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Evaluation of immune responses induced by a novel candidate vaccine against whooping cough in a human pre-clinical model

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INTRODUCTION

1.1 Dendritic cells

Since their discovery by Steinman and Cohn in 1973, dendritic cells (DC) have become increasingly recognized for their crucial role as special type of leukocytes able to alert the immune system for the presence of infections and responsible for the activation and control of both innate and adaptive immune responses (Zitvogel, 2002). DC are especially distributed in tissues that interface the external environment, such as the skin, the gut and the lungs (Nestle et al., 1993), where they can perform a sentinel function for incoming pathogens (Fernandez et al., 1999). DC do not constitute a unique cell population, rather they comprise a large collection of subpopulations, located in both lymphoid and non-lymphoid tissues, that can be distinguished by the expression of specific cell surface markers and functional properties, reflecting a selective specialization in their response to infection (Shortman & Liu, 2002; Ardavi, 2005).

Two main DC subsets have been identified: conventional (''myeloid'') $CD11c^+$ DC (mDC) and $CD11c^-$ $CD123^+$ plasmacytoid DC (pDC). mDC include Langerhans cells, dermal DC and interstitial DC, they are made up of at least two subsets: the more common mDC-1, which is a major stimulator of T cells and the extremely rare mDC-2, which may have a function in fighting wound infection. Furthermore, mDC are able to produce high levels of the immunomodulatory cytokines IL-12p70 and IL-10. pDC play a crucial role in antiviral immunity, they selectively express toll-like receptors (TLRs) 7 and 9, which enable them to sense single stranded RNA and DNA viruses, respectively, producing vast amounts of type I interferons (IFNs) (Liu, 2005; Asselin-Paturel & Trinchieri, 2005).

As the most potent antigen-presenting cells (APCs), DC are specialized for the uptake, processing, transport and presentation of antigens to T cells and are capable of priming naïve T cells (Mellman & Steinmen, 2001). Although the development of DC from early hemopoietic precursors is not fully understood (Ardavin et al, 2000), terminal stages of DC development and their life cycle during an immune response are well defined. Immature DC reside in peripheral tissues and constantly capture antigens from the local environment, process and then present them in association with surface major histocompatibility complex (MHC) molecules. In presence of microbial products or tissue

damage in the environment, DC initiates their migration to peripheral lymphoid organs and their transition from antigen-capturing cells into APCs. The migration and functional transition of DC correlate with decreased antigen uptake and increased half-life of surface MHC-peptide complexes. As a result, antigens captured by DC are, in the cell bound form, transported to and concentrated in the peripheral lymphoid organs for presentation to antigen-specific T cells. Up regulation of co-stimulatory molecules, altered expression of chemokine receptors and production of cytokines are crucial for effector T cell (helper or cytotoxic) differentiation (Banchereau et al., 2000).

1.1.1 Linking innate to adaptive immunity through dendritic cells

The organism bears two arms to fight pathogens: innate and adaptive immunity. Pathogen recognition by the immune system has two major effects. First, it triggers the innate response mediated by the effector cells of inflammation, including macrophages and polymorphonuclear neutrophils, which represent an immediate defence at the sites of pathogen entry. Second, the innate immune system induces adaptive immunity. In this regard, DC play a pivotal role. DC respond to two types of signals: direct recognition of pathogens through pathogen recognition receptors (PRRs) and indirect sensing of infection through inflammatory cytokines, internal cellular compounds, and ongoing specific immune response. In response to these signals, dendritic cells are activated to enter an integrated developmental program called maturation, which transforms dendritic cells into efficient T cell stimulators (Guermonprez et al., 2003). The following paragraphs briefly describe the receptors involved in the induction of dendritic cell maturation and the cell biological modifications resulting in dendritic cell migration toward secondary lymphoid organs and polarization of T helper lymphocytes.

1.1.2 Regulation of dendritic cell functions by toll like receptors

The inflammatory stimuli sensed by DC may be exogenous, such as microbial structures, collectively named PAMPs (pathogen-associated molecular patterns), or endogenous, such as heat shock proteins, hyaluronate and heparan sulphate, fibronectin, high mobility group box 1 protein, and modified low-density lipoproteins (Miyake, 2007). DC perceive these stimuli through germ line-encoded PRRs (Janeway & Medzhitov,

2002), which constitute a large superfamily of receptors located at the cell surface and in the endosomal compartment.

TLRs are the best characterized PRRs. They are type I membrane proteins characterized by an ectodomain composed of leucine rich repeats (LRR) that are responsible for recognition of PAMPs and a cytoplasmic domain homologous to the cytoplasmic region of the IL-1 receptor, known as the TIR domain, which is required for downstream signalling. To date, 13 different TLRs have been identified. TLRs 1-9 are conserved in humans and mice, TLR10 is expressed by B cells and pDC only in humans and TLR11-13 are functional in mice (West et al., 2006).

Although a lot of information is available concerning the functional activities of TLRs 1-9 and 11, basic knowledge on the physiology of TLRs 10, 12, and 13, is still lacking. TLRs 1-6 and 11 are expressed at the cell surface and can be activated by molecules generally located at the surface of bacteria, fungi, or protozoa. TLR 3, 7, 8, and 9 are located within the endosomes and recognize microbial nucleic acids (Kawai & Akira, 2007). TLR12 participates in innate immune responses to microbial agents and is thought to play a role in preventing infections within the urogenital system. TLR13 is a novel and uncharacterized member of the mammalian and is predominantly expressed in the spleen, particularly in dendritic cells and macrophages. Remarkably, cells expressing *tlr13* fail to respond to known TLR ligands but instead respond specifically to vesicular stomatitis virus (Shi et al., 2011).

TLRs can sense various bacterial molecules, including cell wall components such as lipopolysaccharide (LPS) (recognized by TLR4), peptidoglycan (recognized by TLR2), lipoarabinomannan (LAM) (recognized by TLR2), diacyl or triacyl lipopeptides (recognized by TLR2/1 or TLR2/6, respectively), flagellin (recognized by TLR5), and genomic DNA rich in unmethylated CpG sequences (recognized by TLR9). It has recently been shown that group B streptococcus, which resides in the phagosome, induces TLR7-dependent type I interferon, suggesting that the bacterial RNA produced in the lysosomal compartment may be recognized by TLR7 (Kumar et al., 2009).

Following interaction with their agonists, TLRs initiate different signalling pathways that lead to the activation of specific transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Fig.1). This is independent of the nature of the microbial stimulus. All TLR family members, except from TLR3, induce the activation of NF-κB and AP-1 by recruiting the adaptor myeloid differentiation primary response gene 88 (MyD88).

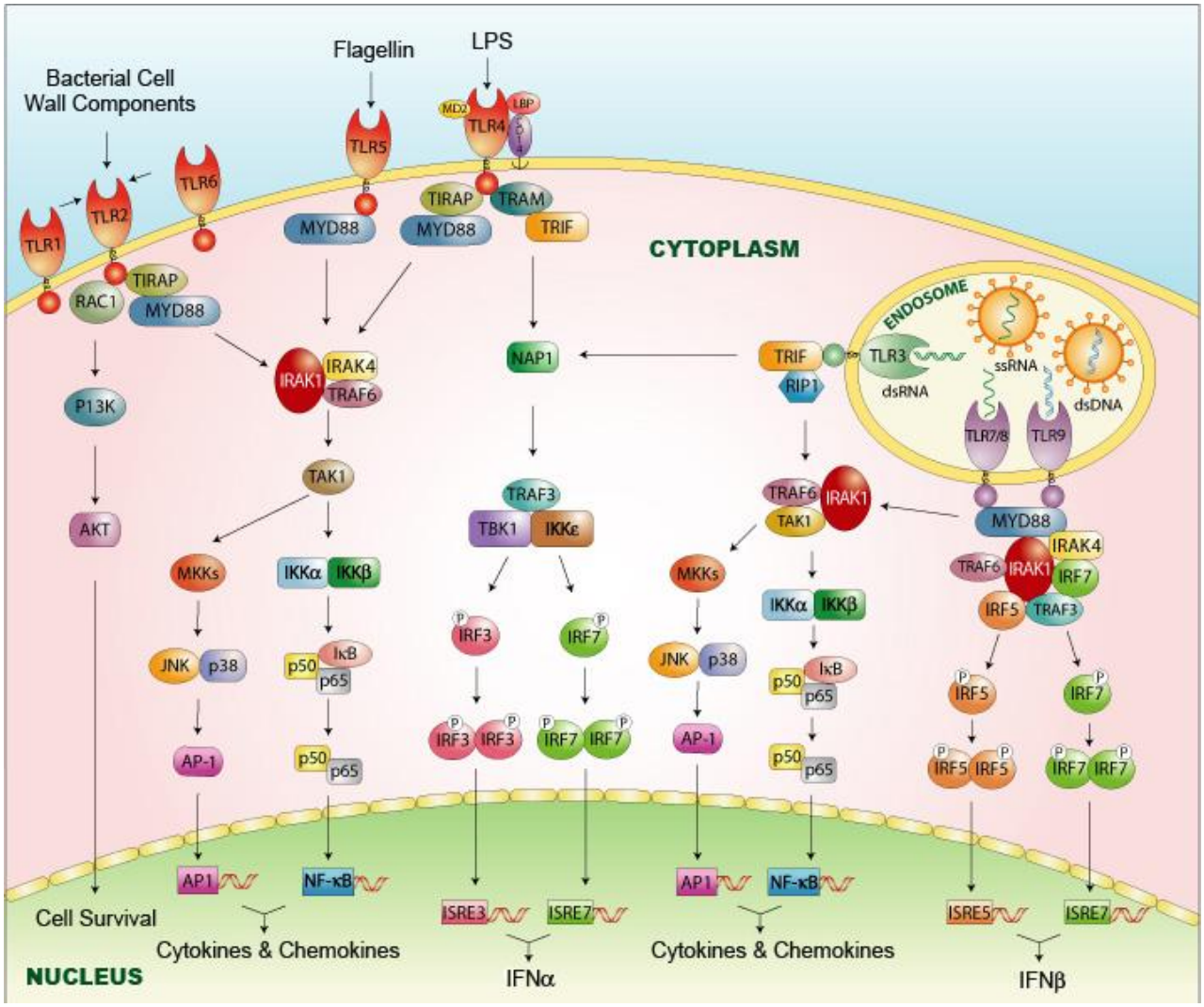

Fig.1: TLR signalling in conventional dendritic cells, macrophages and plasmacytoid dendritic cells. TLR2 (TLR2 in association with TLR1 or TLR6), TLR4, TLR5 are localized on the cell surface for ligand recognition. TLR3, TLR7, and TLR9 are localized in the endosome. All TLRs, except TLR3, recruit MyD88, TLR1, TLR2, TLR4 and TLR6 recruit the additional adaptor TIRAP, which links the TIR domain with MyD88. TLR3 and TLR4 recruit TRIF (Kumar et al., 2009).

Diversely, TLR3 transduces the signal via the adaptor TIR-domain containing adaptor-inducing IFN-beta (TRIF) (Kaisho & Akira, 2006). Whereas the activation of NF-κB and AP-1 is a common feature of the signalling induced by all TLRs, only a subset of them is additionally able to trigger the activation of the transcription factors interferon regulatory factor 3 (IRF3) and IRF7 which largely regulate the expression of type I IFNs (Takeda & Akira, 2004). Type I IFN induction is TRIF-dependent for TLR3, whereas it is MyD88-dependent for TLR7, TLR8, and TLR9. A notable exception is represented by

TLR4, which recruits both MyD88 and TRIF to induce the activation of NF-κB and AP-1 and, similarly to TLR3, TRIF to stimulate the production of type I IFNs though in response to non-nucleic acid ligands. While the MyD88 pathway can start both from the membrane and the endosome, the TRIF pathway always starts from the endosome as well as the MyD88-dependent signalling pathway that leads to type I IFN production (Barton & Kagan, 2009).

TLR-mediated regulation of DC leads to a coordinated transcriptional response that modulates various activities, such as endocytosis, cytoskeleton rearrangements, migration and antigen processing and presentation. This process is generally called DC maturation.

1.1.3 Dendritic cell maturation

DC process microbial antigens into peptides which are loaded onto MHC-I and II molecules and transported to the cell surface for recognition by antigen-specific T cells. Endogenous protein antigens, which are processed onto MHC-I, are first ubiquitinated and degraded into peptides by the proteasome in the cytosol. These are transported for antigen presentation by TAP molecules into the endoplasmic reticulum, where they are loaded onto MHC-I. The peptide-MHC-I complexes (pMHC-I) are then transported to the cell surface for presentation to $CD8^+$ T cells. Exogenously acquired protein antigens, on the other hand, are engulfed and processed in endosomes. Endosomes containing ingested proteins mature and fuse with lysosomes, where proteases degrade the proteins into peptides that are loaded onto MHC-II molecules. Peptide-MHC-II complexes (pMHC-II) are then transported to the cell surface within specialized tubules for presentation to $CD4^+$ T cells (Guermonprez et al., 2002).

Exogenous antigens may also be processed by DC onto MHC-I. This phenomenon, called “cross-presentation” or “cross-priming,” permits DC to elicit $CD8^+$ as well as $CD4^+$ T-cell responses to exogenously acquired antigens (Guermonprez et al., 2003).

Antigen capturing DC become professional APCs that migrate to the draining lymphoid tissue and mature phenotypically, up regulating the expression of co-stimulatory molecules involved in the formation of the immunological synapse such as CD40, CD80, CD86 and MHC-II (Fig. 2). The dominant mediator in the mobilization of DC to lymph nodes via lymphatics is the CC-chemokine receptor 7 (CCR7). The trafficking events that lead DC to their optimal positioning in lymphoid tissue are a key process in the regulation and induction of immune responses.

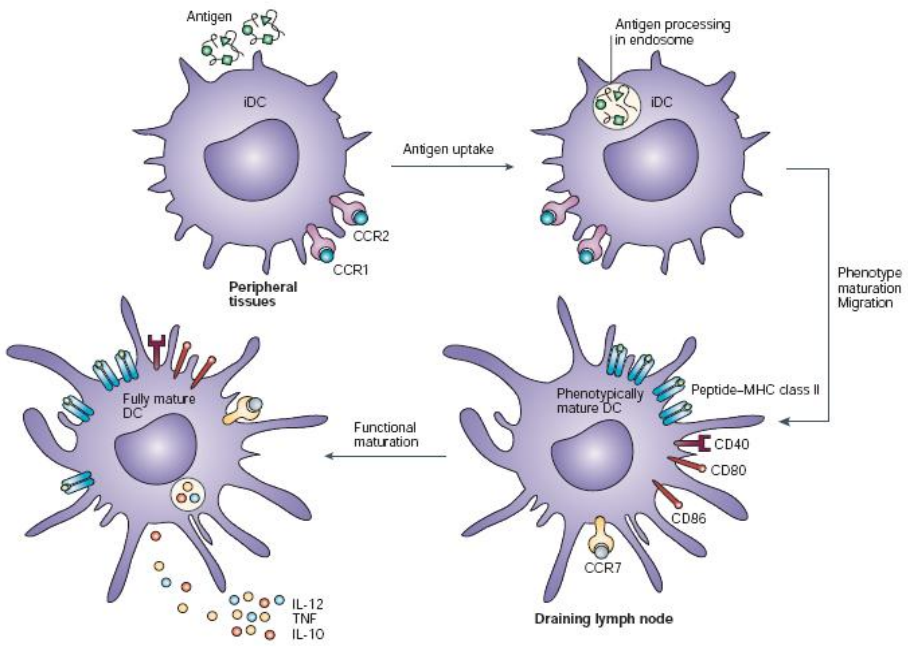

Fig.2: Development of dendritic cell maturation. Haematopoietic stem cells differentiate into immature dendritic cells (iDC) that are recruited to peripheral tissues, where they continuously internalize antigens that can be processed by a MHC class-II-restricted endosomal pathway. After antigen capture, DC migrate to the draining lymphoid tissue and mature phenotypically, In this area, they present peptide-MHC-II complexes on the cell surface, interact with antigen-specific lymphocytes and mature functionally, activating T cells and producing pro-inflammatory or regulatory cytokines, such as interleukin-12 (IL-12p70) and tumour-necrosis factor (TNF), or IL-10 (Hackstein & Thomson, 2004).

CCR7 ligands, CC-chemokine ligand 19 (CCL19) and CCL21 are expressed by lymphatic endothelium and/or within lymph nodes by stromal cells, endothelial cells, and DC themselves, and they each participate in the migration of DC from peripheral tissues to the lymph node (Martin-Fontecha et al., 2003). However, the expression of CCR7 is not sufficient for migration of DC into lymphatics. Instead, CCR7 responsiveness requires coordinated signals to induce a response to its ligands. These triggers include lipids mediators, cysteinyl leukotrienes, and prostaglandin E2 (Randolph et al., 2005). It has been shown that the ecto-enzyme CD38 expressed by mature DC plays a pivotal role in lymphatic migration acting as a receptor delivering into the cell the signals required for the process to take place (Frasca et al., 2006). It is not yet clear how all of these different extracellular triggers work in concert to promote migration via CCR7.

In the lymphoid organs, mature DC act as professional APCs by providing signal 1 (T cell receptor cross-linking) and signal 2 (co-stimulation) to pathogen-specific naive T cells, thus inducing their activation and clonal expansion. In addition, mature DC secrete multiple cytokines and express membrane-bound molecules that drive the development of $CD4^+$ T cells into specific T helper cell types which determine the character of the ensuing immune response (Lanzavecchia & Sallusto, 2001).

1.2 T helper polarization

Effector $CD4^+$ T helper (Th) cells are the key players in steering the immune responses. Th cell differentiation is characterized by the acquisition of cytokine production (Fig. 3).

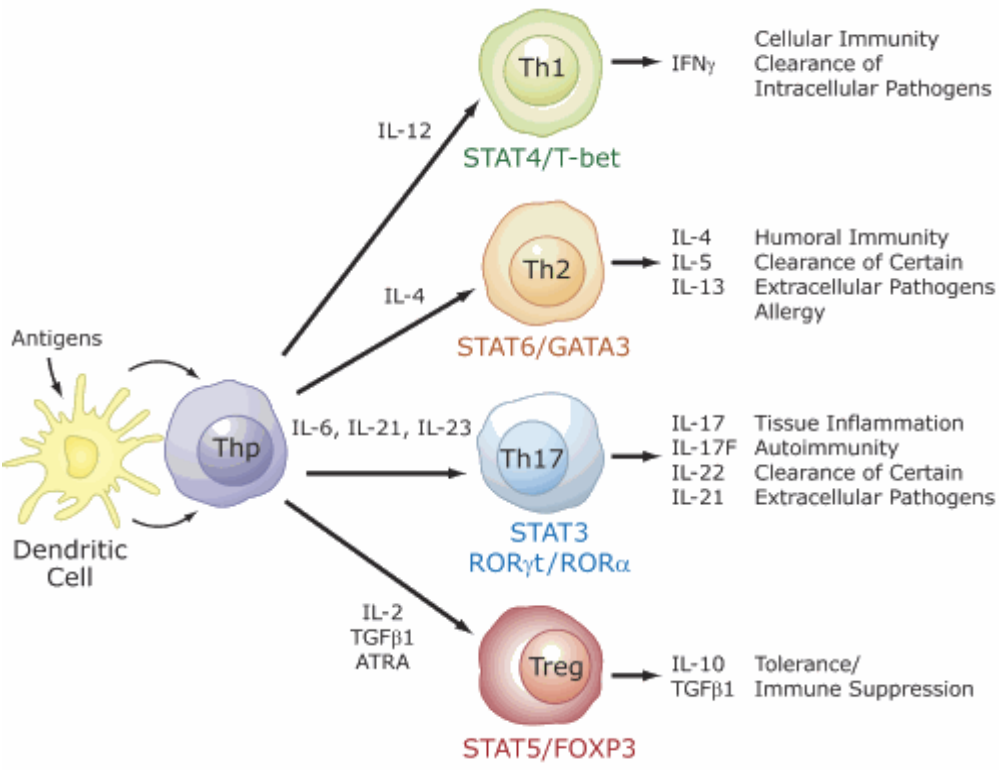

Fig.3: T cell lineage specification Differentiation into different effector $CD4^+$ T cell lineages, Th1, Th2, Th17, and regulatory T (Treg) cells is initiated through the interaction of dendritic cells with uncommitted (naïve) $CD4^+$ Th cells (Jetten, 2009).

Since the establishment of the Th1-Th2 paradigm, the function and regulation of effector T cells has been a subject of intense investigation. Owing to years of collective

efforts, vast knowledge has been gained in identifying new classes of effector T cells and in understanding their function and regulation.

1.2.1 Th1 response

The most important function of Th1 cells is to promote cell-mediated immunity, characterized by cellular cytolytic activity. Th1 cells are important in protection of the host from the obligate intracellular pathogens. Furthermore, they drive the pathway to fight viruses and other intracellular pathogens, eliminate cancerous cells, and stimulate delayed-type hypersensitivity (DTH) in the skin (Kidd, 2003). It is well appreciated that Th1 differentiation can be promoted by DC that are stimulated by particular microbial stimuli and by CD40 ligand (CD40L) to release large amounts of IL-12p70. IL-12p70 comprises two disulphide-linked proteins: the p35 chain and the p40 chain. The activation of human myeloid DC by TLR4 ligands leads to the expression of IL-12p70. IL-12p70 synthesis is also induced in human mDC by ligands for TLR3 and TLR8 (Goriely et al., 2008).

Th1 cells produce interferon-γ (IFN-γ), a dimerized soluble cytokine that is the only member of the type II class of interferons, to stimulate innate and T-cell immune responses. Besides being a Th1 signature cytokine, IFN-γ has been suggested, although debatably, to be important for the differentiation of Th1 cells. The ultimate outcomes of IFN-γ and IL-12p70 signalling are to solidify their Th1 function through promoting the expression of Th1-specific transcription factors. T-bet, belonging to the T-box family of transcription factors and recognized as a master regulator of Th1 differentiation, is rapidly and specifically induced in developing Th1 cells and is critical for initiating Th1 development (Szabo et al., 2000).

1.2.2 Th2 response

T helper type 2 cells were identified at the same time as Th1 cells in the early 1990s. The Th2 response is often associated with the humoral response and is important in resistance against extracellular forms of pathogens. Th2 cells are also important for mucosal immunity in the lung. Aberrant elevation of the Th2 response often leads to chronic inflammatory airway diseases, such as atopic asthma and allergy. In presence of IL-4, DC drive T lymphocytes polarization towards a Th2 differentiation (Swain et al., 1990). Furthermore, DC secretion of IL-10 can act in autocrine and paracrine routes and

has been associated with Th2 T cell phenotypes. The strength of T cell signalling and the cytokine milieu are two crucial determinants for Th2 cell differentiation. Freshly isolated DC expressing low levels of MHC-II and CD80 molecules preferentially induce Th2 cell differentiation. Omega-1, a T2 ribonuclease glycoprotein derived from *S. Mansoni* eggs (SEA), has recently been reported to be a potent Th2 cell-inducing factor. Indeed, DC treated with omega-1 do not produce IL-12p70 and display a resting phenotype (Paul & Zhu, 2010).

Th2 lymphocytes are defined as producers of IL-4, IL-5, IL-9, IL-10 and IL-13. GATA binding protein 3 (GATA-3) is a member of the GATA family of transcription factors. Expression of GATA-3 is sufficient and required for Th2 differentiation (Zhu et al., 2004). Therefore, GATA-3 is regarded as the master regulator for Th2 differentiation. Signal transducer and activator of transcription 6 (STAT-6) activated by IL-4 stimulation is the major signal transducer in IL-4-mediated Th2 differentiation in *vitro*. One of the mechanisms for STAT-6 to promote Th2 differentiation is through inducing high levels of the transcription factor GATA-3 (Kurata et al., 1999).

1.2.3 Th17 response

T helper type 17 is a newly identified class of effector T cells that produce IL-17A and IL-17F. They are found at the interfaces between the external and the internal environment and protect against extracellular bacteria. Th17 cells have been implicated in the induction and propagation of autoimmunity. Indeed, IL-17 expression has been associated with autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, psoriasis and inflammatory bowel disease as well as allergic responses (Yen et al., 2006).

Retinoic acid-related orphan receptors (ROR) are the key transcription factors in Th17 differentiation. Both ROR-α and ROR-γt are critical and somewhat redundant in promoting Th17 differentiation (Ivanov et al., 2006).

Understanding the mechanisms driving the differentiation of human Th 17 cells is of relevance to both immunopathology and vaccination. In mice, DC production of transforming growth factor-β (TGF-β) and IL-6, an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine, co-operate to promote Th17 commitment (Mangan et al., 2006). The differentiation of human naive $CD4^+$ T cells into Th17 cells is promoted by IL-1β and IL-6. In particular, IL-1β is sufficient to induce the expression of ROR-γt and production of both IL-17 and IFN-γ. IL-6, when added to IL-1β, sustains the

expression of ROR-γt and promotes the differentiation of T cells producing IL-17 but not IFN-γ. The addition of TGF-β, which in the mouse has been identified as a cytokine essential for the development of Th17 cells, does not induce and actually suppresses the Th17 differentiation of human $CD4^+$ T cells (Veldhoen et al., 2006; Acosta-Rodriguez et al., 2007). IL-23, a heterodimeric cytokine consisting of two subunits, p40, which is shared with IL-12p70, and p19, seems to modulate the differentiation of human Th17 (Leibundgut-Landmann et al., 2007). Indeed, the differentiation of Th17 cells was inhibited by large doses of IL-2 and was enhanced by IL-23. However, it was subsequently shown that IL-23 is required for IL-17-mediated effector function and the survival but not differentiation of Th17 cells (Veldhoen et al., 2006; Acosta-Rodriguez et al., 2007).

1.2.4 Regulatory T response

Regulatory T cells (Tregs) form a subset of $CD4^+$ T cells that either develop in the thymus (naturally occurring Tregs) or are differentiated from naive T cells following T-cell receptor stimulation (induced Tregs). Unlike other Th cells, which promote an immune response, Tregs are immunosuppressive. Indeed, the most prominent function of Tregs is maintaining self-tolerance and immune homeostasis. Disruption of Tregs function contributes to an excess of autoimmune and inflammatory pathologies. It is also noted that Tregs are important for tempering immune responses against infectious agents and in re-establishing immune homeostasis following pathogen clearance (Belkaid et al., 2008).

The most widely used markers for Tregs are CD25 (also known as IL-2 receptor), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), glucorticoid-induced tumour necrosis factor receptor family-related gene (GITR), CD27 and lymphocyte activation gene-3 (LAG-3). Moreover, Tregs classically express Foxp3, a transcription factor recognized as the master regulator for Tregs function controlling the expression of a wide array of genes including cytokines and surface molecules (Zeng et al., 2007).

1.3 Basic mechanisms of Tregs function

Defining the mechanisms of Tregs function is clearly of crucial importance. Not only would this provide insight into the control processes of peripheral tolerance but it would probably also indicate several potentially important therapeutic targets. Although this quest has been ongoing since interest in Tregs was reignited in 1995 (Sakaguchi et al., 1995),

there has been significant progress in the past few years. From a functional perspective, the various potential suppression mechanisms used by Tregs can be grouped into four basic "modes of action": suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption and suppression by modulation of DC maturation or function (Fig. 4) (Vignali et al., 2008).

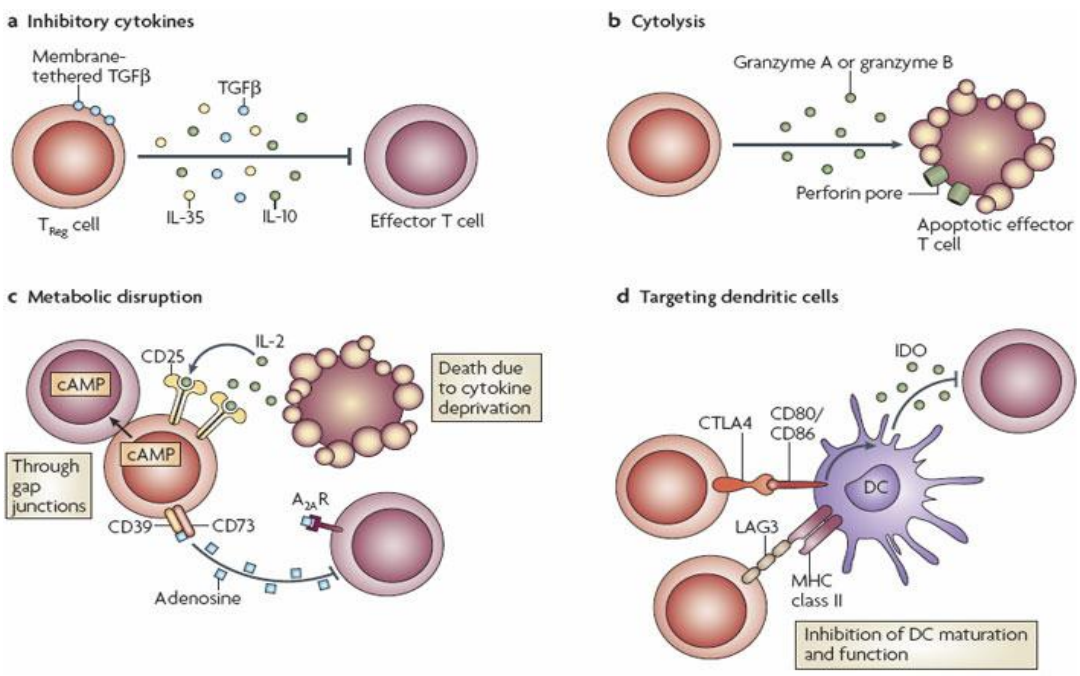

Fig.4: Basic mechanisms of suppression used by Treg cells. A) Inhibitory cytokines include IL-10, IL-35 and TGFβ. B) Cytolysis includes granzymeA- and granzymeB-dependent and perforin-dependent killing mechanisms. C) Metabolic disruption includes high-affinity CD25-dependent cytokine deprivation-mediated apoptosis. D) Targeting dendritic cells (DC) includes mechanisms that modulate DC maturation and/or function (Vignali et al., 2008).

1.3.1 Suppression by inhibitory cytokines

Inhibitory cytokines, such as interleukin-10 (IL-10) and TGF-β, have been the focus of considerable attention as mediators of Tregs-induced suppression (Fig. 4A). There has also been significant interest in their ability to stimulate the development of induced Tregs-cell populations, either *in vivo* or experimentally as a potential therapeutic modality. Although the general importance of IL-10 and TGF-β as suppressive mediators is

undisputed, their contribution to the function of thymus-derived, naturally occurring Tregs cells is still a matter of debate. This is partly due to the general perception that Tregs cells function in a contact-dependent manner rather than through soluble factors (Jonuleit et al., 2001). Indeed, *in vitro* studies using neutralizing antibodies or T cells that are unable to produce or respond to IL-10 and TGF-β suggested that these cytokines may not be essential for Tregs-cell function (Dieckmann et al., 2001). However, this is in contrast with data from *in vivo* studies, showing the potential of allergen specific IL-10-secreting regulatory T cells to provide local allergen-triggered inhibitory mechanisms that allow the safe and long-lasting control of allergic disease (Hawrylowicz & O'Garra., 2005).

Recently in mouse models, a new inhibitory cytokine, IL-35, has been described that is preferentially expressed by Tregs cells and is required for their maximal suppressive activity. IL-35 is a new member of the IL-12 heterodimeric cytokine family and is formed by the pairing of Epstein-Barr virus-induced gene 3 (Ebi3; which normally pairs with p28 to form IL-27), and p35 (also known as *Il12a*; which normally pairs with p40 to form IL-12p70). Both Ebi3 and Il12a are preferentially expressed by mouse $Foxp3^+$ Treg cells but not by resting or activated effector T cells, and are significantly up regulated in Tregs cells that are actively suppressing (Collison et al., 2007).

1.3.2 Suppression by cytolysis

Another potential mechanism for Tregs-mediated suppression of responder T cells would be cytolysis of target cells. Human $CD4^+CD25^+Foxp3^+$ Tregs can be activated experimentally by a combination of antibodies to CD3 and CD46 to express granzyme A and kill activated $CD4^+$ and $CD8^+$ T cells and other cell types in a perforin-dependent, Fas-FasL-indipendent manner (Grossman et al., 2004) (Fig.4B). Noelle and co-workers were the first to report that Tregs from granzyme B-deficient mouse had reduced suppressive activity *in vitro*, and that this granzyme-B-dependent suppression appeared to be a perforin-independent result of Tregs-cell-induced apoptosis of effector T cells (Gondek et al., 2005). The notion that Tregs cells might possess cytolytic activity was supported by studies showing that Tregs cells can kill B cells in a granzyme B dependent and partially perforin-dependent manner that results in the suppression of B cell function (Zhao et al., 2006).

1.3.3 Suppression by metabolic disruption

A long-standing debate in the Tregs-cell field is whether the high expression level of CD25 empowers Tregs cells to "consume" local IL-2 and therefore starve actively dividing effector T cells by depleting the IL-2 they need to survive (de la Rosa et al., 2004). A recent study has reignited interest in this question by suggesting that Tregs cells induce apoptosis mediated through cytokines deprivation (specifically IL-2) (Pandiyan et al., 2007). However, given that a recent report using human Tregs cells suggested that IL-2 depletion alone is not required for Tregs cells to suppress effector T cells (Oberle et al., 2007), more work is clearly necessary to resolve this aspect. Two new Tregs-cell mechanisms have recently been proposed that induce the intracellular or extracellular release of adenosine nucleosides (Fig.4C). Concordant expression of the ectoenzymes CD39 and CD73 was shown to generate pericellular adenosine, which suppressed effector T cell function through activation of the adenosine receptor 2A (A2AR) (Deaglio et al., 2007). Interestingly, binding of adenosine to A2AR appears to not only inhibit effector T cell functions, but also to enhance the generation of induced Tregs cells by inhibiting IL-6 expression while promoting TGF-β secretion (Zarek et al., 2008).

Tregs cells were also shown to suppress effector T cell function directly by transferring the potent inhibitory second messenger cyclic AMP (cAMP) into effector T cells through membrane gap junctions (Bopp et al., 2007). Although these mechanisms represent interesting additions to the list of potential mechanisms used by Tregs cells to mediate suppression, further studies will be required to corroborate these exciting findings and assess the relative use of these mechanisms by Tregs cells.

1.3.4 Suppression by targeting dendritic cells

Another mechanism for Tregs to affect effector T cell activation can be established by modulating DC function. Ligation of CD80/CD86 on DC by CTLA-4 on suppressor cells results in expression and activation of indoleamine 2,3-dioxygenase (IDO), a catabolic enzyme involved in tryptophan degradation. Reduced tryptophan concentration in culture medium has been reported to be associated with decreased activation of T cells and T cell deletion (Mellor & Munn, 2004). Also, in several *in vivo* models for disease disorders, it was demonstrated that CTLA-4 blockade abrogates the suppressive function of murine (Read et al., 2000) and human Tregs (Blansfield et al., 2005). Recent studies

have also suggested that LAG-3 (also known as CD223) may block DC maturation. LAG-3 is a CD4 homologue that binds MHC class II molecules with very high affinity, has a negative regulatory T⁻cell intrinsic function and is required for maximal Tregs-cell suppression (Workman & Vignali, 2004).

1.4 Non classical regulatory T cells

Although naturally occurring Tregs cells originate in the thymus and are controlled by the activity of the transcription factor Foxp3, $CD4^+$ T cells with regulatory activities can also be generated from conventional naive T cells after antigen encounter in the periphery. Antigen induced, IL-10-producing $CD4^+$ T cells arising from $CD4^+CD25^-Foxp3^-$ cells have been found to regulate colitis induced by *Helicobacter hepaticus* infections and to suppress protective Th1 responses to *Bordetella pertussis* in mice (Kullberg et al., 2002; McGuirk et al., 2002).

While the role of transient Foxp3 expression in T effector cells is poorly understood, it is becoming clear that it does not correlate with suppressor function (Roncarolo & Gregori, 2008). Furthermore, recent evidence suggests that Foxp3-independent mechanisms, mediated by IL-10, contribute to the induction and suppressor functions of Tr1 cells (Roncarolo & Gregori, 2008; Conrad et al, 2012).

Much less attention has been given to the potential contribution of regulatory sublineages of $CD8^+$ cells. $CD8^+$ Tregs were first observed by Gershon and colleagues in the early 1970's. The ability to characterize T cell subsets using the CD8 surface molecule suggested that suppressive activity by a subpopulation of $CD8^+$ cells specifically inhibited T helper responses by $CD8^-CD5^+$ T cells. Suppressive activity of $CD8^+$ T cells in autoimmune disease was first demonstrated in experimental autoimmune encephalomyelitis, a murine model of multiple sclerosis. The regulatory role of $CD8^+$ T cells was also observed in other autoimmune disease models, including autoimmune Herpes Stromal Keratitis and myocarditis (Lu and Cantor, 2008).

Recently, a novel subset of $CD4^-CD8^-$ (double negative) T cells has been described to specifically supress T cell response in human, by cell contact-dependent mechanisms (Voelkl et al, 2011)

1.5 Human monocytes-derived dendritic cells

The ability of monocytes to differentiate into DC was originally demonstrated by Sallusto and Lanzavecchia, who reported the generation of DC from human peripheral monocytes (also called monocyte-derived dendritic cells, MDDC) after *in vitro* culture with GM-CSF and IL-4 (Sallusto & Lanzavecchia, 1994). In both murine and human systems, monocytes differentiate into immature DC, characterized by their low expression of MHC class II and co-stimulatory molecules. DC differentiation from mouse bone marrow monocytes has been reported to occur after 24-48 h (Leon et al., 2004). Under equivalent experimental conditions, human blood monocytes appear to take longer (5-6 days) to generate DC, as described by different research groups,

Generally, peripheral blood mononuclear cells (PBMCs) are collected by aphaeresis, and monocytes obtained by either elutriation, CD14 antibody selection, or selection of adherent cells after overnight culture on plates. Protocols to generate immature DC from circulating monocytes most frequently use granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), although the concentrations of these factors and time in culture varied among studies.

Several strategies have been used to produce mature DC, which are characterized by high immune cell activation potential. Factors used to mature immature DC included lipopolysaccharide (LPS), CD40 ligand (CD40L), tumour necrosis factor-α (TNF-α), IFN-α, and IFN-γ. Cocktails combining several factors to better recreate the inflammatory environment have also been used. Factors used in maturation cocktails include prostaglandin E2, IL-1β, IL-6, and polyinosinic:polycytidylic acid (poly I:C) (Castiello et al., 2011).

Over the last 10 years, this method has prompted numerous studies on human DC that were previously hampered by the difficulties in working with *ex vivo*-isolated human DC. It has proven to be an extremely powerful tool for the study of human DC differentiation and maturation processes, especially to understand how DC regulate crucial aspects of host response to microbial infections. Moreover, *in vitro* DC differentiation from monocytes constitutes the current methodological basis for obtaining DC for their use in DC-mediated cancer immunotherapeutic treatments (Banchereau & Palucka, 2005).

1.6 *Bordetella pertussis*

Bordetella pertussis is a Gram-negative bacterial pathogen that infects the human respiratory tract and causes the disease called pertussis or whooping cough. While nine species of *Bordetella* have been identified to date, only three additional members, *B. bronchiseptica*, *B. parapertussis*, and *B. holmesii*, have been associated with respiratory infections in humans and other mammals (Mattoo et al., 2001). *B. bronchiseptica* infects a wide range of hosts and occasionally causes cough illnesses in humans; in particular, severe infections have been noted in persons who are immunocompromised such as patients with AIDS. Human-adapted *B. parapertussis* causes a milder pertussis-like disease and, like *B. pertussis*, lacks an environmental reservoir (Cherry & Heininger, 2004). *B. holmesii*, the most recent of the *Bordetella* species associated with human respiratory tract infection, has been found in the blood of young adults and occasionally in the sputum. Little is known about the biology, virulence mechanisms, and pathogenic significance of *B. holmesii* (Mattoo & Cherry, 2005).

Although broad immunization campaigns have significantly reduced pertussis-caused child mortality, infections with *B. pertussis* still pose a significant health burden. Even in highly vaccinated populations, annual infection rates can reach 1-7% in the general population (Ward et al., 2006). Thus, pertussis remains one of the least well-controlled vaccine-preventable diseases in the world, with as many as 300.000 deaths each year, worldwide. The majority of these deaths occur in non- or insufficiently vaccinated children (WHO, 2006). Interestingly, pertussis has not only persisted in vaccinated populations, but has even resurged in recent years and, although it is traditionally considered a childhood disease, the observed increase in pertussis incidence is seen mainly in adolescents and adults (Mooi, 2010). This shift of pertussis infections towards older age categories raises major health concerns, as these individuals provide a reservoir of *B. pertussis* for transmission to infants, who are at the highest risk of developing severe pertussis (de Greeff et al., 2010).

1.7 Regulation of *Bordetella pertussis* virulence

Infection of the host by *B. pertussis* is initiated through contact with respiratory secretions from an infected individual. Following the inhalation of these particles, bacteria

enter the upper respiratory tract and adhere to ciliated epithelial cells in the nasopharynx and trachea. Once attached to the mucosal surface, *B. pertussis* produces a large array of virulence factors, including adhesins and toxins both exerting also immunomodulatory effects. The concerted expression of these factors prevents rapid clearance of the bacteria and enables replication and dissemination to the lower areas of the respiratory tract, causing pneumonia.

Since the recognition of *B. pertussis* as the causative agent of whooping cough in humans (Bordet & Gengou, 1906), research has focused on understanding its pathogenic lifestyle. In particular, the identification of bacterial factors that contribute to the development of disease has been comprehensively investigated.

One of the initial, intriguing observations was that *B. pertussis* displayed variation in the expression of surface antigens (designated antigenic modulation at that time) in response to environmental signals (Lacey, 1960). It is now known that these phenomena can be explained by the existence of a master regulator that controls transcription of nearly all known virulence genes: the *Bordetella* master virulence regulatory system (bvgASR) locus.

1.7.1 The bvgASR virulence regulon

The transcription of many of the *B. pertussis* genes known to be involved in virulence is controlled by the activity of BvgS and BvgA (Stibitz et al., 2007). These two proteins form a typical two-component system (TCS) that allows dynamic variation of gene expression in response to changes in extracellular signals from the surrounding environment. BvgA is a 23-kDa DNA-binding response regulator and BvgS is a 135-kDa transmembrane sensor kinase that contains a periplasmic domain, a linker region, a transmitter, a receiver, and a histidine phosphotransfer domain. The BvgAS system is responsive to several known environmental signals that can modulate its expression, at least under laboratory conditions. For instance, growing *B. pertussis* at 37 °C induces the expression of BvgAS, while the presence of millimolar amounts of sulphate or nicotinic acid, or growth at or below 25 °C, suppresses BvgAS production (de Gouw et al., 2011). *B. pertussis* strains cultured under so-called 'nonmodulating', virulent conditions are referred to as Bvg^+ phase bacteria.

During the virulent Bvg^+ phase, the periplasmic domain of BvgS relays environmental signals through the membrane to the transmitter domain, which then

autophosphorylates. Phosphorylated BvgS subsequently transfers its phosphate group to BvgA (BvgA-P), which then becomes activated and binds to specific cis-acting promoter sequences, thus inducing the transcription of Bvg^+ phase-specific genes, commonly designated *vags* (for 'vir-activated genes'). Simultaneously, the transcription of *vrgs* ('vir-repressed genes') is repressed by a third protein that is expressed from the bvgASR locus, the 32-kDa cytoplasmic repressor protein BvgR. Based on *in vitro* growth experiments, *vags* have been classified into three different temporal classes: early, intermediate and late genes (Cummings et al., 2006). Early genes, encoding the adhesins filamentous hemagglutinin (FHA) and fimbriae (Fim) respond rapidly to activation and require a lower concentration of BvgA-P for induction. Late genes, which include those encoding adenylate cyclase toxin (ACT) and pertussis toxin (PT), show relatively slow induction kinetics and require higher concentrations of BvgA-P for expression. The gene encoding pertactin (PRN) has been suggested to belong to the class of intermediate genes (de Gouw et al., 2011).

1.7.2 Adhesion factors

The major *B. pertussis* adhesin is filamentous hemagglutinin (FHA), a 220 kDa protein expressing at least three different binding activities, including carbohydrate binding, heparan sulphate binding and integrin binding via an RGD site, which comprises the amino acid sequence Arg-Gly-Asp. These activities allow *B. pertussis* to bind to a variety of cells and extracellular structures in the respiratory epithelium, including epithelial cells and macrophages. FHA is initially produced as a large 367 kDa precursor, which undergoes both amino-terminal and carboxy-terminal maturation. The biosynthesis of FHA depends on an outer-membrane-associated accessory protein named FhaC. This protein is able to form channels through which FHA is believed to cross the outer membrane, most likely in an extended conformation (Jacob-Dubuisson et al., 1999) (Fig. 5).

In addition to FHA, *B. pertussis* produces fimbriae that are composed of the major subunits Fim2 or Fim3, depending on the *B. pertussis* serotype (2 or 3) and of the minor subunit FimD located at the tip. FimD binds to the VLA-5 integrin of macrophages and to sulphated sugars which are ubiquitously present in the respiratory tract. Binding of FimD to VLA-5 activates CR3, the receptor for FHA, which thereby assures cooperativity between fimbrial and FHA binding. This tight coordination is also reflected in the

biogenesis of these two structures. Fimbrial biogenesis depends on at least two accessory proteins encoded by genes that are located within the same operon as *fhaC*. These accessory proteins include a periplasmic chaperone, named FimB, and an outer membrane usher protein, named FimC, as a minimal requirement for biogenesis (Fig. 5).

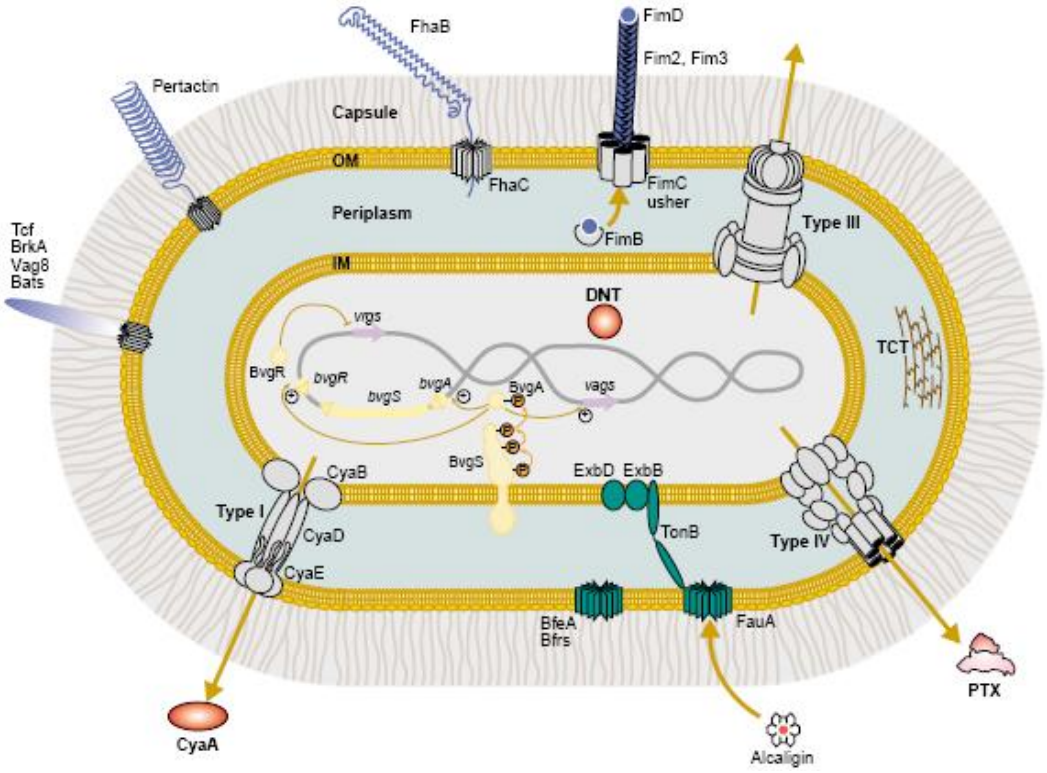

Fig.5: The *B. pertussis* virulence factors: *B. pertussis* is depicted as a Gram-negative organism with inner and outer membranes, periplasm and a capsule. The adhesins Fim, FhaB, PRN, Tcf, BrkA, Vag8 and Bats are shown in blue; the toxins, PT, ACT (CyaA), TCT and DNT are in red; the accessory proteins FhaC, FimB, FimC, Type III, Type IV and Type I are in grey (Locht et al., 2001).

In addition to *fimB* and *fimC*, the *fhaC* operon also contains *fimD*. Studies using epithelial cell lines derived from the human respiratory tract have indicated that the fimbriae play a role in infection of the laryngeal mucosa, whereas FHA is important for colonization of the entire respiratory tract (Van den Berg et al., 1999).

Pertactin (PRN) is the first member of the autotrasporter family to be identified and characterized in *Bordetella*. Mature PRN is a 68-kDa protein in *B. bronchiseptica*, a 69-

kDa protein in *B. pertussis*, and a 70-kDa protein in *B. parapertussis* (Fig. 5). It has been proposed to play a role in attachment since all three PRN proteins contain an RGD tripeptide motif as well as several proline-rich regions and leucine-rich repeats, motifs commonly present in molecules that form protein-protein interactions involved in eukaryotic cell binding (Henderson & Nataro, 2001).

1.8 *Bordetella pertussis* toxins

In addition to adhesins, *B. pertussis* also produces a number of toxins (Fig. 5). Most of them are proteins, except for lipooligosacharide and the tracheal cytotoxin (TCT), which is a fragment of the bacterial peptidoglycan. These factors jointly facilitate the adhesion of *B. pertussis* to the human respiratory tract.

1.8.1 Pertussis toxin (PT)

Among the protein toxins, pertussis toxin (PT) is the most complex one. It is composed of five different subunits, named S1 through S5 according to their decreasing molecular weights, and arranged in an A-B structure. The B moiety, responsible for binding of the toxin to the target cells is composed of subunits S2 to S5, and the A moiety, an ADP-ribosyltransferase, corresponds to S1. After binding to the target cell receptors, the toxin most likely follows the retrograde transport to the endoplasmic reticulum, where S1 probably translocates into the cytosol. In the cytosol, S1 expresses its ADP-ribosyltransferase activity using NAD as the ADP-ribose donor and trimeric signal-transducing G-proteins as acceptors, disrupting signalling pathways with a wide range of downstream effects (Locht et al., 2001).

PT has long been known to cause systemic symptoms associated with pertussis disease, such as lymphocytosis, insulinemia/hypoglycemia and histamine sensitivity, but it was unclear from previous studies whether PT contributed to local events of respiratory infection and disease. However, recent studies using the mouse intranasal infection model indicate that PT is important in the very early stages after inoculation of bacteria. PT-deficient mutant strains showed reduced levels of airway infection 24 h post inoculation and, whereas co-administration of purified PT enhanced infection by the mutant strain, PT administration 24 h after inoculation had no enhancing effect. One possible mechanism of action for PT is the delay of neutrophil recruitment and influx to the airways, and this

response occurs earlier after infection with a PT-deficient strain than with a wild type strain (Carbonetti et al., 2003).

Recently, it has found that depletion of airway macrophages by intranasal administration of clodronate liposomes (Van Rooijen & Sanders, 1994) not only enhances infection by wild-type *B. pertussis*, but also by the PT-deficient strain (up to the level of infection seen with wild-type) (Carbonetti et al., 2007), suggesting that resident airway macrophages may be the primary target cells for PT in its ability to promote infection. Interestingly, ADP ribosylation of airway macrophage G proteins after intranasal treatment of mice with PT lasted longer than two weeks (correlating with the longevity of its infection-promoting activity) (Carbonetti et al., 2003), suggesting that the effects of PT on host cells in the airways may be particularly long-lived.

Furthermore, PT exerts multiple suppressive effects on the immune system beyond those observed on innate immune cells; for example, other studies have shown PT-mediated suppression of serum antibody responses to *B. pertussis* antigens after infection (Carbonetti et al., 2004; Kirimanjeswara et al., 2005), and reduction of MHC-II molecules on the surface of human monocytes (Shumilla et al., 2004). In our laboratory, it was demonstrated that DC cultured in presence of PT generate high levels of IL-12p70, promoting T helper type 1 responses (Ausiello et al, 2002). Furthermore, it was shown that PT acts through TLR4/TLR2 engagement and through a crucial role played by MAPK and IL-10 favours the expansion of a mixed Th1/Th17 immunity (Nasso et al., 2009).

1.8.2 Adenylate cyclase (AC) toxin

Another secreted toxin with a key role in *B. pertussis* pathogenesis is adenylate cyclase toxin (ACT), a 200-kDa polypeptide that is secreted by a type I secretion system. It contains two major domains: a catalytic domain that includes the active site as well as a calmodulin binding site and a haemolytic binding domain that is composed of a hydrophobic channel domain and calcium binding RTX repeats (Vojtova et al., 2006). This domain also mediates binding and internalization of the toxin into target cells. ACT does not involve a cleavable signal peptide and a periplasmic intermediate, but requires the accessory proteins CyaB, CyaD and CyaE. The toxin is synthesized as an inactive precursor and then converted to its active form by CyaC-mediated palmitoylation of Lys983. This palmitoylation is required for binding to the target cells and for the formation of a pore through which the catalytic subunit can penetrate into the cell. Additionally, post-

translational acylation is important for the apoptotic and cytotoxic effect of ACT (Boyd et al., 2005).

The majority of ACT remains associated with the bacterium in an inactive form; however a small amount of active toxin is secreted into the environment (Gray et al., 2004). Although an ACT deletion mutant was also attenuated in colonization, the phenotype was significantly different from the PT mutant. The ACT mutant was able to colonize as efficiently as the wild-type parental strain during the early phase of infection, but was unable to persist beyond the first 4 days post infection. In contrast to PT, providing soluble ACT together with the deletion mutant did not have any significant effect (Carbonetti et al., 2005). However, because ACT is an integral membrane protein that is functional only upon direct contact with its target cell (Basler et al., 2007), it cannot be excluded that the protein production and purification procedure may have rendered ACT inactive. Thus, while PT already contributes during the initial phases of infection, the effects of ACT do not become apparent until later during infection. This suggests that these two toxins serve complementary functions in the pathogenesis of infection, by contributing firstly to the initial establishment of infection and secondly to persistence in the respiratory tract.

In our laboratory it was demonstrated that, ACT expressed by *B. pertussis* strongly interferes with MDDC functions, by reducing the expression of phenotypic markers and immunomodulatory cytokines, and blocking IL-12p70 production. *B. pertussis*-treated MDDC promoted a mixed Th1/Th17 polarization, and the activity of ACT altered the Th1/Th17 balance, enhancing Th17 and limiting Th1 expansion (Spensieri et al., 2006). We also demonstrated that Th1 effectors are induced by *B. pertussis*-MDDC in the absence of IL-12p70 through an ERK1/2 dependent mechanism, and that p38MAPK is essential for MDDC-driven Th17 expansion (Fedele et al., 2010).

1.8.3 Dermonecrotic toxin

A third protein toxin produced by *B. pertussis* is the dermonecrotic toxin (DNT). Unlike the other toxins, DNT is not secreted by the organism, and its role in the pathogenesis of pertussis is not clear. In its purified form, however, it is highly lethal when injected intravenously into mice (Locht et al., 2001). *Bordetella* DNT is a typical A-B toxin, composed of a 54-aminoacid N-terminal receptor-binding domain and a 300-amino-acid C-terminal enzymatic domain. While the receptor for DNT has not yet been identified,

in vitro assays using fibroblast and osteoblast-like cell lines determined that on receptor binding, DNT is internalized via a dynamin-dependent endocytosis. Translocation is independent of acidification of endosomes and retrograde vesicular transport and requires the N-terminal region of the DNT enzymatic domain, which includes a putative transmembrane domain. On endocytosis, DNT undergoes proteolytic nicking by mammalian proteases such as furin, which is necessary for the cellular activity of DNT (Matsuzawa et al., 2004).

1.8.4 Tracheal cytotoxin

Tracheal cytotoxin (TCT) corresponds to a disaccharide-tetrapeptide monomer of peptidoglycan that is produced by all Gram-negative bacteria as they break down and rebuild their cell wall during growth. Its structure is *N*-acetylglucosaminyl-1,6-anhydro-N-acetylmuramyl-(L)-alanyl-(D)-glutamyl-esodiaminopimelyl-(D)-alanine. While the vast majority of bacteria recycle this peptidoglycan fragment by transporting it back into the cytoplasm via an integral cytoplasmic membrane protein called AmpG, *Bordetella* releases it into the environment due to the lack of a functional AmpG (Parkhill et al., 2003). As such, TCT is constitutively expressed and is independent of BvgAS control.

It is hypothesized that, *in vivo*, TCT stimulates IL-1α production in nonciliated mucus-secreting cells, which positively controls the expression of inducible nitric oxide synthase, leading to high levels of nitric oxide (NO) production. NO then diffuses to neighboring ciliated cells, which are much more susceptible to its damaging effects; TCT also functions synergistically with *Bordetella* endotoxin to induce the production of NO within the airway epithelium (Flak & Goldman., 1999).

Recent studies have shown that TCT stimulates responses in immune cells through microbial pattern-recognition molecules that interact with peptidoglycan and its derivatives. In mice, these responses were dependent upon the intracellular receptor Nod1; however, human Nod1 detected TCT poorly (Magalhaes et al., 2005). Instead, TCT binds human and *Drosophila* peptidoglycan recognition proteins, which (at least in *Drosophila* cells) potently activates an immune response pathway (Chang et al., 2006; Kaneko et al., 2006). Therefore, TCT might also contribute to overall immunomodulation during *B. pertussis* infection.

1.9 Modulation of the host immune response by *Bordetella pertussis*

Infection with *B. pertussis* leads to the induction of an innate response and the subsequent development of specific immunity. The respiratory epithelium, together with resident antigen-presenting cells (APCs) such as alveolar macrophages (AMs) and DC are the primary host innate immune cells that sense and shape initial local immune responses towards *B. pertussis*. As the infection progresses, *B. pertussis* is challenged by an additional host defence mechanism: the adaptive immune system where the main effectors are the T and B lymphocytes, which eventually mediate clearance through the development of protective immunity against the pathogen (Mills, 2001). However, *B. pertussis* has evolved a number of strategies to prevent or modulate specific aspects of this response, thus delaying clearance and improving survival chances and transmission to the next host (Fig. 6).

In contrast to most Gram-negative bacteria, *B. pertussis* produces a lipooligosaccharide (LOS) that has a branched core structure with a nonrepetitive trisaccharide, rather than a long repeating O-side chain (Caroff et al., 2000). The lipid A moiety of the *B. pertussis* LOS activates TLR4 signalling pathways in MDDC, albeit significantly less efficiently than the typical lipid A domain present on the lipopolysaccharide structure of enteric Gram-negative pathogens (Fedele et al., 2007; Fedele et al., 2008). The lipid A and the oligosaccharide core domain of lipopolysaccharide, can also be recognized by the surfactant proteins A and D (SP-A and SP-D, respectively), hydrophilic lipid-binding lectins that are ubiquitously expressed in the lower respiratory tract of humans (Chaby et al., 2005).

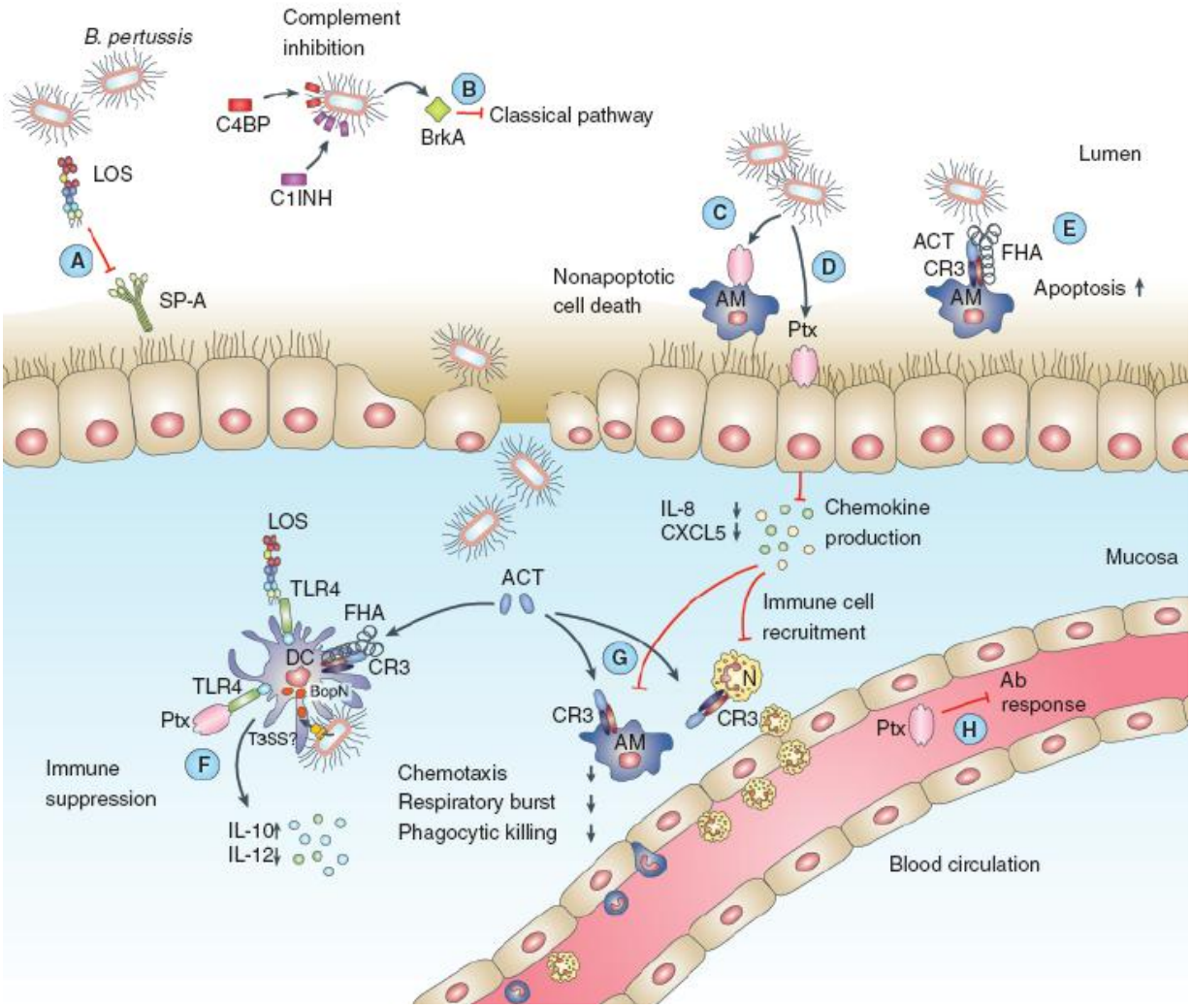

Fig.6: *Bordetella pertussis* virulence factors modulating the host immune response. The most relevant aspects of host immune manipulation in the respiratory tract are schematically illustrated. Detailed information are described in the text (de Gouw et al., 2010).

Binding of SP-A to lipopolysaccharide induces agglutination, destabilizes the bacterial membrane, and facilitates phagocytosis (McCormack & Whitsett, 2002). Interestingly, the terminal trisaccharide of *B. pertussis* LOS prevents access of SP-A and SP-D to the lipid A domain through steric hindrance, and thereby protects the bacteria from surfactant-mediated clearance (Schaeffer et al., 2004a) (Fig.6 A).

Furthermore, *B. pertussis* inhibits complement-induced phagocytosis through *Bordetella* resistance to killing A (BrkA), a virulence factor that interferes specifically with the classical pathway of complement activation (Fernandez & Weiss, 1994). More

recently, two BrkA-independent mechanisms of complement resistance have been identified. *Bordetella pertussis* has been found to bind and recruit the C4b-binding protein (C4BP) as well as the human C1 esterase inhibitor (C1INH), both major inhibitors of the classical complement pathway (Berggard et al., 1997; Marr et al., 2007) (Fig.6 B).

Colonization of the mucosal surfaces of the respiratory tract by *B. pertussis* initiates a local chemokine response, resulting in the recruitment of immune cells to the site of infection. Usually, neutrophils are the first immune cells to arrive, followed by a second wave of natural killer (NK) cells, macrophages, DC, and lymphocytes.

PT has been shown to affect chemotaxis indirectly by suppressing the release of chemokines from resident airway cells. It was demonstrated that inactivation of G proteins by PT strongly impairs lymphocyte migration *in vitro* and causes defective homing to spleen, lymph nodes, and Peyer's patches *in vivo* (Wettschureck & Offermanns, 2011). Indeed, using a mouse model of infection, it was shown that a mutant *B. pertussis* strain lacking PT induced significantly higher levels of keratinocyte-derived chemokine and macrophage inflammatory protein 2, i.e. the murine functional equivalents of human IL-8, as compared with the wild-type strain. Further, lipopolysaccharide-induced CXC chemokine, the murine homolog to human CXCL5/ENA-78, was also inhibited by PT (Andreasen & Carbonetti, 2008) (Fig.6 D). Furthermore PT is able to intoxicate alveolar macrophages (AMs) by ADP ribosylation of its G-proteins (Fig.6 C) and also it is able to enter the circulation and suppress antibody responses to *B. pertussis* antigens (Fig.6 H).

Phagocytic uptake of bacteria is generally followed by fusion of the phagosome with the lysosome, generating a new compartment called the phagolysosome. Bacteria present in this compartment are then exposed to an acidified environment as well as a number of antibacterial molecules, including ROS and proteolytic enzymes that break down the bacterium. Besides survival through entering the endosomal compartment, *B. pertussis* uses several other strategies to suppress phagocytic activity. *B. pertussis* also evades phagocytosis by suppressing the bactericidal activity of phagocytes (Weingart et al., 2000). ACT rapidly induces cellular cAMP levels in $CR3^{+}$ phagocytes, including PMNs (Guermonprez et al., 2001). The rapid elevation of cAMP signals causes a transient and selective inactivation of RhoA, a member of the Rho family GTPases, which are key regulators of actin cytoskeletal dynamics. This inactivation of RhoA causes massive actin cytoskeletal rearrangements that coincide with phagocytic ruffling and the loss of macropinocytic fluid-phase uptake (Kamanova et al., 2008).

To summarize, ACT is able to subvert cAMP signalling in phagocytes and thereby affects their bactericidal activity by inhibiting chemotaxis, the production of superoxides (and hence the respiratory burst), and killing (Fig. 6 G). Furthermore, ACT induces the activity of the effector caspases 3 and 7 in macrophages, which are the key factors triggering apoptosis (Cheung et al., 2009) (Fig.6 E).

Another *B. pertussis* virulence factor that affects apoptosis is FHA. It has been shown that purified FHA induced dose-dependent apoptosis in human phagocytic and epithelial cells (Abramson et al. 2001) (Fig.6 E).

Cellular immunity is suppressed by the concomitant action on dendritic cells of LOS and PT on TLR4 and TLR2, FHA, and ACT on CR3, and the type III secretion system (T3SS) secreted protein BopN, which mediate the suppression of IL-12p70 and the induction of IL-10 (Fig. 6 F).

1.10 Development of improved vaccines against whooping cough

Before childhood vaccination pertussis was a major cause of infant death throughout the world. The high rate of illness and death caused by bacterium stimulated the early development of whole-cell pertussis vaccines (Pw). All Pw vaccines contain whole *B. pertussis* bacteria that have been killed and detoxified by various methods, like treatment with glutaraldehyde or formaldehyde. These vaccines were introduced in many countries in the 1950s and 1960s and although are effective at preventing whooping cough in infants, they can induce local and systemic reactions in a high proportion of immunized infants. More significantly, convulsions and encephalopathy have been reported to be temporally associated with Pw administration (Cherry et al., 1988; Edwards et al., 1999).

The desire to avoid the side effects of whole-cell vaccines has stimulated the development of less reactogenic acellular pertussis vaccines (Pa) (Halperin et al., 1999). Acellular vaccines consist of up to five specific *B. pertussis* antigens, including inactivated PT, FHA, PRN and two fimbrial antigens FIM2 and FIM3. These new vaccines, which have considerably reduced side effects, have been introduced into routine paediatric vaccination programs in many Western countries.

The success of pertussis vaccines has led to the opinion that the disease is essentially under control, however, worldwide 200.000 to 400.000 pertussis-linked deaths are still recorded annually, and the disease still ranks third among the causes of childhood mortality due to infectious agents (WHO, 2006). Although mostly prevalent in developing

countries, the disease is also re-emerging in the developed world, including the United States, where the incidence has increased nearly 10-fold over the last 20 years (Berbers et al., 2009).

Among the proposed reason for pertussis resurgence, attention was focused on changes in the epidemiology of the disease which were unexpectedly recorded in countries with high vaccine coverage, where cases of adolescent and adult pertussis are increasingly frequent (Wirsing von Konig et al., 2002). As a consequence, infected adults constitute an important reservoir for transmission of the disease to very young children, too young to be fully vaccinated, and therefore at risk to develop severe disease associated with high mortality rates. Furthermore, atypical (and therefore difficult to diagnose) pertussis is generally not life-threatening in adults and in many cases remains unnoticed.

Changing in pertussis epidemiology has been proposed to be linked to massive vaccination campaigns. In the pre-vaccination era, the disease occurred most often in pre-school children, infant pertussis was less frequent and adulthood pertussis very rare. Anti-pertussis immunity was naturally boosted due to repeated exposure to *B. pertussis* and immunity induced by disease was long-lasting. Since widespread vaccination of infants, whooping cough almost disappeared in the classical age group but increased in adolescents and adults. This could be probably due to faster waning of immunity after vaccination than after disease and reduced boosting exposure to *B. pertussis*. In fact, some studies suggest that duration of immunity after Pw vaccines or Pa immunization is not significantly different and lasts 4-12 years in children (Wendelboe et al., 2005). However, other study report that immunity induced by efficacious Pw vaccines persists longer than Pa vaccines (Gustafsson et al., 2006).

The re-emergence of pertussis has been attributed to other various factors, including increased awareness, improved diagnostics, suboptimal vaccines, waning immunity and pathogen adaptation (Mooi et al., 2007). Waning immunity in combination with pathogen adaptation are probably the main factors which contribute to the continued circulation of *B. pertussis* strains but the relative contribution of these factors may differ between countries and is the subject of ongoing debate.

Pertussis vaccination usually begins at 2 month of age, and optimal protection requires at least three immunizations. Generally, the three doses are given at 1- to 2-month intervals, implying that optimal protection is only achieved at the age of 6 months. To reduce the incidence of pertussis in the very young and most vulnerable age groups, early immunization, possibly at birth, would thus be highly desirable. However, numerous

studies in humans and in animal models have suggested that the neonatal immune system is too immature to effectively induce vaccine-mediated protective immunity (Lewis et al., 1991; Siegrist, 2001). IFN-γ production especially, which is indicative of a Th1 response that is essential to the development of protective immunity to pertussis (Mills, 2001), appears to be significantly reduced in human newborns, compared to older children or adults (Lewis et al., 1986). This notion is confirmed by the fact that significant amounts of antigen-specific IFN-γ are only produced after several months (at least 6) in children vaccinated with pertussis vaccines, especially with Pa vaccines (Ausiello et al., 1997).

Natural infection with *Bordetella pertussis* has long been considered to induce strong and long-lasting immunity that wanes later than vaccine-induced immunity (Wirsing von Konig et al., 2002). Furthermore, infection with *B. pertussis* induces measurable antigen specific Th1-type immune responses even in very young children (as young as 1 month of age) (Mascart et al., 2003). These observations suggest that live vaccines applicable by the nasal route, in order to mimic as closely as possible natural infection, may be attractive alternatives over the currently available vaccines.

In 2006, the group of Dr. Camille Locht at the Institut Pasteur in Lille (France) described the development of a live candidate vaccine through genetic attenuation of *B. pertussis* to diminish pathogenicity while maintaining the ability to colonize and induce protective immunity.

1.10.1 Construction of a novel live attenuated pertussis vaccine: BPZE1

Recent advances in the understanding of *B.pertussis* virulence have allowed developing a highly attenuated strain, named BPZE1, where three virulence factors were genetically targeted: TCT, PT and DNT.

As already mentioned, TCT is a breakdown product of peptidoglycan in the cell wall of Gram-negative bacteria, which generally internalize it into the cytosol by the AmpG transporter protein in order to be re-utilized during cell wall biosynthesis. *B. pertussis* AmpG is inefficient in the internalization of peptidoglycan breakdown products. Therefore, *B. pertussis ampG* gene was replaced by *E.coli ampG*. The resulting strain expressed less than 1% residual TCT activity (Fig.7).

PT is a major virulence factor responsible for the systemic effects of *B. pertussis* infections and is composed of an enzymatically active moiety, called S1, and a moiety responsible for binding to target cell receptors. Allelic exchange was used to first delete the

ptx operon, and then to insert the mutated version replacing Arg-9 by Lys, and Glu-129 by Gly in S1 (Fig. 7), two key residues involved in substrate binding and catalysis, respectively.

Finally, allelic exchange was used to remove the *dnt* gene (Fig. 7). Although the role of DNT in the virulence of *B. pertussis* is not certain, it has been identified as an important toxin in the closely related species *Bordetella bronchiseptica* and displays lethal activity upon injection of minute quantities (Mielcarek et al., 2006).

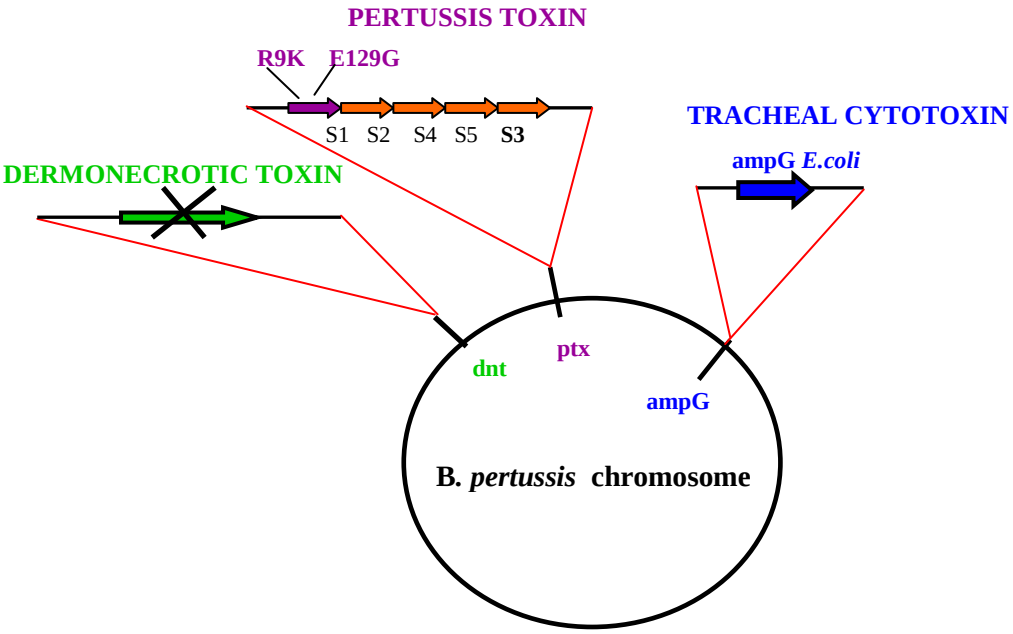

Fig.7: Cartoon of construction of BPZE1 strain. Three major *B. pertussis* toxins were genetically mutated: tracheal cytotoxin (TCT), pertussis toxin (PT) and dermonecrotic toxin (DNT) Details are mentioned in the text (adapted from description in Mielcarek et al., 2006).

1.10.2 Protection to *B. pertussis* challenge after vaccination with BPZE1

In mice, it has been shown that BPZE1 although is highly attenuated, yet able to colonize the respiratory tract and to induce strong protective immunity after a single intranasal administration. Protection against *B. pertussis* was comparable to that induced by two injections of Pa vaccine in adult mice, but was significantly better than two administrations of Pa vaccine in infant mice. Moreover, BPZE1 protected against

*Bordetella parapertussi*s infection, whereas Pa did not (Mielcarek et al., 2006). BPZE1 is thus an attractive vaccine candidate to protect against whooping cough by nasal, needle-free administration early in life, possibly at birth.

Furthermore, the long-term immunogenicity and protective efficacy induced by a single intranasal dose of BPZE1 was also evaluated in mice. Up to 1 year after immunization, BPZE1 showed significantly higher efficacy to protect adult and infant mice against *B. pertussis* infection than two administrations of an acellular pertussis vaccine (Pa).

These data highlighted the potential of the live attenuated BPZE1 candidate vaccine as part of a strategy to solve the problem of waning protective immunity against *B. pertussis* observed with the current Pa vaccines.

1.10.3 Immune response induced by BPZE1 vaccination

Analysis of *B. pertussis* antigen-specific cytokine patterns induced by BPZE1 or Pa vaccination of 8-week old mice confirmed that BPZE1 administration favours a stronger Th1-type response than Pa vaccination. This was revealed by the fact that the ratios of IFN-γ over IL-5 produced by splenocytes stimulated with FHA or PT were significantly higher in BPZE1 vaccinated mice than in Pa vaccinated mice (Mielcarek et al., 2006).

A recent study showed that, one year after immunization with BPZE1, a pertussis-specific persistent response, with high levels of IFN-γ, could be detected from spleen cells restimulated with inactivated *Bordetella pertussis*. BPZE1 induced low levels IL-17 and no IL-10 or IL-5. *B. pertussis*-specific antibodies were induced by live BPZE1 with increasing amounts during the first 6 months post-immunization before a progressive decline (Feunou et al., 2010). BPZE1 immunization induced long-lasting, efficacious memory B-cell and specific antibody responses dominated by IgG2a, which were boosted by subsequent challenge (Skerry & Mahon, 2011).

AIM OF THE STUDY

Resurgence of whooping cough is observed worldwide both in infants and adults, despite high pertussis vaccine coverage, and has been attributed to changes in the epidemiology of the disease at least partly due to the waning of vaccine induced protective immunity over the time (Wendelboe et al., 2005). Increasing the longevity of vaccine-induced immunity against *B. pertussis* might therefore have a profound public health impact. Moreover, early immunization, possibly at birth, would be highly desirable to reduce the incidence of pertussis in the very young and most vulnerable age groups.

Recently, a live highly attenuated pertussis candidate vaccine, named BPZE1, has been developed by eliminating or detoxifying three crucial bacterial virulence factors: pertussis toxin, dermonecrotic toxin, and tracheal cytotoxin. Preliminary studies in mice were promising, since showed that BPZE1 is able to induce a long-term protection in infant mice, superior to that provided by the current acellular pertussis vaccines (Mielcarek et al., 2006).

BPZE1 is at the centre of a project named ChildINNOVAC that represents the common effort of seven different European laboratories in order to pave the way for the development of a novel live attenuated nasal vaccine to protect infants and young children against whooping cough by nasal, needle-free administration early in life, possibly at birth.

In this framework, my specific aim was to perform pre-clinical studies to evaluate the features of BPZE1 induced immunity in a human *ex-vivo* system. This approach was mandatory, since *B. pertussis* is an exclusively human pathogen and rodent models of infection are inherently flawed and may not provide sufficient information. To achieve this goal, I used a human monocyte-derived dendritic cells model, a powerful tool that allows studying several critical aspects of host response against pathogens, from recognition of the infectious agent to the modulation of innate and adaptive immunity.

MATERIALS AND METHODS

2.1 Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. All blood donors provided written informed consent for the collection of samples and subsequent analysis, and the blood samples were processed anonymously.

2.2 Reagents

Polymyxin B, brefeldin A, phorbol 12-myristate 13-acetate (PMA), ionomycin and 2-β-mercaptoethanol were purchased from Sigma Chemicals (St. Louis, MO). Purified *E. coli* LPS was from Cayla-InvivoGen Europe (Toulouse, France). Human rGM-CSF, rCCL21, rIL-4, monoclonal anti- human IL-10 antibody and monoclonal anti-TGF-β antibody were from R&D Systems (Minneapolis, MN). rIL-2 was obtained from Roche (Basel, Switzerland). RPMI 1640 medium was from Life Technologies Invitrogen (Paisley, UK). Sodium pyruvates, nonessential amino acids, L-glutamine, HEPES, penicillin, streptomycin, were all from Hyclone Laboratories (South Logan, UT) (Sigma). D-PBS Dulbecco's Phosphate buffered Saline (Euroclone, Milan, Italy), Lympholyte-H (Cedarlane, Burlington, Ontario, Canada). CD14 mAb-conjugated magnetic microbeads (Miltenyi Biotec), Bromodeoxyuridine (BrdU) and anti-BrdU mAb (BD Biosciences).

2.3 Bacterial strains and growth conditions

The strains used in this study are all derived from *B. pertussis* Tohama I. BPSM (Menozzi et al., 1994), BPZE1 and BPQJ20, deficient in TCT release (Mielcarek et al., 2006), have been described previously. BPRA (PT^-) has a deletion of the PT gene (Antoine & Locht, 1990). BPSA175 (dPT) produces genetically inactivated PT and was constructed by inserting pPT-RE (Alonso et al., 2001) into the *ptx* locus of *B. pertussis* BPRA by homologous recombination. *B. pertussis* BPSMDN was constructed by deleting the DNT gene in *B. pertussis* BPSM using the pJQmp200rpsL18 derivative described in (Mielcarek

et al., 2006). All *Bordetella* strains were grown on charcoal agar plates supplemented with 10% sheep blood (Oxoid, Basingstoke, UK) at 37°C for 48 h. Bacteria were then collected and resuspended in 2 ml PBS, and the concentration was estimated by measuring the optical density at 600 nm. The bacterial suspensions were adjusted to a final concentration of 10^9 CFU/ml. For an accurate measurement of the multiplicity of infection (MOI), the *B. pertussis* suspensions were serially diluted onto charcoal agar plates and CFU were counted up to days of culture.

2.4 Purification and culture of MDDC

Human monocytes were purified from peripheral blood of healthy blood donors (courtesy of Dr. Girelli, "Centro Trasfusionale Policlinico Umberto I," University "Sapienza" Rome, Italy) after Lympholyte-H gradient. $CD14^+$ cells were further purified by positive sorting through CD14 mAb-conjugated magnetic microbeads (Miltenyi) and cultured in flasks ($75cm^2$, Costar, Corning Life Sciences, Lowell, MA) RPMI 1640 medium (Life Technologies Invitrogen, Paisley, U.K.), supplemented with heat-inactivated 10% LPS-screened FCS, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin, all from Hyclone Laboratories (South Logan, UT), and 0.05 mM 2-βmercaptoethanol (Sigma) (hereafter defined as complete medium) in the presence of GM-CSF (25 ng/ml) and IL-4 (25 ng/ml). After 6 days, immature MDDC were washed with RPMI 1640 medium and analyzed by cytofluorimetry (FACScan, BD Biosciences) for the expression of surface markers CD1a, a human dendritic cell marker related to class MHC molecules, CD14 a typical monocytes marker, CD83 and CD38 two maturation markers of dendritic cells. MDDC were used in the experiments whether >80% CD1a and <10% CD14.

2.5 MDDC infection and maturation

MDDC (10^6 cell/ml) were resuspended in complete medium without penicillin and streptomycin and treated with *B. pertussis* cells at bacterium-to cell rations of 20:1, 100:1 and 500:1. After 2 h, cells were extensively washed in the presence of polymyxin B (5 mg/ml) to kill adherent extracellular but not internalized bacteria and incubated at 37°C, 5% CO_2 for 48 h in complete medium to induce MDDC maturation. After 48 h, the treated

MDDC were harvested for immunophenotypic analysis, and the supernatants were collected for cytokine measurement by ELISA.

2.6 Immunophenotypic analysis

Cells were washed and resuspended in PBS containing 3% FBS and 0.09% NaN_3, then incubated with a panel of fluorochrome-conjugated mAbs (obtained from BD Biosciences, San Jose, CA) specific for MDDC (anti-CD14, CD1a, CD80, CD83, and CD38) or specific for T cells (anti-CD3, anti-CD45RA, anti-CD25, and anti-Foxp3). Isotype-matched Abs were used as negative controls. Cells were analyzed with a FACScan or FACSCanto (BD Biosciences). Fluorescence data were reported as percentage of positive cells when the treatment induced the expression of the marker in cells that were negative; median fluorescence intensity (MFI) was used when the treatment increased the expression of the marker in cells that were already positive.

2.7 Detection of apoptosis

MDDC apoptosis was detected by using APOPTEST-FITC (Dako Cytomation, Glostrup, Denmark). Briefly, iMDDC were treated with stimuli at different ratios for either 24 or 48 h, harvested, and double-stained with FITC-conjugated annexin V and iodide propidium (PI) for 20 minutes. Cells were washed in binding buffer and analyzed by flow cytometry in a FACScan using CellQuest software.

2.8 Chemokine-driven chemotaxis

Chemotaxis experiments were performed as described elsewhere (Frasca et al., 2006). Briefly, 600 µl complete medium containing 10 ng/ml CCL21 was added to the lower chamber of polycarbonate filters of 5-mm-pore size, 24-Transwell chambers (Costar, Corning Life Sciences, Lowell, MA). Then, $1.25x10^5$ MDDC (cell input) challenged with *B. pertussis* for 48 h were added to the upper chamber in 100 µl complete medium and incubated for 3 h. Migrated cells were counted by flow cytometry in a FACScan, acquiring events for 60 s using CellQuest software (BD Biosciences).

2.9 Isolation of T lymphocytes and MDDC–T cell allogeneic MLR

To measure allogeneic proliferation, CD3 T cells were purified from PBMCs by negative sorting with magnetic beads (Pan T-cell Kit, Miltenyi Biotec, Auburn, CA). Purity of cell preparations was assessed by cytofluorimetric staining. MDDC were cultured with *B. pertussis* strains for 48 h, washed extensively, and cultured in an MLR with T cells ($5x10^5$) at different MDDC/T cell ratios in 48-well cell culture plates for 6 days. Cell proliferation in MLR was measured by BrdU incorporation evaluated by direct immunofluorescence with an FITC-conjugated anti-BrdU mAb (BD Biosciences). Briefly, BrdU (BD Biosciences) was added to MDDC and T cell MLR at 3 mg/ml final concentration on day 3 and on day 5 of culture. Cells, collected on day 6, were fixed in 0.5% paraformaldehyde, permeabilized and stained for intracellular BrdU by direct immunofluorescence with an FITC-conjugated anti-BrdU mAb (BD Biosciences). Cells were examined by flow cytometry, and T cell proliferation was evaluated. Data were expressed as percentage of positive cells.

2.10 Polarization of T lymphocytes

To evaluate T-lymphocyte polarization, experiments were performed using $CD45RA^+$ naive T cells purified from T cells by negative sorting with anti-CD45RO-conjugated magnetic beads (Miltenyi Biotec). MDDC ($0.5x10^5$) were treated with *B. pertussis* for 48 h and then co-cultured with $CD3^+$ or $CD3^+/CD45RA^+$ T cells ($0.5x10^6$) in 24-well plates (Costar, Corning Life Sciences). On day 6, rIL-2 (50 U/ml) was added to the cultures. On days 12, supernatants were harvested for cytokine measurement. On day 12, cells were activated with PMA (40 ng/ml) and ionomycin (1 mg/ml) for 5 h in the presence of brefeldin A, a compound that blocks proteins in the endoplasmic reticulum, thus inhibiting cellular secretion. Cytokine production in T cells was measured by intracellular staining.

2.11 Analysis of T suppressor function

Purified naive T cells were exposed to allogeneic MDDC treated with *B. pertussis* (MDDC/T cell ratio 1:10) (primary MLR) to generate T suppressor (Ts) cells. Six days later, T cells were recovered and cultured at different numbers with syngeneic T cells ($5x10^5$) in the presence of allogeneic LPS-matured MDDC (mDC) ($0.5x10^5$) in 48-well

cell culture plates (secondary MLR). In some experiments to determine TGF-β production, MDDC and T cells were co-cultured in serum-free medium using a substitute of bovine serum (BIT 9500; Stemcell Technologies, Vancouver, BC, Canada). In some experiments, the cultures were performed in the presence of blocking anti IL-10 (10 mg/ml) and anti-TGF-β (10 mg/ml) Abs. Results were reported as proliferation index calculated as the percentage of BrdU incorporated by the MLR in the presence of Ts with respect to control MLR set as 100%.

To determine the cell-contact dependence of the regulatory response, syngeneic T cells ($5x10^5$) and allogeneic mDC ($0.5x10^5$) were cultured in the bottom well of polycarbonate filters of 0.4-mm-pore size, 24-Transwell chambers (Costar, Corning Life Sciences), which does not allow cell passage in the lower chamber. The T cells ($5x10^5$) recovered from the primary MLR with allogeneic mDC were added in the upper Transwell chamber. After 6 days, the proliferative response of the T cells in the lower chamber was measured by means of BrdU incorporation.

2.12 Determination of cytokine levels by ELISA

To measure cytokine production, MDDC were cultured in the presence of the indicated stimuli in 14-ml round-bottom tubes (Falcon; Becton Dickinson, Lincoln Park, NJ) at 37°C and 5% CO_2. Supernatants were collected after 48 h, and IL-10, IL-12p70, IL-1β, IL-6 (Quantikine; R&D Systems), and IL-23 (Bender MedSystem, Burlingame, CA) production was assessed by ELISA with a sensitivity of 1.0 pg/ml for IL-1β, 0.7 pg/ml for IL-6, 3.9 pg/ml for IL-10, 5.0 pg/ml for IL-12p70, and 20.0 pg/ml for IL-23. OD obtained was measured with a 3550-ultraviolet Microplate Reader (Bio-Rad, Philadelphia, PA) at 450 nm. Cytokines in the supernatants from polarized T cells were assayed by ELISA specific for IFN-γ, IL-5 and IL-17, IL-10 and TGF-β (Quantikine; R&D Systems). The lower detection limits were 8.0 pg/ml for IFN-γ, 3.0 pg/ml for IL-5, 15.0 pg/ml for IL-17, and 4.6 pg/ml for TGF-β.

2.13 Intracellular cytokine staining

Intracellular staining was performed using cells activated with PMA and ionomycin for 5 h in the presence in presence of brefeldin A (5μg/ml), a compound that blocks proteins in the endoplasmic reticulum thus inhibiting cellular secretion and preventing binding of secreted

cytokines to the cell surface. Cells were then fixed in paraformaldehyde (PFA) 2%, permeabilized using a solution containing saponine 0.5% and stained with a predetermined optimal concentration of fluorochrome-conjugated anticytokine Abs (CD3,IFN-γ, IL-4, IL-17, Foxp3, CD25) or appropriate isotype controls. After a 20-min incubation RT, cells were washed and analyzed by flow cytometry in a FACScan or FACSCanto flow cytometer.

2.14 Statistical analysis

Statistical descriptive analyses were carried out using the SPSS statistical package (SPSS, Chicago, IL). Differences between mean values were assessed by two-tailed Student t test and were statistically significant for p values 0.05.

RESULTS

3.1 Phenotypic maturation, viability, and cytokine production in MDDC challenged with different doses of BPZE1

The transition from an immature to a mature stage is well known to endow DC with the capacity to couple innate to adaptive immune responses. Resting immature DC (iDC) reside in the periphery, where they sense pathogens through PRRs. Upon pathogen recognition, a signalling cascade initiates the DC maturation process, characterized by the up regulation of MHC class II and co-stimulatory molecules. In our laboratory an experimental protocol for MDDC challenge with *B. pertussis* has been developed (Fedele et al., 2005). Those studies showed that bacteria were scarcely phagocytosed and rapidly killed intracellularly, and that bacterial contact rather than internalization induced the onset of the maturation program (Fedele et al., 2005).

The optimal BPZE1 dose to challenge MDDC (i.e., the ratio that allows maximization of MDDC activation without affecting MDDC viability) was determined in dose-response experiments ranging from 20 to 500 bacteria per MDDC, and the induction of phenotypic maturation, cell viability, and cytokine production were assessed. Phenotypic maturation was evaluated 48 h after treatment with bacteria by monitoring the surface expression of the co-stimulatory molecule CD80 and the typical dendritic cells maturation markers CD83 and CD38. At all bacteria-to-cell ratios tested, both the vaccine strain, BPZE1 and the virulent parental strain BPSM, induced a significant up regulation of the maturation markers as compared to untreated MDDC (Fig. 8).

MDDC viability was assessed by annexin V and propidium iodide (PI) staining. Annexin V is used as a probe to detect cells that have expressed phosphatidylserine on the cell surface, a feature found in apoptosis as well as other forms of cell death. PI is a fluorescent dye that stains DNA. The plasma membrane of cells that are viable or in the early stages of apoptosis are not permeable to PI, while cells in the late stages of apoptosis or already dead are permeable to PI, having lost membrane integrity.

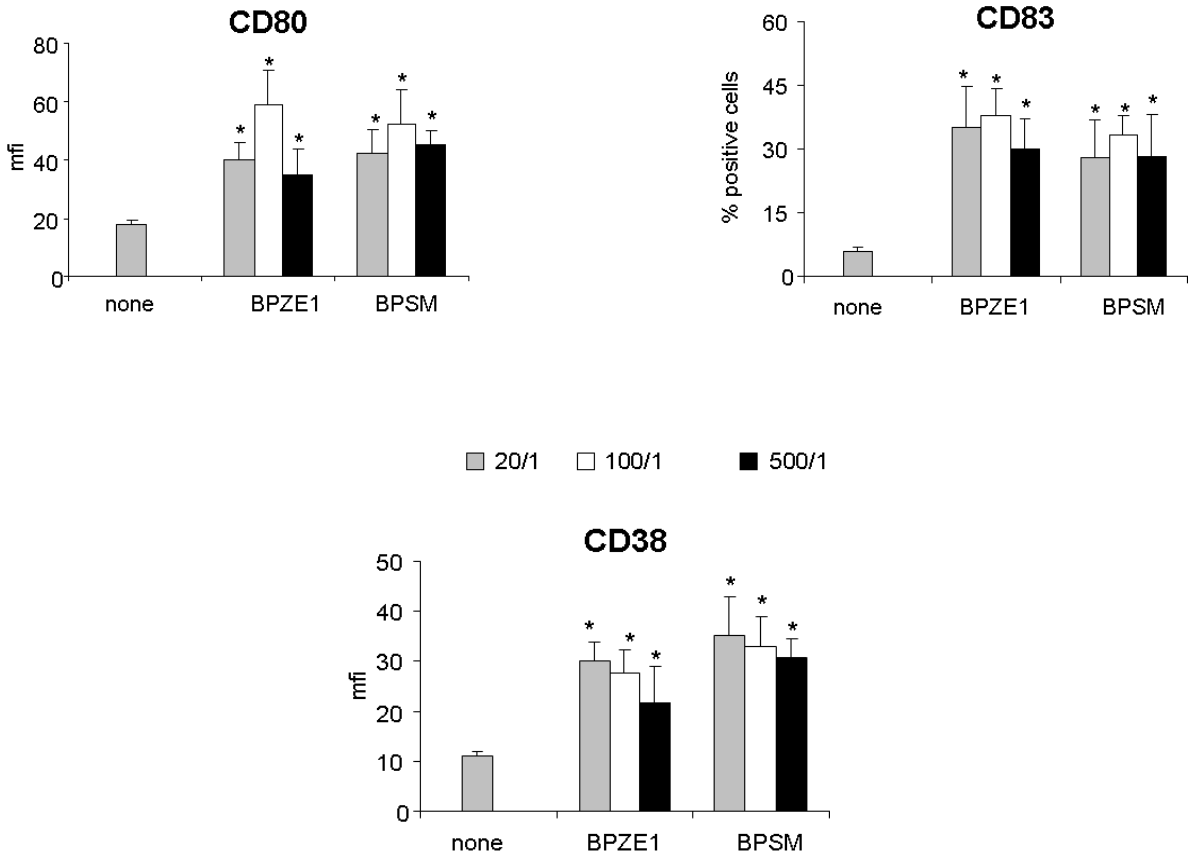

Fig.8: Induction of human MDDC maturation. Fluorescence data are reported as median fluorescence intensity (mfi) when treatment increased the expression of the marker in cells that were already positive (CD80, CD38); otherwise, percentage of positive cells is used (CD83). Mean expression ± SE of 11 independent experiments performed with MDDC obtained from different donors is indicated. *$p<0.05$ *vs.* none.

In preliminary experiments, a time-course analysis of apoptosis induction in MDDC was performed, showing that both BPZE1 and BPSM did not protected cells from spontaneous apoptosis at 8 h, while inducing protection at 24 h and 48 h time-points (Fig. 9). Therefore, the following experiments were performed after 24 h MDDC stimulation.

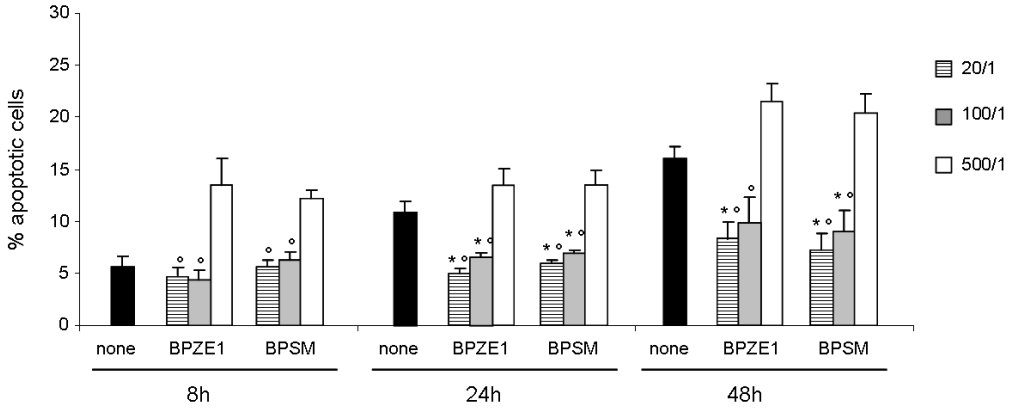

Fig.9 Resistance of MDDC to spontaneous apoptosis: time-course. MDDC were either untreated (none) or challenged with BPZE1 or BPSM at 20:1, 100:1 and 500:1 ratio for 8, 24 and 48 h. Percentage of apoptotic cells is shown. Mean expression ± SE of three independent experiments performed with MDDC obtained from different donors is shown. *p<0.05 *vs.* none; °p<0.05 *vs.* 500/1 ratio.

An increase in the percentage of apoptotic and necrotic cells was induced by both BPZE1 and BPSM bacterial strains when the 500:1 dose was used (Fig. 10).

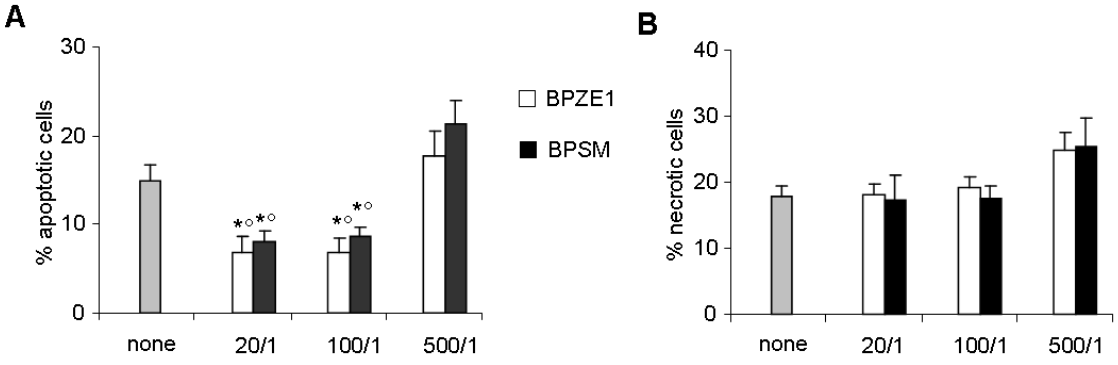

Fig.10: Resistance of MDDC to spontaneous apoptosis. Cells were stained with FITC-conjugated annexin V to assess apoptosis (A) and propidium iodide to assess necrosis (B) and analyzed by flow cytometry. Percentage of apoptotic or necrotic cells is shown. Mean expression ± SE of eight independent experiments performed with MDDC obtained from different donors is indicated. *p<0.05 *vs.* none, °p<0.05 *vs* 500/1 ratio.

In contrast, incubation at 20:1 and 100:1 ratios significantly promoted the resistance of MDDC to apoptosis, spontaneously occurring when the cells were not stimulated (Fig. 10A). Moreover, treatment with either 20:1 or 100:1 ratio did not enhance necrosis (Fig. 10B).

To determine the impact of BPZE1 on the host response, we studied the induction of relevant cytokines produced by MDDC. In preliminary experiments, MDDC were challenged with two different ratios, 20:1 and 100:1, and the levels of IL-12p70, and IL-10 were measured by ELISA assay (Table I).

Stimuli	**IL-10 (pg/ml)**	**IL-12p70 (pg/ml)**
none	34.6 ± 20.3	0.0 ± 0.0
BPZE1 20:1	3027.6 ± 711.9*	0.0 ± 0.0
BPZE1 100:1	5479.7 ± 367.9*	0.0 ± 0.0
BPSM 20:1	4279.2 ± 154.7*	3.0 ± 2.7
BPSM 100:1	5806.0 ± 225.5*	13.3 ± 11.9

Table I: IL-10 and IL-12p70 secretion by MDDC challenged with different doses of BPZE1 and BPSM. Values are expressed as mean ± SE from four independent experiments performed with MDDC obtained from different donors and expressed as pg/ml of cytokine released. * $p < 0.05$ *vs.* none.

IL-10 was produced in a dose-dependent fashion, without statistically significant differences between BPZE1 and BPSM. For IL-12p70, we only recorded a very modest production by BPSM-treated MDDC challenged with 100 bacteria per cell. These data confirmed that optimal MDDC stimulation was achieved when 100 bacteria per cell were used, in accordance with previously published data (Fedele et al., 2005), and showed no differences between BPZE1 and BPSM. Thus, to maximize the challenge of *B. pertussis* on MDDC, the 100:1 ratio was chosen.

3.2 Migratory ability of MDDC treated with BPZE1

In order to initiate the adaptive immune response, DC travel through the lymphatics to the draining lymph node, where they arrive as fully mature DC, able to promote the activation of naive T cells through antigen presentation. Migration of DC from the periphery to the lymph node is regulated by the expression of the lymphatic chemokines CCL19 and CCL21 in the secondary lymphoid organs and by expression of their receptor, CCR7, by mDC. *In vitro* chemotaxis experiments were undertaken to verify whether BPZE1-challenged MDDC were able to perform lymphatic migration. MDDC were either untreated or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h. Cells were then added to the upper chamber of a Transwell system, and CCL21-containing medium was placed in the lower chamber. Cells migrated in the lower chamber were counted after 3 h

BPZE1-treated MDDC efficiently sensed CCL21 gradients. In contrast, BPSM-treated MDDC, although expressing the same cell-surface levels of CCR7 as those of BPZE1-treated MDDC (data not shown), were completely blocked in their ability to migrate in response to CCL21 (Fig. 11A).

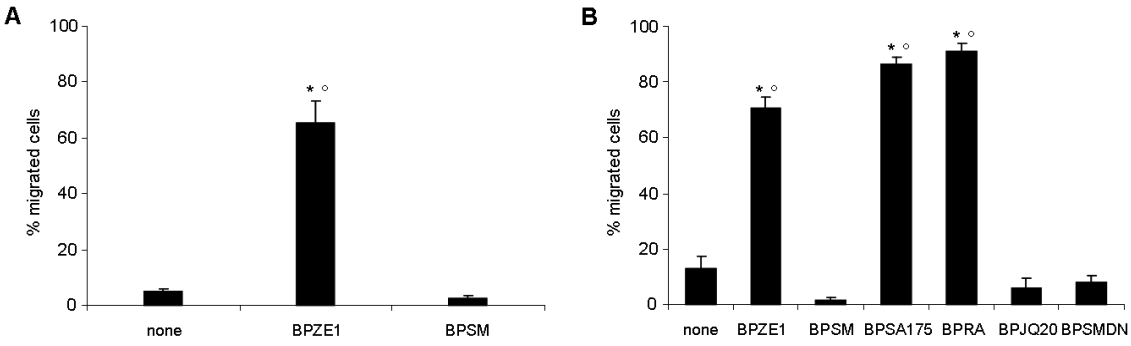

Fig.11: Lymphoid organ-derived CCL21-driven chemotaxis of MDDC. A) Percentage of migrated MDDC challenged with BPZE1or BPSM with respect to the cell input ($1.25x10^5$ cells). Results are the mean values ± SE of five independent experiments performed with MDDC obtained from different donors. B) Percentage of migrated MDDC challenged with BPZE1, BPSM, BPSA175 (dPT), BPRA (PT^-), BPQJ20 (deficient in TCT release), or BPSMDN (DNT^-). Results are the mean values ± SE of three independent experiments performed with MDDC obtained from different donors. *$p < 0.05$ *vs.* none, °$p < 0.05$ *vs.* BPSM.

It is well known that the enzymatic activity of PT specifically inhibits G proteins and blocks cell migration induced by chemokines (Gierschik, 1992). Furthermore, a recent study has been shown that the B oligomer (binding domain) of PT might inhibit chemokine receptor signalling (Schneider et al., 2009). We have hypothesized that PT expressed by the virulent BPSM strain may impair chemotaxis of MDDC and that BPZE1-treated MDDC were allowed to migrate due to the PT detoxification. To test this hypothesis and rule out the possible involvement of PT B-oligomer or other virulence factors not expressed by BPZE1, chemotaxis experiments were performed with MDDC challenged with *B. pertussis* mutants either lacking PT (BPRA) or producing genetically detoxified PT (BPSA175), deficient in TCT production (BPJQ20) or lacking DNT (BPSMDN). As expected, MDDC incubated with either BPSA175 or BPRA migrated in response to the CCL21 gradient, whereas neither BPJQ20-treated nor BPSMDN treated cells were able to respond (Fig. 11B), showing that inhibition of MDDC lymphatic chemotaxis was due to the enzymatic activity of PT.

3.3 Cytokine profile elicited in MDDC challenged with BPZE1

Cytokine production is a key step in the regulation of the immune response exerted by DC, we therefore assessed the production by *B pertussis* treated MDDC of Th1-polarizing IL-12p70, IL-10, involved in Th2 and Tregs/Ts induction, and IL-23, IL-1β, and IL-6, all involved in Th17 polarization. Previous studies performed in our laboratory have shown that *B. pertussis* promotes the production by MDDC of IL-23, IL-1β, and IL-10, whereas IL-12p70 is barely detectable (Fedele et al., 2005; Spensieri et al., 2006; Fedele et al., 2010). The cytokine profile elicited in MDDC by BPZE1 or BPSM was similar and characterized by high levels of IL-10, IL-1β, and IL-6. IL-23 was also produced by MDDC treated with either strain, without any statistically significant difference (Fig. 12).

Concerning the production of IL-12p70, the mean values measured were at best modest and appeared to be influenced by a very high variation between the different MDDC donors. As shown in Fig. 12, BPZE1 induced IL-12p70 only in 4 and BPSM only in 5 of 15 independent experiments performed with different donors. Statistically significant differences for IL-12p70 production with respect to untreated MDDC were reached by BPZE1-challenged but not by BPSM-challenged MDDC. IL-10, IL-12p70, IL-23, IL-1β, and IL-6 release in culture media was assessed by ELISA 48 h post treatment with bacteria.

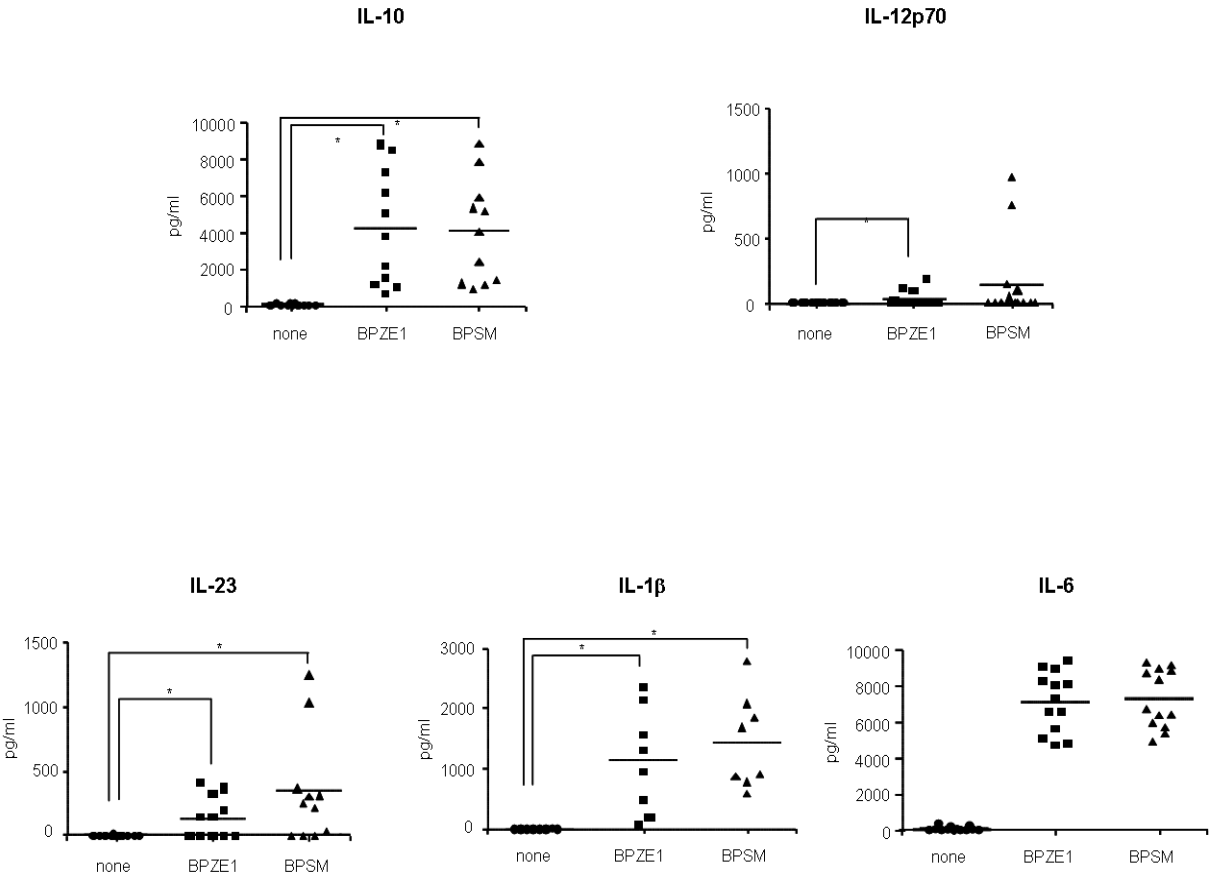

Fig. 12: Analysis of cytokine secretion by MDDC. MDDC were either untreated (none) or challenged with BPZE1 or BPSM at a 100:1 ratio. Values are expressed as mean ± SE from 15 (for IL-12p70), 13 (for IL-6), 11 (for IL-10 and IL-23), and 8 (for IL-1β) independent experiments performed with MDDC and expressed as pg/ml of cytokine released. *p <0.05 *vs.* none

3.4 Ag presentation and polarization of T helper lymphocytes by BPZE1-challenged MDDC

We next investigated key functions of mature DC, such as antigen presentation to T lymphocytes and polarization of the immune response. MDDC either untreated or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h were co-cultured with allogeneic purified T cells ($5x10^5$) in different numbers ranging from $50x10^3$ (10:1) to $1.56x10^3$ (1:320) for 6 days. Proliferation was assessed by BrdU incorporation. BrdU is a

synthetic analogue of thymidine that can be incorporated into the newly synthesized DNA of replicating cells during the S phase of the cell cycle. Intracellular staining with an appropriate mAb directed towards BrdU allows detecting proliferating cells.

MDDC stimulated with BPZE1 efficiently induced allogeneic T cell proliferation, with a maximum proliferation induced at 10:1 T cell/MDDC ratio. Similar results were obtained with BPSM-treated MDDC (Fig. 13).

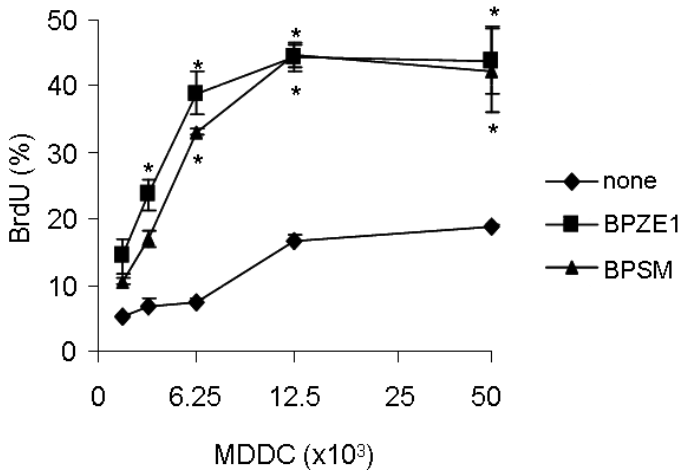

Fig.13: MDDC either untreated (none) or challenged with bacterial strains were co-cultured with allogeneic purified T cells in different numbers and the BrdU incorporation was evaluated. Results are reported as percentage of positive cells (mean ± SE) of four independent experiments performed with MDDC obtained from different donors. *$p < 0.05$ *vs.* none.

Given the cytokine profile induced by BPZE1 in MDDC, we analyzed their capacity to polarize purified T lymphocytes and, in particular, the possibility of Th1/Th17 induction, already described in human (Fedele et al., 2010) and mouse models (Feunou et al., 2010; Andreasen et al., 2009) and considered to contribute to protection induced by whole-cell pertussis vaccination (Higgins et al., 2006). We assessed the capacity to induce polarization both in naive $CD45RA^+$ T cells and in already primed $CD3^+$ T cells.

MDDC either untreated or challenged with BPZE1 or BPSM for 48 h were co-cultured with purified allogeneic $CD3^+$ T cells or $CD45RA^+$ naive T cells. On day 12, supernatants were collected, and secreted cytokines were measured by ELISA.

Fig. 14 shows that both BPZE1-treated and BPSM-treated MDDC drove the expansion of IFN-γ-and IL-17-producing effector T cells, both in total $CD3^+$ T cells (Fig. 14A) or in naive $CD45RA^+$ T cells (Fig. 14B).

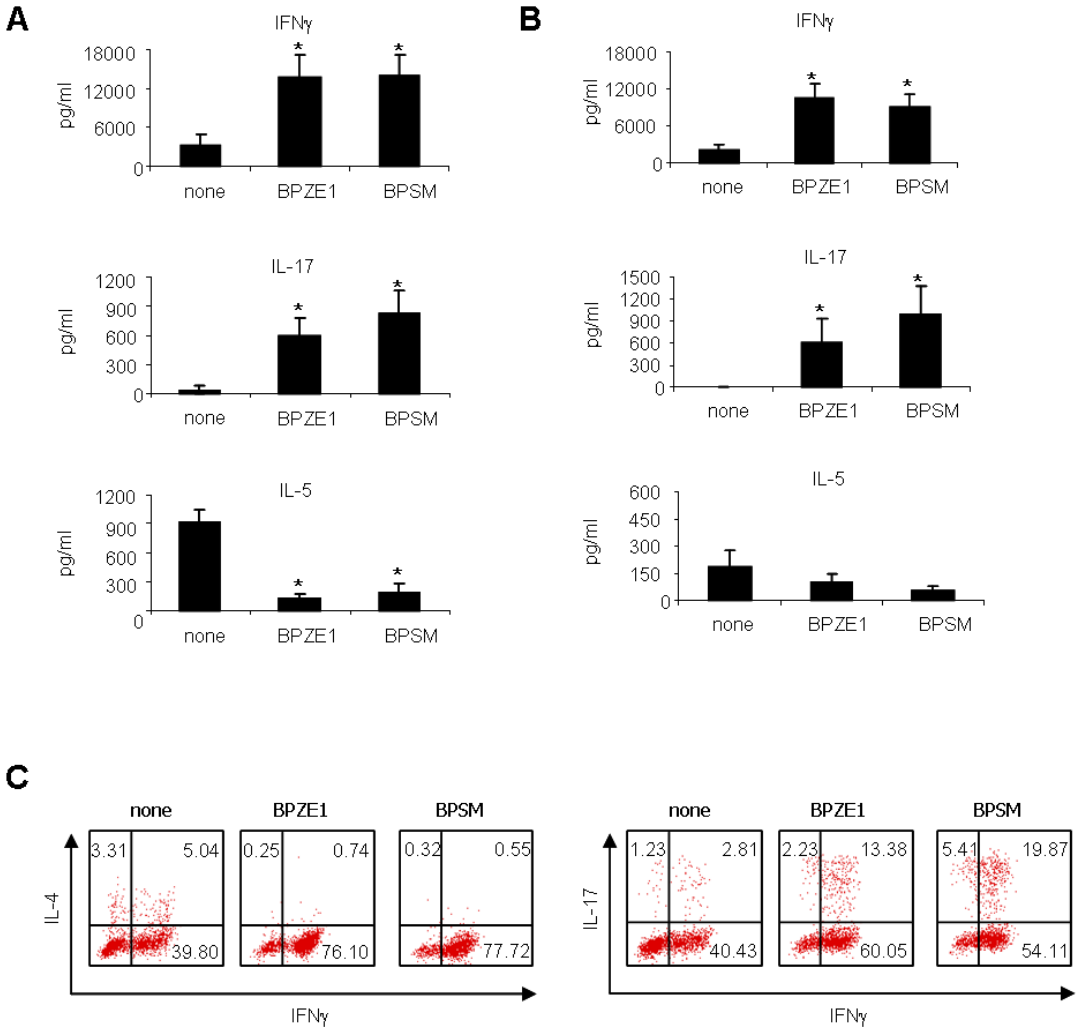

Fig.14: T lymphocyte polarization. MDDC either untreated or challenged with BPZE1 or BPSM for 48 h were co-cultured with purified allogeneic $CD3^+$ T cells (A) or $CD45RA^+$ naive T cells (B). Results are expressed as mean ± SE of five independent experiments performed with MDDC obtained from different donors. C) MDDC either untreated or treated as in A were co-cultured with purified allogeneic $CD3^+$ T cells. Numbers in each quadrant indicate the percentage of positive cells. A representative experiment out of three performed with MDDC obtained from different donors is shown. *$p < 0.05$ *vs.* none

A trend toward higher IL-17 production by BPSM-treated MDDC-driven T cell polarization was found, but the differences were not statistically different between the two bacterial strains. In contrast, the production of IL-5, typical of Th2 polarization, was decreased compared with that of T cells cultured in the presence of untreated MDDC.

These results were further confirmed by intracellular cytokine staining. To this end, MDDC either untreated or treated as in Fig. 14A were co-cultured with purified allogeneic $CD3^+$ T cells.

On day 12, cells were stimulated for 5 h with PMA/ionomycin in the presence of brefeldin A. Ionomycin is an ionophore produced by the bacterium *Streptomyces conglobatus* is used in research to raise the intracellular level of calcium (Ca^{2+}). It is also used to stimulate the intracellular production of the cytokines, interferon, perforin and usually in conjunction with PMA. PMA (Phorbol 12-myristate 13-acetate) is the most commonly used phorbol ester, it binds to protein kinase C and leads to a strong activation of T cells proliferation.

Intracellular staining showed a dramatic reduction of IL-4^+ cells accompanied by an increase of IFN-γ^+ and IL-17^+ cells when $CD3^+$ T cells were co-cultured with MDDC treated with either BPZE1 or BPSM (Fig. 14C). Remarkably, Th1/Th17 double positive cells accounted for the great majority of IL-17 effectors expanded. Similar results were obtained when $CD45RA^+$ naive T cells were used, although lower percentages of cytokine-producing cells were detected when intracellular staining of naive T cells was performed (data not shown). Overall, BPZE1 and its virulent parent strain BPSM behave similarly as far as the capacity to strongly induce allogeneic T cell stimulation and polarization of Th cells are concerned, retaining the same properties already shown in our previous study using *B. pertussis* strain 18323 (Fedele et al., 2010).

3.5 Induction of functional suppressor T cells in vitro by BPZE1-challenged MDDC

Previous studies in mice have shown that IL-10 produced in response to *B. pertussis* infection may in turn promote IL-10-producing Ts (Higgins et al., 2003). Moreover, a reciprocal relationship between Th17 and Ts has been demonstrated (Beriou et al, 2009; Koenen et al., 2008). We therefore decided to investigate the possibility that BPZE1- or BPSM-challenged MDDC drive the induction of regulatory/Ts activity. To determine whether naive T cells exposed to *B. pertussis* treated MDDC become functional

regulatory/suppressors, MDDC either untreated or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h were co-cultured with purified allogeneic $CD45RA^+$ naive T cells at a 10:1 ratio. On day 6, Ts were collected and co-cultured at different numbers, as indicated in the x-axis, with syngeneic T cells ($5x10^5$) in the presence of allogeneic mDC ($5x10^4$). T cell proliferation was assessed by BrdU incorporation. Both BPZE1-Ts and BPSM-Ts inhibited proliferation in the secondary MLR in a dose-dependent manner with statistical significance compared with that of T cells primed with immature MDDC (Fig. 15A).

To rule out an inhibitory role of TGF-β present in the FCS added to the culture medium, we performed the same experiments with a serum-free medium. Also in this case, a marked reduction of T cell proliferation was induced by BPZE1-Ts and BPSM-Ts (data not shown), consistent with the opinion that suppression of proliferation was mediated directly by T cells.

Classical regulatory T cells (Tregs) are $CD4^+/CD25^+/Foxp3^+$ (Zhu et al., 2010; Sakaguchi et al., 2009). After 6 days of co-culture, MDDC and naive T cells were collected and stained for intracellular Foxp3 and CD4 and CD25 cell surface expression in a flow cytometry analysis. We have found that classical Tregs were not induced either in BPZE1-Ts or in BPSM-Ts (Fig. 15B).

The inhibitory effect on T cell proliferation might be mediated through soluble factors produced by Ts or cell contact (Zhu et al., 2010). To correlate the regulatory/suppressor activity of the Ts with cytokine secretion, we further characterized BPZE1-Ts and BPSM-Ts. MDDC and naive T cells were co-cultured as described before.

On day 6, supernatants were collected and cytokine production measured by ELISA. High levels of IFN-γ, IL-17, and IL-10 were induced by BPZE1-Ts and BPSM-Ts. IL-5 was barely detectable, whereas the levels of TGF-β were similar to those produced by T cells co-cultured with untreated MDDC (Fig. 15C).

IL-10 and TGF-β play a crucial role among the soluble factors produced by Ts. Thus, to evaluate the role of these cytokines, BPZE1-Ts or BPSM-Ts, syngeneic T cells ($5x10^5$) were co-cultured with allogeneic mDC ($5x10^4$) (control MLR) and added with Ts ($5x10^5$) to a secondary MLR (control MLR+Ts) in the presence of both anti-IL-10 and anti-TGF-β neutralizing mAbs. As shown in Fig. 15D, the proliferation was still inhibited when neutralizing Abs were added, suggesting a minor role of these soluble factors in the Ts activity.

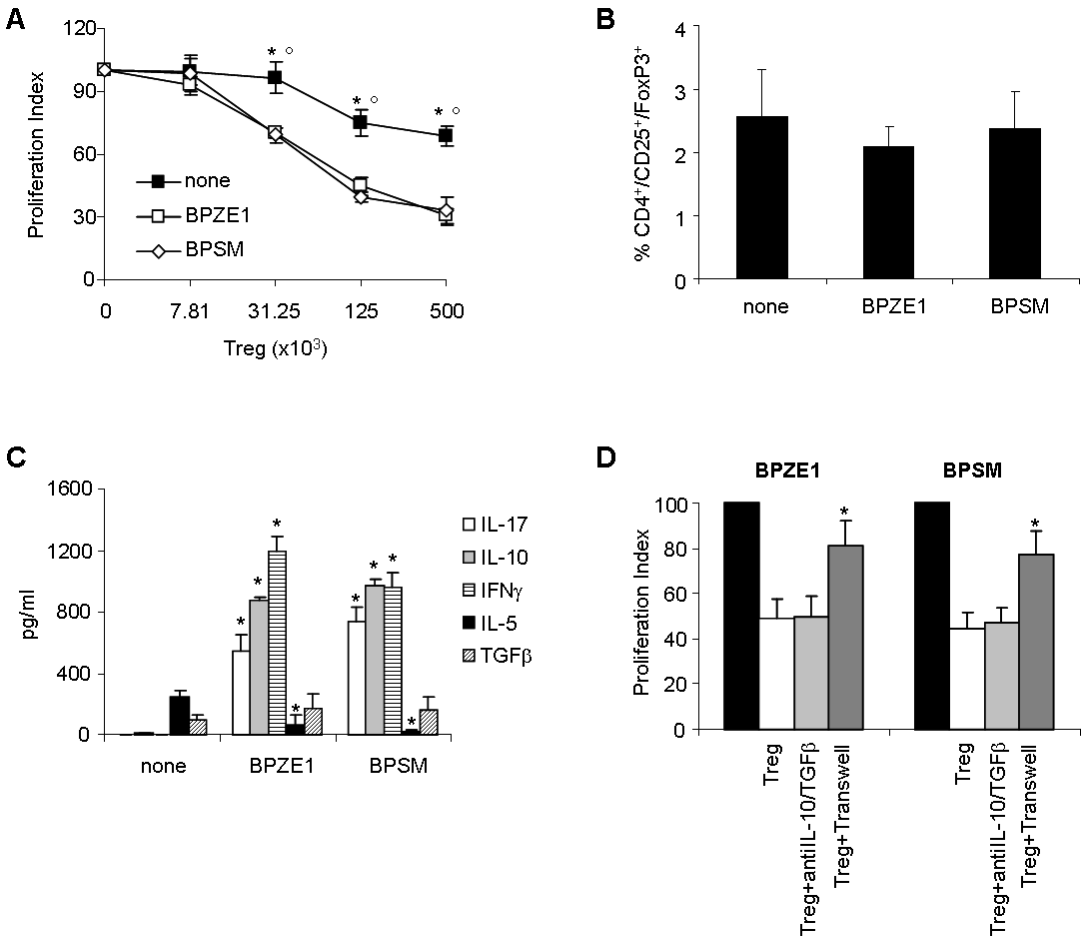

Fig.15: Characterization of Ts activity. A) MDDC either untreated or challenged with BPZE1 or BPSM at a 100:1 ratio for 48 h were co-cultured with purified allogeneic $CD45RA^+$ naive T cells at a 10:1 ratio to generate Ts. Results are reported as proliferation index (mean ± SE) of six independent experiments performed with MDDC and T cells obtained from different donors. B) MDDC and naive T cells were co-cultured as in A. Results are reported as percentage of positive cells (mean ± SE) of three independent experiments performed with MDDC and T cells obtained from different donors. C) MDDC and naive T cells were co-cultured as in A. Results are reported as pg/ml (mean ± SE) of three independent experiments performed with MDDC and T cells obtained from different donors. D) Syngeneic T cells were co-cultured with allogeneic mDC and added with Ts in the presence of blocking anti-IL-10 and blocking anti-TGF-ß mAbs or separated from Ts with a transwell membrane Results are reported as proliferation index (mean ± SE) of three independent experiments. * $p < 0.05$ *vs.*Ts.

To demonstrate that the inhibitory effect on T cell proliferation was mediated by cell-to-cell contact and not through soluble factors, suppression experiments were performed in a Transwell system separating BPZE1-Ts or BPSM-Ts from syngeneic T cells by a polycarbonate membrane that allows exchange of soluble factors but excludes direct cell

contact. The elimination of cell contact almost entirely restored the proliferation index, indicating that the inhibitory effect was mainly mediated by direct cell contacts (Fig. 15D).

DISCUSSION

Although extensive immunization campaigns have significantly reduced pertussis-caused child mortality worldwide, infections with *B. pertussis* still pose a significant health burden. Even in highly vaccinated populations, annual infection rates can reach 1-7% in the general population. Thus, pertussis remains one of the least well-controlled vaccine-preventable diseases in the world, with as many as 300,000 deaths each year, worldwide. The majority of these deaths occur in non- or insufficiently vaccinated children. Interestingly, pertussis has not only persisted in vaccinated populations, but has even resurged in recent years.

The persistence and resurgence of pertussis in countries with highly vaccinated populations has been attributed to various factors including increased awareness, improved diagnostics, suboptimal vaccines, waning of vaccine-induced immunity, and pathogen adaptation. Although pertussis is traditionally considered a childhood disease, the observed increase in pertussis incidence is seen mainly in adolescents and adults. The shift of pertussis infections towards older age categories poses significant health concerns, as these individuals provide a reservoir of *B. pertussis* for transmission to infants, who are at the highest risk of developing severe pertussis.

Knowledge about the molecular mechanisms by which *B. pertussis* infects the host and evades clearance by the immune system will provide insight into its survival strategies and may facilitate the rational design of novel therapeutic and/or preventive strategies that target the 'weak' spots of this versatile pathogen.

Immunity to *B. pertussis* is complex. While single correlates of protection have been proposed (Taranger et al., 2000), protective immunity requires both a strong cellular and antibody response. The whole-cell (Pw) and acellular (Pa) pertussis vaccine appear to protect through slightly different mechanisms, with Pw or natural infection inducing Th1 type response, whereas Pa typically induce more Th2-like immunity in humans (Ausiello et al, 1997) Follow-up studies from clinical trials evaluating Pa-induced immune responses in children have indicated that 15-33 months after a complete course of vaccination, specific antibodies were almost undetectable, suggesting that booster vaccinations are needed to prolong the duration of immunity (Gustafsson et al., 2006). In mice, the pattern of cytokines production indicated that Th1 and Th17 cells are activated by vaccination with Pw, and Th1 and Th2 cells are involved in the immune response upon vaccination

with Pa (Higgins et al., 2006; Mills et al., 1998). Studies in murine models designed to probe the longevity of vaccination showed that vaccine-mediated protection persist for more than 44 weeks following immunization with Pa or Pw (Mahon et al., 2000).

In contrast, naturally acquired immunity to *B. pertussis* has been proposed to be long-lasting (at least 30 years). Several parameters might explain these differences in the duration of immunity induced by bacterial infection and vaccination. While Pa consist of two to five *B. pertussis* antigens, natural infection induces immune responses against a much wide range of antigens, some of which may contribute to the induction of long-lasting protective immunity. In addition, since *B. pertussis* is a strictly respiratory pathogen, it is likely that mucosal or local immunity in the respiratory tract plays an important role in the long-term protective immunity. None of the current pertussis vaccines target the mucosal immune compartment. These observations led to hypothesize that a pertussis vaccine delivered by the nasal route and mimicking as closely as possible natural infection may perhaps induce long-lasting protective immunity. Furthermore, the use of an intranasal, needle-free vaccine could have an important impact on the administration of vaccination in very young children.

To this end, the group of Dr. C. Locht developed the construction of a live candidate vaccine named BPZE1, a genetically engineered *B. pertussis* strain obtained by removing or altering genes involved in the production of three major bacterial toxins, pertussis toxin (PT), dermonecrotic toxin (DNT) and tracheal cytotoxin (TCT). A murine infection model was used to compare protective efficacy of BPZE1 to that of Pa over a period of 1 year. During this entire period, BPZE1 conferred high levels of protection against *B. pertussis* infection, both in mice vaccinated during adulthood or during infancy. At early time-points (3 months after vaccination), protective efficacy was similar to that induced by Pa. However later on, in particular 9 and 12 months after vaccination, BPZE1-induced protection was still strong, whereas Pa-induced immunity waned (Fenou et al., 2010). Furthermore, other studies showed that the longevity of the BPZE1-induced protection was similar to that induced by infection with virulent *B. pertussis* strain (Skerry & Mahon, 2011).

Taken as foundation the studies in mice showing that the live attenuated *B. pertussis* strain BPZE1 is a promising candidate vaccine, the evaluation of this strain in a human experimental setting is mandatory.

We used a well-established model of MDDC challenged with *B. pertussis* to analyze several aspects of BPZE1-driven immune responses in humans. We show that BPZE1,

despite being strongly attenuated, maintains the capacity to promote DC maturation and, similar to BPSM, is able to protect cells from apoptosis. This is a crucial step in DC activation, since the enhancement of the circulating DC longevity ensures a prolonged life span necessary to perform their functions.

A unique feature of mature DC is the ability to migrate toward the secondary lymph nodes, where naive T helper cells are encountered and activated. In this study, we add an important piece of information on the influence of *B. pertussis* on the modulation of the host's immune response and on its immune subversion capacity. Attenuated BPZE1 lacks the inhibitory effect that virulent *B. pertussis* exerts on the lymphatic migration of DC.

PT has been known to prevent chemokine receptor signalling through the enzymatic activity of the A subunit. More recent studies revealed a novel mechanism by which PT may affect migration of T lymphocytes via the B subunit, which is mediated by interaction with the TCR (Schneider et al., 2009). This process leads to desensitization of CXCR4; it occurs within a few minutes and it is reversible.

Our data show that DC challenged with *B. pertussis* mutants producing genetically detoxified PT (BPSA175) or lacking PT (BPRA) migrated in response to a CCL21 gradient. These results confirm that a crucial role in inhibiting cell migration is played by the enzymatically active A subunit, whereas the active B subunit, present in BPSA175, has a marginal role, if any, at least in DC lymphatic migration. *B. pertussis* mutants deficient for DNT or TCT inhibited DC migration to the same extent as BPSM, suggesting that these two toxins do not influence migration in our settings. These results imply that PT, in addition to its role in bacterial colonization and inhibition of an early inflammatory response, mediates an immune evasion mechanism that strongly interferes with DC functions. More importantly, these findings highlight a crucial difference between BPZE1 and BPSM and suggest that one of the main advantages that the vaccine strain may have *in vivo* is the capacity to rapidly and efficiently activate the acquired immune response by allowing the migration of DC to lymph nodes.

In our laboratory previous studies have shown that *B. pertussis* promotes in DC the production of Th17-driving IL-23 and IL-1β and regulatory IL-10, whereas IL-12p70 is barely detectable (Spensieri et al., 2006; Fedele et al., 2010). BPZE1 and BPSM elicited a similar profile, with the exception of detectable levels of IL-12p70 produced by roughly one third of DC donors. This discrepancy could be ascribed to the different genetic background of the *B. pertussis* strains used in this study compared with that of the previous studies. BPSM and BPZE1 are both derivatives of the Tohama I strain, whereas in our

previous studies we used the BP18323 strain, which differs from typical *B. pertussis* strains in many aspects (Gerlach et al., 2001).

Attenuation of the bacterium did not preclude the ability of BPZE1-challenged DC to perform Ag-presenting activity and induce the expansion of polarized Th1 and Th17 effectors, in keeping with the results of our previous studies. Remarkably, IFN-γ was produced by T cells at high levels, independently of the amounts of IL-12p70 produced by polarizing MDDC, confirming our previous finding that *B. pertussis*-driven Th1 immune responses may occur in the absence of IL-12p70.

Several studies have focused on the role of PT in modulating the host immune response, and evidence is accumulating on the role of PT in T cell polarization. Recently, in our laboratory it was shown that MDDC stimulated with genetically detoxified PT induced a mixed Th1/Th17 response (Nasso et al., 2009), and in another study PT-deficient *B. pertussis* was described as a poor inducer of both IFN-γ and IL-17 in mice (Andreasen et al., 2009). The findings presented here demonstrate that detoxification of PT in BPZE1 results in two interesting properties: it abrogates toxicity and the inhibitory effects on chemotaxis while the immunomodulatory properties are retained.

Notably, the induction of both IFN-γ and IL-17 by *B. pertussis* in T cells has been shown in mice (Feunou et al., 2010; Andreasen et al., 2009; Higgins et al., 2006) and humans (Fedele et al., 2010), and these cytokines appear necessary to achieve protection in mice vaccinated with Pw vaccines (Higgins et al., 2006). In our study, intracellular staining of polarized T cells highlights the presence of a double positive Th1/Th17 population. Of note, Th1/Th17 cells have been recently shown to be induced by PT toxin in mice (Andreasen et al., 2009). Although the host defence against extracellular bacteria is widely considered pertaining to the Th17 arm, some evidence indicates that efficient protection requires synergy between the Th1 and Th17 lineages (Lin et al., 2009). Whether these double-positive cells represent a subset of cells in a transient stage of Th development or a new population derived from a distinct but unknown differentiation program remains unclear. However, the latter hypothesis is strengthened by our observation that Th1/Th17 cells are expanded by *B. pertussis*-challenged MDDC independently of the use in polarization experiments of naive or already committed T cells and might suggest that, also in this model, these cells are involved in protection.

The fact that both BPZE1-primed and BPSM-primed MDDC produced high levels of IL-10 argues for the induction of Ts. Our experiments show that BPZE1-treated or BPSM-treated MDDC induce *in vitro* a population of T lymphocytes that are functionally able to

suppress the proliferation of syngeneic T cells, and the suppressor activity was mediated mainly by cell contact rather than by soluble factors. These cells were not classical $CD4^+CD25^+Foxp3^+$ Tregs. Other phenotypes for Ts subsets have been described, such as IL-10-producing CD4+Foxp3$^-$ type1 Tregs or $CD8^+$ Ts (Sakaguchi et al., 2009).

The findings presented in this study indicate that the interactions between *B. pertussis* and the human host have evolved a mechanism of suppression to limit T cell responses. Whether this represents an immune evasion mechanism for the bacterium or has a role in dampening exacerbated immune responses dangerous for the host still requires further investigation. However, it may be relevant in view of recent findings showing that the administration of BPZE1 strongly reduces the cytokine storm induced by influenza A virus infection in mice (Li et al., 2010).

Since BPZE1 and BPSM showed the same capacity to promote Ts induction, bacterial products involved in this process are necessarily shared by the two strains. Studies are needed to unveil the role of other components, including filamentous hemagglutinin, adenylate cyclase toxin, and lipooligosaccharide, all described as inducers of DC with a tolerogenic phenotype (Spensieri et al., 2006; Fedele et al., 2007, Fedele et al. 2008, McGuirk et al., 2002, Ross et al., 2004).

Our data demonstrate that BPZE1 behaves similarly to BPSM in the modulation of DC functions. This observation is not surprising, as the adenylate cyclase toxin and the lipooligosacharide, that, together with PT, were demonstrated to have a direct effect on DC functions (De Gouw et al., 2011), are present in both strains, although PT present on BPZE1 does not express its toxicity. The other genetic differences between BPZE1 and BPSM concern the DNT and TCT, but our data show that these two toxins do not appear to have a relevant effect on functions that were analyzed.

In conclusion, our studies enable us to predict that in humans, the BPZE1 strain is a good candidate vaccine; indeed it strongly activated the maturation of DC with full-blown activity, including the acquisition of a mature phenotype, resistance to apoptosis, and capacity to prime naive Th cells. Attenuation provides to BPZE1-challenged DC the capacity to survive death signals and migrate from the site of infection to the lymph nodes. This allows priming of Th cells and mounting of the adaptive immune response. BPZE1-committed DC have the ability to orchestrate a broad spectrum of protective, albeit proinflammatory, Th1/Th17 responses and Ts responses, which likely balance each other to restore local homeostasis.

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