Ex vivo peptide-MHC II tetramer analysis reveals distinct end-differentiation patterns of human pertussis-specific CD4+ T cells following clinical infection

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Abstract

Pertussis is occurring in highly vaccinated populations, suggesting insufficient protective memory CD4+ T cells to Bordetella (B.) pertussis. P.69 Pertactin (P.69 Prn) is an important virulence factor of B. pertussis, and P.69 Prn7–24 is an immunodominant CD4+ T cell epitope in mice and broadly recognized in humans. P.69 Prn7–24 peptide-MHC II tetramers (DRB4*0101/IVKT) were designed to ex vivo interrogate the presence and differentiation state of P.69 Prn7–24 specific CD4+ T cells in six symptomatic pertussis cases. Cases with relatively more CD45RA−CCR7+ central memory CD4+DRB4*0101/IVKT+ T cells secreted Th1 cytokines, while cases with more CD45RA−CCR7− effector memory CD4+DRB4*0101/IVKT+ T cells secreted both Th1 and Th2 cytokines upon peptide stimulation. CD45RA+CCR7- terminal differentiation pattern was associated with low or non-functionality based on cytokine secretion. This study provides proof of principle for further peptide-MHC II tetramer guided approaches in the elucidation of limited immunological memory to B. pertussis and the resurgence of pertussis.

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Abbreviations: HLA, human leukocyte antigen; MHC, major histocompatibility complex; Tcm, central memory T cells; TD, terminally differentiated effector memory T cells; Tem, effector memory T cells; Th, T helper; TM, tetramer.

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1. Introduction

*Bordetella (B.) pertussis* infection can cause pertussis (whooping cough), an acute infection of the upper respiratory tract, and is most severe in young children [1]. Despite high vaccination coverage, in many countries resurgence of pertussis has been observed since the 1990s, affecting not only non- or partially vaccinated neonates but also adolescents, adult and elderly vaccinees [2–7]. Factors that play a role in this phenomenon are pathogen adaptation and insufficient long-term effectiveness of pertussis-specific adaptive responses. In addition to antibodies, pertussis-specific Th1 and Th17 type CD4+ T cells are essential for protective immunity against *B. pertussis* [8–11]. Estimates of the duration of immunity against *B. pertussis* range from 4 to 12 years after vaccination and 4 to 20 years after infection [12]. For long-term immunity, pertussis-specific memory CD4+ T cells should have good self-renewal capacity under steady state conditions and potent lymphoproliferative capacity and effector functionality in a recall response [13,14].

Circulating human memory CD4+ T cells have distinct differentiation stages and corresponding functionality. Based on CD45RA and CCR7 expression, CD4+ T cells can be distinguished in naïve, central memory T cells (Tcm), effector memory T cells (Tem) and terminally differentiated effector memory T cells (TD) [15,16]. While long-term memory potential is superior in Tcm compared to Tem and TD, multifunctional cytokine production is a hallmark of Tem. TD are short-lived and have Tcm compared to Tem and TD, multifunctional cytokine specific CD4+ T cells. However, studies investigating these CD45RA and CCR7 expression, CD4+ T cells can be distinguished vaccination and 4 to 20 years after infection [12]. For long-term immunity, pertussis-specific memory CD4+ T cells should have good self-renewal capacity under steady state conditions and potent lymphoproliferative capacity and effector functionality in a recall response [13,14].

Circulating human memory CD4+ T cells have distinct differentiation stages and corresponding functionality. Based on CD45RA and CCR7 expression, CD4+ T cells can be distinguished in naïve, central memory T cells (Tcm), effector memory T cells (Tem) and terminally differentiated effector memory T cells (TD) [15,16]. While long-term memory potential is superior in Tcm compared to Tem and TD, multifunctional cytokine production is a hallmark of Tem. TD are short-lived and have low cytokine production capacities [17]. Memory phenotype analysis could provide information on the fitness of the pertussis specific CD4+ T cells. However, studies investigating these properties are dependent on in vitro restimulation for the amplification of the low-frequent pertussis specific T cells and on cytokine production for their identification [18–20]. Culture conditions could influence memory phenotype analysis. Thus, knowledge on the quality and fitness of circulating memory CD4+ T cells to *B. pertussis* is largely elusive due to lack of direct interrogation. Epitope specific MHC class II tetramers can aid in such line of studies by direct staining or enrichment for epitope specific CD4+ T cells [21,22].

P.69 Pertactin (P.69 Prn) is an important virulence factor of *B. pertussis*, and P.69 Prn7–24 is an immunodominant CD4+ T cell epitope in mice [23]. Furthermore, this N-terminal epitope is broadly recognized in the human population [23,24]. In this study, we directly ex vivo interrogated the presence, differentiation state and function of P.69 Prn7–24 specific CD4+ T cells, by combining direct peptide-MHC II tetramer (DRB4*0101/IVKT TM) analysis and peptide-specific Th cytokine measurements in HLA-DR53 positive symptomatic pertussis cases.

2. Materials and methods

2.1. Ethics statement

Blood from volunteers was obtained in accordance with principles expressed in the Declaration of Helsinki and with Dutch regulations in two clinical studies. In one study citrated buffy coat donations from HLA-typed healthy blood bank donors were received after approval by the Sanquin Ethical Advisory Board (trial BS03.0015-x). In another study, blood samples were collected in sodium citrated tubes from pertussis cases after approval by the accredited Review Board STEG (Stichting Therapeutisch Evaluatie Geneesmiddelen) and METC UMC Utrecht (Medisch Ethische Toetsingscommissie Universitair Medisch Centrum Utrecht) (CCMO nr: NL16334.040.07, SKI-study), and after accordance of practicability by the Review Boards of collaborating hospitals. All participants provided written informed consent for the collection of samples and subsequent analysis. Written informed consent for minor cases was provided by both parents of the participants.

2.2. Clinical cohort

The clinical cohort under investigation consisted of twenty-five (16 female, 9 male) HLA-DRB4*0101-positive participants selected from the larger SKI study, a cross-sectional observational study investigating pertussis specific immunity in symptomatic (ex-)pertussis cases, recruited in the Dutch population via General Practitioners and Pediatricians. Selected cases had an average age of 34.6 years (range 10.3–78.9) at blood sampling, which was at a known time interval since laboratory confirmation of their symptomatic pertussis episode (median time since diagnosis 1.2 months; average 48.2; range 0.5–451). History of pertussis vaccination of participants was verified.

2.3. Isolation of PBMC and plasma, and generation of EBV-BLCL

Peripheral blood mononuclear cells (PBMC) from buffy coats from healthy blood bank donors were isolated by centrifugation on a Ficoll-Hypaque gradient (Pharmacia Biotech, Uppsala Sweden), for use as feeder cells in T cell cultures. PBMC and plasma from the clinical SKI cohort were separated on the day of blood collection after centrifugation in Vacutainer cell preparation tubes (CPT) (BD) containing sodium citrate, for use in immunological assays. PBMC were used directly, or as indicated after cryopreservation (in 90%FCS/10%DMSO) at −135 °C. Plasma was stored at 80 °C.

EBV transformed lymphoblastoid B cell lines (EBV-BLCL) from participants were generated by incubating 1 × 10⁶ PBMC with 500 μl B95-8 EBV supernatant for 1 h in a CO2 incubator at 37 °C. Subsequently, cells were cultured in RPMI-1640 medium (Gibco), supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 2.92 mg/ml L-glutamine (Invitrogen), and 10% FCS (HyClone) (referred to as complete RPMI medium), and 1 μg/ml PHA in a 48 well flat bottom plate. EBV-BLCL were cultured and split when necessary for at least 2 weeks before cryopreservation and used as antigen presenting cells in T cell assays.

2.4. HLA-typing

Molecular typing for HLA class-I and II alleles using subjects’ cells was performed at intermediate resolution (two digits) based upon the PCR-SSO technique in combination with Luminex using commercial reagents and following the instructions of the manufacturer (OneLambda Inc., Canoga Park, CA, USA).
2.5. Pertussis-specific peptide, antigen and MHC class II tetramer

Synthetic P.69 Prn\textsubscript{7–24} peptide was prepared by FMOC solid phase synthesis using a SYRO II simultaneous multiple peptide synthesizer (MultiSynTech GmbH, Witten, Germany). The purity and identity of the synthesized peptides were assessed by reverse phase high performance liquid chromatography (HPLC) and was >70% pure.

Recombinant B. pertussis P.69 Prn1 (Prn) was expressed and purified from an Escherichia coli construct as described in literature [25]. Molecular weight and purity of these antigens were verified using SDS-PAGE, and the presence of detectable impurities of E. coli LPS in P.69 Prn was ruled out in a Limulus Amebocyte Lysate (LAL) test (hence endotoxin levels were <0.015 EU/ml).

The following MHC class II tetramers were obtained through the NIH Tetramer Facility (Atlanta, Georgia, USA): P.69 Prn\textsubscript{7–24} peptide loaded HLA-DRB4*0101 proteins conjugated as tetramers through APC streptavidin (DRB4*0101/IVKT TM). An APC-conjugated DRB4*0101 tetramer containing the CLIP peptide (DRB4*0101/CLIP TM) was provided as a negative control and was used to optimize staining conditions in a limited set of pilot experiments.

2.6. Generation of a P.69 Prn\textsubscript{7–24} specific CD4\textsuperscript{+} T cell clone

The human P.69 Prn\textsubscript{7–24} Specific CD4\textsuperscript{+} T cell clone 629-A2 was established by seeding PBMC from subject 629 at 10\textsuperscript{6} cells per well in a 96-wells round-bottom plate in AIM-V medium (Gibco), containing 100 units/ml penicillin, 100 units/ml streptomycin, 2.92 mg/ml L-glutamine (Invitrogen) and 2% human AB serum (complete AIM-V culture medium) in the presence of synthetic P.69 Prn\textsubscript{7–24} peptide at 1 \muM. After 6 days of incubation at 37 °C and 5% CO\textsubscript{2} in a humidified incubator, lymphoblasts were split over new wells containing complete AIM-V culture medium supplemented with 5 ng/ml rIL-2. At day 14 lymphoblasts were cloned by limiting dilution in a 96-well round-bottom plate (0.3 cell/well) in the presence of 5 \times 10\textsuperscript{4} irradiated (20 Gray) allogeneic PBMC/well in complete AIM-V culture medium supplemented with 5 ng/ml rIL-2 and 1 \mug/ml PHA (Sigma). To maintain clones, cells were alternately stimulated in an antigen specific and a-specific manner. Specific stimulation was performed by adding irradiated HLA-DR matched PBMC as feeder cells (1 \times 10\textsuperscript{5} cells/well) to the T cell clones (2–8 \times 10\textsuperscript{4} cells/well) in the presence of synthetic P.69 Prn\textsubscript{7–24} peptide and 5 ng/ml IL-2. A-specific stimulation was performed by plating the T cell clone together with irradiated allogeneic PBMC as feeder cells in complete AIM-V culture medium in the presence of 5 ng/ml IL-2 and 1 \mug/ml PHA. Functionality of T cell clones was tested in rested and washed cells, minimally 10–18 days after being stimulated. CD4\textsuperscript{+} T cell clone 629-A2 was selected from a panel of identical clones after being tested positive for P.69 Prn\textsubscript{7–24} specificity (see 2.7).

2.7. Stimulation of P.69 Prn\textsubscript{7–24} specific T cells

Freshly isolated PBMC were stimulated at 10\textsuperscript{5} cells per well, 3 or 6 wells per condition, in a 96-well round-bottom plate in complete AIM-V culture medium, in the presence of either P.69 Prn\textsubscript{7–24} peptide at 1 \muM, P.69 Prn protein at 1 \mug/ml, PHA at 1 \mug/ml as a positive control, or medium only as a negative control, for 6 days at 37 °C and 5% CO\textsubscript{2} in a humidified incubator. At day 6, 100 \mul supernatant volumes per well were removed for cytokine analysis. Day 6 supernatant was optimal for analyzing Th cytokines, except for the early cytokines IL-2 and IL-4 (own observations). Proliferation of PBMC was assessed at day 7 after in vitro stimulation as further indicated in section 2.8.

For characterization of clone 629-A2, rested cells (see 2.6) were used. Memory subset phenotyping by flowcytometry was performed on 629-A2 cells as described in section 2.10. To assess functionality of the 629-A2 cells, rested cells were washed and subsequently stimulated at 5 \times 10\textsuperscript{4} cells per well with 1 \times 10\textsuperscript{5} autologous or HLA-DR typed APC (EBV-BLCL), as indicated, in complete ALM-V culture medium in the presence or absence of P.69 Prn\textsubscript{7–24} peptide or P.69 Prn protein at the indicated dose, in a 96-well round bottom plate at 37 °C and 5% CO\textsubscript{2} in a humidified incubator. Proliferation of 629-A2 cells was assessed 2 days after in vitro stimulation as further described in section 2.8. For cytokine analysis, supernatants and cells were collected after o/n incubation of 629-A2 cells with P.69 Prn\textsubscript{7–24} peptide pulsed APC, and analyzed as described in section 2.9. For HLA-restriction analysis, P.69 Prn pulsed APC were pre-incubated in the presence or absence of anti-HLA-DR (B8.11.2, culture supernatant 1:200) or anti-HLA-DQ (SPV-L3, culture supernatant 1:100) monoclonal antibodies for 15 min before co-culture with 629-A2 cells for 2 days and testing for specific proliferation, as described in section 2.8.

2.8. Proliferation assay

To assess specific proliferation, 0.5 \muCi \textsuperscript{3}H]thymidine was added to a culture 18 h before harvesting and determination of \textsuperscript{3}H]thymidine incorporation as counts per minute (CPM) in a LKB/Wallac 1205 Betaplate Liquid Scintillation Counter. Every PBMC sample investigated showed proliferation to P.69 Prn\textsubscript{7–24} peptide or P.69 Prn protein at the indicated dose, in a 96-well round bottom plate at 37 °C and 5% CO\textsubscript{2} in a humidified incubator. Proliferation of 629-A2 cells was assessed 2 days after in vitro stimulation as further indicated in section 2.8. For cytokine analysis, supernatants and cells were collected after o/n incubation of 629-A2 cells with P.69 Prn\textsubscript{7–24} peptide pulsed APC, and analyzed as described in section 2.9. For HLA-restriction analysis, P.69 Prn pulsed APC were pre-incubated in the presence or absence of anti-HLA-DR (B8.11.2, culture supernatant 1:200) or anti-HLA-DQ (SPV-L3, culture supernatant 1:100) monoclonal antibodies for 15 min before co-culture with 629-A2 cells for 2 days and testing for specific proliferation, as described in section 2.8.

2.9. Cytokine analysis

Concentrations of cytokines in culture supernatants were determined using combined Bio-plex human Th1/Th2 and Th17 cytokine luminex kits (Bio-rad), according to the manufacturer's instructions. Data acquisition was performed on a Biorad Bio-Plex200. The fluorescence intensities (FI) of the tested analytes measured in complete AIM-V culture medium (containing 2% human AB serum), were all at the lower detection limits of the assay, indicating absence of significant amounts of cytokines in this medium only. Background cytokine levels released by cells in the presence of complete AIM-V culture medium alone were subtracted from cytokine levels released by cells after peptide stimulation. Cytokine concentrations in supernatants were
2.10. Ex vivo phenotyping of P.69 Prn\textsubscript{7–24} specific CD4\textsuperscript{+} T cells by DRB4*0101/IVKT TM

Binding of DRB4*0101/IVKT TM to a P.69 Prn\textsubscript{7–24} specific HLA-DR53 restricted CD4\textsuperscript{+} T cells was optimized with respect to reagent concentration, staining temperature and time of incubation, using 629-A2 cells. Spiking titrating amounts of 629-A2 cells in HLA-DR unmatched PBMC revealed a detection limit of 0.01% (Supplementary Fig. 2). Ex vivo analysis of P.69 Prn\textsubscript{7–24} specific CD4\textsuperscript{+} T cells was performed using frozen PBMC. Briefly, 3 to 11 million PBMC were incubated (1 \times 10^6 cells per well in 96-well V-bottom plates) in complete RPMI medium containing 50 nM Dasatinib (Selleckchem), a protein kinase inhibitor, for 30 min to enhance TM staining [26]. The cells were then stained with 6 \mu g/ml APC-conjugated DRB4*0101/IVKT TM in complete RPMI medium for 1 h at 37 °C. Subsequently, cells were stained with Pacific blue-conjugated anti-CD4 (eBioscience), PerCP-Cy 5.5-conjugated anti-CCR7, blue-conjugated anti-CD4 (eBiosciences), PE-Cy7-conjugated anti-CD8, and FITC-conjugated anti-CD8/CD20/CD40 (PeliCluster Sanquin, SouthernBiotech, BioRad, respectively), and LIVE/DEAD\textsuperscript{® } Fixable Aqua Dead Cell Stain Kit (Invitrogen) for 30 min at 4 °C in FACS buffer (PBS (pH 7.2) supplemented with 0.5% BSA (Sigma Aldrich) and 0.5 mM EDTA (ICN Biomedicals)). Data were acquired on FACS Canto II and analyzed using FlowJo software (Tree Star), gating on singlets (FSC-A/FSC-H) and acquiring data at 4 °C in FACS buffer (PBS (pH 7.2) supplemented with 0.5% BSA (Sigma Aldrich) and 0.5 mM EDTA (ICN Biomedicals)). Data were acquired on FACS Canto II and analyzed using FlowJo software (Tree Star), gating on singlets (FSC-A/FSC-H) and excluding CD8\textsuperscript{+}, CD20\textsuperscript{+}, CD40\textsuperscript{+} and Aqua\textsuperscript{-} (dead) cells. Per standard curve (log\textsubscript{24}), 1.5 \times 10^6 CD4\textsuperscript{+} cells were analyzed for 629-A2 specific CD4\textsuperscript{+} T cells in PBMC samples or low-frequent DRB4*0101/IVKT\textsuperscript{TM} cells between 0.01 and 0.1% of CD4\textsuperscript{+} T cells at a desired level of reliability (coefficient of variation between 5 and 10%) (Guide to Flow Cytometry, Rare-Event Detection by Terry Hoy).

2.11. Ptx serology

Levels of specific human IgG antibodies against the pertussis specific antigen Pertussis Toxin (IgG-Ptx), were measured as a component of a human multiplex immunoassay (MIA), as described in detail by Van Twillert et al. [27]. Briefly, Ptx antigen was coupled to fluorescently labeled microspheres of a distinct bead region (Bio-Rad Laboratories, Hercules, USA). Plasma samples were prepared in two dilutions; 1/300 and 1/3600 in PBS containing 3% BSA, 0.1% Tween-20 (Merck, Germany) (referred to as PBST20-BSA). International standardized pertussis reference serum WHO NIBSC 06/140 was prepared in eight steps of 3-fold dilutions (1/30 to 1/65610) in PBST20-BSA. Each dilution of plasma or reference serum (25 \mu l) was mixed with an equal volume of conjugated microspheres (4000 beads/region/well) in a 96-well multi-scan HTS filter plate (low-protein binding, Millipore, USA) and incubated 45 min at RT. Then, beads were washed (PBS) and bound antibodies were detected by Goat anti-human IgG, R-PE (Jackson ImmunoResearch, USA) in PBS. Analysis was performed with a Bio-Plex 100 in combination with Bio-Plex Manager software version 5 (Bio-Rad Laboratories, USA). Fluorescent intensity (FI) for the Ptx bead region was converted to IU/ml by interpolation from a 5-parameter logistic standard curve (log–log). Data were expressed in IU/ml.

3. Results

3.1. HLA-DR restriction and phenotype analysis of cloned CD4\textsuperscript{+} T cells recognizing the immunodominant pertussis epitope P.69 Prn\textsubscript{7–24}

In an unique clinical cohort of symptomatic pertussis cases, strong proliferative T cell response to the immunodominant P.69 Prn\textsubscript{7–24} epitope was observed in subject 629 [24], a 10 year old male child having experienced pertussis at the age of 3 years. PBMC from this participant were used to generate the CD4\textsuperscript{+} T cell clone 629-A2, recognizing P.69 Prn\textsubscript{7–24} peptide. Dose-dependent P.69 Prn\textsubscript{7–24} peptide specific proliferation of clone 629-A2 was observed (Fig. 1A). This epitope-specific proliferation could be blocked by anti-HLA-DR mAb (Fig. 1B). Since the HLA-DR background of the donor of clone 629-A2 was HLA-DR4,8, we analyzed the HLA-restriction of the clone by using various (partially) HLA-DR matched and unmatched APC. APC sharing HLA-DR8 with the donor did not induce peptide-specific proliferation, while APC sharing HLA-DR4 did (Fig. 1C). The HLA-DRB4 gene encoded HLA-DR53 supertype is linked to the HLA-DRB1 gene encoded HLA-DR4, HLA-DR7, and HLA-DR9 haplotypes. Therefore, peptide specific proliferation of clone 629-A2 in the presence of HLA-DR4-positive APC could also be HLA-DR53 restricted. Indeed, peptide specific proliferation of the clone was observed when various HLA-DR7 positive APC, sharing only HLA-DR53 with the donor, were used. Thus, we obtained a P.69 Prn\textsubscript{7–24} specific HLA-DR53 restricted CD4\textsuperscript{+} T cell clone which could be used for optimizing phenotypic and functional CD4\textsuperscript{+} T cell assays at the single pertussis epitope level.

First we assessed the differentiation stage of resting P.69 Prn\textsubscript{7–24} specific 629-A2 cells, based on CD45RA and CCR7 expression by flow cytometry. 629-A2 cells were CD45RA\textsuperscript{-} CCR7\textsuperscript{-}, indicating an effector memory phenotype (Tem) (Fig. 2A, left panel). We also investigated the functionality of the P.69 Prn\textsubscript{7–24} specific HLA-DR53 restricted CD4\textsuperscript{+} T cell clone by analyzing cytokine production after P.69 Prn\textsubscript{7–24} peptide stimulation in culture supernatants. Production of TNF\textalpha, IFN\gamma, typical Th1 cytokines, and low amounts of IL-13 and IL-10, typical Th2 cytokines were detected in culture supernatants of the P.69 Prn\textsubscript{7–24} specific CD4\textsuperscript{+} T cell clone (Fig. 2B, left panel). No significant amounts of IL-2 and IL-17 were detected. Together these data indicate that cloned 629-A2 CD4\textsuperscript{+} T cells recognize P.69 Prn\textsubscript{7–24} in a HLA-DR53 restricted manner, have a Tem phenotype in rest and produce both Th1 and Th2 cytokines upon stimulation.

3.2. Correlating differentiation pattern and function of circulating and cloned Prn\textsubscript{7–24} specific CD4\textsuperscript{+} T cells

Culture conditions required to generate and maintain CD4\textsuperscript{+} T cell clone 629-A2 could have affected its memory phenotype and cytokine profile. To be able to characterize HLA-DR53 restricted P.69 Prn\textsubscript{7–24} specific CD4\textsuperscript{+} T cell responses directly ex vivo, a MHC class II tetramer was developed based on the epitope specificity and HLA-DR restriction of clone 629-A2, DRB4*0101/IVKT TM. The validity of the DRB4*0101/IVKT TM to stain P.69 Prn\textsubscript{7–24} specific CD4\textsuperscript{+} T cells in PBMC samples was investigated by spiking 629-A2 cells in a background of HLA-DR unmatched PBMC. Spiked 629-A2 cells can be categorized as low (10–100 pg/ml), medium (100–1000 pg/ml) or high (>1000 pg/ml).

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detected as DRB4*0101/IVKT+ in a background of negative cells (shown for one condition, Fig. 2C, left and middle panels). When tested on PBMC of case 629, DRB4*0101/IVKT TM stained 0.11% of CD4+ T cells (Fig. 2C, right panel). Thus, the DRB4*0101/IVKT TM is able to specifically identify P.69 Prn24 specific CD4+ T cells ex vivo in PBMC of a responsive HLA-DR53 subject.

By using the DRB4*0101/IVKT TM we were able to directly assess the differentiation pattern of circulating P.69 Prn24 specific CD4+ T cells from case 629 and compare it to the phenotype of cloned 629-A2 cells. Ex vivo, the majority of CD4+DRB4*0101/IVKT+ T cells was either CD45RA+CCR7+ (Naïve, 43.9%) or CD45RA−CCR7+ (Tem, 39.0%), and a smaller percentage was CD45RA−CCR7+ (Tcm, 8.78%) or CD45RA+CCR7+ (TD, 8.31%) (Fig. 2A, right panel). Hence the Tem phenotype of 629-A2 cells reflects the largest non-naive phenotype of the ex vivo P.69 Prn24 specific CD4+ T cells. Functionality of circulating P.69 Prn24 specific CD4+ T cells from case 629 was investigated by stimulating fresh PBMC with P.69 Prn24 peptide for 6 days and determining concentrations of epitope-specific Th cytokine production in the supernatant. The cytokine profiles of ex vivo stimulated and cloned P.69 Prn24 specific CD4+ T cells were similar, both producing Th1 and Th2 cytokines after P.69 Prn24 peptide stimulation (Fig. 2B). Together these results indicate that although clone 629-A2 represents a major phenotypic and functional component of the P.69 Prn24 specific T cell response from case 629, DRB4*0101/IVKT− TM are a useful tool to study differentiation patterns of P.69 Prn24 specific CD4+ T cells in the peripheral blood of responsive subjects without in vitro bias.

### 3.3. Enumeration of P.69 Prn−24 specific CD4+ T cells in HLA-DR53 positive pertussis cases

Twenty-five HLA-DR53 positive participants of the SKI study, sampled at various time intervals after their symptomatic pertussis episode, were tested for P.69 Prn and P.69 Prn−24 specific lymphoproliferation (Fig. 3). Ten cases had lymphoproliferative responses to whole Prn protein (data not shown) and had S.I.’s > 2 in the presence of peptide, indicating a detectable population of P.69 Prn−24 specific CD4+ T cells. From these ten cases, six subjects, including case 629, were eligible for tetramer analysis based on time points of samples after clinical infection and availability of sufficient amounts of PBMC for rare event detection. Two cases were sampled in acute phase, two cases were sampled in late phase and two cases gave paired acute and late samples (Table 1). Four cases experienced pertussis as adult and two cases were infected with B. pertussis as a child. Subject 629 received 3 months after his pertussis diagnosis an aP booster vaccination. Plasma levels of IgG antibodies against Pertussis Toxin (IgG-Ptx), ranged in acute samples from 84.2 to 672.1 IU/ml and in late samples from 15.5 to 51.2 IU/ml, in line with the typical decay pattern of this diagnostic parameter [27]. Overall, we observed P.69 Prn−24 specific CD4+ T cell frequencies that range from 106 to 1111 cells per million CD4+ T cells (0.01–0.11%, Table 1). Subject 629 had the highest frequency of CD4+DRB4*0101/IVKT− T cells (1111 cells per million CD4+ T cells). In the two longitudinal samples, P.69 Prn−24 specific CD4+ T cell frequencies were of the same order of magnitude.

### 3.4. Differentiation hallmarks of P.69 Prn−24 specific CD4+ T cells in pertussis cases

Co-expression of CD45RA and CCR7 was investigated on CD4+DRB4*0101/IVKT+ T cells from the six cases. Cells with a naïve (CD45RA−CCR7−) phenotype within the CD4+DRB4*0101/IVKT+ T cell population were seen in all subjects (Supplementary Fig. 3). Comparison of the percentages of CD4+CCR7− cells (both naïve and Tcm) to total CD4+ T cells and CD4+DRB4*0101/IVKT+ cells of samples indicated a significantly lower percentage of CCR7− cells in P.69 Prn−24 specific CD4+ T cells (Fig. 4A), suggesting a bias towards the CCR7-negative Tem and TD differentiation stage. Analyzing the memory phenotype of CD4+DRB4*0101/IVKT− T cells in all 6 cases, we observed
Figure 2  Phenotype and function of case 629 P.69 Prn7-24 specific CD4+ T cells after in vitro cloning and ex vivo. (A) Analysis of CD45RA and CCR7 expression on resting P.69 Prn7-24 specific HLA-DR53 restricted CD4+ T cells, 629-A2 cells (left panel), and ex vivo on DRB4*0101/IVKT+CD4+ T cells in PBMC of case 629 (right panel). (B) Cytokine production by 629-A2 cells (left panel) and ex vivo by PBMC of case 629 (right panel) after P.69 Prn7-24 peptide stimulation measured in o.n. and day 6 culture supernatants, respectively. Cytokine concentration was categorized as low (1 = 10–100 pg/ml), medium (2 = 100–1000 pg/ml) or high (3 >1000 pg/ml). (C) P.69 Prn7-24 specific 629-A2 cells were added at 1% to PBMC (left panel) or not (middle panel) and stained with DRB4*0101/IVKT TM followed by CD45RA and CCR7 phenotyping antibodies. PBMC of case 629 (right panel) were stained with DRB4*0101/IVKT TM followed by CD45RA and CCR7 phenotyping antibodies. Dotplots show live CD4+ cells and the percentages DRB4*0101/IVKT+ cells of CD4+ cells are indicated.
various distributions of CD45RA CCR7+ Tcm, CD45RA CCR7− Tem, and CD45RA CCR7− TD cells in both acute and late samples (Fig. 4B). The two paired samples showed relatively stable differentiation patterns within P.69 Prn7−24 specific CD4+ T cells in time (Fig. 4B).

In earlier studies we found that freshly tested human pertussis epitope-specific T cell responses to mostly contain Th1 and Th2 components, without significant amounts of IL-2, IL-10 and IL-17 in culture supernatants [24]. When analyzing epitope specific cytokine secretion patterns of the six cases ranked on declining percentage of the CCR7+ memory phenotype of CD4+DRB4*0101/IVKT+ cells (Tcm Tem TD), a trend in cytokine secretion capacities became visible. PBMC from cases having relatively more CD4+DRB4*0101/IVKT+ cells of the Tcm phenotype secreted Th1 cytokines (IFN\(_\gamma\) and TNF\(_\alpha\)) while subjects with relatively more CD4+DRB4*0101/IVKT+ Tem secreted in addition to Th1 cytokines also Th2 cytokines (IL-13 and IL-5) (Fig. 5). Notably, the far-end differentiated (TD) phenotype of specific cells seen in two cases (151 and 158) was associated with low or non-functionality based on cytokine secretion. These results indicate that distinct end-differentiation stages of pertussis-epitope specific CD4+ T cells correlating with particular cytokine secretion capacities could be observed in subjects after clinical infection.

![Figure 3](P.69 Prn7−24 specific lymphoproliferation in PBMC of HLA-DR53 positive pertussis cases. Fresh PBMC of HLA-DR53 positive (ex-):pertussis cases (n = 25) were stimulated with 1 \(\mu\)M P.69 Prn7−24 peptide for 7 days and [\(^{3}\)H]thymidine incorporation was assessed in the last 18 h. The responsiveness of subjects to PHA was 100% (data not shown). Epitope specific proliferation is shown as stimulation index (S.I. = mean CPM peptide / mean CPM medium) and epitope specific responsiveness with a S.I. \(\geq\) 2 (dashed line) was regarded as positive.)

Table 1: Clinical and immunological parameters of MHC II tetramer probed pertussis cases.

<table>
<thead>
<tr>
<th>Case (phase)</th>
<th>Age at sampling (years)</th>
<th>Pertussis vaccination history</th>
<th>Time since infection (months)</th>
<th>Age at confirmed pertussis (years)</th>
<th>IgG-Ptx (IU/ml)</th>
<th>Frequency of DRB4*0101/IVKT+ cells (number per 10(^6) CD4+ T cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>151 (acute)</td>
<td>37</td>
<td>wP primary infant series b</td>
<td>0.9</td>
<td>37</td>
<td>336.2</td>
<td>201</td>
</tr>
<tr>
<td>158 (acute)</td>
<td>51</td>
<td>wP primary infant series b</td>
<td>0.7</td>
<td>51</td>
<td>672.1</td>
<td>411</td>
</tr>
<tr>
<td>158 (late)</td>
<td>54</td>
<td>wP primary infant series b</td>
<td>42</td>
<td>51</td>
<td>40.7</td>
<td>208</td>
</tr>
<tr>
<td>201 (acute)</td>
<td>64</td>
<td>Not vaccinated</td>
<td>1.0</td>
<td>64</td>
<td>230.1</td>
<td>278</td>
</tr>
<tr>
<td>201 (late)</td>
<td>68</td>
<td>Not vaccinated</td>
<td>56</td>
<td>64</td>
<td>51.2</td>
<td>564</td>
</tr>
<tr>
<td>511 (acute)</td>
<td>47</td>
<td>wP primary infant series b</td>
<td>1.1</td>
<td>47</td>
<td>84.2</td>
<td>180</td>
</tr>
<tr>
<td>603 (late)</td>
<td>11</td>
<td>wP primary infant series b</td>
<td>78</td>
<td>4</td>
<td>15.5</td>
<td>106</td>
</tr>
<tr>
<td>629 (late)</td>
<td>10</td>
<td>wP primary infant series and aP booster at 4 years c</td>
<td>79</td>
<td>3</td>
<td>17.3</td>
<td>1111</td>
</tr>
</tbody>
</table>

\(a\) Phase after infection.

\(b\) Whole cell pertussis vaccination at 3, 4, 5 and 11 months of age.

\(c\) Whole cell pertussis vaccination at 3, 4, 5 and 11 months of age and acellular booster vaccination at 4 years of age.
4. Discussion

Studies on the characteristics and differentiation of human pertussis-specific CD4+ T cells are needed to understand why pertussis immunity wanes relatively fast. To our knowledge this study is the first example of direct ex vivo interrogation of human pertussis-epitope specific CD4+ T cell responses by using peptide-MHC II tetramers. DRB4*0101/IVKT TM reagents were based on the recognition pattern of CD4+ T cell clone 629-A2, specific for the immunogenic P.69 Prn7–24 epitope in the context of a supertypic HLA-DR molecule HLA-DR53, which is encoded for its β chain by the HLA-DRB4 gene. Earlier we described P.69 Prn7–24 responses in a panel of healthy blood bank donors at the polyclonal level to be associated with HLA-DQ1, an HLA-DQ background (HLA-DQ5, –6) not in linkage with the HLA-DR4, −7, and −9 haplotypes expressing HLA-DR53. Hence immunogenicity of the N-terminus of P.69 Prn seems based on the presence of multiple binding motifs for different HLA class II molecules. Analyses performed here were on PBMC of (ex-)pertussis cases from a unique clinical cohort after symptomatic infection (SKI-study), in which pertussis-specific CD4+ T cells were expected to be detectable. With the clinical material available for DRB4*0101/IVKT TM analysis, we were able to directly study P.69 Prn7–24 specific CD4+ T cells in six cases, including two subjects sampled in acute phase, two cases sampled in late phase and two cases with paired acute and late samples. Several characteristics were revealed.

First, frequencies of P.69 Prn7–24 specific HLA-DR53 restricted CD4+ T cell in the tested (ex-)pertussis cases ranged from 106 to 1111 DRB4*0101/IVKT+ cells per million CD4+ T cells (Table 1). Subject 629 had the highest number of epitope specific CD4+ T cell (1111 cells per million CD4+ T cells). This was not related to a recent sub-clinical infection since this subject’s IgG antibody level against Ptx was not elevated. In general a serodiagnostic cut-off of 62.5 IU/ml is used to identify recent pertussis infection [28]. The fact that this individual had clinical pertussis at 3 years of age and 3 months thereafter received a booster aP vaccination, may have resulted in the high frequency of P.69 Prn7–24 specific CD4+ T cells 6 years later. The measured frequencies of P.69 Prn7–24 specific cells in the CD4+ T cell population (0.011–0.11%) are in the order of magnitude of ex vivo enumerated epitope-specific CD4+ T cells reported after various viral or bacterial infections or vaccinations. Yellow Fever Virus (YFV) vaccination induced epitope-specific CD4+ T cells ranging from 0 to 100 cells per million CD4+ T cells [29], while hemagglutinin (HA) epitope-specific CD4+ T cells after vaccination with trivalent inactivated influenza vaccine ranged from 33 to 1667 cells per million CD4+ T cells [30]. In hepatitis C virus (HCV) patients, the typical HCV-epitope specific CD4+ T cell frequencies were in the order of magnitude of 10 – 1000 cells per million CD4+ T cells [31,32]. Human immunodeficiency virus (HIV)-epitope specific CD4+ T cell ranged 80 – 1400 cells per million CD4+ T cells in HIV-infected patients [33,34]. In healthy long-term Epstein–Bar virus (EBV) carriers,
epitope-specific CD4+ T cells frequencies of 8–400 cells per million CD4+ T cells were found [35]. Mycobacterium tuberculosis-epitope specific cells ranged from 300 to 6200 per million CD4+ T cells in tuberculosis patients and BCG vaccinees [36]. In Lyme arthritis patients, the frequency of Borrelia burgdorferi-epitope specific CD4+ T cells is near or below detection limit in peripheral blood with frequencies in the order of magnitude of 5 cells per million CD4+ T cells [37,38]. Typical frequencies of tetanus toxin-epitope specific CD4+ T cells in healthy immunized subjects are < 100 cells per million CD4+ T cells [39]. It should be noted that in our study the selection of the tested subjects was based on measurable lymphoproliferation after epitope-specific stimulation, introducing a bias for ‘high responders’ and subjects with borderline responses might be missed. In the two subjects with longitudinal samples, frequencies of CD4+ T cells specific for the immunogenic epitope P.69 Prn7 remained stable over time (3–4 years). Whether CD4+DRB4*0101/IVKT+ T cells can also be detected in HLA-DR53 positive cases without P.69 Prn7 lymphoproliferative activity, and whether loss of epitope specific CD4+ T responsiveness with increasing age [24] has a quantitative or a qualitative basis are important questions for future peptide-MHC II tetramer guided research.

Secondly, differentiation of P.69 Prn7 specific CD4+ T cells in an individual can be arrested in various stages, Tcm, Tem and TD. In some subjects (cases 201, 603, 629) all memory differentiation stages could be identified in Tem and TD. In some subjects (cases 201, 603, 629) cells in an individual can be arrested in various stages, Tcm, Tem and TD phenotype within CD4+DRB4*0101/IVKT+ T cells is indicated. Cytokine production of fresh PBMC of the corresponding samples after P.69 Prn7 peptide stimulation measured in day 6 culture supernatant is depicted. Cytokine concentration was categorized as low (10–100 pg/ml), medium (100–1000 pg/ml) or high (>1000 pg/ml).

Thirdly, the distinct end-differentiation patterns of P.69 Prn7 specific CD4+ T cells seem to correlate with epitope specific cytokine secretion capacities at the bulk level (Fig. 5). We found in the current and previous studies, that pertussis epitope-specific CD4+ T cell responses can contain mixed Th1 and Th2 cytokine profiles [24]. Notably, no significant amounts of IL-2, IL-10 and IL-17 could be detected in culture supernatants. The absence of IL-2 may be due to the sub-optimal day of analysis after stimulation (own observations). IL-10 and IL-17 are detectable at day 6 after PHA stimulation of PBMC (data not shown). Thus, we have no indications that P.69 Prn7 specific Th17 cells are present in the six tested (ex-)pertussis cases. Interestingly in parallel with the TD linear differentiation pathway, cytokine secretion capacity seems to follow a path of Th1 > Th1 > Th2 > low/non-functional phenotype. The quality, determined by proliferation- and cytokine/chemokine secretion capacities, of T cell responses is important for protective immunity against infections. Based on several
studies it was proposed that CD4+ T cells progressively gain cytokine secretion functionality with further differentiation, until they reach the stage that is optimized for their effector function (Tem). Continued antigenic stimulation can lead to progressive loss of cytokine production, resulting in terminally differentiated CD4+ T cells that are short-lived [17]. For Th1 responses, TNFα and/or IL-2 production is associated with Tcm, Tem are multifunctional cells that produce IFNγ in addition to TNFα and/or IL-2 and TD cells become single-positive IFNγ-producing cells [17]. Our results are in line with this linear differentiation model and also seem to apply for Th2 cytokine production, since an increased epitope specific Th1 and Th2 cytokine production in supernatants was observed in subjects with a pronounced Tem differentiation phenotype of TM+CD4+ T cells (Fig. 5).

For cases 151 and 158 from our SKI-cohort, one could ask what factors might have driven the predominant TD phenotype of DRB4*0101/IVKT+ CD4+ T cells. No remarkable difference in severity or duration of the symptomatic period between these and other infected subjects was observed (data not shown), yet this does not exclude individual differences in infectious load. Notably, in subjects 151 and 158 also the overall memory CD4+ T cell population had a relatively large proportion of TD cells (CD45RA+CCR7) (Supplementary Fig. 3). This could perhaps indicate a more general progress towards end-stage differentiation in these individuals, possibly related to chronological age and/or to the presence of persistent viral infections, notably with Cytomegalovirus [41]. Yet, not only relevant to ‘immunosenescence’, distinct differentiation stages of pertussis specific CD4+ T cells, especially the non-functional TD memory phenotype, are of interest in current children’s vaccination programs. Recently it was noted that the efficacy of acellular pertussis vaccination is more rapidly lost than that of whole cell pertussis vaccination [42,43]. Immunization with (high-dose) pure aP vaccine antigens initially induces stronger T cell responsiveness in children than vaccination with whole cell vaccine [44]. However, lower maintenance of cytokine functionality is seen in aP vaccinated cohorts with time [18,44], a possible mechanism leading to the observed increased susceptibility of aP vaccinated teenagers. Identifying differentiation patterns of specific T cell responses in vaccine studies could perhaps help understand T cell dysfunctionality, but currently applied methods may introduce in vitro artefacts [18,44]. These could be avoided by using peptide-MHC II tetramer based approaches.

In summary, differentiation of human pertussis-specific CD4+ T cells can be arrested in various stages (Tcm, Tem and TD) which seem to correlate with cytokine secretion capacities. Due to the limited available clinical material, the presented results are descriptive. Nevertheless, this study analyzes for the first time human pertussis-epitope specific CD4+ T cell memory phenotype using a peptide-MHC II tetramer approach providing proof of principle for such investigations. We envisage that development of combinatorial encoded tetramers to multiple pertussis-specific epitopes of different proteins with a broad HLA-restriction element usage could facilitate high-throughput parallel detection of antigen-specific CD4+ T cells in material from relevant cohorts [45]. Pathogen-specific ex vivo peptide-MHC II-based analysis, such as applied here, may provide more insight in the CD4+ T cell features annotated with limited immunological memory to B. pertussis and the resurgence of pertussis in the era of pertussis vaccination.

Conflict of interest statement
The author(s) declare that there are no conflicts of interest.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2015.02.009.

References
Peptide-MHC II tetramer guided approaches reveal end-differentiation patterns of CD4+ T cells


