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Immunomodulatory effects of Pidotimod in patients hospitalized for Community-Acquired Pneumonia

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ABSTRACT

Background: *Several studies have been made about pidotimod (PDT) and encouraging results have been collected. PDT is a synthetic dipeptide molecule that seems to have immunomodulatory activity on both innate and adaptive responses. Until now, the effects of PDT on the immune system have only been studied in vivo after long-term administration to evaluate whether its immunomodulatory activity might prevent the development of infections. This study was planned to evaluate the immunomodulatory activity of PDT administered together with standard antibiotic therapy in patients hospitalized for community-acquired pneumonia (CAP).*

Methods: *A total of 36 patients, including 20 children and 16 adults hospitalized for community-acquired pneumonia (CAP) were randomized at a 1:1 ratio to receive either standard antibiotics plus pidotimod (PDT) or standard antibiotics alone to evaluate the immunomodulatory activity of PDT. In Children blood samples for the evaluation of immunological parameters were draw at the time of recruitment before therapy administration (T0), at 3 and 5 days after the initiation of therapy (T3 and T5), and 7 days after the therapy ended (T21). While adults blood samples were taken at T0 before therapy administration, T1, T3 and T5 (respectively after 1, 3 and 5 days from the beginning of the therapy). Isolated PBMC were stimulated for 3 hours for gene expression analysis, and 18 hours for cytometric analysis.*

Results: *Following pneumococcal polysaccharide stimulation, in both groups, the percentage of dendritic cells (DCs) expressing activation and costimulatory molecules was significantly higher in patients receiving PDT plus antibiotics than in the controls. A significant increase in tumor necrosis factor-α and/or interleukin-12 secretion and expression of toll like receptor 2 was observed in PDT-treated children compared with controls. In adults results shown an increase of both TLR2 and TLR4, whereas no consistent effects of pidotimod on IL12 producing immune cells could be detected, TNFα producing monocytes and DCs were robustly reduced in pidotimod patients. In the PDT-treated groups, mRNA expression of antimicrobial peptides and genes involved in the inflammatory response were also augmented in comparison with the controls.*

Conclusions: *These results confirm that supplementation of antibiotic therapy with Pidotimod in patients with CAP results in a potentially beneficial modulation of innate immunity.*

SOMMARIO

Introduzione: *Il pidotimod (PDT) è una molecola dipeptidica di sintesi ad azione immunomodulante. Tale composto è studiato già da diversi anni con incoraggianti risultati a carico sia del sistema immunitario innato che adattativo. Studi in vitro ed in vivo ne hanno già dimostrato i benefici effetti nel trattamento di molte patologie. Col nostro studio abbiamo voluto porre l'accento sugli effetti del PDT associato alla terapia antibiotica standard in pazienti ospedalizzati per polmonite contratta in comunità (CAP).*

Metodi: *36 pazienti ospedalizzati per CAP, di cui 20 bambini e 16 adulti, sono stati arruolati nello studio secondo specifici criteri di inclusione. I pazienti sono stati suddivisi in due gruppi di trattamento, al primo è stato somministrato PDT associato alla terapia antibiotica standard, al secondo la sola terapia antibiotica. I campioni di sangue sono stati prelevati a diverse tempistiche, i bambini sono stati prelevati al momento dell'arruolamento (T0), a 3 e 5 giorni dall' inizio della terapia (T3 e T5), e a 7 giorni dalla fine della somministrazione farmacologica (T21), Gli adulti sono stati invece prelevati al momento dell'arruolamento (T0), e rispettivamente a 1, 3, e 5 giorni dall'inizio della terapia (T1, T3, T5). Le PBMC sono state isolate da sangue periferico e stimolate in presenza di pneumococco. Dopo 3 ore di stimolazione una parte delle cellule è stata utilizzata per l'estrazione dell'RNA per la valutazione dell'espressione di specifici geni coinvolti nella risposta antibatterica; mentre dopo 18 ore di stimolazione le restanti cellule sono state marcate per le analisi citofluorimetriche.*

Risultati: *I risultati ottenuti nei bambini hanno evidenziato un'azione del PDT su cellule denritiche, dove modula l'espressione di marker di attivazione e molecole costimolatorie quali HLA DRII, CD80 e CD86; e monociti, sui quali incrementa l'espressione del TLR2. Inoltre il gruppo trattato con PDT manifesta anche un'incremento nella secrezione di citochine proinfiammatorie TNFα e IL-12. Negli adulti è stato anche osservato un aumento nell' espressione del TLR4 a livello dei monociti, mentre nessuna differenza è stata osservata nella secrezione delle citochine proinfiammatorie. In aggiunta, in entrambi i gruppi si è osservata una modulazione nell' espressione di geni coinvolti nella risposta infiammatoria e antimicrobica.*

Conclusioni: *Questi risultati confermano gli effetti immunomodulanti del PDT già descritti in letteratura, nonché i benefici della molecola associata alla terapia antibiotica standard nei pazienti affetti da CAP, grazie alla sua azione a livello dell'immunità innata.*

LIST OF ABBREVIATION

ACIP: Advisory Committee on Immunization Practices
AECs: Airway epithelial cells
AMP: Antimicrobial peptide
APCs: Antigen presenting cells
ARTIs: Acute Respiratory Tract Infections
BCG-PSN: Bacillus Calmette-Guerin polysaccharide nucleic acid
B.I.D.: Bis in Day (twice a day)
BMDMs: Bone marrow-derived macrophages
CAP: Community-Acquired Pneumonia
CLRs: C-type lectin receptors
COPD: Chronic obstructive pulmonary diseases
CTLs: Cytotoxic T lymphocytes
CY: Cyclophosphamide
CRP: C-reactive protein
CWPS: Pneumococcal cell wall
D53: Ribomunyl
DCs: Dendritic cells
DS: Down syndrome
EDTA: Ethylenediaminetetraacetic acid
ERK1/2: Extracellular-signal-regulated kinase
GIT: Gastrointestinal tract
GM-CSF: Granulocyte-monocyte secretion factor
HBEC: Human bronchial epithelial cells
IL-1: Interleukine-1
IL-6: Interleukin 6
IL-8: Interleukin 8
IL-10: Interleukin 10
ILCs: Innate lymphoid cells
INF: Interferon
LPS: Lipopolysaccharide
LW 50020: Luivac
IECs: Intestinal epithelial cells
MENK: Methionine encephalin
MRSA: Methicillin-Resistant Staphylococcus Aureus
NF-kB: Nuclear factor-kappa B
NK: Natural killer cells

NLRs: (NOD)-like receptors
NTHi: Non typeable *H. influenza*
OM: Otitis media
OM-85 BV: Broncho Vaxom
PBS: Phosphate buffered saline
PCT: Procalcitonin
PPV23: Pneumococcal polysaccharide vaccine
PVC: Pneumococcal conjugate vaccine
PRRs: Pattern-recognition receptor
PMBL: Polyvalent mechanical bacterial lysate
PDT: Pidotimod
PSI: Pneumonia Severity Index
RLRs: RIG-I-like receptors
RT: Reverse transcriptase
RU 41740: Biostim
TH1: T helper type 1
TH2: T helper type 2
TH17: T helper type 17
TLRs: Toll-like receptors
TGFβ: Transforming growth factor-β
TNF: Tumor Necrosis Factor
TRM: Tissue-resident memory T cells
VBNC: Viable-but-non-culturable state
WBC: White blood cell
WHO: World Health Organization

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INTRODUCTION

1. RESPIRATORY INFECTIONS

Respiratory infections are the most common infectious diseases. They have been known and studied for many years, but the high incidence of hospitalization and the spread of resistant microorganisms make it necessary to find new preventive and therapeutic approaches. Furthermore respiratory infections are associated with high morbidity and mortality, especially in immunocompromised patients [1].

Acute Respiratory Tract Infections (ARTIs) are the most common diseases at the pediatric age [2]; indeed children are more susceptible to developing respiratory infections due to the vulnerability of their immune system [3]. In fact, it is estimated to be the cause of death in 19% of cases in children under five years. Moreover, ARTIs are the main cause of morbidity in industrialized countries. Data collected from the World Health Organization (WHO) showed that ARTIs are responsible for 20% of medical consultations, 30% of days lost from work, and 75% of antibiotic prescriptions [4].

Among the risk factors described for childhood, there are: contact with elder siblings, smoking at home, overcrowding, daycare centres and lack of breast feeding. However, even in a healthy population, in the absence of any risk factor, there is a subgroup of people with a higher incidence of ARTIs [4].

1.1 Community Acquired Pneumonia (CAP)

Community-Acquired Pneumonia (CAP) is a leading respiratory infection. Currently it represents one of the most common infections in children worldwide.

Risk factors are represented by a history of upper respiratory tract infections, poor socioeconomic conditions, passive smoke, asthma or other comorbidities and poor nutrition [5]. 90% of death due to pneumoniae occurs in adults over 65 years old. Other risk factors in adults are: alcoholism, immunosuppressive conditions, cardiovascular disease, cerebrovascular disease, chronic liver or renal disease, diabetes mellitus and dementia [6].

Children require hospitalization, and in some cases adults too. 2-14% of the hospitalized patients will die, and 12% will go in to relapse within 30 days of hospital discharge. Surprisingly, non-recurrent cases still are associated with a reduced survival rate. The reasons are unclear, probably it is due to other predisposing factors. CAP also has an important economic impact on society, both directly and indirectly [7].

1.2 Etiology

CAP is generally caused by intracellular bacteria, which varies from country to country. Intracellular bacteria associated with pneumonia are: *Legionella pneumophila, Mycoplasma pneumonia, Chlamydophila pneumoniae, Chlamydophila psitiacci, and Coxiella burnetii.* Generally, these pathogens are associated with extrapulmonary manifestations, but clinical features are characterized by classical bacterial agents such us *Streptococcus pneumonia,* and *Haemophilus influenziae* [8,9].

Staphylococcus aureus and *Klebsiella pneumonia* are associated with a smaller percentage of CAP, in particular, *K pneumoniae* has been associated with a higher incidence of mortality than *S. Pneumonia* [10].

1.2.1 *Legionella pneumophila*

Legionella pneumophila is a Gram-negative intracellular pathogen that causes atypical pneumonia, and presents some similar clinical and radiographic symptoms of *S. pneumonia* [11]. Several serotypes have been identified; the most common in Europe is serotype 1, which is responsible for 90% of pneumoniae cases [8].

Incubation period spans between 2 and 14 days. Then, the disease manifests itself with headache, myalgia, asthenia and anorexia.

The infection occurs through inhalation of a contaminated aerosol from lungs of infected people. *L. pneumophila* enters in alveolar macrophages where it first replicates. The presence of chronic lung disease increases illness susceptibility [11]. In nature, *L. pneumophila* lives in a water environment in low percentage, while it increases in human-made aquatic habitat, at 25-55°C. At these temperatures, *Legionella* is able to replicate in some protozoa, amoebae and slime moulds, thanks to the presence of biofilms. After an extended stay in water, *Legionella* enters a viable-but-non-culturable state (VBNC), and in this form it can survive for a long time and infect the host cell [11].

1.2.2 *Mycoplasma pneumonia*

Mycoplasma pneumoniae is one of the most common causes of atypical pneumonia, particularly prevalent in children and young adults, but it can produce also ear infections such as *otitis media* [12]. It may cause up to 35% of hospitalizations of CAP cases [13].

M. pneumoniae is an intracellular pathogen without cell wall structure, which confers resistance to β-lactam antibiotics [8]. It is a very small pathogen, so infection can occur between people in close contact via droplets, as a result of coughing.

M. pneumonia can infect both upper and lower respiratory tracts, and in some cases the infection can last very long time [13]. The incubation varies from 1 to 3 weeks, and furthermore, the spread of the pathogen is possible a long time after infection, prolonging the epidemic [8].

1.2.3 *Chlamydophila pneumonia*

Chlamydophila pneumonia is a widespread intracellular pathogen of respiratory tract infection. It is estimated that over a lifetime approximately everyone has entered in contact with the bacterium. 70% of respiratory infections due to *C. pneumonia* are asymptomatic, and 2,2-8% of CAP cases are caused by *C. pneumonia* [14]. Further, this kind of infection worsens the symptoms in chronic diseases, such as asthma and chronic obstructive pulmonary diseases (COPD) [9,14]. The incubation lasts about 3 weeks, and the infection occurs through inhalation of aerosol contaminated with bacteria. *C. pneumonia* is able to survive for a long time in quiescent phase which means that it can cause persistent infection. [8].

1.2.4 *Chlamydophila psitiacci*

Chlamydophila psitiacci is an atypical respiratory pathogen that produces psittacosis, a very dangerous lung disease [8].

Contagion is possible both by human–human and human-bird contact. Person-to-person transmission is uncommon, while birds are a very dangerous reservoir. In fact, veterinarians, bird breeders and animal shopkeepers are at high risk.

Transmission spreads through direct contact with infected birds, dried feces, plumage, respiratory secretions, the cloacae and conjunctiva secretions of infected birds [15,16].

1.2.5 *Coxiella burnetii*

Coxiella burnetii is a causative agent of Q fever. It is zoonotic and is mainly present in livestock but also in domestic animals.

Infections occur through contact with urine, feces and infected animal milk where the pathogen is excreted.

In animals the infections are asymptomatic, while, in humans, the bacterium causes acute Q fever with non-specific febrile illness pneumonia and hepatitis [17].

Q fever is most common in men, and there is a high incidence of CAP cases caused by Q fever [8].

1.2.6 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive bacterium, a member of micrococcaceae family [18]. It is an opportunistic pathogen, naturally present in human resident flora of the nasopharynx in 20% of healthy population [19] . It can produce acute infections; in fact, it is responsible for both hospital and community-acquired infections [20]. Healthy carriers have a higher risk of developing the infection because often, nasal *Staphylococcus* and infecting bacterium have the same genotype [19]. This pathogen is extremely versatile and it is able to express different kind of virulence factors that help the bacteria to elude the host immune system [21]. In particular, biofilm development is an important mechanism for defence against the host immune system. Biofilms hide the pathogen, masking the phenotype, so it can defend itself from external attacks such as antibiotic treatment and immune escape [22].

1.2.7 *Klebsiella pneumonia*

Klebsiella pneumonia is one of the most common Gram-negative bacilli, member of Enterobacteriaceae family [23,24]. It is an opportunistic pathogen and therefore represents a risk for immunocompromised patients, which often leads to hospitalization. *K. pneumonia* is naturally present not only in human resident flora of nasopharynx, but also colonizes the gastrointestinal tract and skin, and it is able to infect the urinary tract [24,25].

Virulence factors have a key role in severity of infections, and they are represented by capsular polysaccharide and type 1 and 3 pili. Capsule defends bacteria from phagocytosis, while Pili are involved in biofilm production, and this production is directly related to antibiotic resistance [24].

1.2.8 *Haemophilus influenzae*

Another commensal of both the upper and lower respiratory tract is *Haemophilus influenza*. It is a gram-negative catalase-positive coccobacillus. To date, 2 types are known: non-capsular and capsular polysaccharide; in particular, non-capsular types or non-typeable *H. influenza* (NTHi) include a wide variety of genotypes and phenotypes [26].

NTHi is implicated not only in the majority of chronic infections of the lower respiratory tract such as COPD and cystic fibrosis, but also is associated with respiratory tract infections such as *otitis media* (OM), conjunctivitis, sinusitis, and CAP [27].

1.2.9 *Streptococcus pneumonia*

Streptococcus pneumonia (Pneumococcus) is an encapsulated lanceolate Gram-positive, catalase-negative diplococcus, characterized by a surrounding polysaccharide capsule [28]. Moreover, the pneumococcus is a

static, asporigenous and facultatively anaerobic bacteria, that is alpha-hemolytic in the prensence of oxygen and beta-hemolytic in its absence [29].

Streptococcus pneumonia is naturally located in the upper respiratory tract [30]. It colonizes the nasopharynx and spreads horizontally causing different diseases such as *otitis media*, sinusitis and pneumonia. Pneumococci can migrate in the blood stream causing meningitis, peritonitis, skeletal and vascular infections [28,31].

Pneumococcal disease is preceded by asymptomatic colonization.

The host's immune system plays a key role in the pathogenesis; indeed, a low immune response is due to recurrent infection.

The production of inflammatory factors causes the transition from asymptomatic colonization to overt disease. These factors changes the type and number of receptors on target epithelial and endothelial cells, increasing affinity for pneumococcal proteins. The bonds between bacterial proteins and host cell receptors determine pneumococcal internalization, resulting in migration in respiratory epithelium and vascular endothelium [30].

Invasive Pneumococcal diseases are particularly common in younger children and in older adults [28,32].

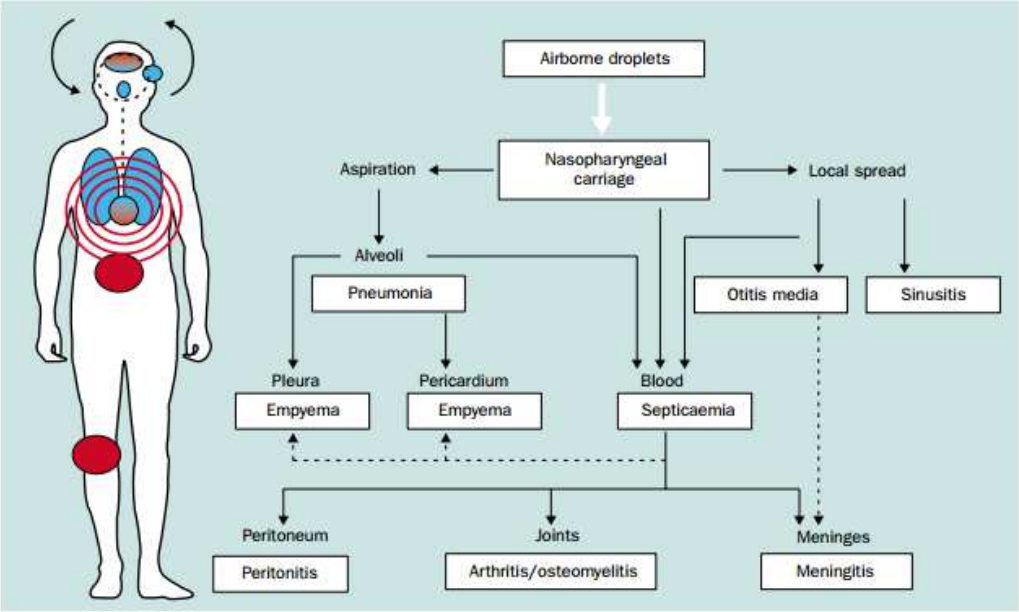

Fig 1 Pathogenetic route for *S. pneumoniae* infection
Organs infected by Pneumococcus through the airways. The infection may become systemic [30].

Virulence factors of *S. Pneumonia* not only cause damage to the host, but also help the pathogen to evade immune defenses. The main virulence factor consists in the polysaccharide capsule [32,33]. The structure of the capsule is composed of oligosaccharides chains, which in turn consist in monosaccharides structures containing from two to eight units [34,35]. After invasion it causes attachment to respiratory epithelial cells, and protects the pathogen thanks to the important antiphagocytic action on neutrophils [33]. Moreover it blocks activation of the alternative complement pathway. Different serotypes are characterized by different types of capsule, and this determine their different ability to cause invasive disease [28,34].

An important role in the pathogenesis is played by pneumococcal cell wall (CWPS). CWPS induces Interleukine-1 and Tumor Necrosis Factor

secretion by monocytes, causing inflammation, inducing activation of alternative complement pathway and mast cell degranulation [34]. Another important virulence factor is represented by Pneumolysin, an intracellular protein with toxic effects on epithelial cells, which stimulates the production of inflammatory cytokines and activates the classical complement pathway [34,35].

Pneumococcal infection induces phagocytosis activation through neutrophils and monocytes. Their recruitment occurs by intra-alveolar chemiotaxis. Pneumolysin secretion can inhibit phagocitosys [35]. Pneumococcal surface antigens induce antibodies secretion which in turn induce opsonization. Opsonization, besides inducing phagocytosis, activates the classical and alternative complement pathway [35].

Table 1. Pneumococcal virulence factors

Virulence factors	Activity
Polysaccaharide capsule	Attachment to respiratory epithelial cells, blocks activation of alternative complement pathway, evasion of host defense
CWPS	Causes inflammation, induces activation of alternative complement pathway and mast cells degranulation
Pneumolysin	Toxic effect on epithelial cells, induces inflammation, inhibits phagocitosys

2. IMMUNE RESPONSE

2.1 Immune responses and respiratory infections

Immune responses in respiratory tract have a key role in removal pathogens introduced by breathing. These dangerous inhalants need to be eliminated quickly by the immune system to avoid spreading of infection airways and inflammatory response.

In respiratory tract, the defense mechanism follows a stepwise program that ensures minimum necessary response against a microorganism. When a pathogen manages to enter in the airway, its initial detection by sensor cells that are localized at the site of infection, acting as the first tier of defense; the sensor cells promote innate immune responses to clear limited infections, and they release first-order cytokines that activate local tissue-resident lymphocytes, that include innate lymphoid cells (ILCs), innate-like lymphocytes, natural killer (NK) cells and tissue-resident memory T (TRM) cells. These lymphocytes, in turn, respond to first-order cytokine signals by producing second-order cytokines. At each stage of the process, effector mechanisms are also activated; these can potentially control the infection and thus prevent the activation of subsequent immune responses, limiting inflammatory damage [36].

2.1.1 Mucosal immune system

The respiratory system is composed of upper and lower respiratory tract and respiratory zone.

In respiratory system, four cell types produce physical and chemical barrier against infections: ciliated cells, mucus-secreting goblet cells, club cells and basal cells. Ciliated cells and mucus-secreting goblet cells create the barrier defense in the larger airways, whereas club cells and basal cells

function as regional progenitor cells to replenish the other cell types [37]. The defense mechanism is related to airway diameter [36].

The respiratory zone is formed by alveoli, among which alveolar type 1 cells facilitate gas exchange, while alveolar type 2 cells secrete pulmonary surfactant.

Alveolar macrophages reside in the airway space of the alveoli and within the mucus of the larger conducting airways. This keeps the respiratory mucosa in a state of quiescence. Indeed, alveolar macrophages constantly receive negative regulatory cues from airway epithelial cells (AECs).

Alveolar macrophages are regulated by the airway epithelium through their interactions with CD200 (OX-2 membrane glycoprotein), which is expressed by type II alveolar cells, with transforming growth factor-β (TGFβ) and with interleukin-10 (IL-10). Therefore, in the steady state alveolar macrophages function to clear particulates, apoptotic cells and cellular debris from the airways in order to maintain homeostatic tissue functions [36,38].

2.1.2 Activation of immune response in respiratory tract

Upon infection by virus, bacteria, protozoa or fungi, the negative regulatory signal due to CD200 and TGFβ are lost. The immune system is activated also by inhalation of allergens or venoms [36].

2.1.2.1 Antibacterial innate immune responses

Recognition of conserved features of microbial pathogens by the innate immune system is mediated by pattern-recognition receptors (PRRs), which detect conserved pathogen-associated molecular patterns (PAMPs). PRRs are expressed by different immunocompetent cells, among which phagocytic cells, natural killer cells (NK), dendritic cells (DCs). Several classes of PRRs have now been identified and characterized including Toll-

like receptors (TLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), cytosolic DNA sensors and nucleotide oligomerization domain (NOD)-like receptors (NLRs). Toll-like receptors (TLRs) belong to a family of transmembrane glycoproteins that serve as PRRs for a variety of microbe-derived molecules and stimulate innate immune responses against microbes expressing these molecules [39-41].

PRRs are characterized by their tissue-specific expression, they can be expressed on the cell surface, in intracellular compartments, or secreted into the blood stream, that determines two general modes of recognition by the innate immune system and immune responses: cell-intrinsic recognition and cell-extrinsic recognition. Principal functions of PRRs include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis [40,41].

Intracellular pathogens bind PRRs expressed intracellularly and induce immunological responses by activation of NF-κB and MAP kinase signaling pathways, which leads to the induction of the antiviral type-I IFN genes.

Cytosolic sensors detect viral RNA and viral DNA in infected cells [42].

Indeed, the detection of extracellular pathogens is mediated by PRRs expressed on the plasma membranes of macrophages, DCs and other cell types. PRRs express on the surface of immunocompetent cells are: TLR1, TLR2, TLR4, TLR5 and TLR6, and CLRs, including dectin-1, dectin-2 and mincle. These receptors recognize bacterial components and fungal cell walls, such as lipopolysaccharides, bacterial lipopeptides, lipoteichoic acids, flagellin, glycolipids and β-glucans.

Particularly, TLR2 and TLR4 are involved in antimicrobial responses.

TLR2 binds the largest number of bacterial PAMPs. After recognition, TLR2 forms heterodimers with TLR1 or TLR6. Instead, TLR4 binds LPS. The TLR signaling pathway is divided into MyD88-dependent and TRIF-dependent

pathways, but both TLR2 and 4 induces proinflammatory cytokine secretion.

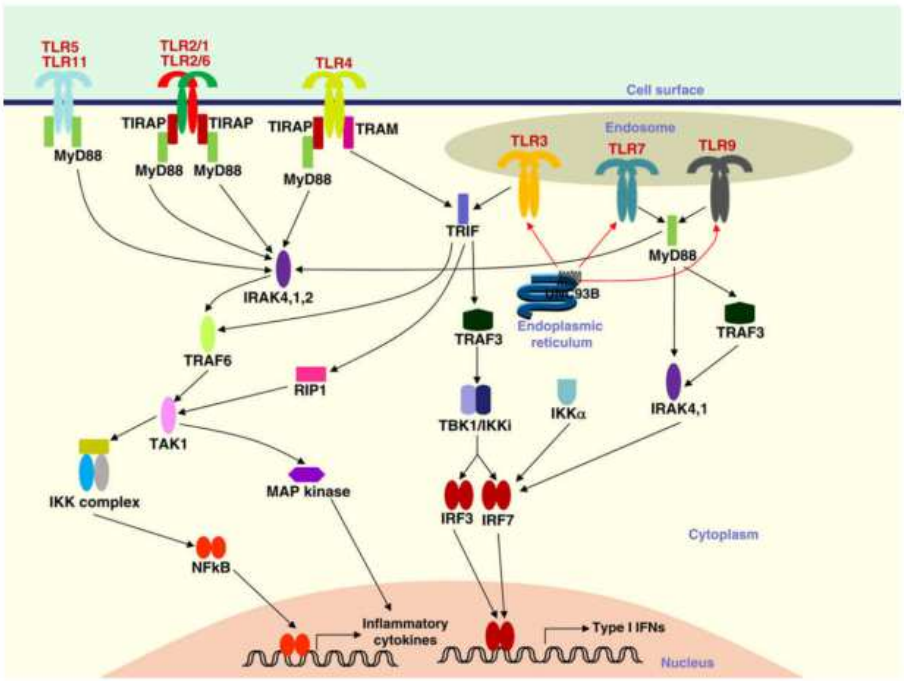

Fig 2 TLRs Activation

TLRs are activated from different bacterial structures. Their activation induces transcriptional factors activation producing pro-inflammatory cytokines secretion [39].

2.1.2.2 Antibacterial adaptive immune response

Adaptive immune responses to microbes induce effector cells that eliminate the microbes and memory cells that protect the individual from subsequent infections. CD4 helper T cells produce cytokines that stimulates B cell responses, antibody production, macrophage activation, and local inflammation. In addition to cytokine secretion, the adaptive immune response can also induce cell death through CD8+ cytotoxic T lymphocytes (CTLs) activation. CD4 T cells and CD8 T cells function cooperatively in defense against intracellular bacteria. Immune system against intracellular bacteria and protozoa requires responses by CD8+ T cells and T helper type 1 cells (TH1 cell), which develop as a consequence of the engagement of TLRs by PAMPs that leads to the production of IL-12 and TNF [40]. Against allergens and helminthes, CD4 differentiate into TH2 secreting IL-10, IL-4, IL-13; the first scenario leads to macrophage activation, the second their inhibition [43,44]. On the other hand, the TH17 subset of helper T cells is specialized in eliminating extracellular bacteria and fungal pathogens. The priming of TH17 cells begins with the engagement of CLRs such as dectin-1 and dectin-2 expressed by DCs, Langerhans cells and macrophages. In addition, the phagocytosis of bacteria-infected apoptotic cells induces the production of TGF-β and IL-6 by DCs, which promotes TH17 cell differentiation [41,45].

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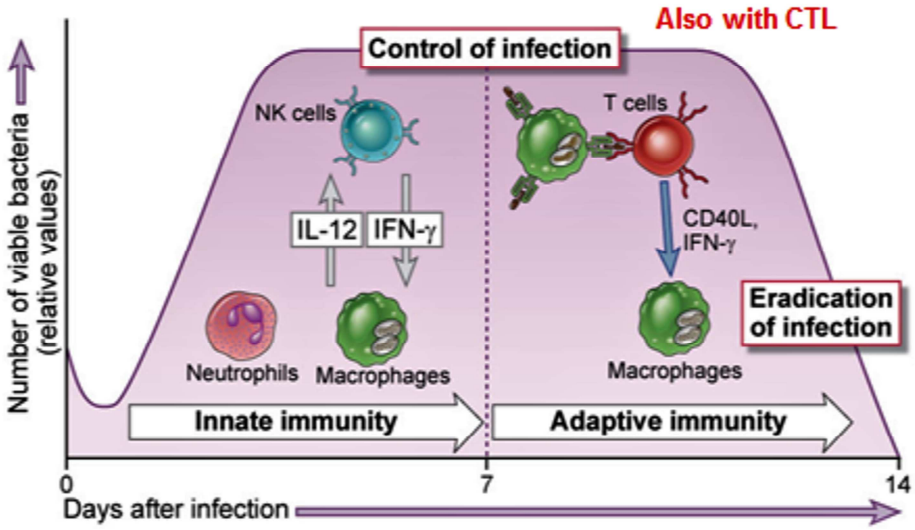


Fig 3 Innate and adaptive immunity to bacteria

Innate immunity is the first defense against infection, proinflammatory cytokines secretion induce adaptive immune response activation. (Abbas A. K, *Cellular and Molecular Immunology*. 2005)

2.2 Antibiotic treatment

Antibiotic treatment of CAP is chosen according to the causative pathogen and its antimicrobial resistance. In cases where it is difficult to identify the pathogen, it is recommended that antibiotic treatment should cover typical and atypical pathogens [8].

Mainly, intracellular bacteria are treated with macrolides, tetracyclines and quinolones. In particularly, severe cases of *M. pneumoniae* require corticosteroid, while for *Coxiella burnetii* doxycycline is the treatment of choice for acute Q fever [8].

The drug of choice for *S. aureus* infections remains penicillin. However, the main problem is represent by MRSA, in this case the drug used is vancomycin [18].

Klebsiella pneumophila is an important nosocomial bacterium. The main problem of *Klebsiella* infections is multidrug resistance, in particular to beta-lactam. Therefore it is necessary to find appropriate therapeutic options. Nowadays the recommended therapy consist in third and fourth generation of cephalosporins, which are more resistant to bacterium beta-lactamase [23].

Haemophilus influenzae pneumonia is generally treated with macrolides, in particular with azithromycin. Furthermore, also in *H. influenzae* antibiotic resistance due to β-lactamase production is very common. Therefore, the treatment of CAP patients with beta-lactams would not be effective [46].

In case of Pneumococcal CAP, the treatment of choice is beta-lactam-macrolide combinations or fluoroquinolone therapy [32][33].

2.3 Pneumococcal immunization strategies

Available anti-pneumococcal vaccines contain a mix of purified capsular polysaccharides. There are 2 types of vaccine: pneumococcal polysaccharide vaccine (PPV23), and pneumococcal conjugate vaccine (PVC) [33].

PPV23 is an unconjugated vaccine composed of pneumococcal capsular polysaccharides from 23 different serotypes [33]. This kind of vaccine is recommended from the Advisory Committee on Immunization Practices (ACIP) for several risk groups, such as children under 2 years of age or people aged between 2 and 65 years old with chronic illness, chronic cardiovascular disease or chronic pulmonary disease [28,30]. Vaccination strategy also involves the administration of PPV23 in older adults (65-79 years old) [7].

Conjugate vaccines are more recent, and there are several types. Pneumococcal conjugate vaccine 7 (PCV7) is recommended for pediatric

age groups, in particular infants under 23 months, but also in older children with higher risk such as immunocompromised patients or those affected by chronic disease [28]. The coverage by pediatric vaccine PCV7, has brought a decrease in cases of invasive pneumococcal disease also in adults [7].

These compounds activate some receptors that identify bacterial products or receptors that induce additional stimulation for activation, such as Toll-like receptors (TLRs) [4]. TLRs belong to a family of transmembrane glycoproteins that serve as pattern-recognition receptors (PRRs) for a variety of microbe-derived molecules and stimulate innate immune responses against microbes expressing these molecules [39,41].

Innate immunity acts as a sentinel of the immune system and it is activated after recognition of different pathogens. It comprises cells and mechanisms that defend the host from infection by other organisms in a non-specific manner, including physical barriers, complement cascade, phagocytic cells, natural killer cells (NK), dendritic cells (DCs) and cytokines [41].

These mechanisms that enhance the innate immune responses also stimulate the adaptive immune response [4].

Several studies have indicated that many infections are reduced after immunostimulant treatment [47]. Different kinds of molecules have been studied so far and they can be varied in nature.

3. IMMUNOMODULATORS

Immunomodulators are drug or substance able of stimulating immune response against infection. They try to mimic the immune responses normally induced by pathogen. This effect is important for their use in the

treatment of several disease, especially those related to a decrease in immune defenses. Nowadays, many immunostimulants are known and marketed.

Many studies in literature describe their beneficial effects in a great number of diseases, in particular, in a meta-analisis study, Del Rio *et al*. evaluated that immunostimulant reduce the incidence of ARTIs by 40% in susceptible children [4].

The action of immunomodulant on innate and adaptative immune system, make them suitable as adjuvant in the antibacterial and antiviral therapy, because modulate both local and systemic inflammatory responses to improve patient outcome to the treatment [44].

They can be of various nature: naturals or synthetics or of bacterial origin, and each is particularly indicated for the treatment of specific pathological condition.

3.1 Bacterial-derived immunomodulators

Bacterial immunomodulators can contain killed bacteria or bacterial lysate; these compounds mimic the intrusion of a pathogen into the body, activating both innate and adaptive immunity [48].

Bacterial antigens reach Peyer's patches in Gut lumen, where they induce dendritic cells (DCs) activation, increase phagocytosis and B and T cells stimulation. Simultaneously they cause humoral response through cytokine secretion [48].

Among bacterial immunomodulators, different compounds are known.

- Broncho Vaxom (OM-85 BV) is a bacterial immunomodulator composed of lysates of 8 of the most common bacteria strains in upper respiratory tract infection, including *Haemophilus influenza*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Klebsiella ozeanae*, *Staphilococcus*

aureus, *Streptococcus viridans*, *Streptococcus pyogenes*, and *Neisseria catarrhails* [48,49]. It regulates innate immunity by acting on macrophages, neutrophils and proinflammatory cytokines production [48], while it acts on acquired immune response by stimulating lymphocytes proliferation and immunoglobulin synthesis [49]. Luan *et al.* demonstrated that OM85-BV stimulated IL-1β, IL-6, and TNF-α production. IL-1β, IL- 6, and TNF-α are critical inflammatory cytokines predominantly produced by macrophages, and have pleiotropic effects on regulating the immune response and acute-phase reaction [49].

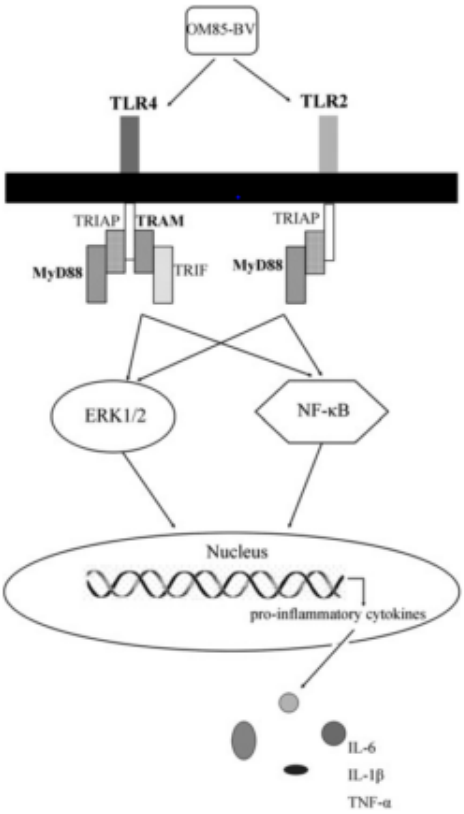


Fig 4 Broncho-Vaxom: mechanism of action
OM-85 induces proinflammatory cytokine secretion through TLR2 and TLR4 activation [49].

- Luivac (LW 50020) is a bacterial lysate immunomodulator consisting of several bacteria that typically cause respiratory infections such as *Staphylococcus aureus, Streptococcus mitis*, *Streptococcus* pyogenes, Streptococcus *pneumoniae*, *Klebsiella pneumoniae*, *Moraxella (Branhamella) catarrhalis*, *Haemophilus influenza*.

This immunostimulant induces T lymphocytes proliferation, and causes an up-regulation of numbers of CD4 and CD8 and memory cells . As regards cytokinesecretion, [48]. Animal studies also demonstrated that oral

administration of LW 50020 resulted in increased production of specific IgA [50].

- Polyvalent mechanical bacterial lysate (PMBL) is a mix of different bacteria, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Hemophilus influenzae*, *Moraxella catarrhalis* and *Klebsiella pneumoniae*. The main action of this compound is observed in maturation of both dendritic cells (DCs) and plasmacytoid DCs. Furthermore, it induces secretion of interleukin 2 (IL-2), interleukin 10 (IL-10), interleukin 12 (IL-12) and interferon gamma (IFN-γ) [51]. Ricci *et al.* reported that treated patients were able to avoid not only infectious disease associated to the microbes present in the administered mixture, but also other infections, such as virus infections [51].

- Ribomunyl (D53) is a ribosomal extract containing immunogenic ribosomes of four bacteria responsible for recurrent respiratory infection: *Klebsiella pneumoniae, Streptococcus pneumoniae, Streptococcus pyogenes* and *Haemophilus influenza*. Ribosomal components are associated with the proteoglycans from cell membrane of *Klebsiella pneumoniae.* D53 stimulates lymphocyte, natural killer (NK) and DCs. Particularly on DCs, D53 up-regulates CD83, CD86 and HLA II expression [48,52]. D53 increases the production of specific antibody against each of the germs for which the ribosomes were present in D53. Pujol *et al.* demonstrated that D53 stimulates mouse spleen cells to produce IL-1, induces differentiation of mouse B cells into immunoglobulin-secreting cells, and enhances NK activity [52].

- Biostim (RU 41740) is an immunomodulator composed of glycoproteins complex extract from *Klebsiella pneumophila*. The compound stimulates

the activity of phagocytic cells and increase antibody secretion by B cells [53].

In vitro studies conducted by Aloui *et al.* have also shown its effect on human bronchial epithelial cells (HBEC), where it induces secretion of granulocyte-monocyte secretion factor (GM-CSF), IL-1, IL-6 and IL-8 [54].

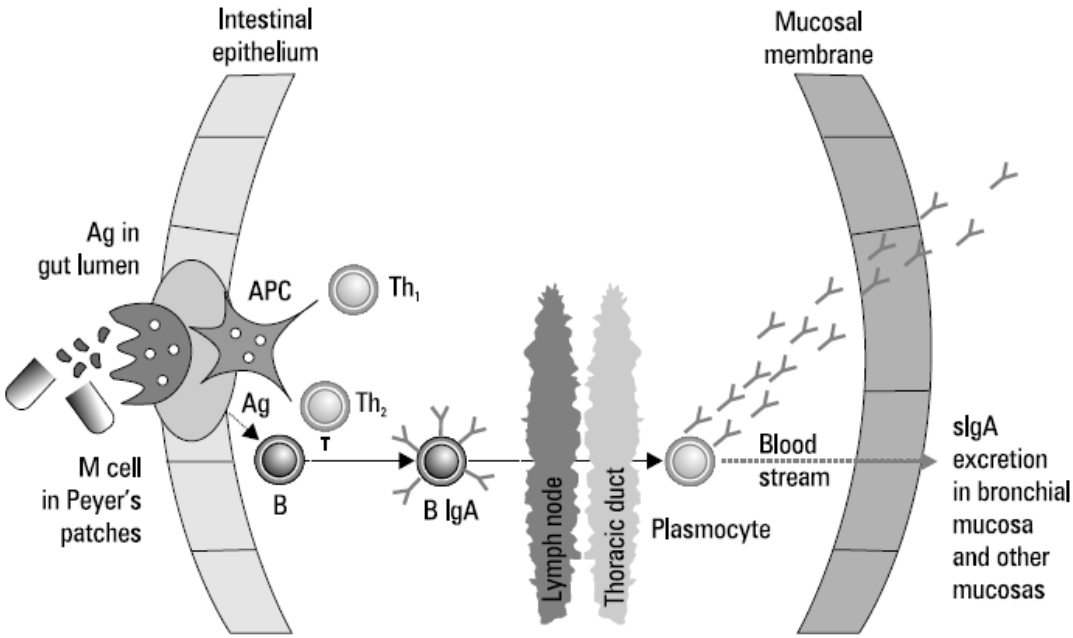


Fig 5 Mechanism of action of the oral immunostimulators [48]**.**

3.2 Natural substances

Natural immunomodulators are compound normally present in nature. They can be extracted from the plants or introduced with alimentation. Among these, **Pleuran** is an insoluble β glucan isolated from *Pleutorus ostreatus*. Its immunomodulatory properties render the host more resistant to infections. Pleuran treatment induces physiological maturation of the humoral immune response and an increase of NK cell number [55]. Results of Jesenak *et al.* showed a significant decline in respiratory morbidity, including a decrease in the number of RRTIs in the treated group compared to placebo group. In particular, they observed significant result after several months from the beginning of the therapy. They observed an increase of IgG and IgM in treated group of children, that is maintained for all duration of the study [55].

- Vitamin D influences innate immune function through a number of mechanisms that include production of antimicrobial peptide (AMP) and of intracellular reactive intermediates. It acts on the maturation of DCs, and induce B-cell differentiation and proliferation. It has also been found to affects T-cell differentiation, shifting polarization to preferentially TH2 cell development (with IL-4, IL-5, and IL-10 production) [56]. Hoe *et al*. investigated the effects of 1,25(OH)2D3 (Vitamin D) on *ex vivo* PBMCs stimulated with Gram-positive (pneumococci) or Gram-negative (LPS) bacterial ligands. In the study, they found that pre-treatment with 1,25(OH)2D3 reduced the levels of TNF-α, IFN-γ, IL-1β, and IL-8 in supernatants in response to heat-killed pneumococcal serotype 19F (HK19F) and LPS stimulation, while only TNF-α and IL-1β were reduced in monocytes [57].

- Probiotics including Lactobacillus and Bifidobacterium, produce different types of antibacterial substances and stimulate host immune system through diverse mechanisms. They interact with intestinal epithelial cells (IECs), mucosal dendritic cells (DCs) and macrophages through diverse way. Pattern recognition receptors (PRRs) including Toll-like receptors, (TLRs) play essential roles in recognition and delivery of signaling cascades, which mediate different gene expression profiles [58]. The anti-inflammatory effect of probiotics is generally attributed to their direct interactions with pathogenic microbes or their cross-talk with host cells. Castillo *et al.* showed effects of *L. casei* administration in mice infected by *Salmonella*. Probiotics administration reduced mortality in infected mice. Post infection, they analysed pro-inflammatory cytokines production in cells isolated from Peyer's patches. After infection, treated mice increased TNF-α and IFN-γ. At day seven and ten post infection, the probiotic administration was able to maintain this production. The same trend was maintained from IL-6 and IL-10. Furthermore, they analysed also probiotic effects on TLRs expression. Results showed that treated mice increase TLR2, TLR4, TLR5 and TLR9 expression after seven days of infection [59].

3.3 Synthetic compounds

The synthetic immunomodulators are completely alien to the natural world, they are obtained by chemical synthesis from industrial processes.
Several studies demonstrated their safety and absence of adverse effects.

3.3.1 Pidotimod

Among synthetic immunostimulators Pidotimod has long been studied.
Pidotimod (3-L-pyroglutamyl-L-thiaziolidine-4 carboxylic acid) is a synthetic dipeptide molecule with immunological activity.

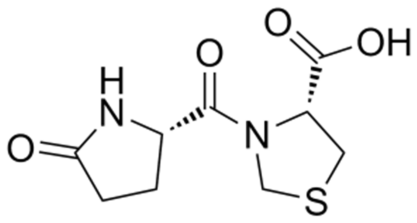


Fig 6 Pidotimod structure

In vivo and *in vitro* studies show that Pidotimod (PDT) acts on both adaptive and innate immunity [60]. Pidotimod is rapidly absorbed by the gastrointestinal tract with a bioavailability of 45%. The plasma half-life is four hours with poor metabolism and renal elimination of the unmodified molecule [61]. Giagulli *et al.* have shown by in vitro studies that PDT induces dendritic cells (DCs) maturation, up-regulates the expression of HLA-DR and of co-stimulatory molecules such as CD83 and CD86, stimulates DCs to release pro-inflammatory molecules driving T-cell proliferation and differentiation towards TH1 phenotype, enhances NK cell functions and promotes phagocytosis [62]. Studies carried out on mouse bone marrow cells, have instead demonstrated that PDT facilitates M2 polarization of bone marrow-derived macrophages (BMDMs) [63]. A second study about BMDMs evaluated synergic effects of PDT and methionine encephalin (MENK) on activation and maturation. When treated with both of them, DCs presents more maturation than those treated with either MENK or PTD alone. Costimulatory molecules CD80, CD86, CD40, followed by

antigen-presenting molecules MHC-II and CD83, increased significantly [64]. DCs have a key role in immune response; indeed, they are potent antigen presenting cells (APCs) with macrophages and B lymphocytes. APCs present antigens to T lymphocytes activating humoral response.

Pidotimod also has effects on different transcription factors; in fact on extracellular-signal-regulated kinase (ERK1/2) and nuclear factor-kappa B (NF-kB), PDT increases the expression of toll-like receptors (TLRs) [65,66]. TLR response also involves mechanisms of acquired immunity, therefore playing a key role in linking innate and adaptive immune responses.

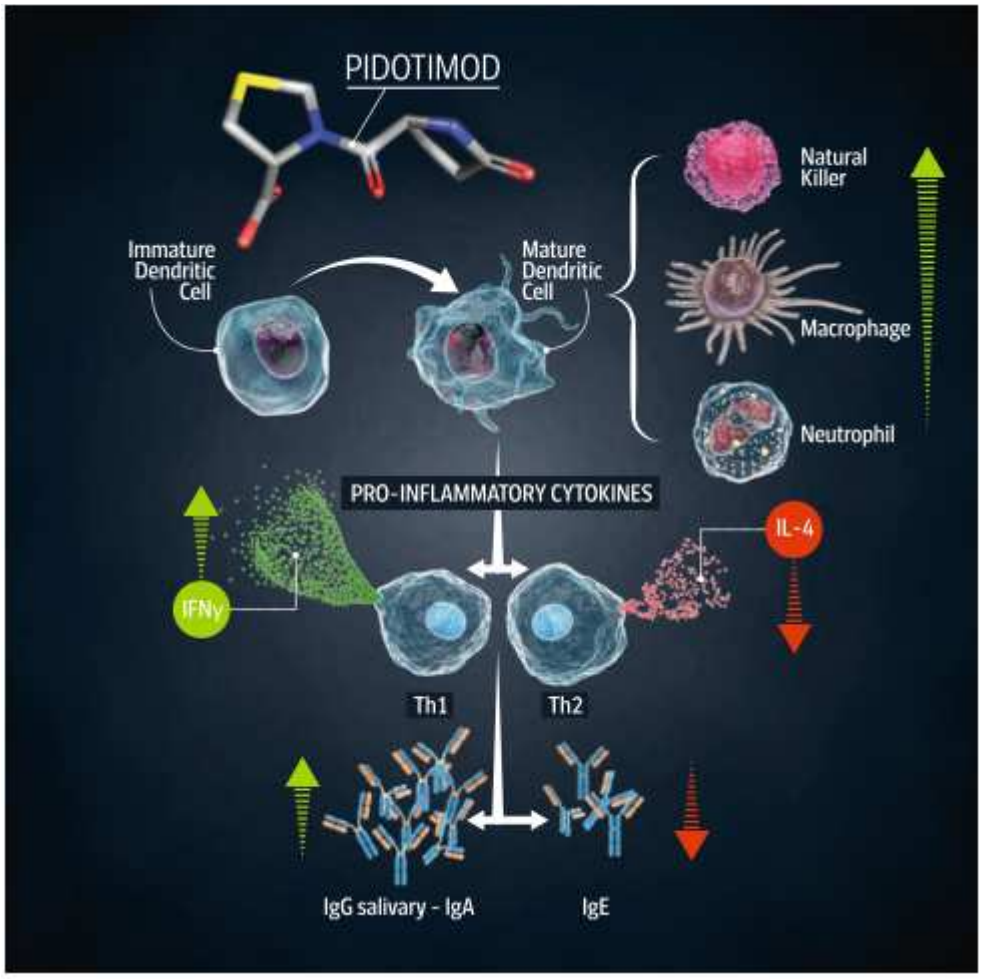


Fig 7 PDT mechanism of action
PDT acts on both, innate and adaptive immunity, activating DCs and proinflammatory cytokines secretion [60].

Zuccotti *et al.* have described PDT effects in children with Down syndrome (DS).

In DS there is a reduction of circulating B-cells, a decrease of CD4 T lymphocytes and an increase of CD8 T cells. Therefore, these children are more susceptible to developing ARTIs. Furthermore, in children with Down's syndrome, up to the age of 18, RTIs are the second leading cause of death [67]. PDT administration reduces the rates of ARTIs in children with DS [2]. Data obtained from Mantia *et al.* demonstrated beneficial effects of pidotimod also in prophylaxis on upper respiratory tract infections in children with DS. In this study, subject treated with pidotimod showed a statistically significant improvement in mucosal hyperemia, nasal secretions and nasal respiratory obstructions [68].

Mameli *et al.* have observed PDT effects in children with predisposing factors of ARTI. Statistical analysis showed a 22% reduction of the rate of ARTIs in children who received PDT compared to those who received a placebo [3]. Pidotimod effects were described in several diseases. Li *et al.* investigated the status of T lymphocytes CD4, CD8, and B lymphocytes NK cells, and the differential secretion of the inflammatory interleukin-6 (IL-6) and interleukin-8 (IL- 8) and tumor necrosis factor-α (TNF-α) in children with Tourette's syndrome (TS). They hypothesize that there is an association between TS and streptococcal infection, therefore an immune therapy may help improve immune dysregulation in these patients. Patients with TS had decreased numbers of CD4+ T cells with a depressed CD4+/CD8+ ratio. By contrast, the numbers of NK cells were increased. Furthermore, they found reduced expression of both IL-6 and IL-8 while the level of TNF-α was increased.

Following 8 weeks of treatment with pidotimod, it was observed that the numbers of T lymphocyte subpopulations and NK cells returned to a more

normal frequency.. Serum levels of pro-inflammatory IL-6 and IL-8 also increased, but still lower than healthy controls [69].

PDT could be a promising treatment for many immunological diseases due to its immunomodulatory properties. Jin *et al.* presented a case report of oral lichen planus treated with PDT.

Oral lichen planus is an immunological disease in which T lymphocytes produce cytotoxic activity against epithelial cells. In the case report the lesion and his symptoms improved remarkably after oral administration of orally PDT 0.4 g twice a day for 1 month, followed by intramuscular injection of bacillus Calmette-Guerin polysaccharide nucleic acid (BCG-PSN), 1 mL, every other day for another month to improve immune function [70].

Huo *et al.*, instead, showed PDT effects on toxoplasmosis prevention. They used a murine model of reactivated toxoplasmosis induced by cyclophosphamide (CY) and investigated the efficacy of pidotimod as an oral preventive agent. Results showed that PDT treatment significantly alleviated the parasitemia induced by the immunosuppressive agent. Thus, the beneficial efficacy of pidotimod administration may prevent the viral reactivation [71].

Data described in literature suggest a promising use of PTD as adjuvant in the vaccines. In particular Zhao *et al.* studied pidotimod effects as an adjuvant on UV-attenuated *Toxoplasma gondii*. Results indicate that co-administration of pidotimod can further enhance the potency of UV-*T. gondii* vaccine [72].

Di Renzo *et al.* evaluated *in vitro* effect of Pidotimod on some cellular immune responses in neoplastic patients. Cancer patients showed an impairment in lymphomonocyte IL-2 production in response to the same mitogens. Reduced IL-2 production might be important because it might contribute to the cancer spreading, especially affecting LAK and NK cells,

which are involved in the immune response against tumors. The *in vitro* addition of Pidotimod increased IL-2 production, However, this data suggest that Pidotimod might be used as an adjunctive immunotherapy in cancer patients [73].

AIM

Several attempts to improve immune function in young children and adults have been made using alternative medicines (e.g., plant preparations), dietary supplements (e.g., vitamins C and D, zinc, cod liver oil and polyunsaturated fatty acids), and other preparations such as inosine pranobex, probiotics, bacterial lysates and pidotimod (PDT). In only a few cases, scientific evidence of positive effects was found. However, encouraging results have been collected with PDT treatment, a synthetic dipeptide molecule (3-l-pyroglutamyl-l-thiazolidine-4carboxilic acid) that seems to have immunomodulatory activity on both innate and adaptive responses. Higher expression of TLR2 proteins, induction of dendritic cell maturation accompanied by an increased release of pro-inflammatory molecules, upregulation of the expression of HLA-DR, stimulation of T lymphocyte proliferation and differentiation toward a TH1 phenotype, inhibition of thymocyte cell death and promotion of phagocytosis have all been demonstrated in *in vitro* studies in both animal and human subjects [66,73,74]. Moreover, *in vivo* studies have demonstrated that long-term prophylactic use of PDT can be of benefit in children with RRTIs, reducing the total number of new infectious episodes and the consequent use of drugs, including antibiotics [75]. A recent Italian study showed that PDT treatment 400 mg/day for 2 months was able of significantly reducing the number of children with upper and lower airways symptoms, and medications use, increasing school attendance, and reducing pediatric visits for RRTIs [76]. Finally, in subjects with Down syndrome, it was found that the response to the influenza vaccine administered at the beginning of a 90 day-PDT course was different from the response in untreated children, suggesting a preferential activation of effector mechanisms and a potential beneficial effect of immunization [2]. So far, the effects of PDT on the immune system have only been studied *in vivo* after long-term administration to evaluate whether its immunomodulatory activity might

prevent the development of infections. No data are available on the immunological impact of PDT when given during an acute disease. Information regarding this could be useful to understand whether this drug could positively influence the clinical course of an acute infection.

This study was planned to evaluate the immunomodulatory activity of PDT administered together with standard antibiotic therapy in children and adults hospitalized for community-acquired pneumonia (CAP).

MATERIALS ANDMETHODS

1. STUDY DESIGN

1.1 Pediatric patients

A randomize double-blind controlled study was conducted in 20 pediatric patients from the Pediatric Highly Intensive Care Unit of the University of Milan, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy between November 1, 2013 and April 30, 2014.

It was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy.

Written informed consent was obtained from the parents or legal guardians of all study participants, and in the case of patients aged ≥7 years, written consent from the children was also collected.

Children enrolled in the study were children aged 3-14 years with clinical signs such us tachypnea and abnormal breath sounds and a chest radiograph consistent with uncomplicated CAP. Complicated CAP was defined as the presence in a chest radiograph of more than one of the following conditions: parapneumonic effusion, defined as loculated pleural fluid; any pleural fluid parameters consistent with empyema; atelectasis; and necrotizing pneumonia [77]. The CAP diagnoses were all confirmed by chest radiographs evaluated by an independent expert radiologist who classified the findings as alveolar pneumonia, non-alveolar pneumonia or no pneumonia in accordance with the World Health Organization criteria for the standardized interpretation of pediatric chest radiographs for the diagnosis of pneumonia [78].

Upon enrollment, detailed information regarding children's demographics, clinical history and the characteristics of the disease were collected together with a blood sample for the evaluation of laboratory variables, including white blood cell (WBC) counts, C-reactive protein (CRP) and

procalcitonin (PCT) levels. A portion of the blood sample obtained at the time of recruitment (T0) (i.e., before therapy administration) was also used for the immunological analysis described below. The enrolled children were randomly assigned in a 1:1 ratio and according to a computer-generated list to receive either standard antibiotic therapy with cefotaxime (100 mg/kg/ day in 3 daily doses, i.v.) plus clarithromycin (15 mg/kg/ day in two daily doses, orally) (control group) according to the guidelines for the treatment of pediatric CAP prepared by the Italian Society of Pediatrics [79] or the same antibiotics plus PDT (Axil®, 400 mg/day in two daily doses, orally) (PDT group). In both groups, cefotaxime was administered for 4 days and then followed by amoxicillin-clavulanate (80 mg/kg/day in 3 daily doses, orally, for 6 days), and clarithromycin was administered at the same dosage for 14 days; in the PDT arm, PDT was given at the same dosage for 14 days. Blood samples for the evaluation of immunological parameters were drawn at T3 and T5 (i.e., 3 and 5 days after the initiation of therapy) as well as at T21 (i.e., 7 days after the therapy ended). All of the information was registered in an electronic database.

Table 2 Main demographic, clinical and laboratory characteristics at baseline in 20 children hospitalized for CAP according to the use of pidotimod plus antibiotics or antibiotics only [80]**.**

Characteristic	All children (n=20)	Pidotimod+ antibiotics (n=10)	Antibiotics only (n=10)	p-value
Demographcs				
Age (yars)				
Mean (SD)	4.6 (1.9)	4.4 (2.0)	4.7 (1.8)	0.70[a]
Sex (%)				
Male	14 (70)	7 (70)	7 (70)	
Female	6 (30)	3 (30)	3 (30)	1
Etnitiсy (%)				

Caucassian	17 (85)	8 (80)	9 (90)	
Non-Caucasian	3(15)	2 (20)	1 (10)	0.99[b]
Parenthal smoking habit[b] (%)				
Both non-smokers (%)	13 (65)	6 (60)	7 (70)	
At least one smoker (%)	7 (35)	4 (40)	3 (30)	0.99[b]
Exclusive breastfeeding≥3 months[c]				
Yes (%)	11 (58)	4 (40)	7 (78)	
No (%)	8 (42)	6 (60)	2 (22)	0.17[b]
Diagnosis of allergy (%)				
Yes	18 (90)	9 (90)	9 (90)	
No	2 (10)	1 (10)	1 (10)	1
Respiratory infections in the previous 6 months (%)				
At least one	13 (65)	5 (50)	8 (80)	
None	7 (35)	5 (50)	2 (20)	0.35[b]
Use of antibiotics for respiratory infection in the previous 6 months (%)				
Yes	4 (20)	2 (20)	2 (20)	
No	16 (80)	8 (80)	8 (80)	1
Clinical characteristics				
Fever (>=37.8°)[c] (%)				
Yes	8 (42)	5 (56)	3 (30)	0.37[b]
No	11 (58)	4 (44)	7 (70)	
Cough[c] (%)				
Yes	18 (95)	8 (89)	10 (100)	
No	1 (5)	1 (11)	0 (0)	0.47[b]
Dyspnea[c] (%)				
Yes	4 (21)	2 (22)	2 (20)	
No	15 (79)	7 (78)	8 (80)	0.99[b]
Rhonchi (%)				
Yes	1 (15)	1 (10)	0 (0)	
No	19 (95)	9 (90)	10 (100)	0.99[b]
Rales (%)				
Yes	16 (80)	7 (70)	9 (90)	
No	4 (20)	3 (30)	1 (10)	0.58[b]
Wheezes (%)				
Yes	4 (20)	3 (30)	1 (10)	
No	16 (80)	7 (70)	9 (90)	0.58[b]
SpO2<92% (%)				
Yes	7 (35)	2 (20)	5 (50)	
No	13 (65)	8 (80)	5 (50)	0.35[b]
Laboratory results				
White blood cell count (cells/µL)				
Mean (SD)	13.154 (6.512)	13.176 (6.708)	13.132 (6.672)	0.97[a]
CRP (mg/L)				
Mean (SD)	5.95 (6.58)	5.82 (5.02)	6.07 (4.25)	0.82[a]
PCT (ng/mL)				
Mean (SD)	0.40 (0.84)	0.42 (0.89)	0.38 (0.84)	0.96[a]

1.2 Adult patients

This pilot multicenter randomized double-blind study was conducted in 16 adult patients with a diagnosis of pneumonia enrolled from two major hospitals in Milano: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, and University of Milan Ospedale San Paolo.

Patients (11 males, and 5 females mean age 56.6 ± 7.7 years) affected by CAP with a pneumonia severity index (PSI) score III or IV and/or a CURB-65 0-2, were included in the study.

Exclusion criteria included: a) hospitalization in the previous 15 days; b) a diagnosis of active tuberculosis or infection with fungi; and c) a condition of immunosuppression, including HIV infection, neutropenia, immunosuppressive therapy, chemotherapy, transplantation, cytotoxic therapy, and chronic systemic steroid therapy. The following data were recorded: demographics; past medical history; severity of symptoms on admission; pneumonia severity index (PSI) and CURB-65 score; physical, laboratory, and radiological findings on admission; microbiological data; empiric antibiotic therapy; time to clinical stability, in-hospital mortality. Nine patients were randomized to the active arm and treated with PDT (Axil® 800mg, 2 daily doses for 10 days) in combination with the standard antibiotic treatment (Levofloxacina 500mg b.i.d); seven patients were randomized to receive the standard antibiotic therapy (Levofloxacina 500mg b.i.d). Immunological analyses were performed at different time points: T0 (at recruitment, before therapy administration) as well as T3 and T5 (3 and 5 days after therapy initiation).

2. SAMPLE COLLECTION

2.1 PBMC isolation

Whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (ethylenediaminetetraacetic acid) (Becton Dickinson, Rutherford, NJ, USA). Samples were centrifugated 1400 rpm for 10 minutes, plasma obtained was collected and stored at -20°C for subsequent analysis.

The remain sample, composed by erythrocytes, PBMCs, granulocytes, and platelets, was diluited in PBS (Phosphate buffered saline) (PBI International, Milano, Italy) and separated thanks to a density-gradient centrifugation on lymphocyte separation medium (Ficoll-Hypaque) (Cedarlane Laboratories Limited, Hornby, Ontario Canada) for 25 minutes at 2300 rpm. PBMCs are localized between the phase of fycoll-hypaque and the phase including granulocytes and eritrocytes, they are collected and washed twice in PBS for 10 minutes at 1900 rpm. Cellular pellet was washed twice in PBS, and cell number and cellular vitality were determinated using an automatic cell counter.

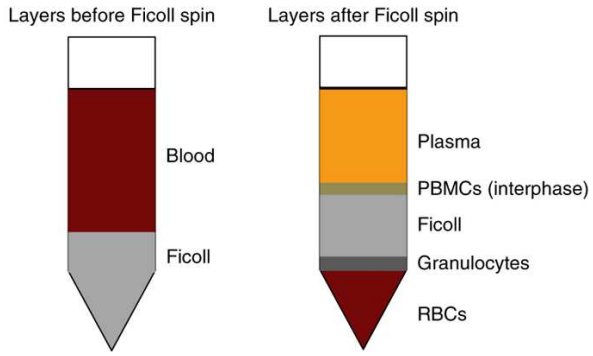


Fig.8 PBMCs separation [81]**.**

2.2 Cell count

Cell count was performed with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc., Corea). ADAM-MC automatic cell counter measures total cell numbers and cell viabilities by cutting edge detection technologies. Instead of tryphan blue staining, which can lead to inaccurate data, ADAM-MC utilizes two sensitive fluorescence dye staining solution, AccuStain Solution T (Propidium Iodide/lysis solution) and AccuStain Solution N (Propidium Iodidel/PBS). AccuStain Solution T allows plasma membrane distruption and nucleus staining for measurement of total cell concentration. AccuStain solution N allows stain of non-viable cells, thus leaving viable cells completely intact. A 532 nm optic laser is automatically focused onto the cell solution inserted into a disposable microchip and cell analysis is made by a CCD detection technology.

The image results were automatically processed, generating the count of non-viable and viable cells and the percentage of viability, which was displayed on the front of the instrument.

3.4 PBMC stimulation

PBMCs were incubated for 18 either without any stimulus, or in the presence of a mix of 8 main Pneumococcal polysaccharides normally present in the vaccine (10µl/ml) (ATCC® Pneumococcal polysaccharide type 23, 4, 14, 9, 57, 6A, 3, 5, LGC Standards, Milan, Italy) or of the lipopolysaccharide (LPS Sigma-Aldrich, St. Louis, MO, USA) (2µg/ml). Anti-CD 28 antibody (R&D Systems, Minneapolis, MN, USA) was added during incubation (2 µg/mL) to facilitate costimulation.

After 3 hours-stimulation, RNA was extracted from PBMCs by acid guanidinium thiocynate-phenol-chloroform method. For cytokine analysis, 10 µg/ml Brefeldin A (Sigma-Aldrich) was added to cell cultures. After 18

hours-stimulation, PBMCs were stained for immunophenotype and intracellular cytokines secretion analyses performed by flow cytometry.

3. FLOW CYTOMETRY ANALYSIS

All flow cytometry analyses were performed using a Gallios flow cytometer (Beckman-Culter, Miami, FL, USA) equipped with a double 15-mV argon ion laser with a wavelength of between 488 and 638 nm, interfaced with an Intercorp computer. Green fluorescence from FITC (FL1) was collected through a 525 nm band-pass filter; orange-red fluorescence from PE (FL2) was collected through a 575 nm band-pass filter; Texas red fluorescence from ECD (FL3) was collected through a 620 nm band-pass filter; red fluorescence from PECy5 (FL4) was collected through a 695 nm band-pass filter; far red fluorescence from PECy7 (FL5) was collected through a 755 nm band-pass filter. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1, FL2, FL3, FL4 and FL5. Samples were first run using isotype controls or single fluorochrome-stained preparations for color compensation. For each analysis, 30,000 events were acquired and gated for CD14 and CD11c expression and SSC properties.

3.1 TLR expression on monocytes

After 18 hours of stimulation, PBMCs were resuspended in PBS and stained for surface mAb CD14 PECy5, TLR4 PE, TLR2 FITC (Beckman-Coulter, Fullerton, CA, USA). After 15 min incubation at room temperature in the dark, cells were washed and fixed in 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS.

3.2 Dendritic cell maturation

Stimulated PBMCs were resuspended in PBS and stained for surface mAb CD11 PECy7, HLA-DR II PECy5, CD86 PE, CD80 FITC (Beckman-Coulter, Fullerton, CA, USA) for 15 min at room temperature in the dark. PBMCs were washed and fixed in 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS.

3.3 Intracellular cytokine expression

After stimulation, PBMCs were washed in PBS and stained for CD14 PECy5, and CD11 PECy5 (Beckman-Coulter, Fullerton, CA, USA). After 15 min incubation at room temperature in the dark, cells were washed and fixed in 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Cells were then permeabilizated with saponin 0.5% (Sigma-Aldrich, St. Louis, MO, USA) and stained for TNF-α PE and IL12 FITC (eBioscence, San Diego, CA, USA). After 45 minutes incubation at 4°C in the dark, cells will be washed and fixed in 1% paraformaldehyde in PBS.

4. GENE ANALYSIS

4.1 RNA extraction

RNA was extracted from $1x10^6$ cultured PBMCs by using acid guanidium thiocyanate-phenol-chlorophorm metod. RNAzol B reagent (Duotech, Milan, Italy), a monophase solution containing phenol and guanidine thiocyanate, was used. Samples were lysed in 200 µl of RNAzol B to which 20% of chlorophorm was added. Samples were centrifugated 13000 g for 15 minutes at 4°, to efficiently remove DNA and proteins from the acqueous phase containing RNA. To the acqueous phase, collected and transferred

to a sterile tube, was added an equal volume of isopropanol, sample were stored at -20°C overnight to allow complete RNA precipitation.

The day after, samples were heated at 4°C for 15 minutes and centrifugated at 13000 g for 15 minutes. Supernatant was removed and RNA pellet was washed twice with 75% ethanol (100 µl).

Once the supernatant was removed, pellet was dried under laminar flow cabin.

4.2 DNasi treatment and retrotranscription (RT)

RNA was dissolved in RNase–free water, and purified from genomic DNA with TURBO DNase (Applied Biosystems/Ambion, Austin, TX, USA), a genetically engineered from of bovine DNase I with greater catalytic efficiency than conventional DNase I at higher salt concentrations and lower DNA concentration. A reaction mixture, containing 1µg of RNA, Turbo DNase 1U and TURBO DNase buffer, were incubated 30 minutes at 37°C. Then DNase was inactivated by EDTA 60 mM.

1 µg of RNA was reverse transcribed into first-strand cDNA in a 20 µl final volume. A reaction mixture, containing 1 µM random hexanucleotide primers, 1 µM oligo dT and the RNA, was heated at 70°C for 5 Minutes to melt secondary structure within the template. The mixture was immediately cooled on ice to prevent secondary structure from reforming. A dNTPs mix, 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 20 U Recombinant RNase inhibitor and M-MLV 5X reaction buffer were added (Promega, Fitchburg, Wi, USA). The reaction mix was incubated 60 minutes at 42°C and then heated 5 minutes at 95° C to inactivate the RT.

4.3 Real Time PCR Arrays

4.3.1 Human Antibacterial Response PCR Array

The antibacterial response signaling pathway was analyzed using a real-time polymerase chain reaction PCR array including a set of optimized real-time PCR primer assays on 96-well plates (SABiosciences Corporation, Frederick, MD, USA). This approach permits the monitoring of the mRNA expression of 84 genes (Table 3) involved in the innate immune response to bacteria plus five housekeeping genes, following the procedures suggested by the manufacturer. Controls were also included on each array for genomic DNA contamination, RNA quality and general PCR performance. The results were analysed by SABiosciences online software. Only targets showing at least a 2-fold modulation were considered significative.

The experiments were performed on pools of patients divided into treatment and stimulation conditions groups. Thus, results represent the mean value of the different targets analysed in each group.

Table3. Complete list of antibacterial responses signaling pathway *(SABiosciences)*

Toll-like Receptor (TLR) Signaling: Receptors & Cofactors: CD14, LY96 (MD-2), TLR1, TLR2, TLR4, TLR5, TLR6, TLR9. MYD88-Dependent: FADD, IRAK1, IRAK3, IRF5, IRF7, MAP3K7 (TAK1), MYD88, TIRAP, TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, TOLLIP, TRAF6. TICAM1 (TRIF) Dependent (MYD88-Independent): IRF5, IRF7, MAP3K7 (TAK1), RIPK1, TICAM1 (TRIF), TICAM2 (TRAM), TLR4, TRAF6. Other: AKT1, CASP8 (FLICE), LBP, PIK3CA (p110a), RAC1.
NOD-like Receptor (NLR) Signaling: Receptors: NAIP (BIRC1), NLRC4 (IPAF), NLRP1, NLRP3, NOD1 (CARD4), NOD2. Inflammasomes: CASP1 (ICE), NAIP (BIRC1), NLRC4 (IPAF), NLRP1, NLRP3, PYCARD (TMS1/ASC). Regulation: BIRC3 (c-IAP1), CARD6, CARD9, CASP8 (FLICE), HSP90AA1, MEFV, PSTPIP1,RIPK2, SUGT1, TNF, XIAP.
Other Bacterial Pattern Recognition Receptors (PRRs): APCS, CRP, DMBT1, ZBP1.
Downstream Signal Transduction: NF?B Pathway: CHUK (IKKa), IKBKB, NFKB1, NFKBIA (IkBa/MAD3), RELA, TNFRSF1A. ERK Pathway: JUN, MAP2K1 (MEK1), MAPK1 (ERK2), MAPK3 (ERK1). p38/JNK Pathway: JUN, MAP2K3 (MEK3), MAP2K4 (JNKK1), MAPK14 (p38 MAPK), MAPK8 (JNK1).
Apoptosis: AKT1, BIRC3 (c-IAP1), CARD6, CARD9, CASP1 (ICE), CASP8 (FLICE), CD14, FADD, IFNB1, IKBKB, IL12A, IL12B, IL1B, IL6, IRAK1, JUN, MAP3K7 (TAK1), MAPK1 (ERK2), MAPK8 (JNK1), MPO, NFKB1, NFKBIA (IkBa/MAD3), PIK3CA, PYCARD, RAC1, RIPK1, RIPK2, TNF, TNFRSF1A, TRAF6.
Inflammatory Response: AKT1, APCS, CCL3 (MIP-1A), CCL5 (RANTES), CD14, CRP, CXCL1, CXCL2, IL1B, IL6, IL8, LBP, LY96 (MD-2), LYZ, MEFV, MYD88, NFKB1, NLRC4, NLRP3, NOD1 (CARD4), RAC1, RELA, RIPK2, SLC11A1, TICAM2 (TRAM), TIRAP, TLR1, TLR2, TLR4, TLR5, TLR6, TLR9, TNF, TNFRSF1A, TOLLIP.
Cytokines & Chemokines: CCL3 (MIP-1A), CCL5 (RANTES), CXCL1, CXCL2, IFNA1, IFNB1, IL12A, IL12B, IL18, IL1B, IL6 IL8.
Antimicrobial Peptides: BPI, CAMP, CTSG, LCN2 (NGAL), LTF, LYZ, MPO, PRTN3, SLPI.

3.4 Statistical analysis

Data were analyzed according to standard statistical tests; t test were performed to compare groups during the treatment. Procedures were based on parametric analysis.

RESULTS

1. PEDITRIC PATIENTS

1.1 Pneumococcal-specific immune responses

The expression of surface molecules and cytokine secretion were assessed upon PBMC stimulation *in vitro* with pneumococcal polysaccharides.

PBMCs were analysed at different time points (T0, T3, T5 and T21) in PDT group compared to controls.

Results showed that PDT modified on dendritic cells and monocytes functions.

1.1.1 Dendritic cell activation

Figure 8 shows the results of dendritic cell (DC) maturation analysis according to treatment arms DCs were characterized by analyzing the expression of surface markers associated with cell activation and maturation (HLA-DRII) and costimulatory molecules (CD80, CD86).

No differences were observed between the two groups at enrolment. An increased CD80 expression on the CD11c+ cells were reported in PDT group compared to control group at T3, T5 and T21, (p<0.05, p<0.001, p<0.01 respectively) (Figure 9, panel A). The percentage of CD86-expressing CD11c+ cells was significantly higher at T5 and T21, with an increase of 22% and 27%, respectively (p=0.05) in PDT-treated patients compared to control patients (Figure 9, panel B). Similar trend was observed for HLA-DRII expression on DCs, at T5 and T21 (p=0.001 and p=0.008) (Figure 9, panel C). Finally, analysis of co-expression of HLA-DRII, CD80 and CD86 on DCs showed similar differences at T5 and T21 between the two groups of CAP patients (p=0.0042, p=0.0017, respectively) (Figure 9, panel D).

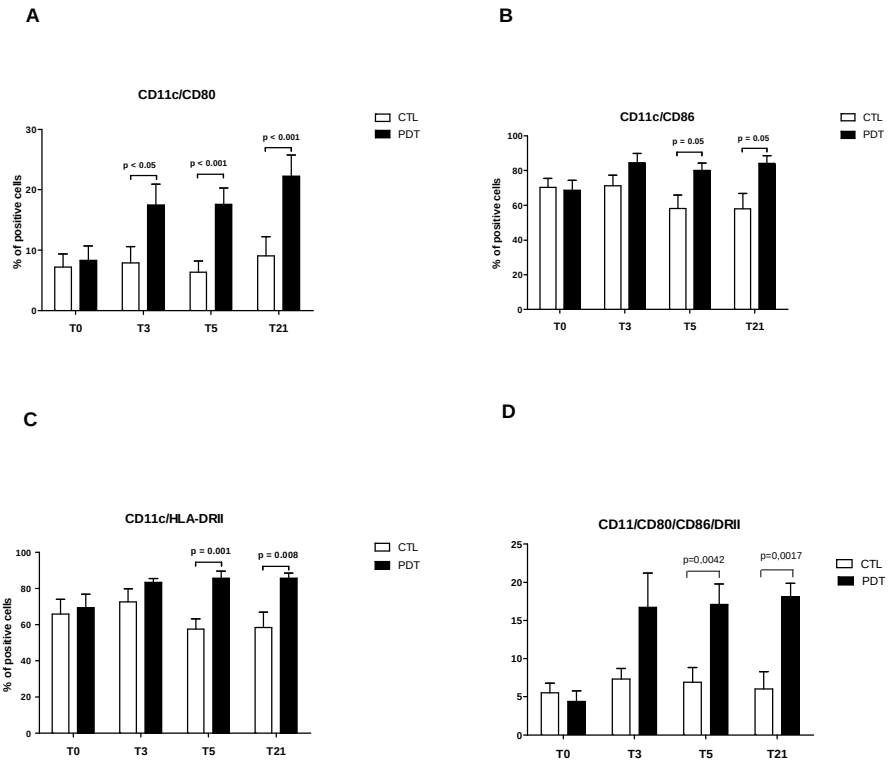


Fig.9 Activation of DCs.
Percentages of pneumococcal-stimulated CD80- (**A**), CD86- (**B**), HLA-DRII- (**C**), and CD80, CD86, and HLA-DRII-expressing dendritic cells (CD11c+) (**D**). Surface molecules are shown at baseline and in response to therapy (T1, T5, T21) in children with community-acquired pneumonia (CAP) treated with antibiotics plus pidotimod and in controls treated with antibiotics only. For each analysis, 30,000 events were acquired and gated on CD11c expression and side scatter properties. Mean values + SEM and statistically significant differences are indicated.

1.1.2 TLRs expression on monocytes surface

Activation of monocytes (CD14+ cells) via TLRs was also evaluated after stimulation of PBMCs with pneumococcal polysaccharides. As shown in Figure 10, TLR2 expression on CD14+ cells was significantly higher in PDT group compared with control group at 5 and 21 days after treatment initiation, with a significant *p* value for the comparison across time-treatment groups (*p*=0.011, *p*=0.049) (Figure 10, Panel A). TLR4 expression showed a statistical significance at T21 with an increase of 23% (*p*=0.017) (Figure 10, Panel B). Data obtained by analyzing TLR2 and TLR4 co-expression on CD14+ cells mirrored the single-marker evaluation, (T21: *p*=0,017) (Figure 10, Panel C).

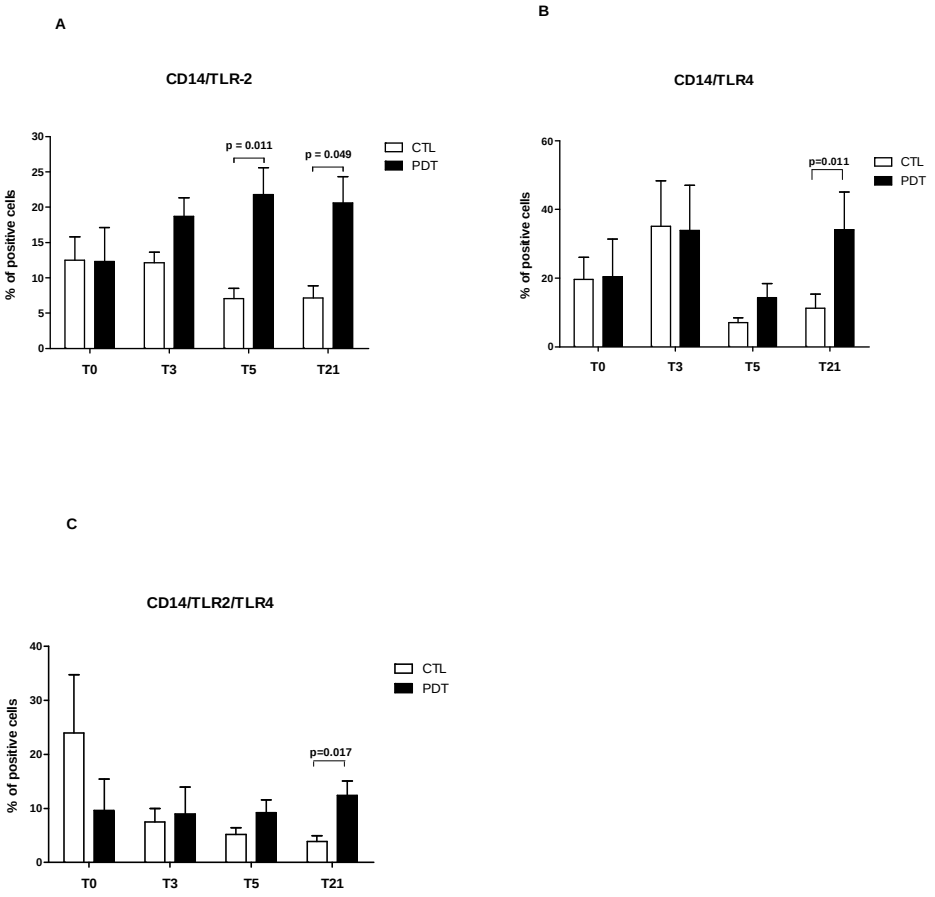


Fig.10 Percentage of TLR expression on monocytes.
TLR2 (**A**), TLR4 (**B**), and TLR2/TLR4-coexpression (**C**) expression on CD14+ cells are shown at baseline and in response to therapy in children with community-acquired pneumonia (CAP) treated with antibiotics plus pidotimod and in controls treated with antibiotics only. For each analysis, 30,000 events were acquired and gated on CD14 expression and side scatter properties. Mean values + SEM and statistically significant differences are indicated.

1.1.3 Cytokine secretion by immunocompetent cells

Analyses of cytokine release from DCs revealed that the percentage of TNFα- or IL12-producing CD11c+ cells was higher in the PDT group compared to the control group at days 5 and 21, with a significant *p* for both cytokines across time (Figure 11, Panels A and B) (*p*=0,010, *p*=0,008, *p*=0,01, *p*<0,001). A similar trend can be observed in TNFα and IL12-producing DCs (*p*=0,049, *p*=0,037) (Figure 11, Panel C).

Cytokines were detected also in monocytes. In CD14+ cells, TNFα and IL-12 producing cells percentage increased at day 5 and day 21 in treated group compared to control group (Panels D and E). TNFα secretion reached statistical significance at T21 (*p*=0,02), while IL-12 is already significant at T3 (*p*=0,043). Such significance was maintained at subsequent time points (*p*=0,041, *p*=0,05). The data is confirmed in coexpression analysis (Panel F), at T5 and T21 cytokines percentage reached statistical significance (*p*=0,024, *p*=0,05).

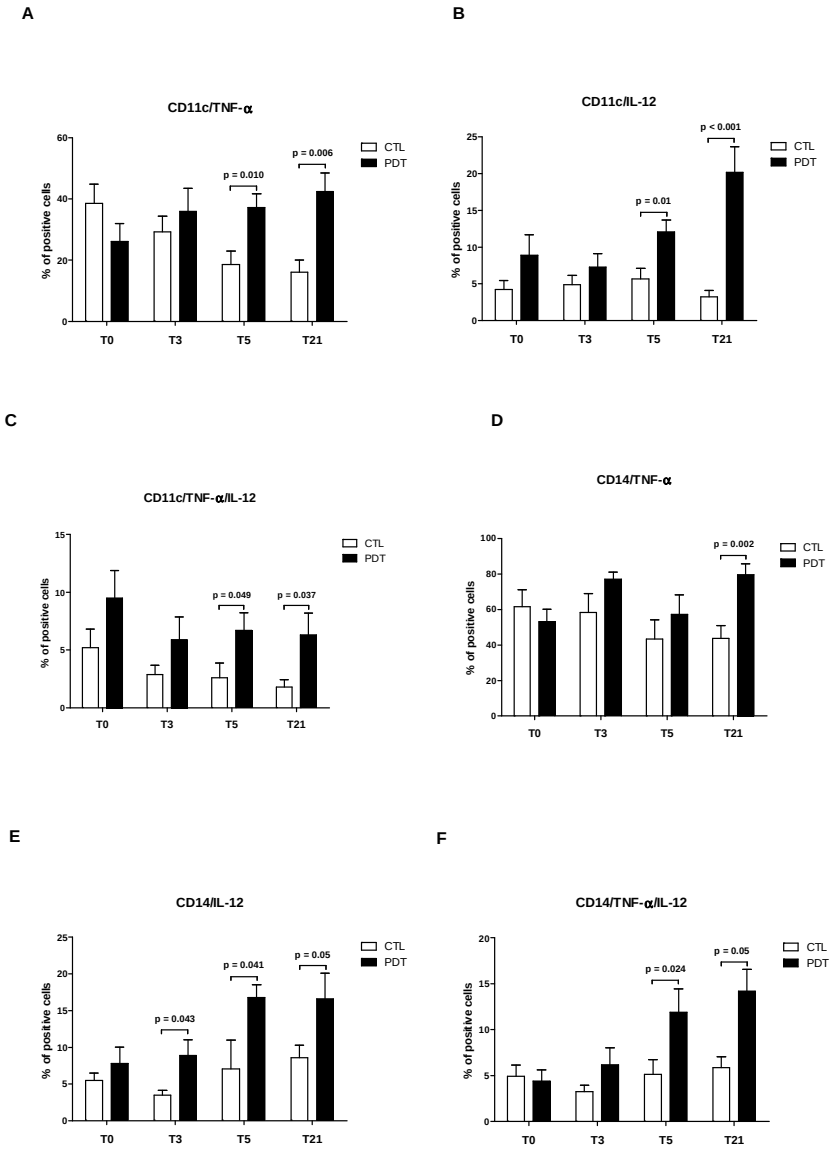


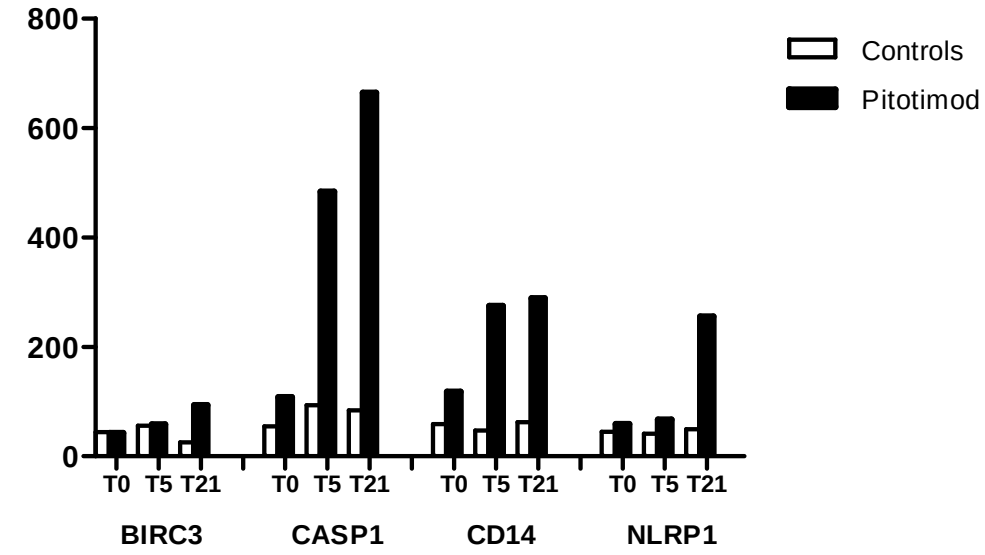
Fig.11 Proinflammatory cytokine secretion. Percentages of pneumococcal-stimulated TNFα- (**A**), IL12- (**B**), and TNF-α/IL-12- (**C**) secreting dendritic cells (CD11c+), percentages of TNFα- (**D**), IL12- (**E**), and TNF-α/IL-12- (**F**) CD14+ cells are shown at baseline and in response to therapy in children with community-acquired pneumonia (CAP) treated with antibiotics plus pidotimod and in controls treated with antibiotics only. For each analysis, 30,000 events were acquired and gated on CD11c and CD14 expression and side scatter properties. Mean values + SD and statistically significant differences are indicated.

1.1.4 Modulation of targets involved in antibacterial response

Gene expression was evaluated by Real Time PCR array technology. Figure 12 summarizes gene expression related to the antibacterial response signaling pathway after pneumococcal polysaccharide stimulation. Expression of antimicrobial peptides reached a peak of expression at day 5 with a subsequent decrease at day 21. A similar trend was observed in both groups of patients, but the increases were more pronounced in the PDT-treated individuals. Amongst the genes that were significantly upregulated by pidotimod: Baculoviral IAP Repeat Containing 3 (BIRC3) encodes a member of the IAP family of proteins that inhibit apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2; Caspasi 1 (CASP1) encodes a protein which is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis; CD14 encodes a protein that is preferentially expressed on monocytes/macrophages; NLR family pyrin domain containing 1 (NLRP1) encodes a member of the Ced-4 family of apoptosis proteins. Ced-family members contain a caspase recruitment domain (CARD) and are known to be key mediators of programmed cell death.

Likewise, an upregulation of inflammatory response genes was also observed in the PDT-treated group in comparison with the control group. The represented genes are those which are modulated following stimulation. Among them, CCL3, CXCL1 and CXCL2 are genes involved in synthesis of chemokines; IL1β, IL6, IL8 and IL18 encode for the inflammatory interleukin; NF-kB and NLRP3 that have a key role in regulating the inflammation.

A


Anrimicrobial peptides
800
600
400
200
0
Controls
Pitotimod
T0 T5 T21
T0 T5 T21
T0 T5 T21
T0 T5 T21
BIRC3
CASP1
CD14
NLRP1

B

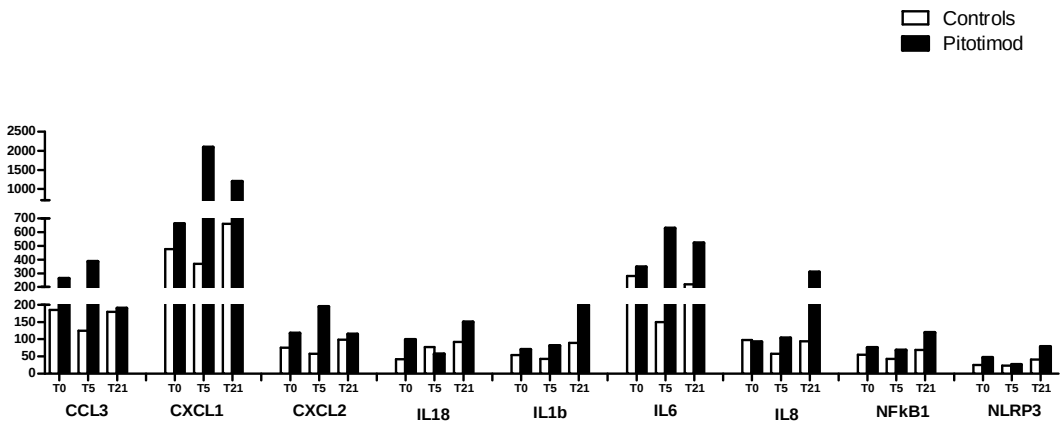


Fig.12 Antibacterial response signaling pathway
Antimicrobial peptides (Panel **A**) and genes involved in the inflammatory response (Panel **B**) at baseline as well as at days 3, 5 and 21 in children with community-acquired pneumonia (CAP) treated with antibiotics plus pidotimod and in controls treated with antibiotics only. Thus, the results represent the mean values of the different targets analyzed in the PDT versus Placebo subjects. Only values > 2-fold were considered significative. Standard deviations (SD) are not shown because the data were obtained by pool of cDNA sample.

1.2 LPS-stimulated immune response

LPS stimulation has been used as positive control. Results on DC and monocyte activation, on proinflammatory cytokine secretion were similar to how obtained upon pneumococcal-specific stimulation.

1.2.1 Dendritic cell activation and maturation

Upon LPS stimulation, DCs undergo maturation and activation through upregulation of HLA DRII and costimulatory molecules CD80 and CD86. In particular, the differences reached statistical significance at each time point (T3: p=0,011; T5: p=0,013; and T21 p=0,015). No significative differences are observed in TNFα and IL12 production, even if in PDT treated group an increased secretion of both cytokines was reported at T3, T5 and T21 (Figure 13).

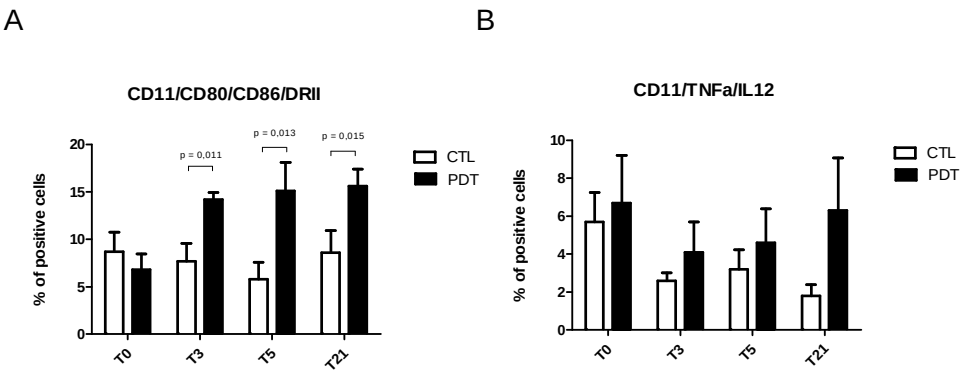
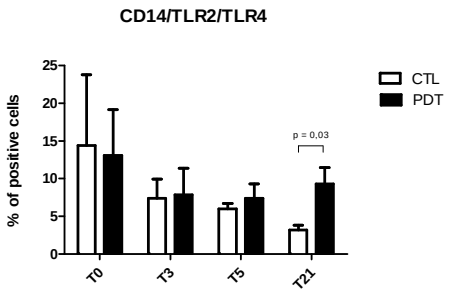


Fig. 13 Effects of LPS on DCs.
Percentage of CD80, CD86 and HLA DRII expression (**A**), and proinflammatory cytokines secretion (**B**). For each analysis, 30,000 events were acquired and gated on CD11c expression and side scatter properties. Mean values + SD and statistically significant differences are indicated.

1.2.2 TLR expression and cytokine secretion in monocytes

We observed monocyte activation upon LPS stimulation as well. TLR2 and TLR4 increased in PDT group at T3, T5 and T21 compared to the control group, in particular 21 days after the initiation of the therapy results showed statistical significance (*p*=0,03). In agreement with previous results, also proinflammatory cytokine secretion (TNFα and IL12) increase in PDT group at each time points but with no statistical significance (Figure 14).

A



B

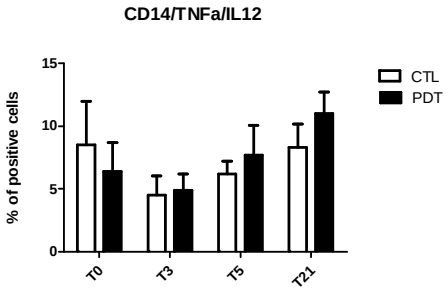


Fig. 14 Effects of LPS on monocytes.
Percentage of TLR2 and TLR4 expression on CD14+ cells (**A**), and proinflammatory cytokines secretion (**B**). For each analysis, 30,000 events were acquired and gated on CD14 expression and side scatter properties. Mean values + SEM and statistically significant differences are indicated.

2. ADULT PATIENTS

2.1 Pneumococcal response

The expression of surface molecules and cytokine secretion were assessed upon PBMC stimulation *in vitro* with pneumococcal polysaccharides.
PBMCs were analysed at different time points (T0, T1, T3 and T5) in PDT group compared to controls.
Results showed that PDT modified dendritic cells and monocytes functions.

2.1.1 Dendritic cells activation

The data regarding DCs maturation according to treatment arm are reported in Figure 15 were characterized by analyzing the expression of surface markers associated with cell activation and maturation, MHC class II molecules (HLA-DRII) and costimulatory molecules (CD80, CD86).
Results indicate that HLA class II expressing DCs were increased, albeit not significantly, at day 5 in the pidotimod group, indicating that antigen presentation might be improved by pidotimod. The percentage of CD80- and CD86-expressing DC was increased as well at day 3 in the treated group compared to the control group; in this case, the differences were statistically significant ($p<0.05$). Because CD80 and CD86 are key proteins in initiating B-T lymphocytes collaboration and in the generation of antibodies, these results suggest that pidotimod has the ability to optimize this phase of the immune response.

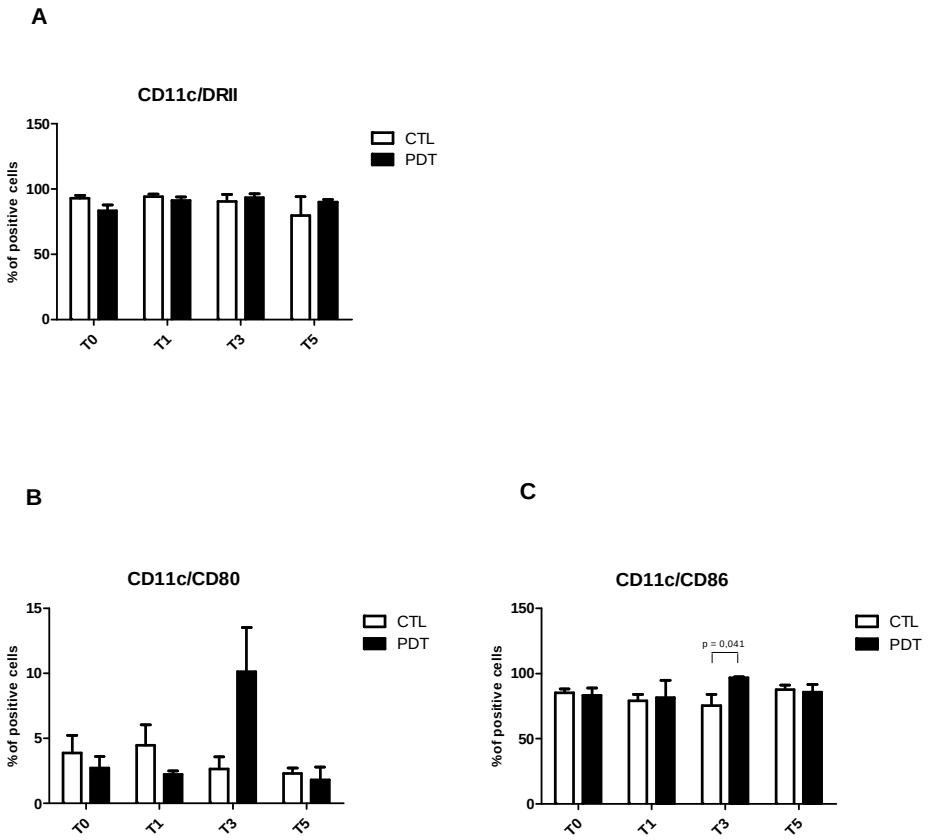


Fig.15 Percentages of pneumococcal-stimulated positive dendritic cells.
CD11c+ expressing HLA-DRII (**A**), CD80 (**B**), and CD86 (**C**). Molecules are shown at baseline and in response to therapy in adults with community-acquired pneumonia (CAP) treated with antibiotics plus pidotimod and in controls treated with antibiotics only. For each analysis, 30,000 events were acquired and gated on CD11c expression and side scatter properties. Mean values + SEM and statistically significant differences are indicated.

2.1.2 TLRs expression on monocyte surface

Microbial infections stimulate innate immunity via binding of PAMPs they express to TLRs. Results showed that TLR2- and TLR4-expressing CD14+ cells (monocytes) were significantly increased in patients in whom pidotimod was added to the standard antibiotics therapy.

Both TLR2 and TLR4 expression on monocytes was significantly higher in the PDT group compared with the untreated group 5 days after treatment initiation, with a significant *p* value (*p*=0,018 and *p*=0,028, respectively) (Figure 16).

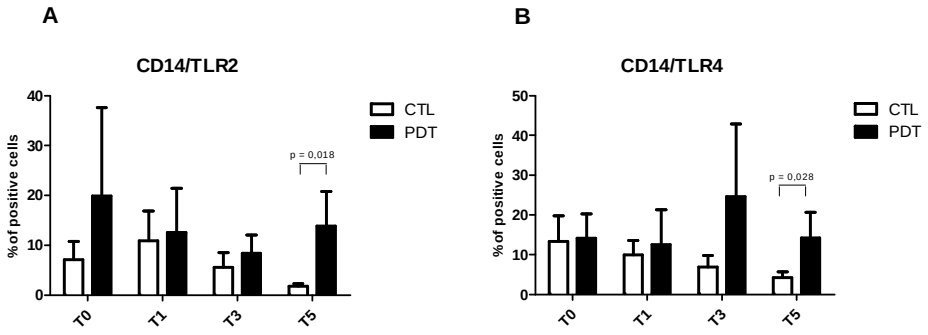


Fig.16 Percentage of TLRs in monocytes.
TLR2 (**A**) and TLR4 (**B**) expressing CD14+ monocytes in control and PDT patients analysed at baseline, 1, 3, and 5 days of therapy. For each analysis, 30,000 events were acquired and gated on CD14 expression and side scatter properties. Mean values + SEM and statistically significant differences are indicated.

2.1.3 Cytokines secretion by immunocompetent cells

To evaluate whether pidotimod could modulate the production of cytokines from immune cells, TNFα- and IL12- producing monocytes and DCs were measured in pneumococcal-stimulated PBMCs. Results in Figure 17 showed that, whereas no consistent effects of pidotimod on IL12-producing immune cells could be detected, TNF-α-producing monocytes and DCs were robustly reduced in pidotimod patients.

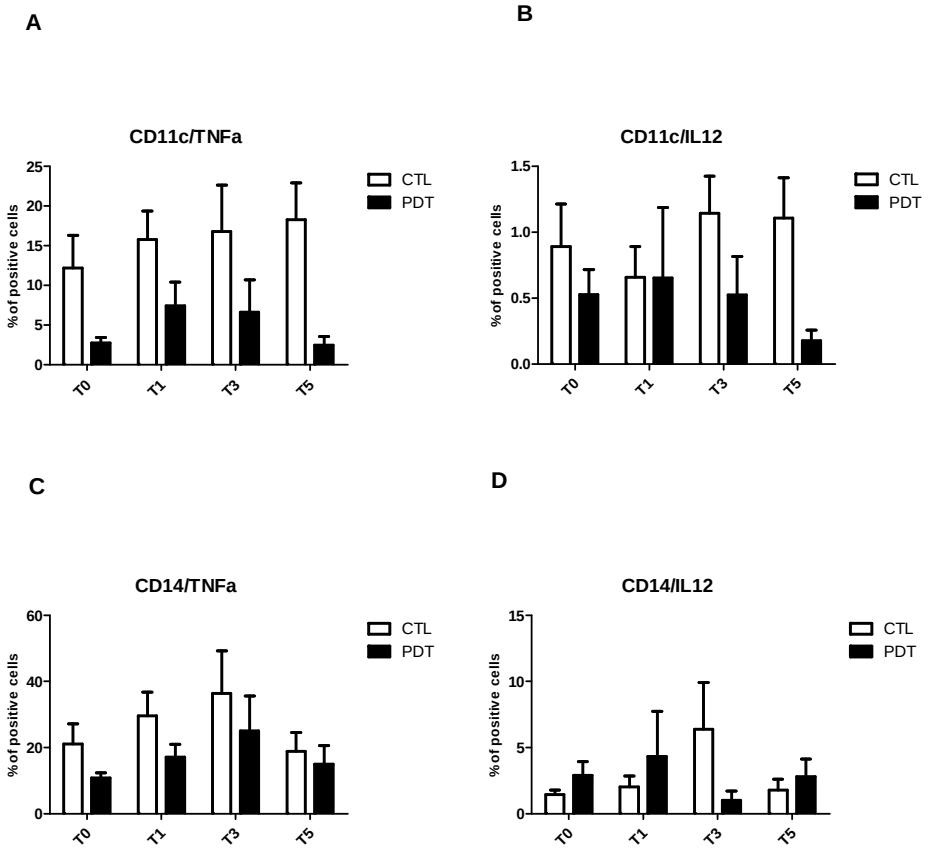


Fig.17 PDT effects in cytokines secretion.
Percentage of TNFα- and IL12-secreting dendritic cells (**A**-**B**) and monocytes (**C**-**D**) in control and PDT patients analysed at baseline, 1, 3, and 5 days of therapy. Mean values, SEM, and *p* values are indicated.

2.1.4 Modulation of targets involved in antibacterial response

The ability of pidotimod to stimulate antimicrobial and immunomodulatory peptides was evaluated in all the individuals enrolled in the study. Data obtained in unstimulated PBMCs 3 days after the initiation of therapy indicated that a number of genes that codify proteins endowed with antimicrobial properties were significantly upregulated in the pidotimod + antibiotic compared to antibiotics alone group (Figure 18). Only those targets showing at least a 2-fold difference in the two groups were considered as those to be significantly modulated. Amongst the genes that were significantly upregulated by pidotimod: Bactericidal/Permeability-Increasing Protein (BPI), cathepsin G (CTSG) and myeloperoxidase (MPO) encode molecules that are associated with the antimicrobial functions of human neutrophil granules; Cathelicidin Antimicrobial Peptide (CAMP), C reactive protein (CRP), and lipocalin 2 (LCN2) encode proteins that elicit and amplify innate and acquired immune responses; lactoferrin (LTF) binds to the bacterial surface and has direct antimicrobial functions; and Secretory Leukocyte Peptidase Inhibitor (Slpi) produces a protein which protects epithelial tissues from attack by endogenous proteolytic enzymes. The effects of pidotimod on inflammatory responses were analyzed as well in unstimulated PBMCs. Results shown in Figure 19, indicated PDT effects on unstimulated PBMC. 3 days after initiation of therapy (Panel A), genes responsible for the generation of cytokines (CCL and CXL) as well as those for the inflammatory cytokines IL1b and TNFα were down regulated. Contrariwise, type 1 IFNs, IL-6 and IL-12 were upregulated in the pidotimod + antibiotic compared to the antibiotics alone group. Notably, at day 5 (Panel B) the picture was different. The addition of pidotimod to the standard therapy resulted in a massive increase in mRNA for both CCL and CXL chemokines, whereas mRNA for type 1 IFN, TNFa, IL-6, IL-12 was greatly reduced as compared to the values observed on day 3.

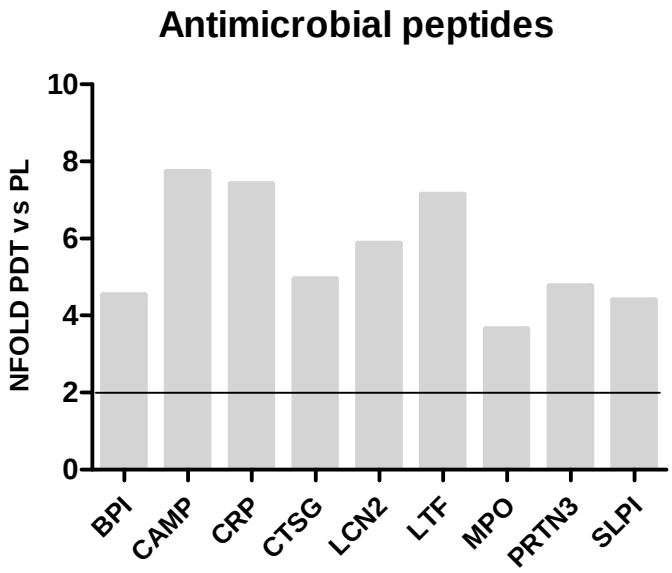


Fig.18 Antimicrobial peptides expression.
Unstimulated PBMCs 3 days after initiation of the therapy; CAP patients treated with antibiotics plus pidotimod (PDT) *vs* controls treated with antibiotics only. Experiments were run on samples from all the subjects included in the study, pooled into two groups: PDT and controls. Thus, the results represent the mean values of the different targets analyzed in the PDT *vs* placebo subjects. Only increases > 2-fold were considered significative.

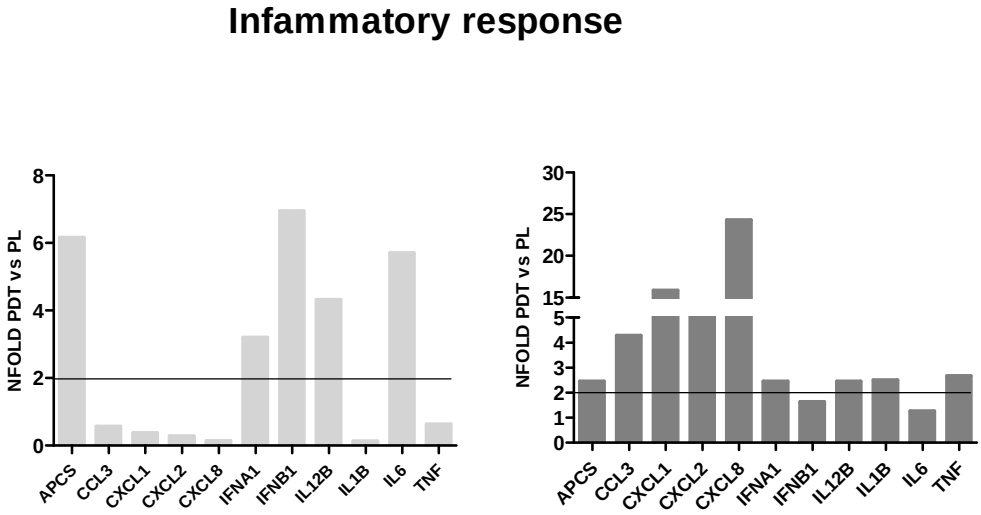


Fig.19 Inflammatory response markers expression.
Unstimulated PBMCs 3 (Panel **A**) and 5 days (Panel **B**) after initiation of the therapy; CAP patients treated with antibiotics plus pidotimod (PDT) VS controls treated with antibiotics only. Experiments were run on samples from all the subjects included in the study pooled into two groups: PDT and Placebo. Thus, the results represent the mean values of the different targets analyzed in the PDT versus Placebo subjects. Only increases > 2-fold were considered significative.

2.2 LPS response

Also in adult patients LPS stimulation led to the similar results (Figure 20). DRII expression showed no difference between two groups (Panel A), while CD80 and CD86 increase their expression in the treated group than control (Panel B and C), in particular at T3, were CD80 reach statistical significance with *p* value of 0,01. It is possible observe the same trend also in cytokine secretion (panel D and E), although it never reached statistical significance, where the secretion of both IL12 and TNFα was increased in treated group. On monocytes, we didn't observe difference with pneumococcal stimulation. TLRs expression increased in PDT group compared to placebo, in particular at T3, while in cytokines secretion there was no difference between two groups at different time points (data not shown).

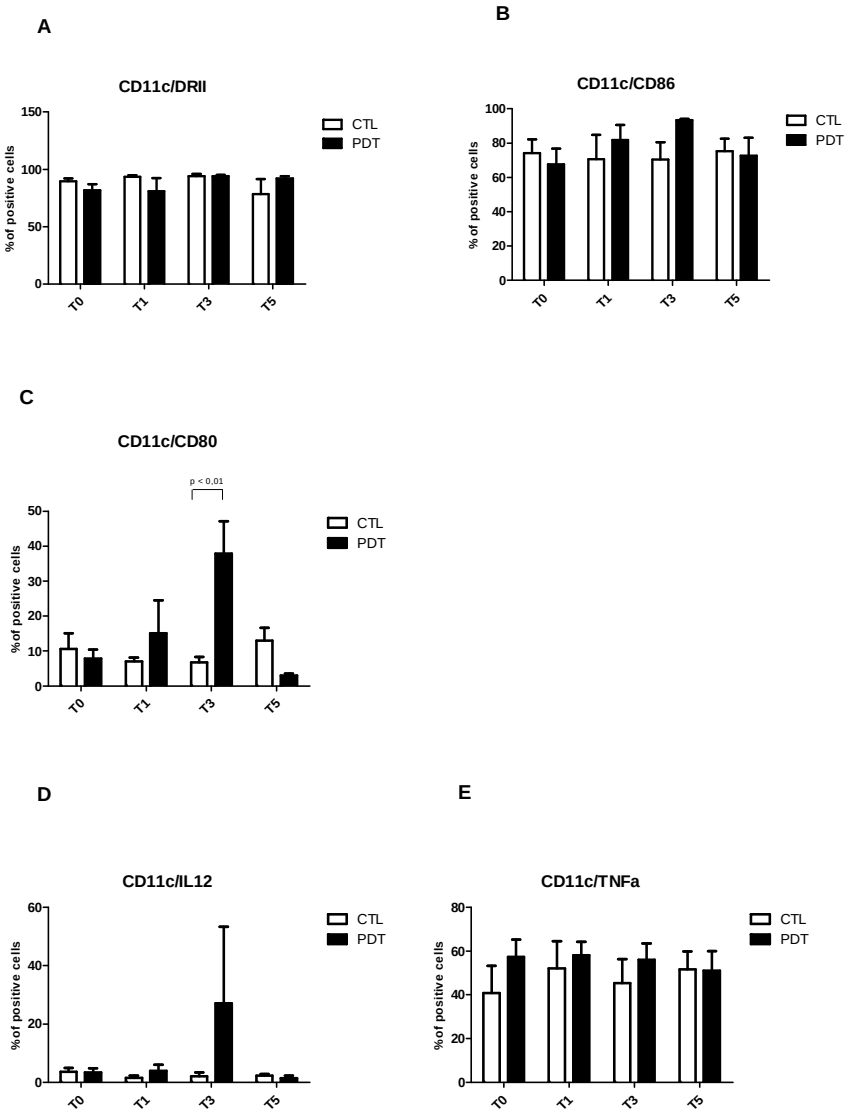


Fig.20 DCs activation.
Percentages of LPS-stimulated positive dendritic cells (CD11c+) expressing HLA-DRII (**A**), CD86 (**B**), and CD80 (**C**), cytokines secretion IL12 (**D**), and TNFα (**E**). Molecules are shown at baseline and in response to therapy in adults with community-acquired pneumonia (CAP) treated with antibiotics plus pidotimod and in controls treated with antibiotics only. For each analysis, 30,000 events were acquired and gated on CD11c expression and side scatter properties. Mean values + SEM and statistically significant differences are indicated.

DISCUSSION AND CONCLUSION

Immunostimulators are known and studied since 90s. In particular, pidotimod was longed studied. Several *in vitro* studies showed its effects on Dendritic cells maturation and activation, and on differentiation of CD4+ naïve T cells towards a TH1-polarizing phenotype [61,76]. PDT promotes upregulation of TLR2 and a significant increase in NF-kB protein expression and NF-kB nuclear translocation [65]. *In vivo* studies confirmed previous results, further it was demonstrated that PDT enhances NK cell function and promotes phagocytosis [77]. In Down Syndrome patients, PDT administration in conjunction with flu vaccine, induces a potentiation of immune responses and, in particular, in innate immunity [2].

Its effects on both, innate and adaptive immunity, have made PDT a good candidate for treatment of several diseases. In this study we analysed PDT effects for the treatment of comunity-acquired pneumonia. Promising results were obtained. Our data indicate that PDT administration in addition to standard antibiotic therapy in children with CAP may significantly increase natural immune system responses to an infectious stimulus via a direct influence on DCs maturation and function, TLR2 expression in monocytes, antimicrobial peptide secretion and upregulation of genes involved in inflammatory response. The positive effect was reported after only few days of PDT administration and remained evident for several days after PDT treatment ended. In agreement with previous data, results obtained in adults confirm that Pidotimod significantly increases the percentage of TLR2 and TLR4-expressing CD14+ cells and the production of chemokines as well as the expression of CD80 and CD86, crucial costimulatory receptor involved in the collaboration between T and B lymphocytes, and the modulation of the generation of cytokines by immune cells.

At steady state, DCs are described as immature, a phenotype characterized by low surface expression of MHC class II molecules and co-

stimulatory molecules such as CD80 and CD86. In response to activation (e.g. infections), DCs undergo a program of maturation that leads to the acquisition of a number of fundamental properties including antigen processing and presentation, migration and T cell co-stimulation. In this context, upregulation of co-stimulatory molecules and HLA-DRII antigens is evidenced together with a significant production of pro-inflammatory cytokines, such as IL-12 and TNF-α, which play an important role in the polarization of T helper cell subsets toward a Th1 profile during priming by DCs [78]. TLR2 plays a major role in promoting protective immunity against respiratory pathogens [79], and its upregulation could reduce susceptibility to further respiratory infections. In PDT-treated children, the increased expression of antimicrobial peptides and genes involved in cell chemotaxis, the upregulation of the inflammatory response and increased apoptosis [80,81], suggest that PDT administration might significantly increase the activity of the immune system for a long period of time, thus reducing the risk of early recurrences during CAP in children.

The observations that TLR2 and TLR4 are up regulated by Pidotimod in CAP patients is important as these receptors recognise molecules that are part of bacterial cell wall and are produced during bacterial replication. TLRs biding to PAMPS initiates innate immune responses that are mediated by cytokines and stimulates antimicrobial peptides production [39,40].

In adults, Pidotimod is associated with a reduced synthesis of TNFa, a proinflammatory cytokine whose excessive production is known as a negative prognostic factor in CAP. These are preliminary data that need to be confirmed in a larger group of patients, but, taken together, combined results of these two cohorts of patients undeline that Pidotimod is endowed with immunomodulatory abilities.

From a clinical point of view no significant differences between the two groups in terms of time to clinical stability and symptoms resolution were recorded. In conclusion, results herein support the proposal to verify the possible clinical benefits of Pidotimod-associated immune modulation in larger populations, possibly including complicated CAP cases. Furthermore, its beneficial effects on both innate and adaptive immunity, make it an excellent candidate as adjuvant in the vaccines. So it would be interesting, as future perspectives, to analyse its efficacy in vaccine formulation.

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SCIENTIFIC PRODUCTS

List of scientific products produced during the PhD Course (January 2014-May 2017).

Publications

Publications about the results presented in the thesis:

- Esposito S, **Garziano M**, Rainone V, Trabattoni D, Biasin M, Senatore L, Marchisio P, Rossi M, Principi N, Clerici M. "*Immunomodulatory activity of pidotimod administered with standard antibiotic therapy in children hospitalized for community-acquired pneumonia".* J Transl Med. 2015 Sep 3;13:288. doi: 10.1186/s12967-015-0649-z. PMID: 26335787

- Trabattoni D, Clerici M, Centanni S, Mantero M, **Garziano M**, Blasi F. *"Immunomodulatory effects of pidotimod in adults with community-acquired pneumonia undergoing standard antibiotic therapy."* Pulm Pharmacol Ther. 2017 Mar 13. pii: S1094-5539(17)30043-3. doi: 10.1016/j.pupt.2017.03.005. PMID: 28302543

Other pubblications:

- Sironi M, Biasin M, Gnudi F, Cagliani R, Saulle I, Forni D, Rainone V, Trabattoni D, **Garziano M**, Mazzotta F, Real LM, Rivero-Juarez A, Caruz A, Lo Caputo S, Clerici M. "*A regulatory polymorphism in HAVCR2 modulates susceptibility to HIV-1 infection."* PLoS One. 2014 Sep

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Abstarct

- **M. Garziano**, S. Lo Caputo,3 M. Sironi, I. Saulle, F. Gnudi, V. Rainone, F. Mazzotta, D. Trabattoni, M. Clerici, M. Biasin. "*A regulatory polymorphism modulates TIM-3 expression and susceptibility to HIV-1 infection"* Oral Presentation. 6° Italian Conference on AIDS and Antiviral Research, ICAR 2014 Roma , 25-27 may 2014.

- I. Saulle, S. Yahyaei, M. Biasin, **M. Garziano**, M. Masetti, A. Berzi, D. Trabattoni, S. Lo Caputo, F. Mazzotta, M. Clerici. "*Plasma and PBMC miRNA Profile in sexually HIV-1 exposed seronegative individuals*" Presenting Author. Poster Presentation. 8° IAS Conference on HIV Pathogenesis, Treatment and Prevention, Vancouver, British Columbia, Canada, 19-22 July 2015.

- **M. Garziano**, I. Saulle, F. Gnudi, M. Masetti, A. Berzi, F. Mazzotta, S.L. Caputo, D. Trabattoni, M. Biasin, M. Clerici."Immune activation is present in HIV-1 Exposed Seronegative Individuals (HESN) and is independent from microbial translocation" Poster Presentation. 8° IAS Conference on HIV Pathogenesis, Treatment and Prevention, Vancouver, British Columbia, Canada, 19-22 July 2015.

- M. Masetti, D. Trabattoni, F. Gnudi, S.V. Ibba, I. Saulle, **M. Garziano**, A. Berzi, M. Biasin, J. Rossignol, M. Clerici."Thiazolides elicit anti-viral innate immunity and drastically reduce HIV replication in vitro" Presenting Author. Oral Presentation 7° Italian Conference on AIDS and Antiviral Research, Riccione (Rimini), Italy 2015.

- A. Berzi, S. Ordanini, M. Masetti, I. Saulle, **M. Garziano**, M. Biasin, D. Trabattoni, A. Bernardi, M. Clerici "A pseudo-glycodendrimer Inhibits DC-SIGN-Mediated HIV Trans-Infection and Interferes with DC-SIGN Signal" Oral Poster Discussion 7° Italian Conference on AIDS and Antiviral Research, Riccione (Rimini), Italy 2015.

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