ion. (F) Candidate epitopes are identified by targeted NanoLC-ESI-MS/MS sequence analysis and database matching. (G) Functionality of the naturally processed and presented MHC class II epitopes is assessed in T cell assays using in vitro restimulation of human T cells with antigen-presenting cells (APC) and synthetic peptides.
was determined as counts per minute (CPM) using a Wallac 1205 beta-plate liquid scintillation counter. Results are expressed as stimulation index (SI) from triplicate (donors from the NVI-243 study) or decuple wells (healthy blood donors), calculated as follows: average cpm of PBMC in the presence of peptide(s)/average cpm of PBMC in medium only (direct proliferation) or average cpm of stimulated PBMC cultures in the presence of peptide-pulsed antigen-presenting cells/average cpm of stimulated PBMC cultures in the presence of mock-pulsed antigen-presenting cells (indirect proliferation). SI values of ≥1.5 were considered positive.

For cytokine analysis, human Th1/Th2 and Th17 cytokine Bio-Plex kits (Bio-Rad) were used to determine concentrations of IL-2, IL-4, IL-5, IL-10, IL-12(p70), IL-13, IL-17, tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) according to the manufacturer’s instructions in (pooled) supernatants taken, as indicated, from those wells out of 40 plated per specific synthetic peptide pool that were visibly activated.
TABLE 1 Naturally processed and HLA-DR2-presented epitopes of *B. pertussis* whole-cell biomass

| Source protein | Epitope | Abundance<sup>a</sup> |
|---------------|---------|----------------------|
| Chaperonin groES protein (groES) (BP3496<sup>b</sup>) | 34KPDQGEVAVGP<sup>64</sup>73K | 80 |
| Putative periplasmic protein (PPP) (BP3341) | 137IALYPNSQLAPT<sup>146</sup> | 175 |
| Adenylosuccinate synthetase (ASS) | 166LAELD<sup>179</sup>LHNPFLTV<sup>179</sup>T | 10 |
| Putative peptidoglycan-associated lipoprotein (PAL) (BP3342) | 105GGAENLALGQRR<sup>116</sup>A | 10 |
| | 105GGAENLALGQR<sup>118</sup>RDA | 350 |

<sup>a</sup>Copies/cell. <sup>b</sup>*B. pertussis* gene identification number (48).

Measurements and data analysis were performed with the Bio-Plex system in combination with Bio-Plex manager software. Results are expressed in pg/ml.

For analysis of cross-reactivity, PBMC from a groES<sub>34–52</sub>-reactive healthy donor were stimulated using groES<sub>34–52</sub> synthetic peptide for 10 days and then further expanded on 10 ng/ml IL-2 and fresh medium. On day 24, expanded T cells were restimulated for 2 days in triplicate wells using medium only, synthetic peptide representing *B. pertussis* groES<sub>34–52</sub> or synthetic peptide representing *Homo sapiens* hsp104<sub>40–58</sub> in the presence of autologous antigen-presenting cells. Then <sup>3</sup>Hthymidine was added and incorporation was measured after overnight incubation, as indicated above. Results are expressed as stimulation index (SI) from triplicate wells.

**Statistics.** The statistical significance of differences in ex vivo lymphoproliferation between groups was calculated with the Student’s t test (two-tailed, unequal variance).

**RESULTS**

Immunoproteomics strategy for sensitive detection of MHC class II-presented *B. pertussis* epitopes. Foreign MHC class II-presented epitopes can be isolated from antigen-presenting cells for NanoLC-ESI-MS analysis, but to distinguish them from thousands of self-derived epitopes in complex peptide mixtures is technically highly challenging. To rapidly recognize *B. pertussis*-specific peptide masses in MHC class II eluates, an immunoproteomics strategy was applied, based on metabolic labeling of the bacterial proteome (schematically illustrated in Fig. 1A to F). Antigenically, the <sup>15</sup>N and <sup>15</sup>N whole-cell biomass, harvested in the virulence (bgv<sup>+</sup>) phase, were comparable, as was confirmed by immunoblotting of the known virulence factors P.69 pertactin (P.69 Prn), pertussis toxin subunit 1 (PtxS1), filamentous hemagglutinin (FHA), and fimbiae 2 (Fim2) (Fig. 2). After processing of 1:1 wt/wt mixed bacterial biomass by MDDC, an HLA-DR-derived peptide sample was obtained, fractionated, and analyzed for the presence of candidate *B. pertussis*-derived epitopes (Fig. 1D to F).

*B. pertussis* PAL, groES, PPP, and ASS identified as dominant source proteins of naturally processed and MHC class II-presented epitopes. An example of such a candidate epitope is given in Fig. 3A and B. Epitope sequencing by targeted nanoscale LC-MS/MS and database matching indeed identified its source protein as a *B. pertussis* protein, groES (Fig. 3C). In total, seven naturally processed and presented and hitherto unknown peptides, representing epitopes from four different *B. pertussis* proteins, including length variants, were identified using isotope-tagged identification (Table 1). A peptide representing as residues 103 to 118 from the envelope PAL protein, PAL<sub>103–118</sub>, was the most abundant epitope, with an estimated MHC-peptide copy number of 350 epitope per MDDC. Lower epitope densities were found for a natural length variant of this epitope and epitopes from groES, PPP, and ASS. No mass spectral doublets were assigned to known *B. pertussis* virulence factors, despite the fact that these proteins were present in the *B. pertussis* biomass preparations that were used for MDDC loading (Fig. 2). Also, data-dependent mass sequencing of peptides present in the eluate did reveal the presence of a wide repertoire of HLA-DR-associated self-sequences (data not shown), but this approach did not yield any additional *B. pertussis* epitopes.

Naturally processed and HLA-DR2-presented epitope regions represent functional CD4<sup>+</sup>T cell targets. To test the immunogenicity of the processed and HLA-DR2-presented pertussis peptides, PBMC from an HLA-DR-matched blood donor were stimulated ex vivo using synthetic peptides representing the eluted groES<sub>34–52</sub>, PPP<sub>132–146</sub>, ASS<sub>166–179</sub>, and PAL<sub>103–118</sub> sequences. In this donor, direct lymphoproliferation could be measured against all four epitopes (Fig. 4).

We reasoned that the identified peptide sequences may not only represent the exact HLA-DR2-presented epitopes, but may also indicate a broader protein region liberated by proteolysis and possibly containing multiple HLA-DR-binding motifs. We also assumed, since the epitopes were in vitro derived from an experimental whole-cell vaccine, that childhood whole-cell pertussis vaccination could be sufficient to prime CD4<sup>+</sup>T cell responses to these epitopes. To evaluate this, PBMC from 20 healthy HLA-DR2-typed adults from a birth cohort associated with whole-cell pertussis vaccination were stimulated with pools of overlapping synthetic peptides representing the epitope regions, including flanking sequences, to encompass adjacent motifs that were predicted to bind a wide array of HLA-DR molecules (46, 47) (data not shown). As summarized in Fig. 5, direct specific proliferation was observed against all four epitope regions with 30 to 40% over-
all responsiveness, not limited to HLA-DR2⁺ donors only. These data suggest that the extended epitope regions of groES, PPP, ASS, and PAL are recognized in a broader immunogenetic context.

To investigate the type of helper T cell responses associated with epitope immunogenicity, PBMC from responding donors 1, 5, 14, and 19 were cocultured in a new set of restimulations with relevant sets of peptides and cytokine analysis on culture supernatants of wells with activated cultures. All epitope regions were associated with both Th1- (IFN-γ and TNF-α) and Th2-type (IL-5 and IL-13) cytokine responsiveness (Fig. 6). No other cytokines, such as IL-10 or IL-17, were detected.

Specific proliferation as well as cytokine production of expanded CD4⁺ T cell cultures from various donors could be blocked by monoclonal antibodies against HLA-DR molecules, confirming the involvement of HLA-DR as a restriction element (data not shown). Collectively, these findings indicate that the four HLA-DR2-eluted epitopes represent favorably processed and presented protein regions immunogenic for CD4⁺ T cells and have a mixed Th1/Th2 cytokine signature.

**Epitope-specific responses observed during clinical pertussis infection.** To test whether epitopes from groES, PPP, ASS, and PAL were naturally exposed during infection, PBMC obtained from pertussis patients within 12 months after their laboratory-confirmed clinical episode (from pertussis patients) were assayed for donors. N- and C-terminal amino acid positions of protein region are represented by 12-mer overlapping 18-mer synthetic peptides. HLA-DR2 was confirmed clinical episode (from pertussis patients within 12 months after their laboratory-PAL were naturally exposed during infection, PBMC obtained to result in cross-reactivity against endogenous tissue.

Using an unconventional immunoproteomics approach, we identified hitherto unknown naturally processed and dominantly presented B. pertussis-specific CD4⁺ T cell epitopes. This approach has two major advantages over strategies aimed at mapping epitopes on predefined protein targets. First, discovery of epitopes is made independent of their protein source, which is especially relevant for pathogens with large proteomes, such as B. pertussis, with more than 3,800 open reading frames (48). Second, relative abundances and natural length variants of epitopes can be determined, which may have an immunological impact.

The B. pertussis epitopes discovered in this study to be MHC class II presented and immunogenic in humans originated from four different bacterial source proteins localizing to two different subcellular compartments of B. pertussis whole cells. PPP (alternative name, YbgF) and PAL, encoded by adjacent genes on the same operon, are both components of the cell envelope Tol-Pal system, specific for the integrity of the bacterial outer membrane (49). The 10-kDa chaperonin groES, involved in protein folding and assembly as a homohexameric ring associated with the chaperonin cpn60, and ASS, catalyzing the first step in the de novo biosynthesis of AMP, are both located in the cytoplasm (48).

Unexpectedly, none of the dominantly presented epitopes originated from known B. pertussis virulence factors, such as FHA, P.69 Prn, PtxS1, or Fim2, while these proteins were abundantly present in the digested bacterial biomass (Fig. 2) and some encode...
known T cell epitopes (29, 35, 50, 51). In fact, preceding B. pertussis harvesting, in-culture gene expression values for groES, PPP, ASS, and PAL were comparably high (groES and PAL) or even lower (PPP and ASS) than those for FHA, P.69 Prn, PtxS1, or Fim2 (52) (B. van der Waterbeemd, personal communication).

Therefore, a high antigen load may be favorable, but it is not sufficient for a source protein to dominate in the MHC class II ligandome of MDDC. It is known that for epitopes to be formed, modest but not destructive proteolysis of source proteins (53) and peptide affinity to MHC class II molecules (54) are prerequisites. The groES, PPP, ASS, and epitope regions all have very high predicted epitope binding scores for either HLA-DRB1*1501 or HLA-DRB5*0101, the two HLA-DR molecules expressed on the HLA-DR2/H11001 MDDC (data not shown). Hence, they can be assumed to be strong competitors for binding with epitopes derived from other proteins. Also, other features driving exclusive presentation by MDDC could be involved, such as those described for the Toxoplasma gondii protein profilin, being immunodominant in the CD4+ T cell response to the pathogen solely because of enhanced and selective TLR11-mediated uptake (55). TLR2 could play a role in the uptake of the lipoprotein PAL (56–62), possibly in association with the other Tol-Pal component PPP. TLR2 might also sense groES, eventually via associated compounds (63–65). It is unknown whether ASS has innate receptor affinity.

Another unexpected observation was the modest average epitope abundance of the pertussis MHC class II ligands, with estimated numbers of MHC-peptide complexes per cell of 5 to 350. This was dissimilar to the range of MHC class II-presented epitopes we described previously, using an MDDC model with meningococcal outer membrane vesicles as the antigen and applying the same degree of sample prefractionation and analytical sensitivity. HLA-DR-associated meningococcal epitopes isolated from either HLA-DR1 homozygous or HLA-DR2 homozygous MDDC ranged from 30 to 10,000 copies per cell (reference 38 and

![FIG 7](clik.png) Clinical infection enhances CD4+ T cell lymphoproliferation to different B. pertussis epitope regions. PBMC of pertussis patients within 12 months after laboratory-confirmed pertussis (n = 30) and of healthy controls (n = 10 noninfected household contacts and n = 20 healthy blood bank donors) were stimulated with pools of 18-mer peptides, representing the entire indicated proteins at 1 μM per peptide. [3H]Thymidine incorporation was determined 7 days after in vitro stimulation. Dots represent SI from triplicate wells of different individuals.

![FIG 8](seq_homology.png) Sequence homology between B. pertussis and H. sapiens 10-kDa chaperonins. Gray letters indicate the amino acid sequence of the longest naturally processed B. pertussis groES34-52 peptide variant and identical amino acid residues in H. sapiens hsp10; dark-gray letters indicate nonhomologous amino-acids in the H. sapiens hsp10 epitope sequence.
H. Meiring, unpublished data). Although we cannot exclude a certain degree of peptide loss, potentially also from unidentified epitopes during the sample isolation procedure, or of undersampling of MHC ligand identification at the mass spectrometry level (66), it is unlikely that this fully accounts for the large difference between the pertussis and the meningococcal epitope repertoire. MDDC pulsed with B. pertussis whole-cell vaccine had comparable levels of HLA-DR molecules at the cell surface, of MHC protein retentate, and of self-peptides in the eluate as did meningococcal outer membrane vesicle matured MDDC (data not shown). Hence, our data indicate a selective and limited breadth and density of B. pertussis MHC class II epitopes on human MDDC, among which additional epitopes from groES, PPP, ASS, and PAL or epitopes from virulence factors were either absent or too low in copy number to be detected by our system. Limited epitope display, especially from virulence factors, could circumvent effective immune recognition and mechanisms crucial for protection. While it has been shown that B. pertussis antigens can interfere with MDDC maturation and function (67), low and highly selective epitope expression on MDDC might be another successful feature of B. pertussis to evade the adaptive immune response. Further immunoproteomics research, extending MHC class II ligandome analyses to other HLA-DR alleles, antigen-preparation and density of targets for CD4+ T cell-dependent antibody responses, which could be tested in an animal challenge model, is not expected, since the antigens are not surface exposed on the bacterium. However, the antigens could otherwise serve as relevant targets of immune mechanisms. The induced specific CD4+ T cell populations could enhance pertussis immunity via the generation of an inflammatory cytokine milieu, or alternatively, could dampen other CD4+ T cell specificities through competition for space (68) or through regulation. The CD4+ T cell responses to the identified epitope regions, observed in a considerable proportion of tested individuals, were associated with a mixed Th1/Th2 cytokine profile, i.e., secretion of IFN-γ, TNF-α, and IL-5 and IL-13, in the absence of IL-10 and IL-17. IFN-γ and TNF-α are both inflammatory cytokines that have been implicated in controlling B. pertussis through potentiating bactericidal activity of macrophages and enhancing phagocytosis by neutrophils (69–71), suggesting a role in protection. Notably, (ex)patients had slightly elevated proliferative responses to three of four naturally processed target proteins, possibly indicating that the epitopes were exposed during infection and could play a role in natural CD4+ T cell immunity to B. pertussis. While the data set was possibly too limited to be conclusive and testing more patients’ samples could help to settle this point, it is also possible that elevation of T cell responses is confined to a very narrow time span after boosting. The majority of patients in this study were adults who were within 2 months after their formal diagnosis. However, since B. pertussis has a relatively long incubation time (14 to 21 days) and adults are often diagnosed only after a period of prolonged coughing, an early boosting effect of the epitopes on T cell levels could have easily been missed.

Responses to the B. pertussis groES epitope were non-cross-reactive to the endogenous human hsp10 homologue. Therefore, dissimilar to the CD4+ T cell epitope cross-reactivities observed for some mycobacterial and human hsp70 protein families, a potential role in autoimmunity or immunoregulation for B. pertussis groES is not envisaged (64).

In conclusion, an unexpected limited epitope breadth and abundance of human MDDC-presented B. pertussis-specific CD4+ T cell epitopes was found, with a role for other proteins than those that are known virulence factors. Clearly, although being a nonroutine and highly technical approach, immunoproteomics can shed light on classes of otherwise elusive T cell epitopes. These results represent a step toward a more complete characterization of the natural immune response to B. pertussis, involving other specificities than hitherto anticipated. Ultimately, a better understanding of the immunological correlates of protection against whooping cough and their flaws is needed to develop new functional assays and more effective vaccines.

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