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Superior *B. pertussis* Specific CD4+ T-Cell Immunity Imprinted by Natural Infection

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Abstract

Pertussis remains endemic in vaccinated populations due to waning of vaccine-induced immunity and insufficient interruption of transmission. Correlates of long-term protection against whooping cough remain elusive but increasing evidence from experimental models indicates that the priming of particular lineages of *B. pertussis* (Bp) specific CD4+ T cells is essential to control bacterial load. Critical hallmarks of these protective CD4+ T cell lineages in animals are suggested to be their differentiation profile as Th1 and Th17 cells and their tissue residency. These features seem optimally primed by previous infection but insufficiently or only partially by current vaccines. In this review, evidence is sought indicating whether infection also drives such superior Bp specific CD4+ T cell lineages in humans. We highlight key features of effector immunity downstream of Th1 and Th17 cell cytokines that explain clearing of primary Bp infections in naïve hosts, and effective prevention of infection in convalescent hosts during secondary challenge. Outstanding questions are put forward that need answers before correlates of human Bp infection-primed CD4+ T cell immunity can be used as benchmark for the development of improved pertussis vaccines.

Keywords

Bordetella pertussis (Bp) · Mechanism of protection · Natural infection · Th1 and Th17 polarized subsets · Tissue residency

Abbreviations

ACV	acellular pertussis vaccine
Bp	*Bordetella pertussis*
FHA	filamentous hemagglutinin
HSPC	hematopoietic stem and progenitor cell
PRN	pertactin
PTX	pertussis toxin
Th	T helper subset
WCV	whole cell pertussis vaccine

1 Introduction

Bordetella pertussis (Bp) is a gram negative encapsulated coccobacillus that colonizes the mucosa of the upper respiratory tract (URT). It is transmitted via airborne droplets and the average incubation time is 7–10 days. Bp is the causative agent of the disease pertussis or whooping cough, and can lead to severe illness, especially in young

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infants and newborns due to bronchopneumonia (Masseria et al. 2017). Pertussis is one of the leading causes of infant death due to respiratory infection (Tan et al. 2015; Muloiwa et al. 2018). The disease proceeds in several stages. It starts with a catarrhal phase, in which patients experience malaise and have symptoms of a cold. This catarrhal phase is followed by the paroxysmal phase, which presents with frequent attacks of coughing and characteristic squeaking inhalation due to breathing problems (2–6 weeks) and subsequently by the convalescent phase, which is characterized by a continuous coughing. Full recovery from disease may take weeks up to several months. However, a host immune response is initiated as soon as Bp attaches to the ciliated epithelial cells in the URT and starts colonizing. Here the bacterium is sensed by resident innate immune cells that shape a complex series of interactions with other recruited innate and adaptive cell types, influenced by both pathogen-derived factors and the local inflammatory microenvironment. In primary infections Bp can modulate virtually all aspects of the immune response and avoid early clearance by producing numerous virulence factors and adapting various bacterial life styles, as reviewed extensively (Locht et al. 2011; de Gouw et al. 2011; Higgs et al. 2012; Melvin et al. 2014; Brummelman et al. 2015; Jongerius et al. 2015; Cattelan et al. 2016; Fedele et al. 2017; Dorji et al. 2018; Gestal et al. 2018). Eventually effector immunity develops and the pathogen is cleared from its nasopharyngeal niche within weeks. Hence, patients are contagious and transmit the bacterium only during the catarrhal phase and the first weeks of the paroxysmal phase (Kilgore et al. 2016). In addition to effector immunity, the convalescent host develops long-lived memory immunity, able to react faster and to prevent secondary Bp infections and transmission. Although symptomatic reinfections have been described (Versteegh et al. 2002), most reinfections likely occur unnoticed and result in boosting levels of naturally acquired immunity that may have waned (Van Twillert et al. 2016). In the era before the introduction of pertussis vaccination, immunity against whooping cough was only naturally acquired and, based on its epidemiology, pertussis was considered a childhood disease (Hewlett and Edwards 2005). Then infant pertussis immunization was introduced in many developed countries in the 40s and 50s, that strongly reduced the mortality and disease load but also shifed its epidemiology to older age groups (Hewlett and Edwards 2005). The first type of pertussis vaccines were whole cell vaccines (WCV), consisting of killed Bp biomass, next to a broad spectrum of bacterial protein antigens these included the reactogenic Bp endotoxin LOS. While WCVs were highly effective, they were associated with side effects and were replaced by safer acellular pertussis vaccines (ACV) in most high income countries since the 90's. ACVs contain one up to five major immunogenic purified proteins of the bacterium that are adjuvanted by Aluminum salts. Despite vaccination, however, the disease reoccurred in many countries and is seen with two- or three-yearly epidemic peaks in the last few decades (Fulton et al. 2016). Various explanations for this resurgence have been postulated, such as waning vaccine-induced immunity, strain adaptation, improved diagnostics and medical awareness (Cherry 2012a, b). Since 2012 epidemiological evidence has accumulated that immune protection induced by ACV is less long-lasting than its WCV-induced counterpart (Klein et al. 2013; Sheridan et al. 2014). This prompted novel interest and research into the differences between correlates of protection against pertussis as induced by the two vaccine types or by natural infection (Diavatopoulos et al. 2018). Infection-primed immunity is regarded to represent the broadest and most durable protective immune response, based on epidemiological data (Wendelboe et al. 2005; Wearing and Rohani 2009). Apart from the multispecificity of the response, interactions with the whole live bacterium within an inflammatory milieu unique for primary infections might underlie the higher effectiveness of naturally-acquired Bp specific immunity.

2 Elusive Correlates of Protection against Pertussis

Basically Bp is regarded a noninvasive pathogen, however the pathogen can also enter and survive inside a number of human cell types (reviewed in (de Gouw et al. 2011)), mostly evidenced for

pulmonary alveolar macrophages (Paddock et al. 2008; Bromberg et al. 1991), monocyte-derived macrophages (Friedman et al. 1992; Lamberti et al. 2010), monocytes (Fedele et al. 2005), and respiratory epithelium (Lamberti et al. 2013). This indicates that the host's defense should be able to act against extracellular as well as intracellular Bp. In agreement herewith, both humoral and cellular immune mechanisms contribute to adaptive immune protection against Bp. The presence of serum antibodies against filamentous hemagglutinin (FHA), pertactin (PRN) and/or fimbriae (Fim2 and 3) have been suggested to protect against colonization based on their adhesin-specificity and potential to opsonize or mediate killing of whole Bp bacteria (Storsaeter et al. 1998; Cherry et al. 1998; Thorstensson et al. 2014). Low levels of antibodies to pertussis toxin (PTX) showed some correlation with susceptibility to disease (Taranger et al. 2000; Storsaeter et al. 2003). Yet, unlike other infectious diseases for which clear protective cut-off levels of antigen-specific antibodies have been identified (Plotkin 2010), a level of 20 IU/ml IgG antibodies to PTX is currently used as just an arbitrary level of protection to pertussis.

As a second protective arm of defense against pertussis, cell-mediated immunity by CD4+ T cells is implied. Moreover, CD4+ T-cell immunity imprinted by natural infection is thought to contain crucial signatures that correlate with the long-term protective capacity of the convalescent immune response. Recently, developments in animal models of primary Bp infection and secondary challenge have shed new light on CD4+ T-cell lineages induced by natural infection and the effector mechanisms they may propagate. Here we will review the insights from animal studies regarding superior cell-mediated immunity in the convalescent host and ask what is the evidence hereof in humans. It is discussed which gaps in knowledge should be closed before we understand hallmarks of human superior CD4+ T cell immunity that may be used as benchmark to improve current pertussis vaccines or vaccination strategies.

3 Clearance of *B. pertussis* Requires CD4+ T-Cells with a Distinct Differentiation Profile

In the past few decades, mouse models have been used extensively to address the protective role of cellular immunity to control an infectious respiratory challenge of Bp, generally inoculated via intranasal or aerosol administration (Van Der Ark et al. 2012). Naïve mice typically clear an infectious dose within 4–6 weeks (Mills et al. 1993; Barbic et al. 1997; Raeven et al. 2014) but recovered mice clear a secondary challenge within several days through the presence of primary infection-acquired memory immunity (Mills et al. 1993; Raeven et al. 2016). The role of T-cells and in particular of CD4+ T-cells producing IFNγ in controlling a bacterial load became clear in studies using athymic *nu/nu* mice (Mills et al. 1993) as well as SCID and IFNγ$^{-/-}$ mice (Barbic et al. 1997), IFNγR$^{-/-}$ mice (Mahon et al. 1997), or mice depleted of the CD4+ T-cell subset (Leef et al. 2000). Transferred CD4+ (and not CD8+) T-cells, derived from convalescent mice 6 weeks after Bp infection, were able to clear an infectious challenge in sublethally irradiated *nu/nu* recipient mice (Mills et al. 1993). Later Bp specific IL-17-producing T-cells were recognized as an additional T-cell lineage implied in protection, since neutralizing anti-IL-17 Ab significantly reduced the protective efficacy of a WCV-induced immune response against Bp (Higgins et al. 2006). Banus et al. found that Th17 responses, additive to Th1 responses, were also driven by infection (Banus et al. 2008) and Bp-infected IL-17A defective mice were found to have significantly higher bacterial loads in the lungs (Ross et al. 2013). Proof for the protective capacity of the infection-induced Th17 cells came from data showing that adoptive cell transfer of cultured splenic Th1 and Th17 lineage cells from convalescent mice reduced, separately and synergistically, the bacterial loads over the course of a Bp infection (Ross et al. 2013). Bacterial toxins PTX and adenylate

cyclase toxin (ACT or CyaA) seem to be involved in triggering protective Th1 and Th17 lineages by virulent Bp, since reduced Th1 as well as Th17 responses are seen in the absence of PTX (Andreasen et al. 2009) and reduced Th17 responses in the absence of ACT (Dunne et al. 2010). However, protective T-cell responses are not absolutely dependent on functional PTX since higher doses of $PTX^{-/-}$ strain or infection with the live attenuated Bp strain BPZE1 expressing a genetically detoxified PTX (and lacking functional tracheal cytotoxin and dermonecrotic toxin) (Mielcarek et al. 2006) can still drive Th17 (Andreasen et al. 2009) or Th1 and Th17 (Feunou et al. 2010a; Kammoun et al. 2012; Solans et al. 2018) type responses, respectively. Infection-primed cell-mediated immune protection seemed long-lasting since adoptive transfer of spleen cells 12 months after intranasal inoculation with BPZE1 protected SCID recipient mice (Feunou et al. 2010b).

Recently, Mills and colleagues applied methodology including *in vivo* labeling of lymphocytes to study local mouse CD4+ T-cells primed by Bp infection (Wilk et al. 2017). Tissue-resident memory T-cells (Trm) are traditionally characterized by the expression of CD69, which inhibits sphingosine-1-phosphate receptor 1 (S1PR1)-mediated egress from tissues (Arnon et al. 2011), and of CD103 (alpha subunit of aEb7 integrin), which docks cells to epithelial E-cadherin (Cepek et al. 1994; Casey et al. 2012). Wilk et al. showed that blocking the capacity of immune CD4+ T-cells to migrate to respiratory tissue through the S1PR1 antagonist, FTY720, abrogated the capacity to rapidly clear bacteria (Wilk et al. 2017) and that clearance of convalescent mice was associated with accumulation of CD4+ T-cells with the CD69 + CD103+ Trm phenotype (and negative for the lymphoid homing receptor CD62L) in lungs and nose. Adoptive transfer of CD4+ T-cells derived from the lungs of 60 days-convalescent mice, but not from vaccinated mice, strongly reduced the bacterial burden in Bp-challenged naïve recipient mice (Wilk et al. 2019). Furthermore, this study indicated that the CD4+ T-cells isolated from lungs, nose and spleen from convalescent mice, like those from WCV-vaccinated but unlike those from ACV-vaccinated mice, produced IFNγ and IL-17 upon stimulation. Notably, Solans et al. found that intranasal infection with the attenuated BPZE1 strain also primed IFNγ-producing and IL-17-producing CD4+ Trm cells in the lungs (Solans et al. 2018), indicating that full virulence of Bp was not required to steer this type of immunity. This study also revealed a probable link between the IL-17-producing CD4+ Trm cells and the induction of protective local secretory IgA responses. Post-infection co-development of Th17 type and IgA responses was also reported earlier in a systems immunology study using a recent clinical Bp strain (Raeven et al. 2014). Together, the studies in the mouse indicate that CD4+ T-cell lineages primed by natural infection, and to some extent by WCV, rapidly protect against an i.n. challenge dose, in profound contrast to CD4+ T-cells primed by ACV. As reviewed earlier (Brummelman et al. 2015) and elsewhere in this volume (chapter 6, Ausiello et al), ACV induce an alternative functional CD4+ T-cell profile, i.e. in mice dominated by the production of IL-4, IL-5 and IL-13 (Th2) or mixed Th2/Th17 cytokines, and prevent disease but do not protect against nasopharyngeal infection and transmission.

4 Bp Infection in Baboons as a Model to Study Hallmarks of CD4+ T-Cell Lineages Protecting against Colonization, Transmission and Disease

Since 'mice don't cough' the question is always raised as to whether the above findings can be translated to humans. Recently the baboon (*Papio anubis*) was identified as a non-human primate species to have a body temperature close to humans and reproducing typical clinical signs of pertussis (Warfel et al. 2012a). When experimentally challenged with strains representing currently circulating Bp clades, baboons develop leukocytosis, paroxysmal coughing, mucus production and heavy colonization of the airway

(Warfel et al. 2012b; Warfel and Merkel 2014; Naninck et al. 2018; Zimmerman et al. 2018). Importantly, transmission of the bacteria between hosts is observed (Warfel et al. 2012b). Based on these similarities with human (histo)pathology, transmission and immunology, this novel non-human primate model is regarded of high translational value to evaluate pertussis vaccine efficacy and correlates of protection. Merkel and coworkers found that baboons recovered from primary infection did not get colonized nor showed symptoms upon secondary challenge and that this convalescent state was associated with detectable peripheral Bp specific Th1 and Th17 responses, persisting for several years (Warfel and Merkel 2013; Warfel et al. 2014). Furthermore, infected animals had significant induction of IL-17 in the nasopharyngeal mucosa, and enhancement of IL-6, IL-23 and IL-1ß, cytokines responsible for the initiation and proliferation of Th17 immune responses in humans, as well as of several Th17 effector molecules, GCSF, IL-8, MCP-1 and MIP1a. Although the model so far was not designed to look at immune cell recruitment to the URT, the data are suggestive of the presence of a local Th17 type response. Further (indirect) evidence for a local immune response triggered by infection came from the detection of serum IgA to PTX, FHA, and PRN after intranasal/intratracheal inoculation of the attenuated BPZE1 strain in baboons. Since serum IgA responses are usually minimally or not at all induced after intramuscular pertussis vaccination in infants but develop at the respiratory mucosal sites after infection, this finding was interpreted to relate to local induction of IgA by BPZE1 (Locht et al. 2017). Local tissue-resident humoral and cell-mediated immunity remain to be studied in the baboon model.

Hence, from the available animal models, the concept emerges that protective infection-induced immunity against a subsequent Bp re-infection relies on the presence of specific CD4+ T-cells in the URT that produce Th1 and predominantly Th17 cytokines, and that have originally migrated to and remain resident in the respiratory tissues. Yet an important question is whether this paradigm holds true for humans as well.

5 Evidence for Bp Infection-Induced Th1 and Th17 Lineages and Tissue Residency in Humans

5.1 Th1 Lineage

The first studies addressing the cytokine profile of human CD4+ T-cells induced by clinical or asymptomatic Bp infection date back three decades. This was just after the discovery of the first Th1 and Th2 CD4+ T-cell subsets (Mosmann et al. 1986; Mosmann and Sad 1996; Del Prete et al. 1991) but well before the recognition of IL-17 producing CD4+ T cells as a separate lineage (Kolls and Linden 2004; Harrington et al. 2005; Wynn 2005; Park et al. 2005; Bettelli et al. 2007). In a series of 'classical' papers several groups interrogated cytokine responses from cases, ex-cases or naturally exposed individuals without history of pertussis vaccination, after stimulation of PBMC with killed whole bacteria or with vaccine antigen, typically (inactivated) PTX, FHA and/or PRN. Altogether these studies demonstrated that also in humans the Th1 cell subset is activated during Bp infection. In 1991 a first observation of Th1-polarization of Bp specific T-cells concerned a number of PTX-specific CD4+ T-cell clones isolated from a single adult donor with a history of whooping cough, which all were found to secrete IFNγ, some IL-2 but no IL-4 in their supernatants in response to antigen (Peppoloni et al. 1991). To extend these observations using uncloned T-cells, Ryan et al. took advantage of a cohort of acutely Bp-infected or convalescent young children (between 2 months and 8 years of age), all unvaccinated. Fresh PBMC were tested for proliferation and cytokine production after in vitro stimulation with Bp antigen. Most cases and ex-cases showed proliferative responses and production of IFNγ but not of IL-5 (Ryan et al. 1997). Hereafter Ausiello et al. showed, using enriched T-cells from a small sample of healthy adults with no history of pertussis vaccination, antigen-specific induction of gene transcripts for IFNγ and IL-2 but not for IL-4 nor IL-5. At the time, these data

were interpreted to reflect natural acquisition of an antigen-specific protective Th1 cytokine response by repeated exposure to Bp in an endemic setting (Ausiello et al. 1998). Also, these investigators described Th1 type Bp specific cell-mediated immunity several years after pertussis diagnosis in unvaccinated children, as opposed to more mixed Th1/Th2 type responses in ACV vaccinated non-infected age-matched controls (Ausiello et al. 2000). Meanwhile in a case report Hafler and Pohl-Koppe (1998) had described a strong proliferative response and IFNγ cytokine secretion to whole killed Bp cells and PT in two clinically infected teenagers, without detectable secretion of the Th2 cytokine IL-4. An important contribution to this series was by Mascart et al., who studied one to 4 months old infants suffering from an acute Bp infection, in comparison to non-immune age-matched controls, prior to their first dose of WCV. High levels of IFNγ in culture supernatants and high numbers of IFNγ spot forming cells were shown in PTX- and FHA-stimulated cultures from the infected infants, which were absent in the control non-immune group. Also, PTX or FHA stimulation did not trigger any Th2 responses, in either group of infants, as evidenced by the absence of IL-13 cytokine release or IL-4 spot forming cells, while all infants could mount Th2 responses to mitogen stimulation (Mascart et al. 2003). Taken together, these pioneering studies indicated that human priming by Bp infection alone, in the absence of any vaccination background, strongly steers Th1 responses. That this seems to be a unique hallmark of natural infection became clear from clinical vaccine studies in the same time frame and thereafter, showing that priming by WCV or ACV vaccination can induce mixed Th1/Th2 type CD4+ T-cell responses, with WCV-induced responses generally being more Th1-dominated (reminiscent of infection-induced responses), and ACV-induced responses being more Th2-dominated (van Twillert et al. 2015; Brummelman et al. 2015; Fedele et al. 2015; Ryan et al. 1998; Mascart et al. 2007; Schure et al. 2012, 2013; van der Lee et al. 2018a) (chapter 6 in this volume, Ausiello C. et al).

5.2 Th17 Lineage

After the discovery of the Th17 T-cell lineage (Kolls and Linden 2004; Wynn 2005; Bettelli et al. 2007) and its importance, in synergy with Th1 cells, to mediate protection in convalescent mice (Dunne et al. 2010; Ross et al. 2013), measurement of Th17 cytokines in human Bp specific T-cell studies became key. Yet, these studies involved vaccine immunogenicity or booster comparisons, or studies in convalescent patients with a history of vaccination (van Twillert et al. 2015; Schure et al. 2013), but none addressed primary Bp infection alone. In cases within 2–-3 months after clinical infection, all with a WCV or ACV priming background, we did not observe IL-17 cytokine secretion but instead mixed Th1 and Th2 cytokine responses, when stimulating PBMCs with synthetic peptides representing epitopes from PTX or PRN (Han et al. 2013, 2015), or with naturally presented Bp epitopes from other proteins (Stenger et al. 2014). Also in our group, Schure et al. and Van der Lee et al. described just low levels of IL-17 in Bp antigen-stimulated PBMC cultures from WCV or ACV-primed pediatric cohorts (Schure et al. 2013; Van Der Lee et al. 2018a). Yet in agreement with recent work by da Silva Antunes et al. (2018), we found that compared to ACV-primed adults, Bp antigen-stimulated PBMC cultures from WCV-primed adults, having received an ACV booster vaccination, not only produced increased levels of Th1 and Th2 cytokines, but also of Th17 cytokines (van der Lee et al. 2018b). In line with these age trends, in samples from a prospective pertussis household study (de Greeff et al. 2012) we found significant levels of IL-17 in Bp antigen-stimulated PBMC cultures from infected adults with a history of vaccination, while these were low or non-detectable in cultures from infected pediatric counterparts (Buisman et al., unpublished data). Whether this implies that Th17 responses are less well developed at younger age or that assays may have to be optimized for detection of children's IL-17 responses needs to be verified. It has been shown that naïve T-cells in cord blood fail to

differentiate to Th17 cells in co-culture with autologous antigen-presenting cells, despite the presence of polarizing cytokines (de Roock et al. 2013) and that newborns lack circulating Th17 cells (Stoppelenburg et al. 2014). In vitro studies using human monocyte-derived dendritic cells indicated that enzymatically active and inactive PTX and enzymatically active ACT can drive Th17-promoting IL-23 responses (Nasso et al. 2009; Fedele et al. 2010), indirectly supporting the in vivo requirements for these factors in human Th17 lineage differentiation, as evidenced in the mouse (Andreasen 2009; Feunou 2010a; Kammoun 2012; Solans 2018).

5.3 Tissue Residency of CD4+ T-Cells

The discovery of Trm-cells in several tissues has added to the scope of local immunity. Their central role is to respond rapidly to mediate clearance of a pathogen on the site of infection (Mueller and Mackay 2016). The phenotype of human CD4+ Trm-cells resembles that of Trm-cells in mice through constitutive expression of CD69 and CD103 (and absence of lymphoid homing receptors such as CCR7) (Kumar et al. 2017). Although human Trm cells are best defined for the CD8+ T cell subset, CD4+ Trm cells, including in the lung, have been described to play a role in protective immunity and immunopathology (Turner and Farber 2014). Human lung-residing CD4+ Trm cells have been studied for pathogens such as influenza virus and *Mycobacterium tuberculosis* (Walrath and Silver 2011; de Bree et al. 2007; Purwar et al. 2011), but not yet in the context of Bp infection.

5.4 Imprinting of T-Cell Lineage Differentiation

Taken together, there is no dispute about the fact that Bp infection in humans strongly triggers the Th1 lineage, and not the Th2 lineage. The additional involvement of the Th17 lineage, and tissue residency of both Th1 and Th17 lineages in humans, however, remain unknown due to the absence of IL-17 data in older studies in unvaccinated subjects primed by natural Bp infection. Based on shared (histo)pathology, transmission and immunology features between the baboon model of Bp infection and humans, it can be hypothesized that in addition to Th1 cells, human Th17 T-cells and tissue residency of the T-cell lineages might be involved as well. Lineage differentiation of CD4+ T-cells during immune responses reflects their priming by dendritic cells, which in turn get polarized by innate signals associated with the immunizing event (Sallusto et al. 2018). CD4+ T-cell programming is regulated by early 'signal transducers and activators of transcription' (STAT) pathways and imprinted epigenetically by networks of regulator proteins and permissive or repressive chromatin signatures (O'Shea and Paul 2010). The presence of IFNγ and IL-12 and of IL-6, IL-23 and IL-1ß, are involved in priming of Th1 and Th17 lineages, respectively (Sallusto et al. 2018). Understanding how CD4+ T-cell heterogeneity is formed and whether a degree of lineage plasticity exists in the context of immunity to Bp is not the topic of this review but an important field to investigate.

6 Protective Effector Mechanisms Downstream of Th1 and Th17 Cell Lineages

Bp has many virulence factors mediating evasion of early host clearance mechanisms and prolongation of survival and growth on the respiratory mucosae. Immune modulation includes interference with the production of antimicrobial peptides, the recruitment of phagocytes, complement- and phagocyte-mediated killing, the secretion of chemokines and cytokines, and development of adaptive B- and T-cell responses (Brummelman et al. 2015). It goes beyond the scope of this chapter to cover in detail which initial host-pathogen interactions and host

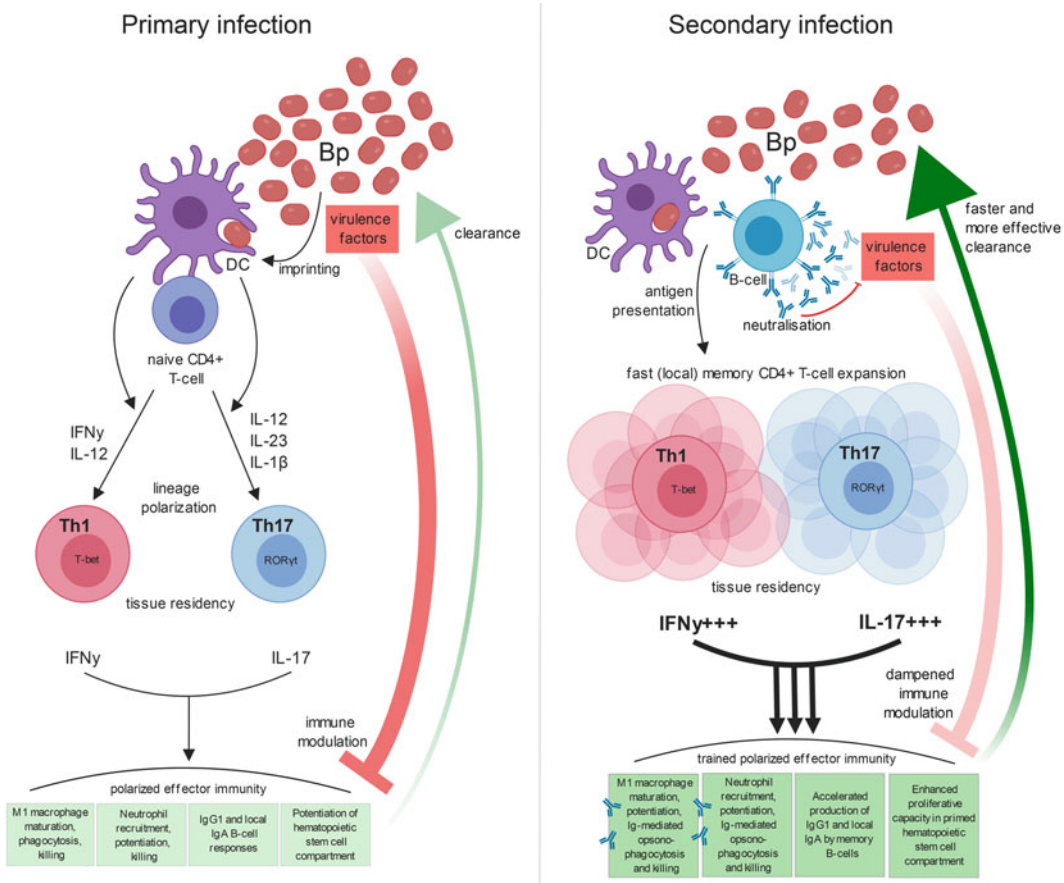

Fig. 1 Model for the role of IFNγ and IL-17 in clearance of Bp infection

Primary infection polarizes towards tissue-resident Th1 and Th17 lineage immunity, resulting in local production of IFNγ and IL-17. These cytokines potentiate macrophage, neutrophil and antibody-mediated mechanisms of clearance. Left panel: clearance in primary infections is significantly delayed by immune modulating virulence factors of Bp. Right panel: Fast, local expansion of infection primed memory responses results in accelerated and more effective Bp clearance during secondary infections. Accelerated production of IFNγ, IL-17 and antibodies enhance effector immunity, as indicated.

NB not illustrated is the contribution of NK cells and γδ T-cells to local IFNγ and IL-17 production during Bp infection, respectively. Red lines represent inhibiting effects and green arrows represent potentiating effects. Abbreviations: Bp, Bordetella pertussis; DC, dendritic cells; T-bet, T-box expressed in T-cells (transcription factor in Th1 lineage cells); RORγt, retinoic acid receptor-related orphan receptor gamma (transcription factor in Th17 lineage cells). Created with BioRender.com.

responses are implied, first delaying and then promoting the clearance of the pathogen. We here focus on four mechanisms by which effector cytokines produced by the Th lineages associated with infection-primed immunity, i.e. IFNγ and IL-17, can arm innate and adaptive cells to successfully clear primary and prevent secondary infections (illustrated in Fig. 1).

6.1 IFNγ- and IL17-Induced Potentiation of M1 Macrophages

It has been well established that macrophage polarization plays a key role in infectious diseases, and that IFNγ triggers 'classical' activation and differentiation of monocyte precursors into M1 macrophages (Benoit et al. 2008; Arora et al. 2018). Activated M1 macrophages promote

enhanced secretion of M1 chemokines, inducible nitric oxide synthase (iNOS) dependent reactive nitrogen intermediates (NO), reactive oxygen species (ROS), high levels of IL-12, IL-23 IL-1β and TNFα, and low levels of IL-10. This creates a pro-inflammatory milieu and NO- and ROS-mediated capacity of the M1 macrophages to kill bacteria engulfed in phagosomes. Bp may end up in phagosomes of phagocytic cells by various pathways including phagocytosis via complement receptors, innate receptors, and, in if antibodies are present, via immunoglobulin receptors (FcRs).

Other cell types than Th1 cells can also produce IFNγ, i.e. CD8+ cytotoxic T-cells, natural killer (NK) cells, antigen-presenting cells (APC) and B cells, and NK cells have indeed been found to be an important early source of IFNγ in the lungs of Bp infected mice, required for the initial polarization of Th1 cells (Byrne et al. 2004). Yet Th1 cells, capable of secreting large amounts of IFNγ, are thought to be the major activators of M1 macrophages. In turn, M1 macrophages secrete large quantities of IL-12 which aids in amplifying Th1 polarization of CD4+ lymphocytes (Martinez and Gordon 2014; Arango Duque and Descoteaux 2014). Macrophage killing of Bp is regarded an important protective mechanism to control Bp infection (Valdez et al. 2016; Bernard et al. 2015). Higgs et al. found that not only IFNγ but also TNFα and IL-17 had potentiating effects on the in vitro bactericidal capacity of murine peritoneal macrophages and a murine alveolar macrophage cell line (Higgins et al. 2006), This suggests that Th1 and Th17 lineage cytokines act in concert to stimulate M1 macrophage function. In fact, this dual M1 potentiating effect may be truly significant to counteract the M2 polarization effect of a high expression ratio of genes encoding the suppressors of cytokine signaling 1 and 3 (SOCS1/SOCS3) (Wilson 2014), observed in macrophages after intracellular infection with Bp (Valdez et al. 2016). Notably, Th2 associated cytokines IL-4 and IL-13 trigger the 'alternative' pathway of macrophage activation. Resultant M2 macrophages secrete high amounts of IL-10 and little IL-12 and IL-23, and lack microbicidal activity (Arora et al. 2018).

6.2 IL-17- Induced Recruitment and Activation of Neutrophils

Neutrophils are the dominant population among granulocytes and play a primary role in the host defense by removing pathogens through phagocytosis. High numbers of neutrophils are maintained in the circulation to facilitate rapid recruitment to infected tissue, mediated by chemotactic host signals and secreted bacterial molecules. Initially, recruitment of neutrophils to lungs after Bp infection is blocked through the action of PTX (Kirimanjeswara et al. 2005; Carbonetti 2015; Locht et al. 2011). Then subsequent host-pathogen interactions initiate neutrophil recruitment, peaking at 10–14 days after inoculation in mice. Th17 cells are known to orchestrate the recruitment of neutrophils to the site of infection, via IL-17A. In IL-17A defective mice neutrophil recruitment and Bp clearance was impaired, compared to wildtype mice (Ross et al. 2013). IL-17 not only mediates neutrophil recruitment but increases their ability to kill phagocytosed Bp through oxygen species generation. Other antimicrobial functions of neutrophils are NET-formation and degranulation (Eby et al. 2015). As discussed by Eby et al., it is likely that Th17 cells are the major source of IL-17 promoting the late Bp clearance by neutrophils. Yet, in parallel with the early polarizing innate IFNγ production by NK cells, early innate IL-17A was found to be produced already 2 h after Bp infection by lung γδ T-cells, promoting a Th17 polarizing milieu (Misiak et al. 2017).

Finally, neutrophils are no longer regarded only as terminally differentiated short-lived cells. Human neutrophils were reported to possess antigen presentation capacity in a MHC class II restricted manner, including the expression of co-stimulatory molecules, and ability to polarize

naive T-cells into Th1 and Th17 lineages (Abi Abdallah et al. 2011; Radsak et al. 2000; Lin and Lore 2017). This interaction between neutrophils and Th17 lineage cells has not been described for Bp infection yet.

6.3 Local Cross Talk Between Th17 Cells and IgA-Producing B Cells

In the naïve host, Bp has ample opportunity to modulate host defense mechanisms and clearance is delayed until the adaptive immune response helps to arm the major phagocytes to eliminate Bp at the site of infection. In the convalescent host, memory immune cells can rapidly mount elevated effector T-cell responses and antibody levels, preferably locally in the respiratory tract. IgA has been widely recognized as the antibody class of mucosal immunity. Cytokine patterns in antigen-specific CD4+ T-cells have been shown to instruct B cells to class-switch to particular isotypes. By programming particular T-cell lineages and effector cytokines, pathogens can modulate class-switching in B-cells (Tarlinton and Good-Jacobson 2013; McHeyzer-Williams et al. 2012). Hence pathogen specific T- and B-cell responses are tightly interlinked, with associations between isotype switching in murine B cells to IgG2a (or IgG2c) guided by Th1 cytokine IFNγ, to IgG1 and IgE guided by Th2 cytokine IL-4, and to IgA guided by TGFß and IL-17 in the Th17 cell milieu (Reinhardt et al. 2009; Christensen et al. 2017; Tarlinton and Good-Jacobson 2013). As revealed by Mills et al. in the mouse in addition to specific Th1 cells, Bp infection steers Th17 lineage cells that become tissue-resident in the lungs and nasal cavity shortly after priming (Wilk et al. 2019). Recently, Locht and coworkers showed interlinkage between these tissue-resident Th17 lineage cells and the capacity to produce IL-17 and protective local IgA in mice (Solans et al. 2018; Solans and Locht 2019), and between local Th17 lineage promoting cytokines and serum IgA in baboons (Locht et al. 2017). Hence, T-cell derived IL-17 production in the URT can now be proposed to promote local IgA-switched B cell responses including rapid boosting of specific IgA levels upon secondary challenge. Local specific antibodies would not only be capable of neutralizing virulence factors, thereby avoiding immune modulation by Bp, but also of enhancing opsonophagocytosis of Bp by local macrophages and neutrophils, thereby accelerating clearance in the convalescent host. Whether a similar link exists between local IFNγ-producing Th1 cells and murine IgG2a (or IgG2c)-switched B-cells (and IgG1-switched B cells in humans) remains to be investigated.

6.4 IFNγ-Induced Training of Myeloid Cells and Hematopoietic Stem and Progenitor Cells

Recently, Varney et al. suggested an important mechanism downstream of IFNγ, explaining how the more durable immune protection could work in hosts recovered from primary Bp infection or immunized with WCV, as opposed to the shorter duration of protection seen in ACV-immunized hosts. It was demonstrated in mice that infection- and WCV-induced immunity impacted responsiveness at the level of Hematopoietic Stem and Progenitor Cells (HSPC) in the bone marrow, especially pointing at myeloid preparedness and rapid expansion of HSPCs and tissue homing upon reinfection (Varney et al. 2018). Gene set enrichment analyses demonstrated that, like WCV-immunized but unlike ACV-immunized mice, Bp-infected mice exhibited unique gene signatures that suggested roles for IFNγ-induced gene expression. Mice exhibiting an IFNγ-priming milieu had relatively large myeloid proportions in the spleen as well as enhanced gene expression in HSPCs, regarding processes such as survival, cell renewal, autophagy and antigen processing and presentation. In line with some of these findings, Raeven et al. already found clusters of genes typically expressed in lungs of mice recovered from primary Bp infection, indicating enhanced activity of 'trained' innate immune cells and involving antigen processing and presentation or MHC

signalling (Raeven et al. 2016; Raeven et al. 2017). Programming at the HSPC level was first described in a model of priming with the live attenuated Bacillus Calmette Guerin strain (BCG) of Mycobacterium tuberculosis bovis (MTB) (Kaufmann et al. 2018), indicating that IFNγ-induced training of innate cells and HSPCs is a biological reproducible and relevant phenomenon.

Clearly, IFNγ and IL-17 are master regulators in various effector mechanisms downstream of infection-primed Bp specific Th1 and Th17 cell responses. As these cells likely also produce other cytokines or soluble mediators and engage in many cell-cell interactions, additional mechanisms contributing to the durable type of Bp specific protective immunity should not be excluded.

7 Conclusions & Future Directions

Pertussis remains a public health problem despite vaccination, affecting all age groups and especially vulnerable infants. To forward the development of future pertussis vaccines with a longer duration of protection and interrupting transmission, correlates of protection against pertussis should be better understood. Over the past three decades, the mouse and baboon models of Bp infection have been exploited to elucidate why convalescent hosts primed by infection are better protected than hosts immunized with pertussis vaccines. The basis seems to lie in the imprinting of CD4+ Th1 and Th17 cell lineages, their tissue residency in the lung and nasal tissue and the steering role of their effector cytokines on mechanisms such as phagocytic clearing, differentiation of IgA B-cell responses and training of myeloid cells and HSPC. Natural infection-acquired immunity in unvaccinated humans has also been found to be associated with the most durable protection against pertussis, based on epidemiological evidence. In view of shared features between human and baboon Bp infection biology, it can be hypothesized that similar CD4+ T-cell lineages and mechanisms play a role as well. However only few aspects of human infection-primed CD4+ T-cell immunity have been explored to date.

Several challenges lie ahead to increase our understanding of the role of CD4+ T-cells in human protective immunity against pertussis, using natural infection as a benchmark. Questions that need answers include:

- Can we detect Bp specific CD4+ T-cell responses in the peripheral blood of unvaccinated humans after natural infection or (likely) exposure, and can functional differentiation of especially Th17 lineages be confirmed? Are these associated with IgA B-cell responses?
- Can human Bp specific CD4+ T-cells be found with a tissue-resident phenotype and a Th1/Th17 lineage profile in cell suspensions obtained from bronchoalveolar lavages or lung surgery biopsies from unvaccinated naturally exposed subjects?
- Can infection-primed human CD4+ T-cells be enriched and in depth explored for candidate biomarkers of durable protective Bp immunity? What are the defining immunological signatures of these cells at the proteome, transcriptome and/or epigenome level?
- Can we develop assays to immunomonitor candidate biomarkers that predict durable protective CD4+ T-cell immunity to Bp in future clinical registration studies?

To address these questions blood and preferably mucosal samples from pertussis cases, representing all ages, without vaccination history are pivotal. Except for older age groups recruiting non-vaccinated younger cases, born after the implementation of immunization programs, may pose a problem. Also, assays to interrogate the Th lineage differentiation of human CD4+ T-cells in depth should be designed with care. First of all, Bp antigen-specificity should be well-defined and perhaps antigen panels need to be optimized for infection-primed immunity. Second, in view of their low frequency, enrichment of Bp specific CD4+ T-cells prior to analysis is likely required, either based on in vitro activation markers (da Silva 2018) or ex vivo labeling with MHC

class II tetramers (Han et al. 2015). Third, depending on the question, methodology to in-depth analyse the enriched Bp specific CD4+ T-cells for candidate biomarkers of protective immunity may be selected at different systems' levels, including multiparameter flow cytometry, CyTOF, RNAseq, genome-wide STAT binding or ATAQ-seq, where possible at the single cell level. Associations between human Th17 lineage immunity and levels of Bp specific IgA determined in serum and mucosal samples such as nasal lining fluid and lung lavages should be addressed. Also a systems approach including other immune cells like myeloid cell types and HSPC could reveal wider mechanisms of imprinting of Bp infection-induced immune protection in humans.

Altogether this will help to understand deeper hallmarks of Bp infection-primed CD4+ T-cell immunity that could be used as benchmark to guide development and implementation of improved pertussis vaccines with a longer duration of protection against disease, and preventing transmission.

Key Issues

- Immunity induced by natural Bp infection is superior as it has higher durability compared to vaccine-induced immunity based on epidemiological data
- Based on animal models, infection-induced superior CD4+ T-cell immunity is characterized by Th1 and Th17 lineages that enhance macrophage killing capacity and neutrophil recruitment and function
- Bp infection in animal models induces tissue-resident memory CD4+ T-cells in lung and nose. These play an important role within the local mucosal immune response, including stimulation of IgA class switching
- Bp infection seems associated with IFN-γ-induced training of innate cells and HSPCs
- It is currently unknown how the Bp specific CD4+ T-cell lineages acquired by natural infection are programmed exactly and to what extent lineage imprinting is reversible
- A great body of evidence discussed in this review comes from animal models, but it remains to be investigated whether findings can be translated to human immunity
- Although studying infection-induced immunity in unvaccinated cohorts is challenging due to the rarity of these cohorts and confounding factors such as age and unknown (subclinical) repeated exposures, it should be given more priority
- Studying human naturally-acquired cell-mediated mechanisms will help to understand immunity that is essential to combat Bp infections and guide or accelerate pertussis vaccine development

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