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Haz que tu vida sea
campana que repique
o surco en que florezca y fructifique
el árbol luminoso de la idea.
Alza tu voz sobre la voz sin nombre
de todos los demás, y haz que se vea
junto al poeta, el hombre.

Llena todo tu espíritu de lumbre;
busca el empinamiento de la cumbre,
y si el sostén nudoso de tu báculo
encuentra algún obstáculo a tu intento,
¡sacude el ala del atrevimiento
ante el atrevimiento del obstáculo!

Nicolás Guillén

A Rolando y Vicky...

...A Loïc.

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ABBREVIATIONS

mDC: myeloid dendritic cells

pDC: plasmacytoid dendritic cells

MoDC: Monocyte-derived dendritic cells

TH: T helper

Treg : Regulatory T cells

HTEC: Human tonsil epithelial cell

N. meningitidis: *Neisseria meningitidis*

Hi: *Haemophilus influenza*

SA: *Staphylococcus aureus*

LPS: Lipopolysaccharide

DAMP: Danger associated molecular pattern

PRR: Pathogen recognition receptor

TLR: Toll-like receptor

MHC: Major histocompatibility complex

IL: Interleukin

IFN: Interferon

TNF: Tumor necrosis factor

TGF: Transforming growth factor

TSLP: Thymic stromal lymphopoietin

G-CSF: Granulocyte colony stimulator factor

GM-CSF: Granulocyte and macrophage colony stimulator factor

MFI: Mean of immunofluorescence intensity

ABSTRACT

Dendritic cells are specialized to initiate and modulate acquired immunity to endogenous (self) and exogenous (non-self) signals. This modulation will depend on the nature of the antigen and the microenvironment where dendritic cells encounter these signals. *Neisseria meningitidis* (*N. meningitidis*) is an extracellular bacterium which colonizes strictly the human mucosa where it lives as a commensal microbe. However, bacteria can become pathogenic if they reach the bloodstream or the brain causing septicemia or meningitis, respectively. Acquired immunity to *N. meningitidis* has been evidenced and related with commensalism at the oro-nasopharynx mucosa. The aim of this work was to evaluate the contribution of dendritic cells in the initiation of *N. meningitidis* immunity and the role of the epithelium in this response using human primary cells as a model obtained mainly from tonsils.

Tonsils are mucosa-associated lymphoid tissues found in the human throat and nose where the epithelium is infiltrated by myeloid and plasmacytoid dendritic cells. Here, we demonstrated for the first time in a human primary cell model that *N. meningitidis* is able to activate both dendritic cells subsets isolated from tonsils. Bacteria induced the secretion of IL-6, IL-10, IL-1β, TNFα, IL-1α and IL-12p70 by

tonsil myeloid dendritic cells while they induced the expression of surface CD86 and the secretion of IFNα, IL-6 and TNFα by tonsil plasmacytoid dendritic cells. The activation of plasmacytoid dendritic cells by *Neisseria* was also confirmed in plasmacytoid dendritic cells isolated from blood.

Then, we evaluated the role of the epithelium in this response. Our results show that the epithelium plays an anti-inflammatory role during dendritic cells response to *N. meningitidis.* In the case of *N. meningitidis*-stimulated myeloid dendritic cells, the level of IL-10 was reduced while the secretion of TNFα and IL-12p70 were completely abrogated by the presence of the soluble factors from *N. meningitidis*-stimulated epithelium. Similar results were obtained in *N. meningitidis*-stimulated plasmacytoid dendritic cells, where IFNα and TNFα secretion was completely inhibited by the soluble factors from *N. meningitidis*-epithelium interaction, while the co-stimulatory capacity of plasmacytoid dendritic cells remained intact. Next, we showed that this response was specific to *N. meningitidis* infection, since plasmacytoid dendritic cells stimulated by others Gram-negative and Gram-positive bacteria induce IFNα secretion by plasmacytoid dendritic cells, but only the IFNα secreted by *N. meningitidis* is inhibited by the soluble factors derived from the epithelium. This study highlights the complex interplay between different cell types at the mucosa (myeloid and plasmacytoid

dendritic cells and epithelial cells) during the immune response and the subtle modulation exerted by *N. meningitidis* on this response.

RESUMEN

Las células dendríticas son especializadas en iniciar y modular la respuesta inmune adquirida contra señales endógenas (propias) o exógenas (no propias). Esta modulación dependerá de la naturaleza y del microambiente donde las células dendríticas encontrarán estas señales. *Neisseria meningitidis* (*N. meningitidis*) es una bacteria extracelular que coloniza exclusivamente la mucosa humana, donde vive como un microorganismo commensal. Sin embargo, la bacteria puede volverse patogénica cuando alcanza la circulación sanguínea o el cerebro, causando septicemias o meningitis, respectivamente. La respuesta inmune adquirida contra *N. meningitidis* ha sido evidenciada y relacionada con la fase comensal de la bacteria en la mucosa oro-faríngea. En este trabajo, nos propusimos como objetivo evaluar la contribución de las células dendríticas en el inicio de la respuesta inmune contra *N. meningitidis* y el papel que desempeña el epitelio en esa respuesta utilizando células primarias humanas como modelo obtenidas principalmente de amígdalas.

Las amígdalas son tejidos linfoides asociados a mucosa ubicadas en la garganta y nariz en humanos. Su epitelio se encuentra infiltrado de células dendríticas mieloides y plasmacitoides. En este estudio, demostramos por primera vez que *N. meningitidis* activa ambos

subtipos de DC aisladas de amígdalas. La bacteria indujo la secreción de IL-6, IL-10, IL-1β, TNFα, IL-1α y de IL-12p70 por las células dendríticas mieloides, mientras que en células dendríticas plasmacitoides aisladas de amígdalas, indujo la secreción de IFNα, IL-6 y TNFα, además del aumentar la expresión de CD86 en la superficie de la célula. La activación de las células dendríticas plasmacitoides por *N. meningitidis* fue confirmada también en células aisladas de sangre.

Posteriormente, evaluamos el papel del epitelio en esta respuesta. Nuestros resultados muestran que el epitelio juega un papel anti-inflamatorio durante la respuesta de las células dendríticas dirigida a *N. meningitidis.* En el caso de la respuesta de mieloides células dendríticas a *N. meningitidis*, el nivel de secreción de IL-10 fue reducido mientras que la secreción de TNFα y de IL-12p70 fueron completamente inhibidas por la presencia de los factores solubles provenientes de la interacción *N. meningitidis*-epitelio. Resultados similares se obtuvieron en las células dendríticas plasmacitoides estimuladas con la bacteria, donde las secreciones de IFNα y de TNFα fueron completamente inhibidas por los factores solubles provenientes del epitelio. Luego mostramos que esta inhibición es específica para *N. meningitidis*, ya que células dendríticas plasmacitoides estimuladas con otras bacterias Gram-negativas y Gram-positivas inducen la secreción de IFNα, pero solo el IFNα inducido por *N. meningitidis* resultó inhibido por los factores solubles proveniente de las

células epiteliales. Este estudio destaca la compleja interacción entre los diferentes tipos celulares en la mucosa (células dendríticas mieloides, plasmacitoides y células epiteliales) durante la respuesta inmune y la fina modulación ejercida por *N. meningitidis* en esa respuesta.

INTRODUCTION

Mammalian immunity is one of the most complexes, organized and multilevel controlled systems in the world of living beings that evolved mainly as a consequence of microbe-selected pressure. It is composed by an intricate interaction of cellular and molecular factors coordinated in time and space to protect the host from exogenous and endogenous signals. In vertebrates, immune response can be divided in innate and acquired immunity.

Cells that participate in innate immunity recognize a limited number of endogenous (self) or exogenous (non-self) signals named Danger Associated Molecular Pattern (DAMP). Exogenous signals may come from microbes such as bacteria, fungi or viruses, while endogenous signals come from host antigens such as nucleic acid from dying cells. The innate immune response acts quickly to eliminate danger signals. This function is carried out by a myriad of cells and soluble factors. Among them are Dendritic Cells (DC), macrophages, monocytes, neutrophils, eosinophils, basophils, mast cells, natural killer cells, complement, coagulation factors and cytokines [1].

Similar to innate immunity, acquired immunity can be triggered by self and non self signals. The major difference resides in that acquired immune cells recognize innumerable antigens besides DAMP signals

recognized by cells of the innate immune system. However, to activate acquired immunity it is necessary to process and present antigens to T lymphocytes. Only DC are able to initiate this type of immune response [2].

1.Dendritic cells

DC are the essential antigen-presenting cells linking innate and acquired immunity. These cells are localized at the site of entry of pathogenic microorganisms and have phagocytic abilities which allow them to capture, process and present antigens from the peripheral tissues to naïve T lymphocytes localized in the secondary lymphoid tissue. This event is known as priming of immune T cells [3].

1.1 Antigen recognition and activation of dendritic cells

DC are scattered through all the tissues sensing the presence of foreign microorganisms. Once detected, DC can phagocyte those microbes and become activated. The detection of microorganisms occurs through specialized receptors expressed on DC called PAMP Recognition Receptors (PRR) which recognize Pathogen-Associated Molecular Patterns (PAMP) or DAMP present on the microbes or altered cells [4]. These PRR are also expressed by non-immune cells and can be localized on the surface, the cytosol or within intracellular compartments. Some PRR such as Toll-Like Receptors (TLR) and C-type lectin receptors are

transmembrane receptors, while Nucleotide-binding and oligomerization domain (NOD)-Like Receptors (NLR) and Retinoic-Inducible Gene I (RIG-I) Like Receptors (RLR) are exclusively intracellular, probably distributed in the cytosol [5]. DAMP includes carbohydrates, lipids, peptides and nucleic acids. TLR are essential to activate acquired immunity and are the most studied PRR [6]. In human up to ten TLR have been described which include TLR-2, TLR-4, TLR5 and TLR-6 that are expressed on cellular surface and TLR-3, TLR-7, TLR-8 and TLR-9 that are expressed in endosomal compartments. Surface TLR mainly recognize external component of pathogens: TLR-2 binds to peptidoglycan of Gram-positive bacteria; TLR-4 binds lipopolysaccharide of gram-negative bacteria; TLR-5 recognizes flagellin and TLR-6 fungi cell wall. On the other hand, endosomal TLR are specialized in the recognition of pathogen nucleic acid. TLR-3, TLR-7 and TLR-8 bind to RNA from virus while TLR-9 detects DNA molecules that contain unmethylated CpG-containing motifs commonly found in bacteria, fungi and virus. The engagement of these PRR induce several signaling pathways that converge in the activation of transcription factors such as activator protein-1 (AP-1), Mitogen-Activated Protein Kinase (MAPK), Nuclear Factor Kappa-B (NFkB) and Interpheron Regulatory Factor (IRF), depending on the molecular signature of the pathogen or tissue damage [5, 7].

DC activation involves a complete modification of the cell physiology characterized by an increase of the expression of co-stimulatory molecules such as CD80, CD40, CD86 and Major Histocompatibility Complex (MHC). Furthermore, activated DC secrete cytokines specialized for cellular communication involved in many physiological functions. The combination of DAMPS expressed by the microbes and the messages from microenvironmental tissue will condition DC function. Hence, once DC arrive to secondary lymphoid tissues, the signals integrated at the periphery will be communicated to T lymphocytes during the priming which in turn will determine the kind of response displayed to limit pathogen burden and tissue damage [3].

1.2 Dendritic cells directing T cell response

Commonly, intracellular antigens from virus or intracellular bacteria are presented on MHC class-I molecules to CD8 T cells while extracellular antigens are presented on MHC class-II molecules to CD4 T cells, also known as T helper (TH) cells. In the presence of IL-12, TH cells can differentiate to TH1 cells that secrete IFNγ and express T-bet transcription factor. On the other hand, in the presence of IL-4, TH cells can differentiate to TH2 that secrete IL-3, IL-4, IL-5, IL-13 and IL-10 and express GATA-3 transcription factor. TH1 cells are involved in immunity against intracellular pathogens, which promote CD8 and NK cell function. TH2 cells induce isotype switching by B cells and bias their

differentiation to IgG-secreting plasma cells which allow extracellular bacteria clearance [8, 9]. Recently, a third subset of T lymphocytes has been described called TH17 cells. In humans, TH17 cells are produced when the differentiation takes place in the presence of IL-1β, IL-6, IL-23 and TGFβ which allows the expression of the transcription factor RORγt [10-12]. Results from different groups show opposed evidences regarding the need of TGFβ for TH17 differentiation *in vitro* [13, 14]. TH17 plays a critical role protecting the host against a variety of bacteria and fungi but it is also involved in autoimmunity and inflammation [15, 16]. Another subset of T cells can differentiate following the interaction with DC. These cells, called induced regulatory T cells (Treg) are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limit chronic inflammatory diseases [17]. Treg are characterized mainly by the expression of the transcriptional factor Foxp3 [18]. In humans, *in vitro* generation of Treg by human DC is still unclear, but the presence of IL-10 and TGFβ seem to be essential in this task [19]. The TH classification is an overview of the complexity of the response triggered by danger signals. The type of T helper and B cell response induced after microbial encounter depends and reflects the nature of the microbe, the tissue where it was found and the subset of DC that senses the signal.

1.3 Dendritic cell subsets

An important factor to consider when studying the activation of the immune response is the existence of different subsets of DC. These subsets are classified depending on its origin and biological properties (phenotype, function, and microenvironment within the body). DC are specialized in sensing different types of pathogens and therefore determine distinct classes of immune responses. There are two different subsets of DC in humans: myeloid (mDC) and plasmacytoid (pDC) [20]. Myeloid or conventional DC express CD11c, while pDC lack this marker. Instead, pDC express CD123, the alpha chain of IL-3 receptor and CD4. Both subsets are lineage (CD3,CD19,CD14,CD16) negative and MHC-II positive [21, 22]. A major difference between mDC and pDC reside in their differential expression of TLR. mDC express TLR-1, TLR-2, TLR-3 and TLR-8 but not TLR-7 nor TLR-9, while pDC express only TLR-7 and TLR-9 [23]. TLR-4 was described to be expressed by *in vitro* monocyte-derived DC (MoDC) but its expression on freshly isolated mDC from blood or tissue remains to be confirmed [24]. The differential expression of TLR has led to a specialization of DC subtypes to respond to different stimulus. mDC are specialized in responding to extracellular antigens like extracellular bacteria. On the other hand, pDC are described to induce a strong inflammatory response to intracellular signals such as viruses and nucleic acids [25]. mDC activation leads to IL-1, IL-6, IL-10,

IL-12p70 and TNFα secretion, while pDC are characterized by the production of type-I interferon in response to virus and CpG. pDC also secrete IL-6 and TNFα [26, 27]. It has been shown that pDC can also be activated by extracellular bacteria as *Staphylococcus aureus* and *S. pyogenes* [28, 29]. However, this aspect of pDC function remains poorly understood. Another difference between both types of DC is that mDC are more efficient in the phagocytosis and T cell priming than pDC. Both subsets of DC are able to direct TH1, TH2, TH17 and Treg differentiation, but a particular role directing TH1 and Treg has been ascribed to pDC [9, 20, 30]. mDC and pDC have been found in the blood and also infiltrating nasal and tonsil epithelium in human [31-33]which will be of consideration in this work.

1.4 DC integrating signals from tissue environment

As mentioned before, the initiation of acquired immune response depends on the nature of the antigen and on the tissue context where the antigen is captured by the DC. This work is focused on tonsil mucosa microenvironment and its potential effect on DCs. Here, we summarize some studies that reveal the impact of the epithelium on DC response. Although, pDC can also infiltrate the mucosa and hence integrate signals from surrounding tissue [31-33], the role of the epithelium on pDC function has never been addressed and will be addressed in this work.

Mucosal epithelium is the first cellular front line to be in direct contact with external antigens, limiting the outside from the inside of the host. Its role as an active player on immunity has been largely ignored, until recently. It is well accepted now that epithelial cells determine the beginning and the fate of an immune response. Physical interaction between epithelium and DC became evident when extension of mDC dendrites through epithelial barrier was observed. These projections appear to sample bacteria present in the lumen of the gut [34, 35]. Tight interaction between epithelium and DC are accomplished without disrupting the epithelium integrity since DC express thigh junction proteins. Interestingly, both pathogenic and non-pathogenic bacteria can induce mDC extensions [36].

Soluble factors play a major role in this interplay between DC and epithelium, skewing acquired immune response. For instance, recent studies show that Thymic Stromal Lymphopoietin (TSLP) from epithelial cells activates mDC without the presence of any TLR-ligand. Particularly TSLP increase OX40L expressed on mDC surface directing the response toward TH2 differentiation [37]. In addition, uncontrolled expression of TSLP in keratinocytes or airway epithelium can lead to TH2-associated pathologies such as atopic dermatitis and allergic asthma [38, 39]. Together these results show a unique capacity for a cytokine to elicit acquired immunity acting through DC during homeostatic condition,

without TLR engagement. This was demonstrated both in mice and human. IL-25 (IL-17E) also appears to be necessary for the induction of TH2 response and together with TSLP can direct immunosuppressive response. This is possible since TSLP impair TH1 response by inhibiting IL-12 from DC whereas IL-25 inhibits TH17 by inhibiting IL-23 production by DC. In mice, intestinal epithelial cells also release TGFβ and retinoic acid responsible for driving CD103+ tolerogenic DC development [40]. In humans, CD103+ DC seem to induce Treg in response to Retinoic acid, TGFβ plus TSLP, all produced by intestinal epithelial cells [41, 42]. Inflammatory cytokines are also produced by the epithelium and can be induced by external factors. This is the case for IL-1α, IL-1β and IL-18, which create a pro-inflammatory phenotype in the mucosa [43].

Epithelial cells produce metabolic enzyme and intermediaries including indoleamine 2,3-dioxygenase (IDO), cyclooxygenase-2 and the arachidonic-acid metabolite prostaglandin E2 (PGE_2), which may regulate innate and acquired immunity. For instance, PGE_2 DC have an impaired capacity to produce IL-12, while producing high levels of IL-10 and inducing the differentiation of T cells towards TH2 cells [44].

Epithelium is infiltrated by several immune cells such as lymphocytes, and may have a modulator role on the immune response. A role for activated epithelial cells in directly regulating B-cell response

was shown through their secretion of B-cell Activating Factors (BAFF) and a Proliferation-Inducing Ligand (APRIL) that favored T-cell independent IgA responses [44]. In contrast, the release of secretory protease inhibitor (SLPI) by epithelial cells control APRIL pathway and limit IgA induction [32]. Together, these data support a complex role for the epithelium as a regulator of the innate and acquired immunity.

How can epithelial cells sense external stimulus? As immune cells, the epithelium expresses different families of PRR: TLR, NLR, RIG-I, CLR that allow them to recognize DAMP from exogenous pathogens. The importance of PRR expressed by the epithelium has been demonstrated in several studies using TLR-4, NOD-1 and NFκB deficient mice. These mice have impaired immunity to bacterial infection. In contrast, increased function of NFκB epithelial cells induces a deregulated pro-inflammatory cytokine response in mice [43, 44].

A fundamental question in the field of mucosal immunology is how the host discriminates between pathogenic and commensal bacteria. To address this question it is important to keep in mind that immunological processes are confined in organized compartments shaped by a network of cellular and soluble factor interaction that include microbial agents. But what is the contribution of each component to trigger an "adequate" immune response? These are aspects that will be addressed in this

thesis, using as a microbial model the versatile commensal/pathogenic bacteria *Neisseria meningitidis*.

2. Neisseria meningitidis

2.1 Between commensalism and pathogenesis

Neisseria meningitidis (*N. meningitidis*), or meningococci, are Gram-negative extracellular bacteria that live as commensal organisms in up to 40% of the adult population, exclusively in human oro-nasopharynx mucosa. The transmission of bacteria occurs by direct contact or saliva droplets between carriers allowing its rapid spread. Occasionally, bacteria initiate pathologies after crossing mucosal epithelium and reach the bloodstream by mechanisms that are still unknown. Once in the blood, bacteria can proliferate and induce septicemia or alternatively cross the blood-brain barrier, proliferate in the cerebrospinal fluid and provoke meningitis. It is still unknown how the same bacteria are converted from non-pathogenic commensal into pathogenic microbes. The exclusive reservoir of *N. meningitidis* is the human oro-nasopharynx converting the bacteria into an obligate human pathogen. This exclusivity is mainly due to three different aspects detailed below.

***Neisseria meningitidis* as an obligate human pathogen**

Adhesion

Adhesion of *N. meningitidis* to host cells is essential for commensalism and infectious stages of the bacteria and this process only occurs on human cells. Carriage of bacteria at the mucosal level is

possible since *N. meningitidis* adhere to epithelial cells. The adhesion of *N. meningitidis* to epithelial cells was revealed using human tissue in organ cultures from nasopharyngeal and tonsil samples [45, 46]. This was also reproduced in vitro using epithelial cell lines and primary bronchial epithelial cells [46]. During the infectious stage, *N. meningitidis* adhere to endothelial cells at the lumen of capillaries located in the infected organs. This was observed by immunohistological study of a meningococcal sepsis [47]. In both, epithelial and endothelial cell adhesion, *N. meningitidis* appears to form aggregates called microcolonies [47]. The key bacterial factor involved in the microcolonies arrangement is the type IV pili exposed at *N. meningitidis* surface [48, 49]. This adhesion process is highly specific to human cells, for instance adhesion does not occur on murine cells.

Iron acquisition

Acquisition of iron and iron complexes has long been recognized as a major determinant in the pathogenesis of *N. meningitidis*. Experiments performed by Holbein et al. in 1980 demonstrate that *N. meningitidis* capture iron complexed to human transferring. In these studies, they inject iron compounds (iron dextran or human ferritransferrin) into mice and challenged them with a subsequent intraperitoneal inoculation of live *N. meningitidis*. This resulted in lethal infection, whereas mice

injected only with *N. meningitidis* suffered a transient bacteremia and recovered quickly.

Complement system

The systemic spread of *N. meningitidis* depends on its capacity to survive within the host circulation. Human complement system is essential to prevent this spread. This is evident in individuals lacking specific components system who have elevated risk of developing meningococcal disease compared with the general population [50]. Nevertheless, *N. meningitidis* express some structures that resist complement mediated-lysis. A large scale analysis of the meningococcus genome has revealed that all the genes required for this resistance are involved in the synthesis of polysialic acid capsule and the lipooligosaccharide (LOS), a short version of the classical lipopolysaccharide [51]. Therefore, *N. meningitidis* is highly adapted to its host which explains the lack of animal model.

Epidemiology and prevention of meningococcemia

N. meningitidis-related diseases develop quickly, after only few hours, limiting the effectiveness of antibiotic treatment. Furthermore, some strains of *N. meningitidis* are responsible for large epidemics in Africa and sporadically in the United States and Europe. For these

reasons, the best way to limit meningococci diseases is by preventing the systemic entrance of the bacteria through vaccination.

Vaccines against *N. meningitidis* have been developed in the past, and are continuously improving. Unfortunately, protection induced by these vaccines is still limited since they have failed to induce long-term immunity and not all the strains of *N. meningitidis* are covered. More importantly, there is still no protection for infants, the most affected population together with adolescents. These are reasons that explain why *N. meningitidis* remain a worldwide health problem [52].

For the development of new vaccines, attention has been centered on the innate immune response to *N. meningitidis* mainly at systemic level. Only few publications explore immune response trigger at mucosal level, the place where *N. meningitidis* enter in contact with the host. The reason for such limited information may be attributed to the lack of animal models which limits the study of mucosal immune response to *N. meningitidis*.

2.2 Innate immunity to N. meningitidis at the systemic level

Once viable *N. meningitidis* reach and proliferate in the bloodstream, the release of endotoxins mainly bacterial LOS induces an exacerbated production of cytokines. It was suggested that patients who develop overwhelming sepsis are highly endotoxin responsive, i.e.

inclined to produce larger amounts of pro-inflammatory cytokines. These cytokines are mainly TNFα, IL-1β, and in low amounts IL-12. IL-10 an anti-inflammatory-related cytokine was also detected during meningococcal septic shock. Contradictory results show that both pro-inflammatory TNFα and anti-inflammatory IL-10 cytokines correlate with the disease severity [53, 54]. The source of these cytokines is not clear, but a contribution in modulating innate and acquired immune response by endothelial cells, monocytes, neutrophils, macrophages and DC has been described [55, 56].

The clearance of bacteria in the bloodstream is achieved mainly by the complement system and phagocytic cells. However, bacteria develop strategies to subvert the innate immune response. *N. meningitidis* can perform post-translational sialylation of its LOS and express sialic acid capsule. As mentioned above, these structures play an important role in the evasion of the complement system. Moreover, they can decrease the phagocytosis and the release of cytokines in several cells of innate immune system and the endothelium and render the bacteria poorly immunogenic [55, 57, 58].

2.3 Naturally acquired immunity to N. meningitidis is associated with its carriage at the mucosal level.

Acquired immune response to *N. meningitidis* has been evidenced by the presence of specific antibodies and appears to be generated naturally, without previous vaccination. This so called naturally acquired immunity is related to the commensal stage of the bacteria at the oro-nasopharynx mucosa. This was demonstrated for the first time by Goldshneider in 1969. In his study, he measured the carriage of *N. meningitidis* in the saliva of military recruits in the beginning and after several weeks of recruitment. After only 24 days, up to 94% of the population acquired the bacteria in the mucosa. Furthermore, this increase in the carriage was correlated with an increase of specific antibodies to *N. meningitidis* [59]. It has been shown that people with higher titer of serum bactericidal antibody were protected from subsequent disease. Then, other groups demonstrated the presence of immunoglobulin (Ig)G and IgA antibodies specific to *N. meningitidis* in the saliva of the carriers [60, 61]. Concordantly, infants younger than 6 months and up to 24 months of age present the lower serum activity and are the population with the highest incidence of the disease [52]. Most recently, studies performed by Heyderman's laboratory showed the induction of TH1, TH2 and Treg response to antigen derived from *N. meningitidis* using human tonsil mononuclear cells, with a predominance

of TH1 responses [62, 63]. Furthermore, a study with *Neisseria gonorrhoeae* (*Ng*) shows the induction of T regulatory cells during vaginal infection of mice [64]. *Ng* is closely related to *N. meningitidis* and share many properties with them, suggesting that similar response can be induced by *N. meningitidis*. Altogether, these data support that *Neisseria* are able to induce an acquired immune response initiated at the mucosal level that results protective to subsequent infections.

Through the evolution, *N. meningitidis* have developed genetic tools to alter their surface structures and evade the immune system. Phase variation and antigenic variation challenge the recognition of specific antigens of acquired immune cells. This was reflected in the modification of molecules associated with the process of adhesion, colonization and also the exposure of DAMP to host cells [65]. Furthermore, *N. meningitidis* can also secrete specific proteases that cleave secreted human IgA, obstructing the activity of specific antibodies to *N. meningitidis* [66]. This reveals the importance of understanding the feature and mechanism by which acquired immunity achieves its protective role against *N. meningitidis*, despite the strategies used by the bacteria to escape this response.

2.4 Interaction of Neisseria meningitidis with host cells

As described before, *N. meningitidis* can form aggregates at the surface of both the epithelium and the endothelium. Epithelial cells from oro-nasopharynx mucosa are the first to contact *N. meningitidis*, the place where *N. meningitidis* live as commensal organisms. Conversely, endothelial cells interact with *N. meningitidis* only when bacteria reach the blood. Furthermore, mucosal and blood tissues, are infiltrated by cells of immune system able to respond to *N. meningitidis*. Here we describe important results found in the literature regarding DC response to *N. meningitidis.* Next, we describe the consequences of the interaction between epithelial and endothelial cells, with particular attention in epithelial cell response.

Dendritic cells and *Neisseria meningitidis*

Interaction between DC and *N. meningitidis* has been studied using different models. Human DC can be generated in vitro by adding IL-4 and GM-CSF to mononuclear cells (MoDC). With those cells, phagocytosis of *N. meningitidis* has been demonstrated and was shown to be followed by an increase of co-stimulatory molecules and IL-1β, IL-6, IL-8, IL-12 and TNFα production. Together, these data show that *N. meningitidis* activate the MoDC. This activation depends on the binding of LOS and outer membrane protein from *N. meningitidis* with TLR-4

and TLR-2 respectively. LOS from *N. meningitidis* can direct TH1 response whereas their outer membrane proteins induce preferentially a TH2 biased response [55, 57, 67-71]. Hence, activation of MoDC by *N. meningitidis* can elicit a TH response. MoDC share some features with freshly isolated mDC from tissue or blood. For instance, both express on their surface the CD11c marker. However, major differences can be found between both cells, as freshly isolated mDC secrete only low levels of IL-12p70 in response to LPS, an essential cytokine involved in TH1 polarization. Furthermore, the expression of TLR4 in freshly isolated mDC from blood is still controversial, since some studies argue that mDC lack TLR-4 expression while other show their expression [6, 24]. Most importantly for the purpose of this thesis, *in vitro* generated DC lack important signals compared with freshly purified DC which integrate signals from the tissue where they come from. This can be decisive in the fate of the immune response to *N. meningitidis* and can differ depending on the tissue where this response is triggered.

On the other hand, the activation of pDC by *N. meningitidis* has never been directly demonstrated in humans. Only two papers approach this type of study and they show an increase in bacteremia and a reduction of mice survival using TLR9-deficient mice, *in vivo*. *In vitro*, stimulation of wild type pDC with *N. meningitidis* resulted in high levels of IL-6, TNFα and IFNα secretion which was abrogated in TLR-9-/- pDC

[72]. Although as mentioned before, a murine model is not optimal to characterize immune response to *N. meningitidis*, which are human obligate bacteria. The second study performed with human blood used *N. gonorrhoeae* bacteria. As *N. meningitidis*, N. *gonorrhea* colonize human epithelium but at the cervix and urethral mucosa. The study revealed an induction of IFNα by human blood cells upon exposure to Ng. This IFNα was inhibited when pDC where depleted from blood cells [73]. Nevertheless, the blood used in this work was from patients infected with human immunodeficient virus and depleted of CD8 cells before being used.

In conclusion, important information is still missing to help us clarify the biological function of human DC during *N. meningitidis* interaction at systemic and mucosal levels. Moreover, the role of the epithelium in DC function to *N. meningitidis* is yet unexplored.

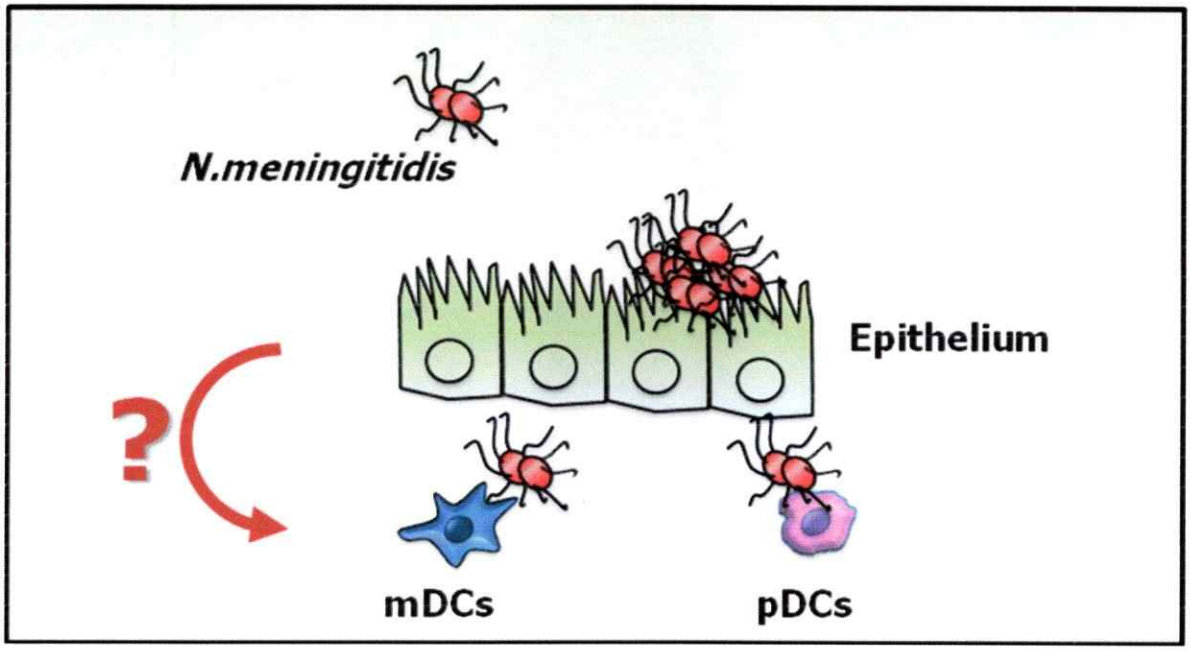
Neisseria meningitidis modifies the physiology of epithelial and endothelial cells.

Interaction of *N. meningitidis* with epithelial and endothelial cells results in a complete modification of the cell physiology. Both cell types share similar cellular response to *N. meningitidis*. This response is characterized by the recruitment of membrane and cytoskeleton

proteins such as CD46, ICAM-1, actin, ezrin under *N. meningitidis* microcolonies, which can be visualized by immunofluorescent microscopy. The rearrangement of the cells also known as cortical plaque is triggered by proteins that integrate the type IV pili expressed at the surface of *N. meningitidis* [49, 74, 75]. In addition to surface changes, *N. meningitidis* adhesion leads to complete rearrangement of the cell transcriptome. Principally, an increase of survival, anti-apoptotic and cytokine genes were found. An increase in the expression of IL-6, IL-8, TNFα and GM-CSF genes has been described [76-78]. An important difference between epithelium and endothelium is the way the bacteria cross these two cellular barriers. The bacteria are transported via a transcellular pathway to the basolateral surface of epithelial cells ([79, 80]. On the contrary, the passage of *N. meningitidis* through brain endothelium occurs by breaking intercellular junction [81].

Hence, at the mucosal level, *N. meningitidis* adhesion to epithelial cells can lead to the passage of the bacteria as well as the release of soluble factors. As a consequence *N. meningitidis*-epithelium interaction may impact the microenvironment and activation of the DC present in the tissue and potentially the fate of immune response to *N. meningitidis*.

HYPOTHESIS



In this work we hypothesized that the bacteria-epithelium interaction modulates the dendritic cell response during *Neisseria meningitidis* challenge at the oro-nasopharynx mucosa.

OBJECTIVES

Characterization of dendritic cells responses to *Neisseria meningitidis* and determination the epithelium role on this response.

SPECIFIC AIMS

1. To characterize the response of myeloid and plasmacytoid dendritic cells stimulated with *N. meningitidis*.

2. To determine the soluble factors released during *N. meningitidis* - epithelium interaction.

3. To evaluate the impact of the *N. meningitidis* -epithelium soluble factors on DC response characterized in objective 1.

4. To determine the specificity of DC response to *N. meningitidis* compared with different bacteria at the mucosa.

METHODS

Bacterial strains and growth conditions

Bacterial strains used in this work were all from human clinical isolates. A serogroup C meningococcal strain 8013, designated 2C43, was used throughout the study. *Haemophilus influenzae* (*H. influenzae*), *Staphylococcus aureus* (*S. aureus*) and α-hemolyitic *Streptococcus oralis* (*S. oralis*) strains were kindly provided by Etienne Carbonell from Clinical Microbiology Department at HEGP. *N. meningitidis* was made to express the green fluorescent protein (GFP) by introducing the pAM239 plasmid by conjugation [47]. Bacteria were stored at -80°C in GCB Glycerol. All the infection assays were done with the same stock of frozen bacteria. The day before the infection, *N. meningitis* and *S. aureus* were grown on GCB/Agar plates (Difco) and *H. influenzae* and *S. oralis* were grown on Blood/Agar plates (BD Diagnostic system) at 37°C, 5% CO_2. The day of infection, bacteria from overnight culture were adjusted to an OD_{600}= 0,05 and incubated for 2h at 37°C, 5% CO_2 in appropriate medium. Bacterial cultures with an OD_{600} between 0,2- to 0,3 were used to stimulate dendritic cells or epithelial cells.

Freshly purified human dendritic cells

Tonsils were recovered from Hospital Calvo Mackenna in Chile and Hôpital Necker in France. Both plasmacytoid and myeloid dendritic cells

were obtained using a modified protocol from (Kadowaki, 2000). Briefly, tonsils were digested with 1 mg/mL of Collagenase-D (Roche Diagnostic) and 25 μg/mL of DNAse (Roche Diagnostic) for 15 min at 37°C, 5% CO_2 and 120rpm. Digestion procedure was repeated three times. Cells were recovered and filtered using a 70μm cell strainer (BD Falcon). Mononuclear cells were isolated by Ficoll gradient (GE Healthcare). Cells collected after the Ficoll were washed 3 times with PBS (GIBCO BRL). Mononuclear cells were depleted of lymphocytes with a mixture of anti-CD3 (Supernatant from clon UCHT1) and anti-CD19 (Biolegend clon HIB19) monoclonal antibodies (mAbs) and magnetic anti-IgG Dynabeads (Invitrogen).

Buffy coats were obtained from healthy adult donors at *Hôpital Européen George Pompidou* following the convention between Inserm U970 and EFS or from healthy adult donors at the Saint-Antoine Crozatier Blood Bank. A Ficoll gradient (GE Healthcare) was performed at 18°C, 2400 rpm for 20 min to obtain mononuclear cells. Cells were carefully collected and washed 3 times with PBS (GIBCO BRL). Mononuclear cells were depleted of lymphocytes, monocytes and NK cells with a mixture of anti-CD3 (Supernatant from clone UCHT1), anti-CD19 (Biolegend clone HIB19), anti-CD14 (Dendricics clone MOPG), anti-CD56 (*Biolegend* clone HIP56) mAbs and magnetic anti-IgG Dynabeads (Invitrogen).

The same protocol was used to positively select both tonsil and blood DC was used. Cell staining was performed using a mixture of anti-CD3-FITC (Miltenyi Biotec), anti-CD19-FITC (BD/Pharmingen), anti-CD14-FITC (BD/Pharmingen), anti-CD16-FITC (BD/Pharmingen), anti-CD56-FITC (BD/Pharmingen) mAbs for lineage markers, anti-CD11c-PE (BD/Pharmingen) and anti-CD4-APC (Miltenyi Biotec) for DC markers. CD4+, CD11c+lin- cells and CD4+, CD11c-lin- were isolated as mDC and pDC respectively by cell sorting using FACS Aria sorter. Purity was checked after each sorting and was >99%.

Stimulation of dendritic cells

Dendritic cells were cultured in 96-well plates at a density of $1x10^6$ cells/mL in RPMI 1640 containing 10% FCS, 1% pyruvate and 1% HEPES. Bacteria were added to cells at a multiplicity of infection (MOI) of 10. As positive controls, mDC were stimulated with 1 μg/mL of LPS and pDC with 10^9 PFU of non-activated influenza virus or 5 μg/ml of CpG-C (Kindly provided by Dr. F. Barrat). In some experiments, supernatants from bacterial-infected or non-infected epithelial cells were added to DC with bacteria. 1% of penicillin/streptomycin antibiotics were added 2 h after infection to prevent bacterial growth. After 24h of culture supernatants were collected and frozen at -80°C until assayed. Cells were recovered to evaluate maturation phenotype of DC.

Primary human tonsils epithelial cell (HTEC) culture

Tonsils from the "Service ORL Pédiatrique of hôpital Necker" in France or "Servicio otorrino-laringología of Hospital Calvo Mackenna" in Chile were recovered following ethical protocols. The protocol used was modified from Pegtel et al, 2004 [82]. Soft tissue was separated from epithelium under sterile conditions, by cutting and scrapping with scalpel and tweezers. Small explants of epithelium (3-5 mm) placed in Petri dishes with DMEM-F12 medium (GIBCO BRL) supplemented with 10% Fetal Bovine Serum (PAA Laboratories), penicillin, streptomycin and Amphotericin B were incubated overnight at 37°C in a humidified incubator under 5% CO_2. Next day, explants were washed with PBS at RT. Explants were cut in smaller pieces (1-3 mm) and placed on cell culture treated dishes (approximately 4 dishes per tonsils). 3 mL of fresh medium supplemented with calcium (100mM) was carefully added to induce adherence of the explants. After 2 days the calcium-rich medium was replaced with Defined keratinocyte-serum free medium plus keratinocyte supplement (GIBCO BRL). Every two days the medium was changed until epithelial cell colonies reached 1-2 cm in diameter. Cells were trypsinized for 10 min at 37°C. A sample of the cells was used to analyze the expression of cytokeratin by flow cytometry. For immunofluorescence assays, epithelial cells were grown overnight at a density of 10^5 cells/cm^2 onto 12-mm diameter glass coverslips coated

with fibronectin (10 μg/ml in PBS for 30 min). For cytokines secretion assays, cells were grown in 24-well plates at a density of $2x10^5$ cells per mL and grown until they reached confluence by changing the medium every two days.

Stimulation of human tonsil epithelial cells

For immunofluorescence assays epithelial cells were infected with a GFP-expressing strain of *N. meningitidis* using a MOI of 100. After 30 min unbound bacteria were washed away with PBS at RT and infection was allowed to proceed. Assays were stopped after 4 to 6h of infection, depending on the *N. meningitidis* colony size. For cytokines secretion assays, indicated bacteria were added at a MOI of 10 to 100. After 2h of infection, 1% of penicillin/streptomycin and amphoterycin B were added to stop bacterial replication and fungi contamination. After 24h of infection, supernatants were recovered from infected cells and centrifuged at 10000 rpm, 4°C for 15 min to pellet residual cells and bacteria. Supernatants were aliquoted at -80°C and then used to quantify cytokines and stimulate DC in culture.

Dendritic cell phenotype

Stimulated-DC were recovered and stained with anti-human CD86 or an IgG1 isotype (BD Bioscience) and were analyzed in a BDLSRII Flow cytometry (BD Bioscience). Dead cells were stained using DAPI.

CD86 expression was analyzed on DAPI-negative gated cells using FlowJo® 7.6.1 Software (Tree Star Inc.).

Human tonsil epithelial cell phenotype

HTEC were permeabilized using Cytofix-cytoperm (BD Bioscience) for 20 min at RT. Cells were washed with PermWash (BD Bioscience) and stained for 20 min with anti-human Pan-cytokeratin (SIGMA). Cells were analyzed with a BDLSRII Flow cytometer (BD Bioscience) using FlowJo® 7.6.1 Software (Tree Star Inc.).

Immunofluorescence of human tonsil epithelial cells

Non-treated and bacteria-treated epithelial cells were washed with PBS (GIBCO BRL). Cells were fixed with 3,7% paraformaldehyde for 20 min at RT and washed with PBS. Fixed cells were permeabilized with 0,1% of Triton x-100 (SIGMA-Aldrich) for 5 min at RT. Unspecific binding was blocked with PBS-0,2% gelatin for 30 min RT. Cells were incubated with anti-ZO-2, anti-occludin, anti-ezrin primary antibodies at RT for 1 hr. Cells were washed with PBS and incubated with Goat-anti-mouse, Goat-anti-rabbit secondary antibodies (Molecular Probes) and/or phallodin for 1 hr at RT. Nucleus was stained with 100 ng/mL of DAPI for 5 min at RT. Finally, cells were washed 3 times in PBS, mounted on MoWiol® and visualized in epi-fluorescent microscope Cannon.

Cytokines measurement in the supernatants

Supernatants from infected-epithelial cell were analyzed by Cytomteric Beads Array (CBA) Flex Set (BD Bioscience) to quantify the following cytokines: IL-1α, IL-1β, IL-3, IL-4, IL-6, IL-7, IL-10, TNFα, IFNγ, IFNα, G-CSF, GM-CSF and TGFβ. The presence of TSLP was measured by ELISA (R&D Systems). IL-1α, IL-1β, IL-6, IL-10, TNFα, IFNγ and IL-12p70 were measured in mDC-supernatants while IL-6, IL-10, IFNγ, TNFα and IFNα were measured in pDC-supernatants by CBA Flex Set (BD Bioscience). CBA analysis was performed on LSRII (BD Bioscience) instruments and analyzed using FCAP Array™ Software (BD Bioscience).

Statistical analysis

A nonparametric two-tailed Wilcoxon test was used for pairwise comparisons of cytokines. P values of 0,05 or less were considered. Analysis were performed using GraphPad Prism® Software.

RESULTS

Experimental strategy: a model of human primary cells

To carry out this project we chose to use primary cells derived or isolated from human tonsils and blood. For dendritic cell (DC) isolation, we took advantage of a protocol that involved depletion of B and T cells followed by a positive selection using FACS sorting to obtain a highly purified population of DC. We were able to isolate two populations of DC based on the expression of the specific markers CD11c and CD4, in cells that lack the expression of lineage markers (CD3, CD14, CD16, CD19 and CD56). One population was $CD11c^{+}$ and the other $CD4^{+}CD11c^{-}$ corresponding to myeloid (mDC) and plasmacytoid (pDC), respectively (**Figure 1A**). On average, we obtained $5x10^{5}$ mDC and pDC for each $1x10^{9}$ mononuclear cells from tonsils (0,0005%). In some experiments, the same protocol was used to isolate pDC from blood of healthy donors obtaining up to $2x10^{6}$ pDC per volume of blood obtained for each donor.

Both mDC and pDC have been described to infiltrate tonsil epithelium [31-33]. However, the effect of mucosal epithelium during DC response to *N. meningitidis* has never been explored before. In order to evaluate the contribution of epithelial cells during *N. meningitidis* immunity, we generated a culture of epithelial cells from tonsils by using

a modified protocol from Pegtel et al. summarized in **Figure 1B** and detailed in Materials and Methods.

In the first part of this thesis we characterized the response of DC to *N. meningitidis*. Next we validated the protocol used to generate tonsil epithelial cells and characterized their response after stimulation with *N. meningitidis.* In order to evaluate the contribution of both epithelial cells and DC during *N. meningitidis* encounter, we generated a model where the three players were presents. To this end, epithelial cells were stimulated with *N. meningitidis* and the soluble factors released during this interaction were collected (**Figure 1C**). Then, these soluble factors were added to freshly isolated DC along with the bacteria. The response of mDC and pDC subjected to different treatments were evaluated separately (**Figure 1D**). Finally, we performed preliminary experiments to analyze the specificity of pDC response to *N. meningitidis* compared with different bacteria.

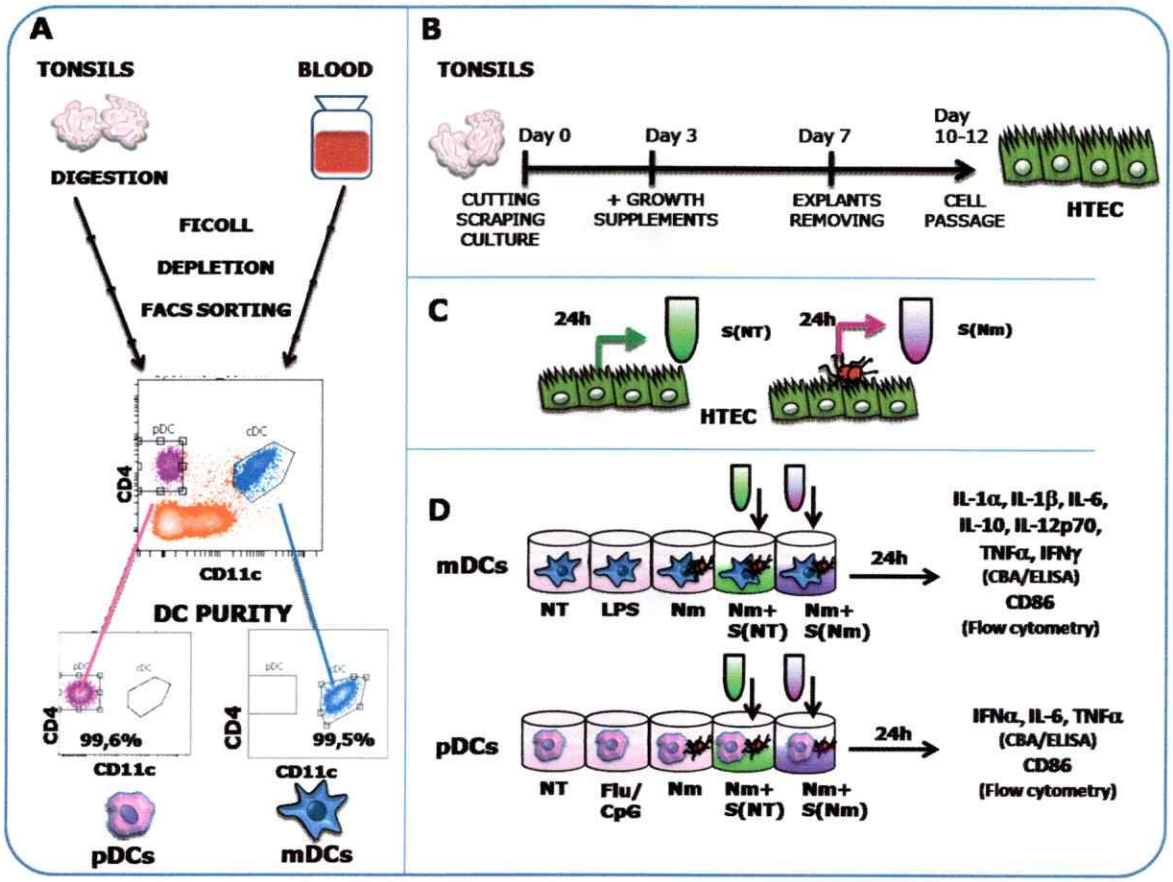


Figure 1. Experimental strategy: a model of human primary cells

A) Human myeloid DC (mDC) and plasmacytoid (pDC) were purified from peripheral blood and tonsils. Blood and tonsil mononuclear cells were obtained using Ficoll-gradient and were depleted of B and T cells. Depleted fraction was stained with Lin (CD3, CD14, CD16, CD19 and CD56), CD11c and CD4 markers. $Lin^-CD4^+CD11c^-$ pDC and Lin^-CD11c^+mDC were purified by FACS sorter (Purity>95%). **B)** Human tonsil epithelial cells (HTEC) were generated using a protocol modified from Pegtel et.al. [82]. Tonsil explants were adhered and cultured with serum-free supplemented medium. After 10-12 days, a homogeneous epithelial cell culture was obtained (Purity>90%). **C)** Soluble factors from *N. meningitidis*-treated (S(*Nm*)) or non-treated (S(NT)) HTEC were obtained after 24h of cultures. Soluble factors were stored and used to quantify cytokines and stimulate DC. **D)** Freshly purified mDC and pDC were stimulated as indicated for 24h. Supernatants from stimulated-DC were recovered to quantify cytokines and the cells were used to analyze maturation phenotype by flow cytometry.

Freshly isolated dendritic cells from tonsils are activated by *N. meningitidis*

N. meningitidis induce myeloid dendritic cell activation

We collected the supernatants released during *N. meningitidis*-mDC interaction and analyzed the levels of cytokine secreted after 24h of stimulation. In samples from up to 15 different donors, the most abundant cytokine detected was IL-6 which reaches a mean of 16 ng/mL compared to 4 ng/mL secreted by non-treated cells. IL-10 induction was also abundant, producing levels that reached 4,5 ng/mL corresponding to a 10-fold increase compared to untreated cells. Significant induction of IL-1β, TNFα, IL-1α and IL-12p70 was also detected in the supernatant of *N. meningitidis*-stimulated mDC. On average, we detected 1,5 ng/mL of IL-1β, 1ng/mL of TNFα, 70 pg/mL of IL-1α and only 20 pg/mL of IL-12p70. In our experimental setting, we used LPS as a positive control, but it appeared to be a poor stimulator compared to *N. meningitidis*, although, there is an important increase of IL-1α, IL-1β, IL-6, IL-10 and IL-12p70. We also analyzed maturation phenotype of mDC. We observed that co-stimulatory molecules such as CD86 were expressed in a high percentage on untreated mDC. This percentage slightly but significantly increased when the cells were stimulated with *N. meningitidis* (**Figure 2**). mDC were also isolated from blood samples.

Results obtained with blood mDC were similar to those obtained with tonsil mDC (**Figure 3**).

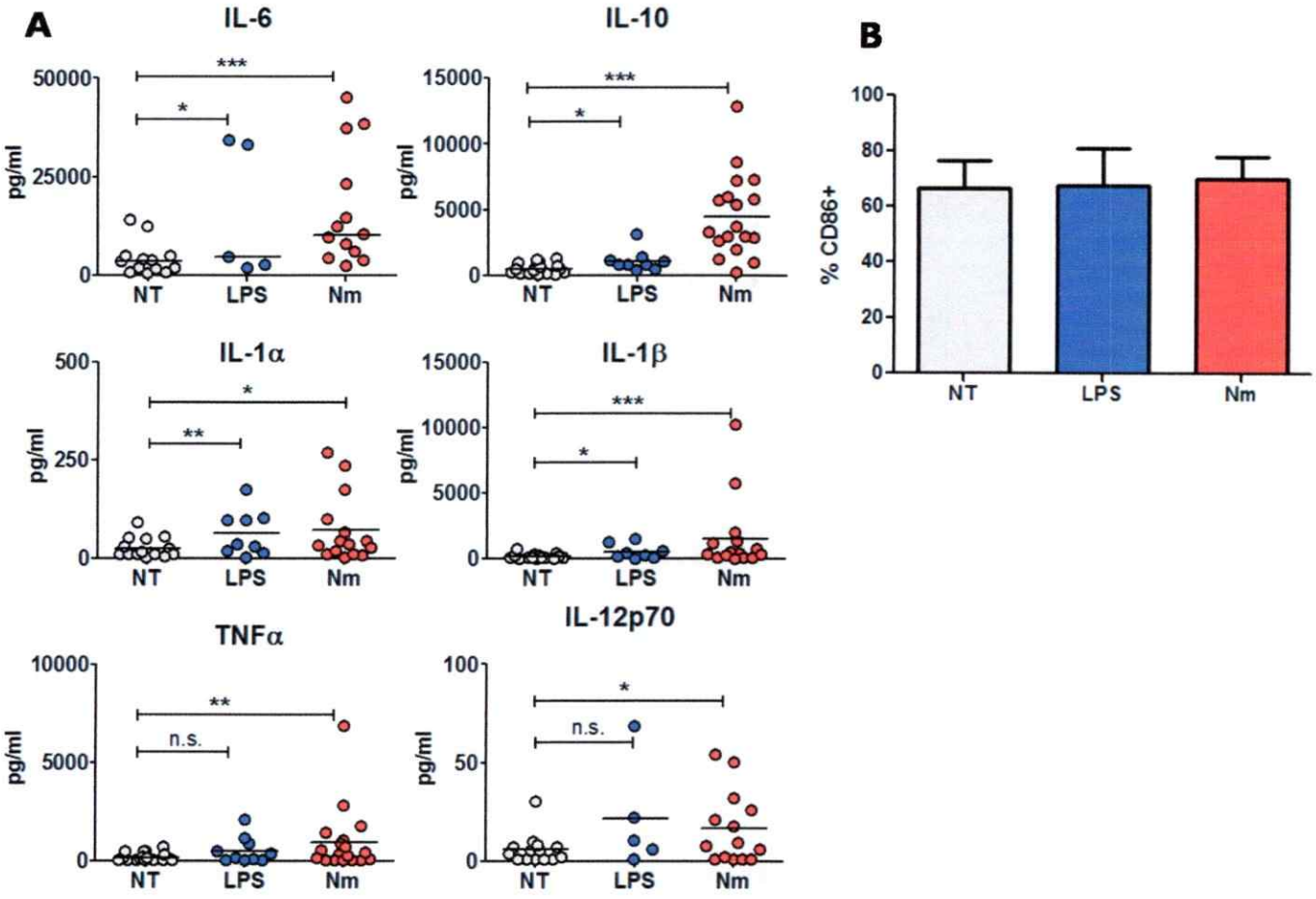


Figure 2. *N. meningitidis* activate tonsil mDC.

Freshly isolated mDC from tonsil were stimulated with *N. meningitidis* (*Nm*) (MOI=10), LPS (1μg/mL) or untreated (NT). **A)** Supernatants were collected after 24h and IL-1α, IL-1β, IL-6, IL-10, TNFα and IL-12p70 cytokines were quantified by CBA. Values and the mean of 10-15 donors are shown. **B)** Stimulated mDC were recovered after 24h and CD86 expression was measured by flow cytometry. Quantification of the percentage of $CD86^{+}CD11c^{+}DAPI^{-}$mDC is shown. Data are mean ± S.D. from 4-8 different donors. Statistical differences were evaluated using Wilcoxon test. Data point present on the x-axis had undetectable levels of cytokines. n.s. non significant differences. *, p≤0,05; **, p≤0,01, ***,p≤0,001.

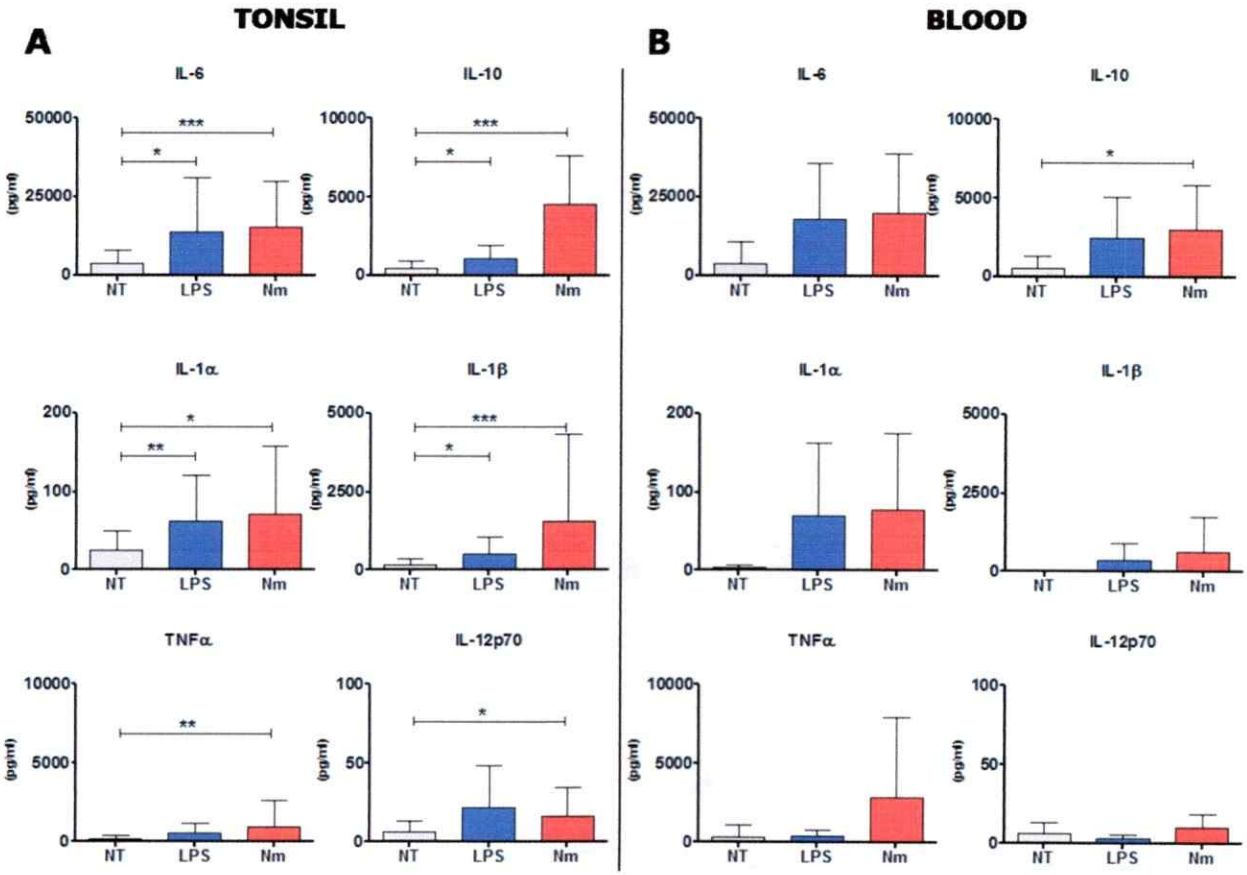


Figure 3. *N. meningitidis* induce similar pattern of cytokine secreted by blood mDC compared with tonsil mDC.

Freshly isolated mDC from tonsil or blood were stimulated with *N. meningitidis* (*Nm*) (MOI=10), LPS (1μg/mL) or untreated (NT). Supernatants were collected after 24h and IL-1α, IL-1β, IL-6, IL-10, TNFα and IL-12p70 cytokines were quantified by CBA. **A)** Values and the mean of 10-15 tonsils donors are shown. **B)** Values and the mean of 3-6 blood donors are shown. Data are mean ± S.D. from 3-6 different donors. Statistical differences were evaluated using Wilcoxon test. n.s. non significant differences. *, p≤0,05; **, p≤0,01, ***,p≤0,001.

N. meningitidis induce plasmacytoid dendritic cells activation

pDC play a major role in immune protection to viral infection but their role on bacterial infection is less known. In tonsils this subset can infiltrate the epithelium. Therefore, we stimulated freshly purified pDC from tonsils with *N. meningitidis* and measured the presence of cytokines in the supernatant of up to 15 donors. Surprisingly, *N. meningitidis* induced an average of 2 ng/mL of IFNα secreted by pDC, i.e. 35-fold more than the negative control. Significant production of IL-6 and TNFα was also detected. We measured 500 pg/mL of IL-6 and 140 pg/mL of TNFα. As expected, pDC activated with the positive control, influenza virus induced stronger IFNα, IL-6 and TNFα secretion than *N. meningitidis*-stimulated pDC (**Figure 4**).

To evaluate if pDC from non-mucosal environment can also be activated by *N. meningitidis* we purified blood pDC from up to 15 healthy donors and stimulated each one with *N. meningitidis*. As in tonsils, pDC from blood secrete higher and significant levels of IFNα compared to untreated cells. IL-6 and TNFα secretion was also significantly induced by *N. meningitidis*. In this case, TLR-9 ligand CpG was presented as positive control since it is more representative of bacterial stimulus. Furthermore, similar levels of cytokines induced by influenza virus were detected with CpG. Blood pDC displayed similar response than tonsil pDC after activation with *N. meningitidis*. The only

difference between both cells is that tonsil pDC secrete higher basal levels of cytokines compared with blood pDC in untreated conditions (**Figure 4 and 5A**). Since it is easier to obtain pDC from blood than tonsils, we used these results to continue our studies with blood pDC.

To evaluate if *N. meningitidis* can induce an increase in co-stimulatory molecules present on the surface of pDC we measured the expression of the CD86 by flow cytometry. We observed that around 20% of pDC expressed CD86 compared to the 3% of untreated cells. CpG-treated pDC showed a higher percentage of CD86 expression compared to *N. meningitidis*-pDC, which is consistent with the higher level of cytokines detected with CpG stimulus. (**Figure 5 B,C**). These results show that the activation of pDC by the bacteria is less efficient than activation by CpG.

These results demonstrate for the first time, that Gram-negative extracellular bacteria *N. meningitidis* activate pDC. This reveals an unexplored role of pDC in the generation of an immune response to *N. meningitidis*.

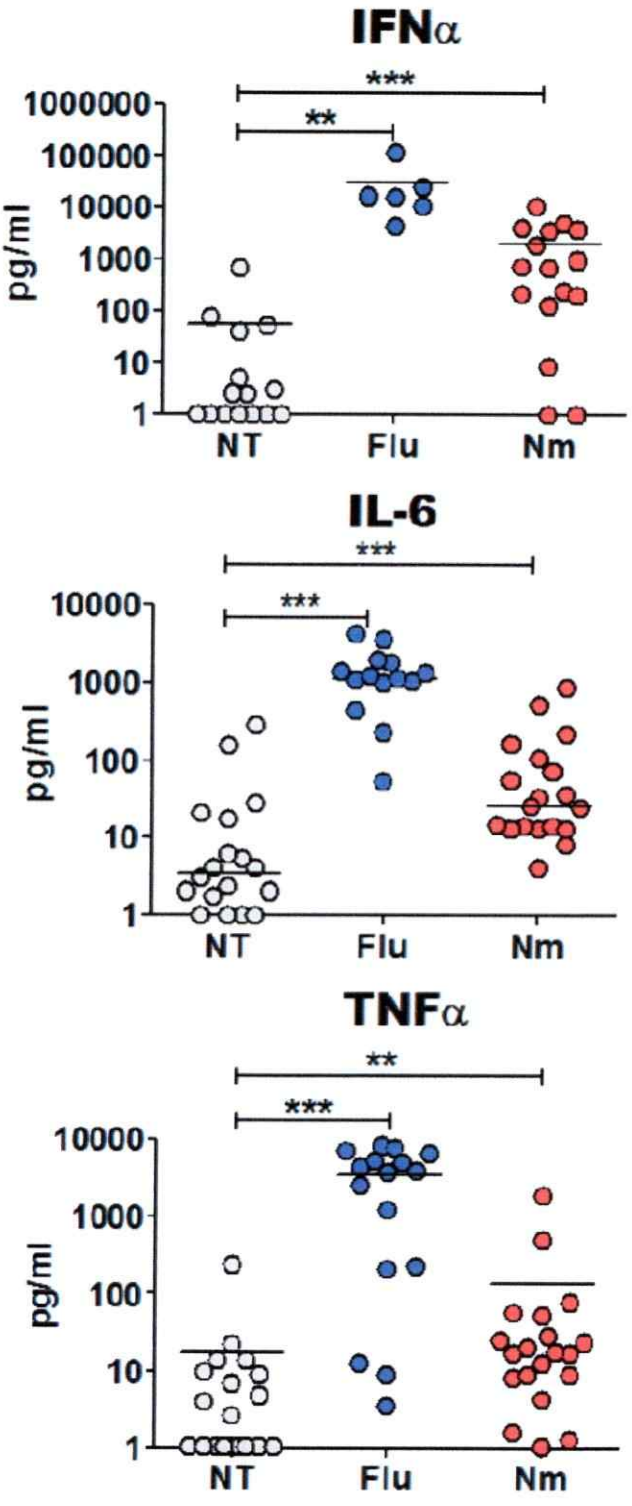


Figure 4. *N. meningitidis* activate tonsil pDC.

Freshly isolated pDC from tonsil were stimulated with *N. meningitidis* (*Nm*) (MOI=10), Influenza virus (Flu) (10^6 PFU) or untreated. Supernatants were collected after 24h and IFNα, IL-6 and TNFα were quantified by CBA. Values and the mean of 6-15 donors are shown. Statistical difference was evaluated using Wilcoxon test. Data point present on the x-axis had undetectable levels of cytokines. *, p≤0,05; **, p≤0,01, ***,p≤0,001.

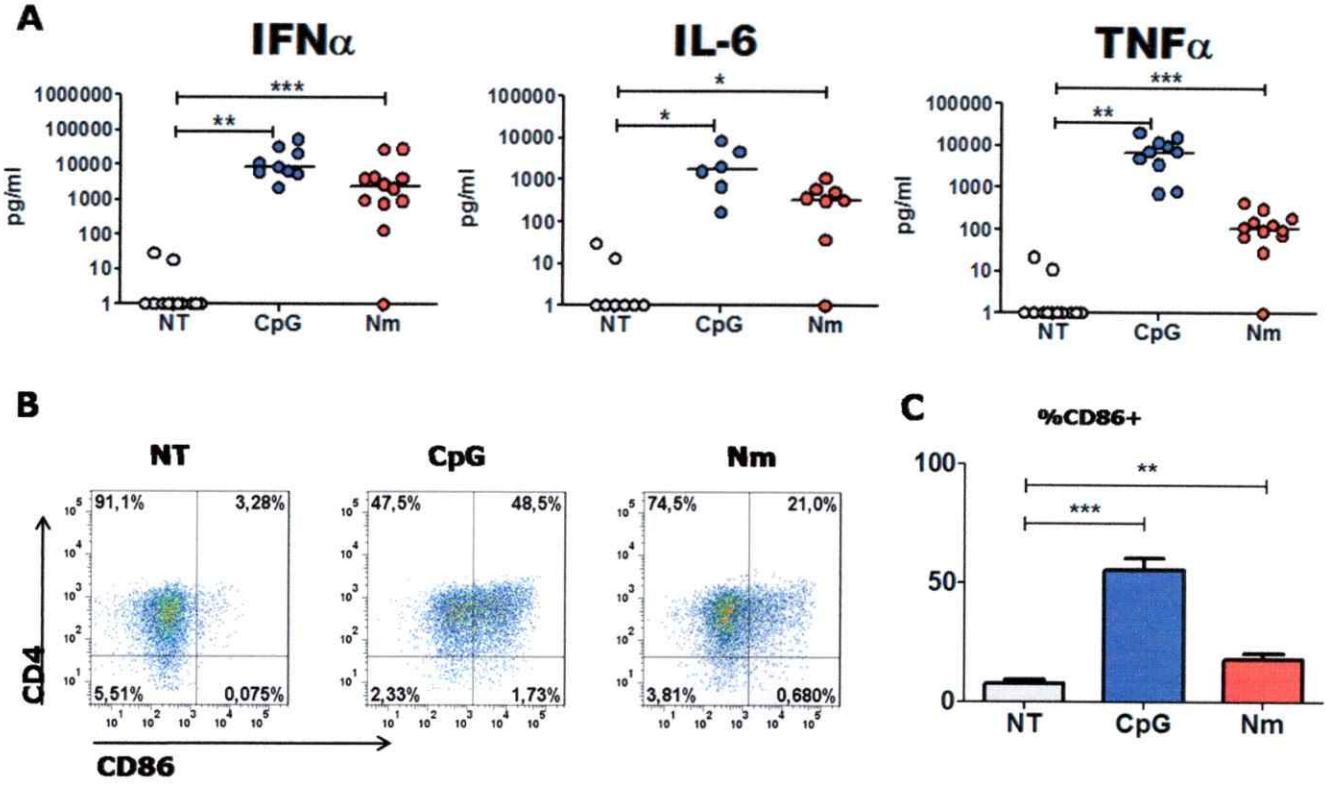
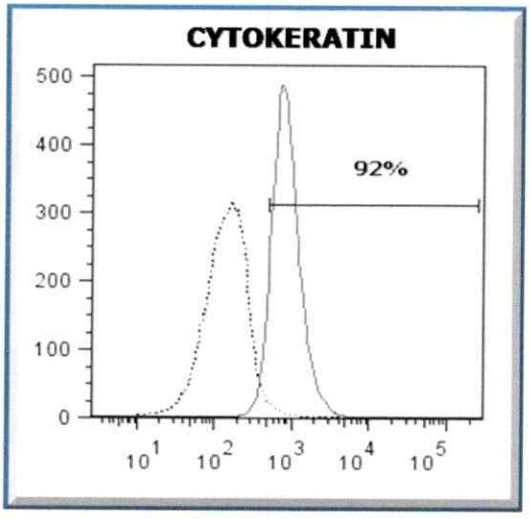


Figure 5. *N. meningitidis* activate blood pDC.

Freshly isolated pDC from blood were stimulated with *N. meningitidis* (*Nm*) (MOI=10), CpG (1μM) or untreated (NT). **A)** Supernatants were collected after 24h and IFNα, IL-6 and TNFα cytokines were quantified by CBA. Values and the mean of 6-15 donors are shown. Data points present on the x-axis had undetectable levels of cytokines. **B)** Density plots of CD86 expressed on the surface of $CD4^+$pDC after 24h of stimulation are shown and were analyzed by flow cytometry. Data are representative of 12 independent donors **C)** Quantification of the percentage of $CD86^+CD4^+DAPI^-$pDC is shown. Data are mean ± S.D. from 12 different donors. Statistical differences were evaluated using Wilcoxon test. n.s. non significant differences. *, p≤0,05; **, p≤0,01, ***,p≤0,001.

Generation of human tonsil epithelial cell culture

We generated human tonsil epithelial cells (HTEC) to evaluate their contribution on immunity to *N. meningitidis*. We first confirmed the epithelial cell lineage of our culture by assessing the expression of cytokeratin, a specific marker of epithelial cells, by flow cytometry. We observed that more than 90% of the cells express this marker (**Figure 6A**). We also performed functional analysis of the epithelial cell culture regarding tight junction expression. Immunofluorescence microscopy shows that effectively, tight junction proteins ZO-2 and occludin are expressed on HTEC at the edge of the cells (**Figure 6C**).



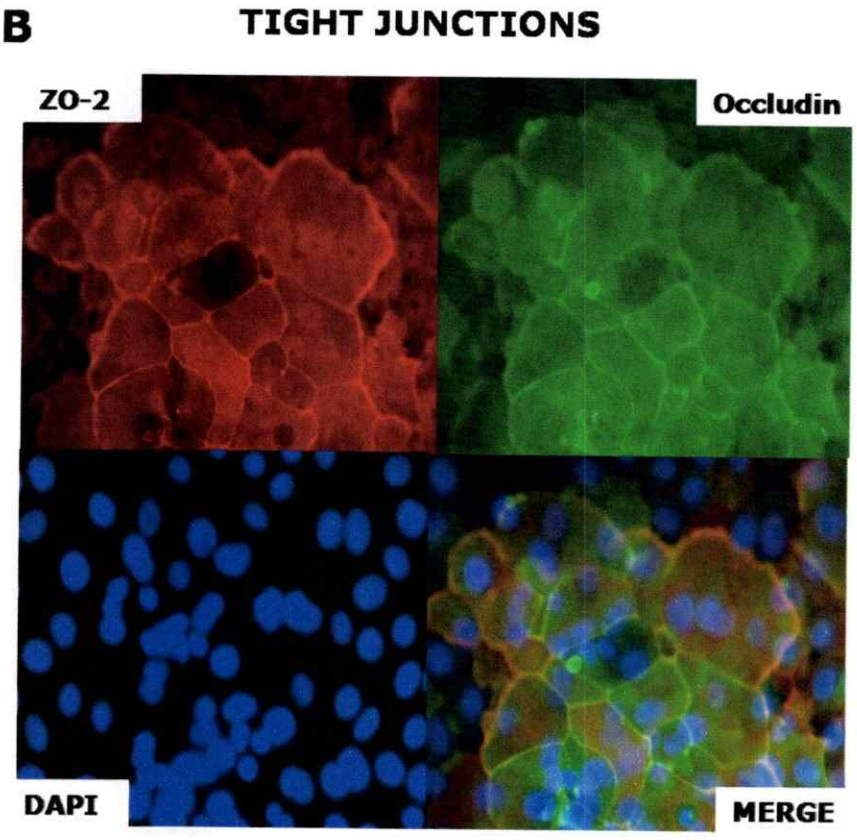


Figure 6. Validation of the protocol to obtain human tonsil epithelial cells (HTEC).

A) Epithelial cell marker expressed by human epithelial cells generated from tonsils was assessed by flow cytometry. Histograms show cytokeratin expression in permeabilized cells. Solid histogram indicates stained cells and dashed histogram, isotype control. **B)** Expression of tight junction proteins was analyzed by immunofluorescence microscopy on HTEC. Picture was taken with an amplification of 20x0,5x1,5x. The expression of ZO-2 (red), occludin (green) is shown. The nucleus was stained with DAPI. Merge of the tree colors is shown. Data are from one experiment representative of three independent experiments.

N. meningitidis induce cellular response and cytokine secretion in human tonsil epithelial cells

N. meningitidis interaction with the epithelium has been described to induce a particular cellular response described in the introduction. To confirm that *N. meningitidis* induces this cellular response in our cell culture; we incubated tonsil epithelial cells with live bacteria expressing GFP protein and assessed cellular response by immunofluorescence microscopy (**Figure 7A**). After 4h of stimulation, we observed the recruitment of ezrin protein under GFP-*N. meningitidis* microcolonies (**Figure 7B**). Percentage of ezrin and actin protein recruited under bacteria microcolonies was quantified and plotted in **Figure 7C**. Up to 90% and 70% of colonies recruit ezrin and actin proteins respectively. These results show that the bacteria adhere to and induce cellular response in HTEC culture as expected.

Previous studies revealed that *N. meningitidis*-epithelium interaction induces a dramatic modification of cellular physiology. We just described the early cellular response once *N. meningitidis* adhere to HTEC. This interaction also includes important changes in epithelium transcriptome and the secretion of soluble factors. Of particular interest for our study is the release of cytokines, able to modify DC microenvironment at the mucosal level. For this reason, we stimulated HTEC with *N. meningitidis* to quantify cytokines secreted in the

supernatant. From a broad panel of cytokines assessed: IL-1α, IL-1β, IL-3, IL-6, IL-7, IL-8, IL-10, G-CSF, GM-CSF, TSLP, TNFα, IFNα, IFNγ, we were able to detect the secretion of a limited number of cytokines. We observed a significant increase of IL-8, which was the most abundant cytokine detected in the supernatants. We also detected significant production of G-CSF and IL-6 and a small production of GM-CSF when HTEC were incubated with the bacteria. This secretion was dependent on the bacteria concentration (**Figure 8A,B**). Importantly, we did not detect IL-10, TSLP nor TGFβ reported to modulate mDC response in gut and skin mucosa [40-42]. In non-treated supernatant, we detected basal levels of IL-8, IL-6 and G-CSF all cytokines associated with a pro-inflammatory response at the epithelium level (**Figure 8 C,D**).

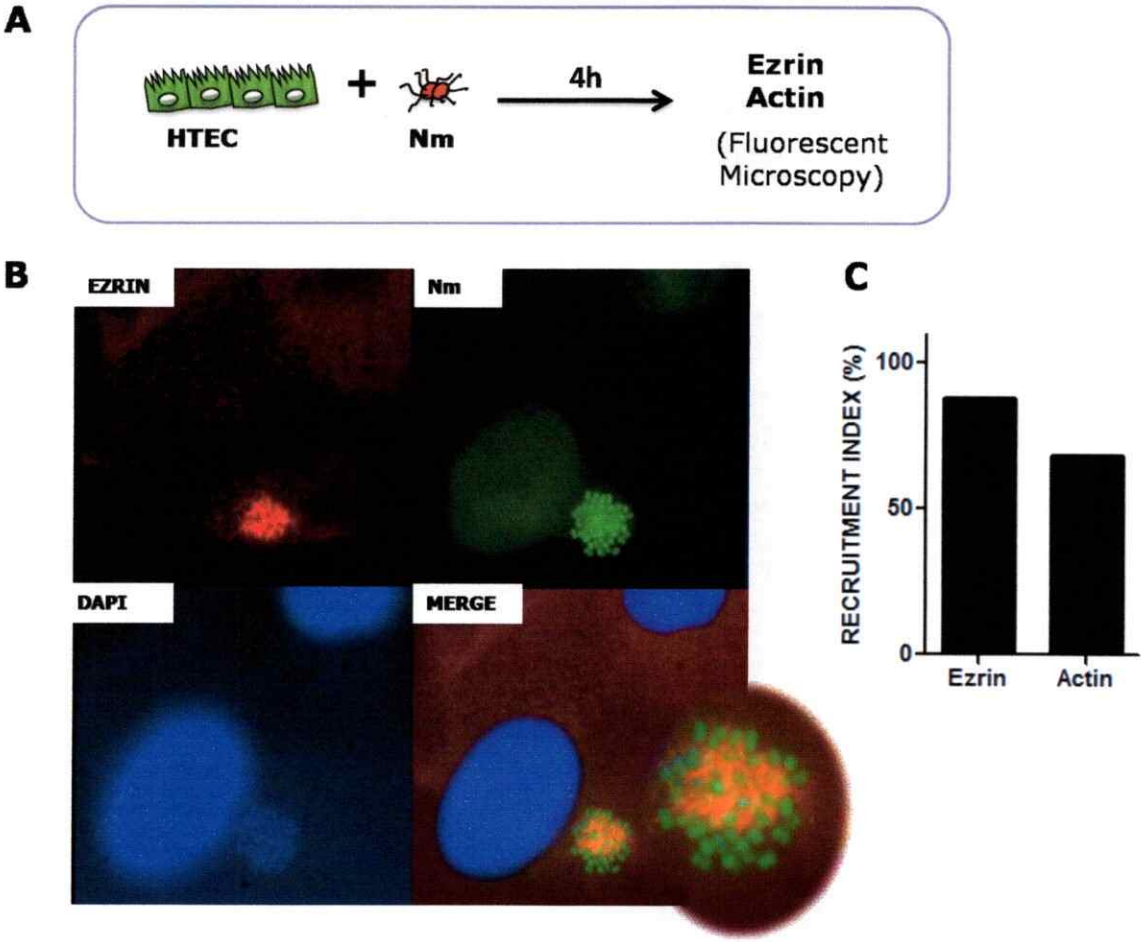


Figure 7. *N. meningitidis* cellular response of human epithelial cells generated from tonsils.

A) Human tonsil epithelial cells were stimulated with *N. meningitidis* expressing GFP (MOI=100). After 4h of infection, cells were fixed and stained to visualize ezrin and actin expression by immunofluorescence microscopy. **B)** The picture shows the expression of ezrin (red), *Nm* (green), nucleus stained with DAPI (blue) and the merge. The picture was taken at 100x2,5x. **C)** Frequency of bacterial microcolonies efficiently recruiting ezrin and actin (Recruitment index) was quantified. Data are from one experiment representative of three independent experiments.

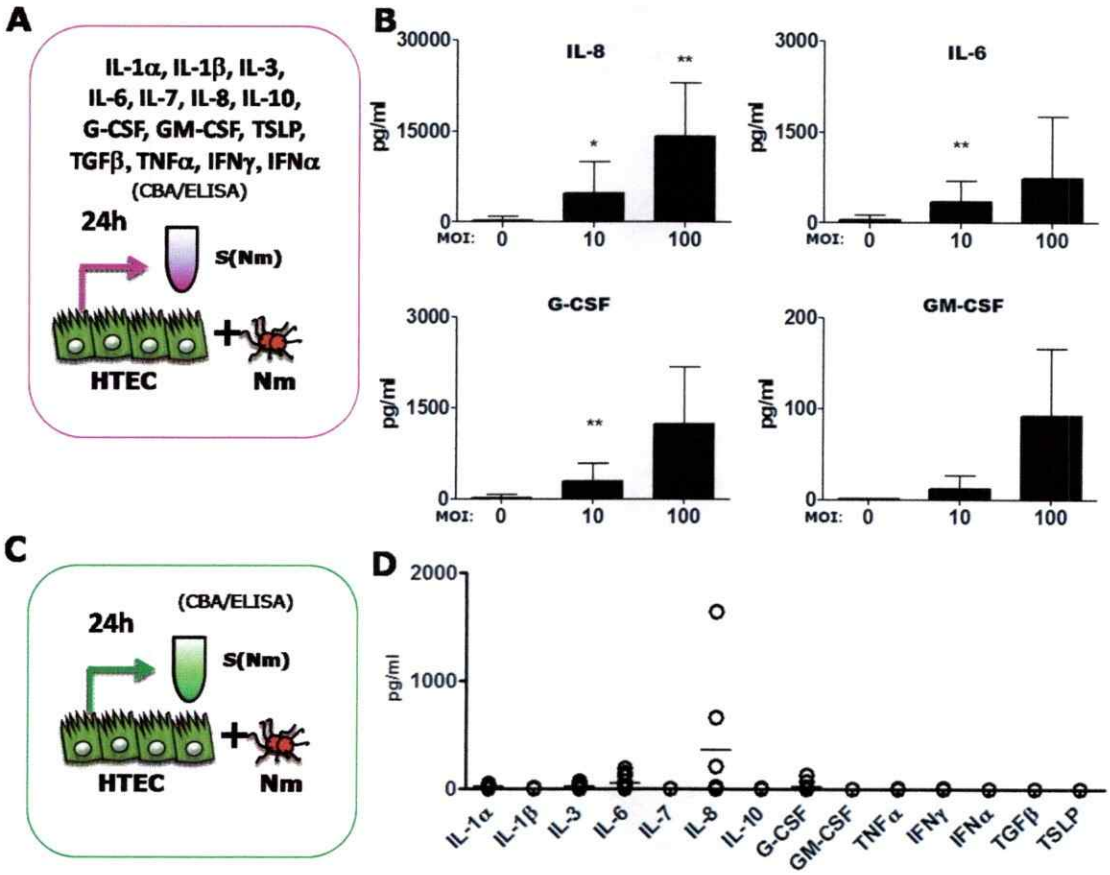


Figure 8. *N. meningitidis* induce cytokine secretion by human tonsil epithelial cells.

A) Human tonsil epithelial cells were stimulated with *N. meningitidis* (MOI= 10-100). Supernatants were collected after 24h and IL-1α, IL-1β, IL-3, IL-6, IL-7, IL-8, IL-10, G-CSF, GM-CSF, TSLP, TGFβ, TNFα, IFNγ, IFNα cytokines were quantified by CBA or ELISA. **B)** IL-8, IL-6, G-CSF and GM-CSF secretion measured by CBA is shown. **C)** Supernatants from untreated human tonsil epithelial cells was recovered after 24h of culture. The presence of IL-1α, IL-1β, IL-3, IL-6, IL-7, IL-8, IL-10, G-CSF, GM-CSF, TSLP, TGFβ, TNFα, IFNγ, IFNα cytokines were quantified by CBA or ELISA. **D)** Basal level of cytokines present in the supernatants of untreated epithelial cells is shown. Data are mean ± S.D. from 6-10 different donors. Statistical difference was assessed using Wilcoxon test. *, p≤0,05; **, p≤0,01, ***,p≤0,001.

Epithelium discriminates between Gram-negative and Gram-positive bacteria

Once we validated and characterized epithelial cell response to *N. meningitidis*, we wondered if this particular pattern of cytokine secretion is specific to *N. meningitidis* or if it depends on microbial stimuli. To analyze this, we used others Gram-negative and Gram-positive bacteria that share the ecological niches with *N. meningitidis* and incubated them with HTEC. We used the Gram-negative bacterium *Haemophilus influenzae* (*H. influenzae*) and as Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) and *Streptococcus oralis* (*S. oralis)* from clinical samples. We collected the supernatant 24 hours after stimulation of HTEC with the different bacteria. Interestingly, we found that Gram-negative bacteria, N. meningitidis and *H. influenzae* preferentially induced IL-6, IL-8, G-CSF, and also GM-CSF to a lesser extent. Gram-positive bacteria, *S. aureus* and *S. oralis* preferentially induced IL-1α, IL-1β and TSLP (**Figure 9**). This shows that the epithelium is able to discriminate between Gram-negative or Gram-positive bacteria.

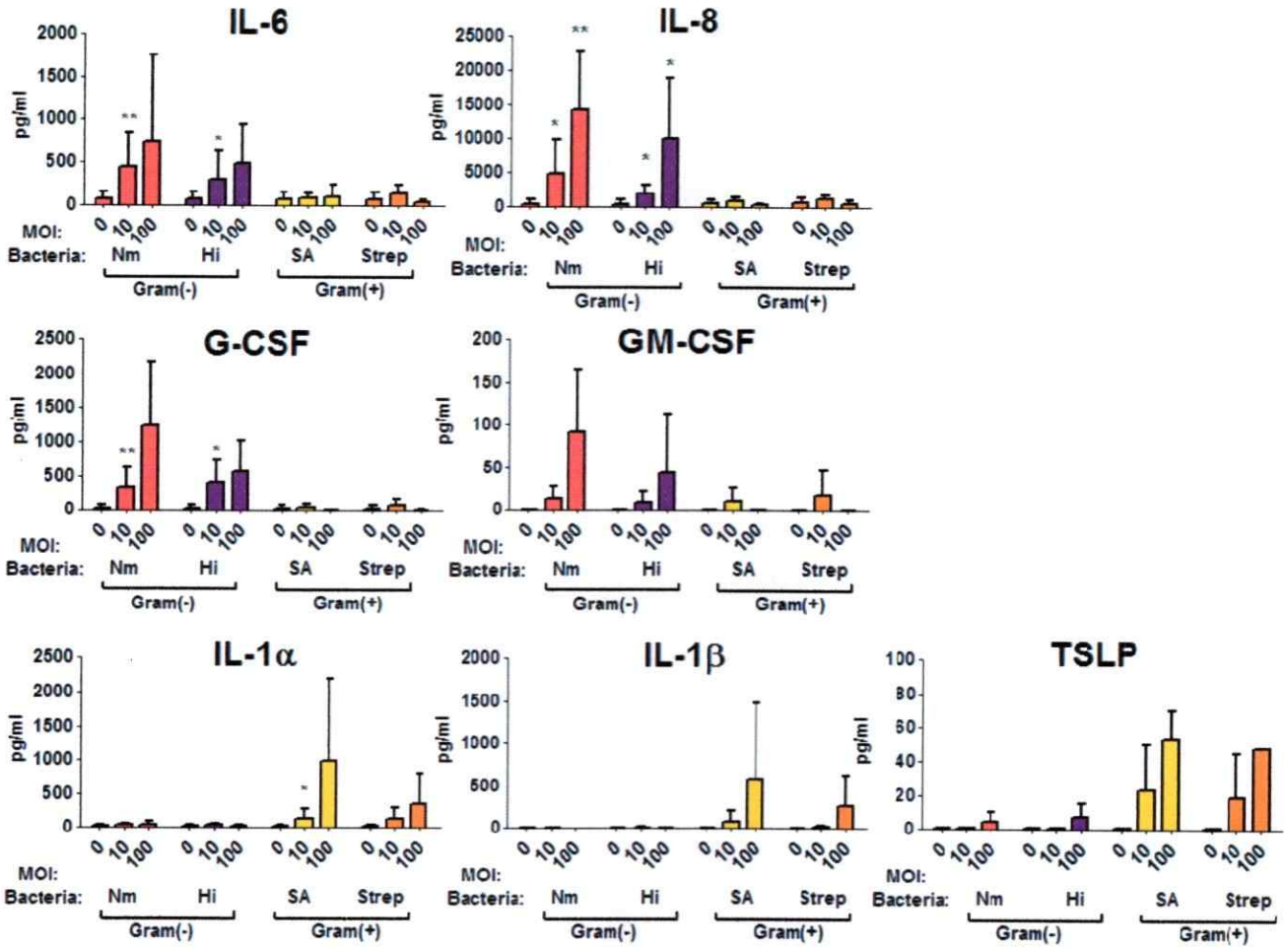


Figure 9. Human tonsil epithelial cells discriminate between Gram-negative and Gram-positive bacteria.

Human tonsil epithelial cells were stimulated with Gram(-) bacteria: *N. meningitidis* (*Nm*) and *H. influenzae* (Hi) or Gram(+) bacteria: *S. aureus* (SA) and *S.pneumoniae* (Strep) (MOI= 10-100). Supernatants were collected after 24h and IL-1α, IL-1β, IL-3, IL-6, IL-7, IL-8, IL-10, G-CSF, GM-CSF, TSLP, TGFβ, TNFα, IFNγ, IFNα cytokines were quantified by CBA or ELISA. IL-6, IL-8, IL-1α, IL-1β and TSLP secretion is shown. Data are mean ± S.D. from 3-9 different donors. Statistical difference was assessed by Wilcoxon test. *, p≤0,05; **, p≤0,01, ***,p≤0,001.

Effect of the epithelium on dendritic cell response to *N. meningitidis*

Our results show that mDC and pDC are activated by *N. meningitidis*. In this work we postulate that *N. meningitidis*-epithelium interaction can modify DC response to the bacteria. Initially, we wanted to generate a model where epithelium and DC contact trough a transwell chamber and then add the bacteria. Surprisingly, we were not able to find the adequate conditions to grow the tonsil epithelial cells on the transwell filters. Hence, in order to by-pass this technical difficulty we decided to stimulate tonsil epithelial cells with *N. meningitidis* and collect the soluble factors released. These soluble factors were filtered and then added to freshly isolated mDC and pDC. The response of mDC and pDC, under the different treatments were compared separately. First we will describe the results found in mDC and then those found in pDC.

Soluble factors from N. meningitidis-infected epithelium limit cytokines secreted by myeloid dendritic cells activated by N. meningitidis

We compared cytokines secreted by tonsil mDC activated by *N. meningitidis* in the presence or absence of soluble factors produced from

N. meningitidis-stimulated epithelium (S(*Nm*)) (**Figure 10A**). We observed that IL-10 produced during *N. meningitidis* encounter diminished significantly from 4 ng/mL to 3 ng/mL when the soluble factors from *N. meningitidis*-treated epithelium were added to mDC. Moreover, TNFα secretion was also inhibited decreasing from 1000 pg/mL to 200 pg/mL when the soluble factors from *N. meningitidis*-treated epithelium were added. Furthermore, the low amount of IL-12p70 produced by N. meningitidis-activated mDC was completely abrogated by the same soluble factors. In contrast to these results, no differences in the secretion of IL-6, IL-1α and IL-1β were observed when *N. meningitidis*-activated mDC were stimulated in the presence of *N. meningitidis*-treated epithelium soluble factors (**Figure 10B**). These results show an inhibitory role of tonsil epithelium which after contact with *N. meningitidis* inhibits a selected pattern of cytokines.

Soluble factors from untreated epithelium impact on myeloid dendritic cell activated by N. meningitidis

Next, we wanted to evaluate if the soluble factors responsible for the inhibitory effect observed on the cytokines secreted by mDC are presents in the supernatants of non-treated epithelial cells. To this end we collected the soluble factors from non-treated epithelium (S(NT))

and added them to *N. meningitidis*-activated mDC. We performed these experiments only three times, therefore they are still preliminary results. However, the results obtained suggest that the IL-1α, IL-1β, TNFα and IL-12p70 cytokines secreted by *N. meningitidis*-activated mDC are inhibited when soluble factors from non-treated epithelium were added. No differences were observed for IL-6 and IL-10 secretion (**Figure 10B**). These preliminary results suggest that the soluble factors responsible for the inhibition of TNFα and IL-12p70 are produced by tonsil epithelium rather than induced by the interaction of *N. meningitidis* with the epithelium.

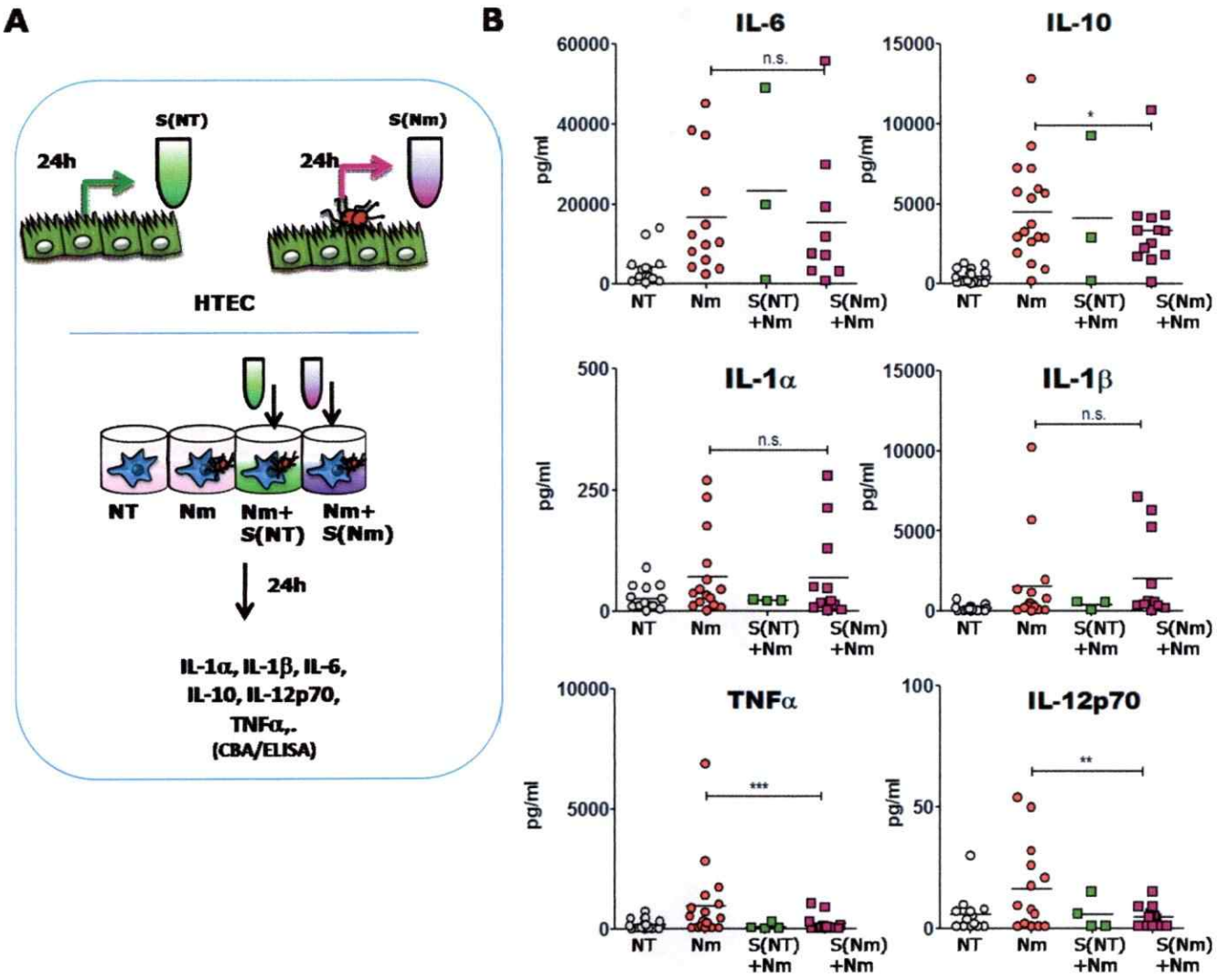


Figure 10. Soluble factors from epithelium selectively inhibit cytokines induced by *N. meningitidis*-stimulated tonsil mDC.

A) Human tonsil epithelial cells were stimulated with *N. meningitidis* (*Nm*) (MOI=10) or were untreated (NT). Soluble factors from bacterial treated (S(*Nm*)) and untreated (S(NT)) epithelial cells were collected after 24h (above). Tonsil mDC were stimulated with *N. meningitidis* (*Nm*), in the presence of soluble factors from untreated epithelium (S(NT)+*Nm*), in the presence of soluble factors from bacterial-treated epithelium (S(*Nm*)+*Nm*) or were untreated (NT). Supernatants of stimulated-mDC were collected after 24h and IL-6, IL-10, IL1α, IL-1β, TNFα and IL-12p70 cytokines were quantified by CBA (below). **B)** IL-6, IL-10, IL1α, IL-1β, TNFα and IL-12p70 secreted by stimulated-mDC are shown. Values and the mean from 3-15 different donors are shown. Data point present on the x-axis had undetectable levels of cytokines. n.s. non significant differences. Statistical difference was assessed by Wilcoxon test. *, p≤0,05; **, p≤0,01, ***,p≤0,001.

Soluble factor from *N. meningitidis-infected epithelium inhibit cytokines secreted by pDC-activated by N. meningitidis*

In the case of pDC activation, we compared *N. meningitidis*-stimulated pDC in the presence or absence of soluble factors produced by *N. meningitidis*-treated epithelium (**Figure 11A**). We measured the response of blood pDC from 6 to 8 different donors. We observed a dramatic and significant inhibition of IFNα and TNFα when the soluble factors from bacterial-treated epithelium were added to pDC. We did not detect significant changes in the secretion of IL-6 (**Figure 11B**). These results show that the soluble factors from *N. meningitidis*-epithelium interaction have an inhibitory effect on the secretion of selected cytokines produced by bacterial-activated pDC, which is in line with the results observed with mDC.

Soluble factors from untreated epithelium impact on pDC response to N. meningitidis

As with mDC, we wanted to know the contribution of non-treated epithelium in the inhibition of the cytokines secreted by *N. meningitidis*-activated pDC. We measured the response of blood pDC from 6 to 8 different donors. We observed that soluble factors from non-treated epithelium not only inhibit the secretion of IFNα and TNFα produced by *N. meningitidis*-activated pDC but also abrogate IL-6 secretion. (**Figure**

11B). Hence, the inhibition of cytokines secreted by *N. meningitidis*-stimulated pDC is an intrinsic property of the epithelium.

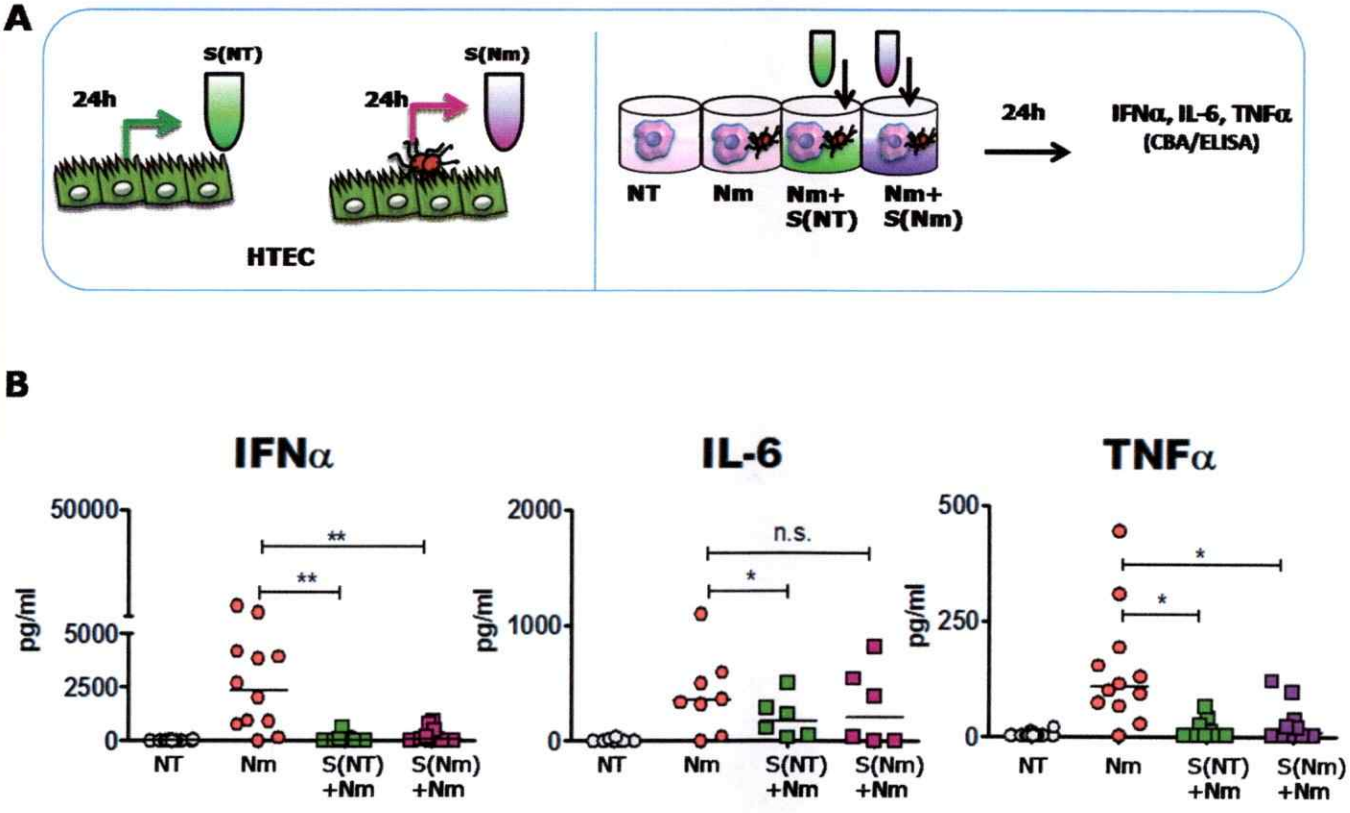


Figure 11. Soluble factors from epithelium inhibit IFNα and TNFα secreted by *N. meningitidis*-stimulated blood pDC.

A) Human tonsil epithelial cells were stimulated with *N. meningitidis* (*Nm*) (MOI=10) or untreated (NT). Soluble factors from bacterial treated (S(*Nm*)) and untreated (S(NT)) epithelial cells were collected after 24h (above). Blood pDC were stimulated with *N. meningitidis* (*Nm*), in the presence of soluble factors from untreated epithelium (S(NT)+*Nm*), in the presence of soluble factors from bacterial-treated epithelium (S(*Nm*)+*Nm*) or were untreated (NT). Supernatants of stimulated blood pDC were collected after 24h and IFNα, IL-6 and TNFα cytokines were quantified by CBA (below). **B)** IFNα, IL-6 and TNFα secreted by stimulated blood pDC are shown. Values and the mean from 6-12 different donors are shown. Data point present on the x-axis had undetectable levels of cytokines. n.s. non significant differences. Statistical difference was assessed by Wilcoxon test. *, p≤0,05; **, p≤0,01, ***,p≤0,001.

Different bacterial species residing on the oro-nasopharynx mucosa exert different effect on plasmacytoid dendritic cell response through interaction on the epithelium

As the activation of pDC by bacterial stimulus remains poorly studied, and no evidences of the impact of the epithelium on this pDC response was reported before, in the final part of this work we focused on pDC response. *N. meningitidis* at mucosal level act as commensal bacteria; therefore we were interested to finding out whether the effects described above were specific to *N. meningitidis*. To this end, we tested the effect of *H. influenza*, *S. aureus* and *S. oralis* bacteria. Like *N. meningitidis* these bacteria share similar lifestyle and pathogenesis living at the oro-nasopharynx mucosa. Major differences between these different bacteria are the virulence factors used to survive the host. We incubated pDC with these bacteria at the same concentration used with *N. meningitidis*. After 24h we analyzed pDC viability. The viability of pDC was maintained with all bacteria except for *S. oralis* therefore we continued with the other strains to stimulate pDC (**Figure 12**).

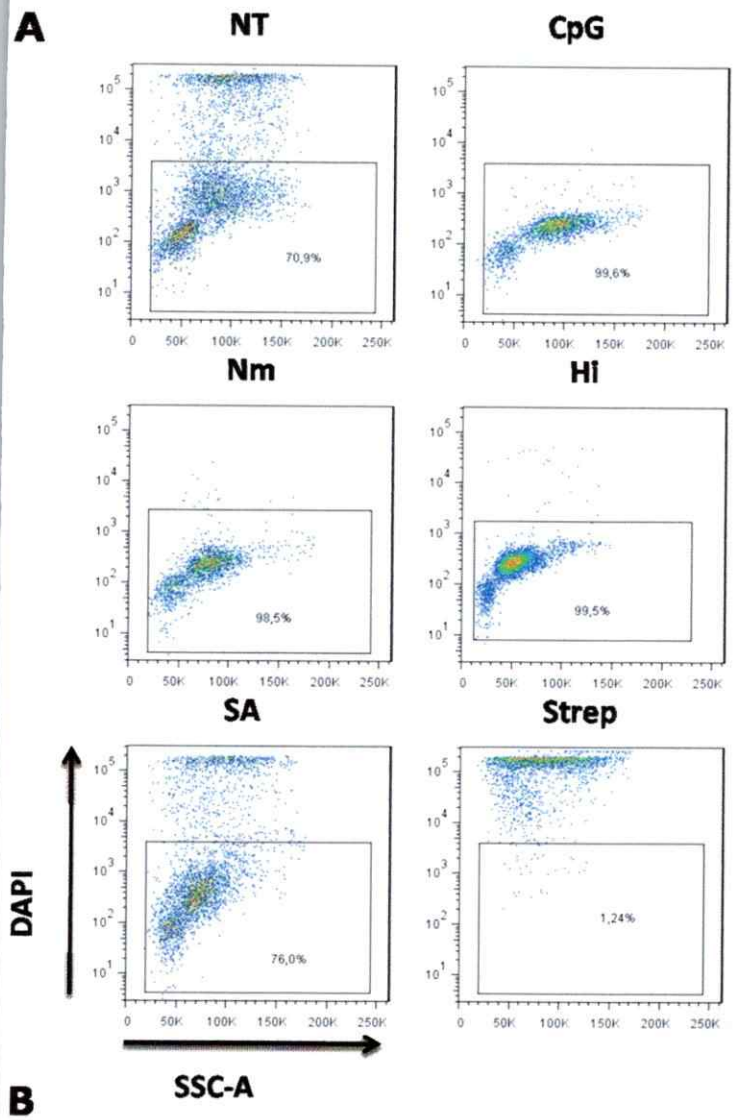


Figure 12. Viability of pDC stimulated with different bacteria.

Blood pDC were stimulated with *N. meningitidis* (*Nm*), *H. influenzae* (Hi), *S. aureus* (SA), *S. oralis* (Strep) bacteria or with CpG. After 24h, viability, considered as the percentage of $DAPI^-$ cells, was analyzed by flow cytometry. **A)** Density plots show the fluorescence of DAPI (y-axis) vs. size scatter (x-axis) of blood pDC. Gates indicate the percentage of live cells ($DAPI^-$). **B)** The quantification of the percentage and mean of DAPI- cells from 3 different donors is shown.

As with the experiments performed with *N. meningitidis*, we collected supernatants from the epithelium infected with the different bacteria. The soluble factors were added to pDC in the presence of the same bacteria used to stimulate epithelial cells. We compared IFNα secreted by bacteria-activated pDC in the presence of soluble factors from non-treated epithelium (S(NT)+X)), in the presence of soluble factors from the epithelium treated with the same bacteria (S(X)+X)), or without any soluble factor (X). As shown in **Figure 13**, only *N. meningitidis*-induced IFNα was inhibited by the soluble factors from non-treated epithelium. In contrast, IFNα induced by *H. influenza* and *S. aureus* was not inhibited by the soluble factors from bacterial-treated nor from non-treated epithelium. These results show that the inhibition of IFNα by the epithelium observed in pDC is specific for *Nm* and not for other bacteria that are more pathogenic, showing specificity in the response of pDC to bacteria at the mucosal level.

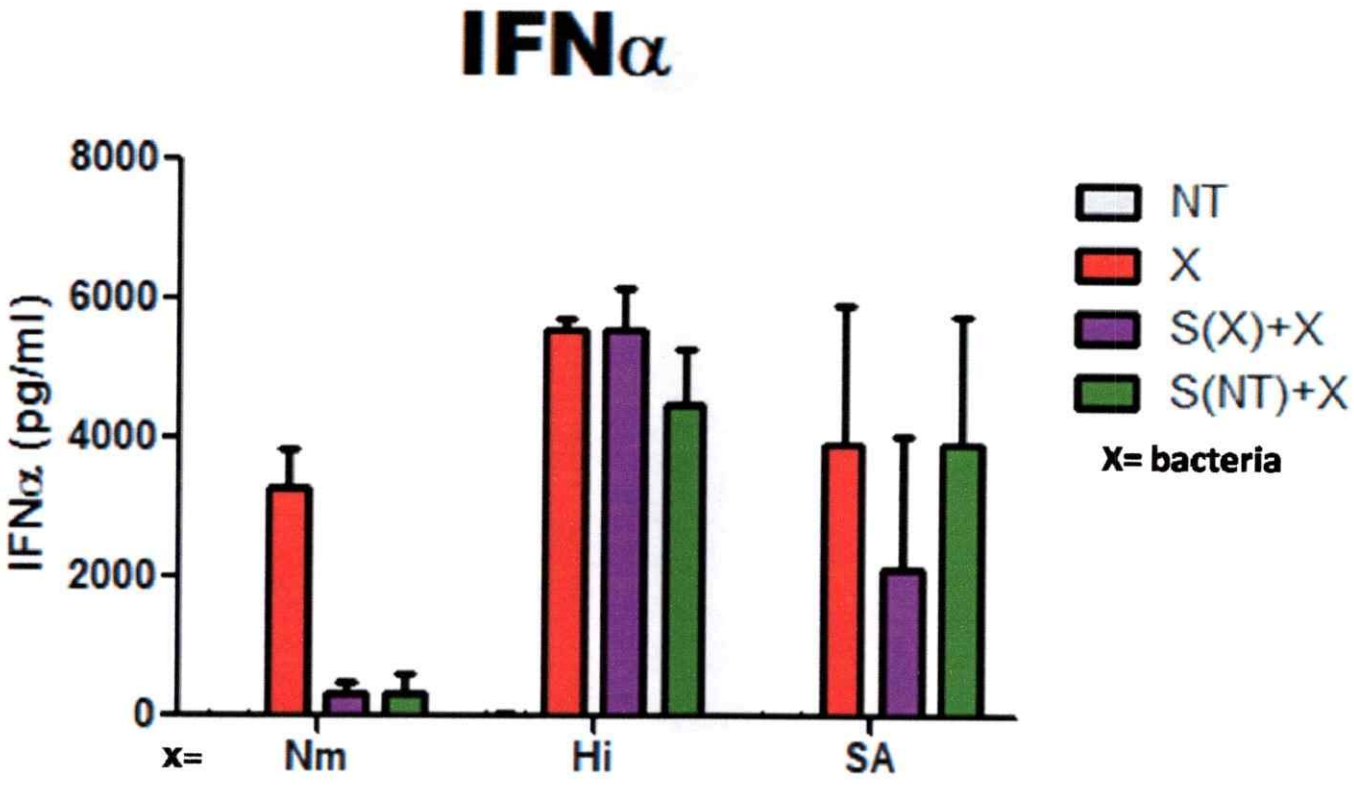


Figure 13. The inhibition of IFNα by epithelial cell soluble factors is specific to N. meningitidis infection.

Blood pDC were stimulated with bacterial stimulus (X) in the presence of soluble factors from bacteria-epithelium interaction (S(X)+X), or from untreated epithelium (S(NT)+X)) or untreated (NT). The same bacteria used to stimulate pDC were used to generate bacteria-epithelium supernatants. X represents the bacteria specified on x-axis: *N. meningitidis* (*Nm*), *H. influenzae* (hi) or *S. aureus* (SA). Data are mean ± S.D. from 2-3 independent donors.

DISCUSION

In this thesis we were interested in evaluating the role of the epithelium in the activation of dendritic cells (DC) by *N. meningitidis*. To this end, we used a model of primary cells isolated from human tonsil to recreate the conditions found *in vivo*. Our strategy was to cultivate epithelial cells with the bacterium, and then we used the supernatant to treat DC which were activated by *N. meningitidis*. To develop the experimental model, both types of cells (epithelial cells and DC) were obtained from human tonsils. In some experiments, DC cells were also isolated from human blood.

The main populations of DC found in human tissues are myeloid (mDC) and plasmacytoid (pDC) [83, 84]. Distinct subpopulations of mDC were also described in these tissues based on surface markers such as CD16, BDCA1 and BDCA3 however, it is not clear if these cells play different function [85, 86]. Recently, a putative equivalent of mouse $CD8\alpha^+$ DC has also been described [87]. Here we isolated mDC and pDC from tonsils and blood by cell sorting after enzymatic digestion of the tissues. With this technique we obtained $CD11c^+$ myeloid (mDC) and $CD4^+$ $CD11c^-$ plasmacytoid (pDC) discarding CD16 cells since this marker is also expressed on natural killer cells, macrophages and neutrophils. At the end of the protocol we obtained small amounts

($5x10^5$ per donors) of both mDC and pDC in tonsils, which limits following isolation of mDC subpopulations. However, it will be interesting to evaluate in the future if other DC subset are involved in the immune response to *N. meningitidis*. Here our first goal was to evaluate if mDC and pDC respond to *N. meningitidis.*

Freshly isolated myeloid dendritic cells vs. monocyte-derived dendritic cells.

In this work, we showed that *N. meningitidis* activate freshly isolated mDC from tonsils. The secretion of cytokines produced by DC in response to *N. meningitidis* was previously studied using monocyte-derived DC (MoDC). Here for the first time we confirmed this using freshly purified DC which as mentioned before have some difference with *in vitro* generated MoDC [83, 88]. We detected a slight induction of IL-12p70 by *N. meningitidis*-activated tonsil mDC which contrasts sharply with the high levels secreted by *N. meningitidis*-activated MoDC published before, [69, 70]. This can be due to differences in TLR-4 expression between freshly-purified mDC and MoDC [24]. Similarly, previous studies on MoDC reported higher levels of TNFα induced by *N. meningitidis* compared with the levels secreted by mDC, in this thesis [55, 67]. On the contrary, the induction of IL-6 and IL-1β by *N. meningitidis* was higher in mDC than the reported in the literature using MoDC [55, 67, 68]. The secretion of IL-1α was previously detected

intracellularly in *N. meningitidis*-activated MoDC, but the secretion has never been reported [67]. Here we found a slight but significant induction of this cytokine after the activation of mDC with *N. meningitidis*. In summary, our results demonstrate that during direct contact of Nm with mDC, pro-inflammatory and anti-inflammatory response will be triggered but differently from the observations obtained with MoDC studies, this response will be dominated by IL-6 and IL-10 while IL-12p70 would play a minor role. This difference can be crucial for the subsequent activation of B and T cells, which will be discussed below.

An intriguing observation was that in our experimental setting, LPS appears to be a poor stimulator compared to *N. meningitidis*, although there is a considerable increase of cytokines. This can be explained because whole bacteria can present more than one PAMP recognition receptor. Moreover, low amounts of IL-12p70 were detected after activation of mDC with LPS. This can be due to the differential expression of TLR-4 between freshly-purified mDC and MoDC as previously mentioned [24].

Plasmacytoid dendritic cells as sentinels of bacterial infection?

The effect of *N. meningitidis* in pDC response has been poorly studied. The major signature of activated pDC is the production of type-

I interferon (IFN) such as IFNα [27]. An important discovering of this thesis was that *N. meningitidis* were able to induce high levels of IFNα in pDC. This demonstrates that pDC are activated by *N. meningitidis* inducing a pro-inflammatory response after bacterial encounter. Moreover, increased expression of CD86 on *N. meningitidis*-stimulated pDC surface suggests that these cells are potentially able to activate T cells. These results introduce a new role for pDC during immune response to *N. meningitidis*. The fact that pDC activation was stronger under influenza virus compared to bacteria can be explained by a more efficient internalization of the virus and therefore an increase of intracellular TLR activation than with bacteria. Another explanation is that the strain of *Neisseria* used here expresses a polysaccharide capsule which, as previously said, can interferes with bacteria phagocytosis limiting pDC response. Both tonsils and blood pDC secrete the same pattern of cytokines in response to *N. meningitidis* revealing that independently of the localization of the both cells they are able to respond to *N. meningitidis*. These results highlight the importance of pDC not only during infection by virus but also by extracellular bacteria such as *N. meningitidis*.

Epithelial cells as sensor of the oro-nasopharynx flora

We generated human tonsil epithelial cell (HTEC) to analyze the consequences of *N. meningitidis*-epithelium interaction on the

functionality of dendritic cells (DC) in response to *N. meningitidis*, mimicking what could be the physiological conditions. Previous studies using endothelial, meningeal and epithelial cell line from cervix, colon and bronchia described the induction of IL-6, IL-8 GM-CSF and TNFα genes by *N. meningitidis* [76, 77]. Here, when we stimulated HTEC with *N. meningitidis*, we found the production of IL-6, IL-8, G-CSF and GM-CSF. G-CSF and GM-CSF have not been associated with *N. meningitidis* carriage or infection at epithelial cell level until now. They are inflammatory cytokines involved in the differentiation and recruitment of neutrophils, which in turn can recruit DC. Recently, G-CSF has been involved in the induction of IL-10-producing T cells and in the recruitment of pDC in the lamina propia of Crohn's disease patients [89]. In turn, GM-CSF has been described as a TLR-independent activator of pDC which direct the response toward TH1 polarization [90]. Thus, *N. meningitidis*-epithelium interaction could modify mucosal microenvironment and potentially impact the function of DC. On the other hand, we did not detect TNFα in the supernatant from *N. meningitidis*-stimulated HTEC. Induction of TNFα by *Neisseria* has been described at gene expression level [76, 78, 91] but only one study confirms this result at the protein level using brain endothelial cell model [92]. This suggests a cellular specificity in the response to *N. meningitidis*, depending on the tissue where bacteria are present. Other

cytokines previously described to be secreted by epithelial cells which modulate the polarization of immune response in gut and skin mucosa are IL-10, TGFβ and TSLP [37, 40, 44, 93, 94]. Here we did not detect the induction of these cytokines by *N. meningitidis*.

We evaluated if the pattern of cytokines secreted by HTEC was specific to *N. meningitidis* using other Gram-negative and Gram-positive bacteria which as *N. meningitidis* colonize the oro-nasopharynx mucosa. Our results show that epithelial cells can discriminate between Gram-negative and Gram-positive bacteria and deliver different messages depending on the nature of the microbe. These results give a major relevance to the epithelial cells during the generation of immune response, showing that they are more than just a cellular barrier for bacteria but also discriminate between pathogens, delivering differential messages that will be interpreted by infiltrating DC present in the surrounding microenvironment.

Crosstalk between epithelium and myeloid dendritic cells

The impact of the epithelial cells on mDC response to microbial stimulus has been addressed before using mainly gut mucosa as an experimental model [41, 42]. However, their role on DC response to *N. meningitidis* has never been explored so far. Here we placed epithelial cells as key players on the immune response during bacterial infection.

We found that soluble factors from *N. meningitidis*-epithelium interaction reduced the inflammatory response of mDC to *N. meningitidis*. IL-10 was reduced while TNFα and IL-12p70 were completely abrogated by the soluble factors. IL-6, IL-1α and IL-1β instead remained unchanged. IL-10 and TNFα have been related to an increase in the severity of septic shock to *N. meningitidis* and IL-12p70 detected in the serum of these patients [54]. This shows the importance of the epithelium in limiting inflammation during *N. meningitidis* infection at the oro-nasopharynx mucosa. However, control experiments using soluble factors from untreated epithelium, manipulated under the same condition than *N. meningitidis*-epithelium soluble factors, revealed that this property can be attributed to the epithelium itself. Although these results are preliminaries, this would suggest that the anti-inflammatory role observed on mDC response is regulated intrinsically by epithelial cells and that this inhibition has to be broken by the pathogens to induce a strong inflammatory response.

Crosstalk between epithelium and *plasmacytoid dendritic cells*

We evaluated if soluble factors from *N. meningitidis*-epithelium interaction can modify the activation of pDC by *N. meningitidis*. The results obtained here demonstrate that soluble factors from *N. meningitidis*-epithelium interaction exert a dramatic inhibition of the inflammatory cytokines produced by *N. meningitidis*-stimulated pDC.

This effect was even more dramatic than the one observed on mDC response to *N. meningitidis*. IL-6 and CD86 remained unchanged by the soluble factors from *N. meningitidis*-infected epithelium. As in mDC, the responsible in inhibiting the cytokines produced by *N. meningitidis*-stimulated pDC must be present at the supernatant of untreated epithelium.

Taken together, the results obtained with freshly isolated mDC and pDC suggest that under physiological condition at the oro-nasopharynx mucosa, the epithelium will limit the inflammatory response produced by both DC subsets during *N. meningitidis* encounter.

What remains to be discovered is the identity of the soluble factor or factors produced by the epithelium responsible for the cytokine inhibition of *N. meningitidis*-stimulated DC. Cytokines produced at basal levels by epithelial cells such as IL-8, IL-6, G-CSF and IL-3 could be involved in this process. Nevertheless, these cytokines are mostly associated with pro-inflammatory response, thus, further experiments need to be performed to clarify this point. Possible candidate not measured here are metabolic enzymes and intermediates like for example, IDO, cyclooxygenase-2 and its arachidonic-acid metabolite PGE_2 and retinoic acid, which have been previously associated with an

inhibitory response and are produced by epithelial cells from different tissues [44].

Potential response of acquired immune cells during *N. meningitidis* infection

As mentioned in the introduction, depending on the pattern of cytokines released by DC during T cell priming, differentiation will be directed to TH1, TH2, TH17 or Treg cells [8, 9]. Here we found that during direct contact of *N. meningitidis* with DC (mDC and pDC) IL-1α, IL-1β, IL-6, IL-10, TNFα, IFNα and IL-12p70 will be released. IL-6 is a potent, pleiotropic inflammatory cytokine that promotes T cell differentiation toward TH2 while it inhibits TH1 polarization [95], it overcomes Treg-mediated suppression of T-cell proliferation [96]and induces B cells differentiation into IgG-secreting plasma cells [97]. IL-10 instead, is associated with anti-inflammatory properties and the differentiation of TH2 and Treg cells [98]. On the other hand, IL-1β and TNFα inflammatory cytokines together with IL-6 can elicit TH17 differentiation [13, 14]. In contrast, IL-12p70 and IFNα are known inductors of TH1 differentiation [8, 99]. Therefore, our results suggest that both mDC and pDC directly stimulated with *N. meningitidis* are potentially able to direct the differentiation of TH1, TH2, TH17 and Treg cells. However, since these cells produced important amounts of IL-6 it is probable that these cells preferentially promote a TH17 cells.

Once soluble factors from the epithelium were added to DC in the presence of *N. meningitidis*, the cytokines released were mainly IL-6, IL-10, IL-1β, and IL-1α. The presence of IL-6 and IL-1β suggest a possible polarization of T cells toward TH17 while IL-10 could induce TH2 or Treg cells. Instead, in this condition, TH1 should be impaired. Furthermore, it could be possible that under these conditions DC participate in B cell differentiation. These observations would be in line with the data published by other groups which described a preferential polarization to TH2, Treg and TH17 instead TH1 in other mucosa such as gut and skin [37, 41, 42, 97]. We tried to confirm this performing co-cultures experiments with naïve T cells. However, no differences between T cells co-cultured with *N. meningitidis*-stimulated DC alone or in the presence of soluble factors from *N. meningitidis*-epithelium were found. Therefore, additional experiments must be performed to confirm these results.

The induction of mucosal Treg, TH1 and TH2 during *Neisseria* infection has been described previously in human tonsil and mice intravaginal infection [63, 64]. Our results agree in part with these studies since we also observed a cytokine pattern related to an induction of TH1, TH2 and Treg cells in response to *N. meningitidis*, although they did not measure the presence of TH17. However, in one study, it is suggested that a TH1-biased response is induced by *N. meningitidis*

antigens which contrast with our observations [63]. This can be due to the nature of the antigens used in this work, since they used outer membrane vesicles from *N. meningitidis* while we use the whole live bacteria. Another explanation could be that after the disruption of the tonsils and the consequent loss of compartmentalization of tissue, especially of epithelial cells, the scenario could be more similar to the blood, emphasizing the importance in considering epithelium in the immune response to bacteria.

Is the case of N. meningitidis unique?

Our results showed that the response of epithelial and pDC was specific to *N. meningitidis*, compared with other bacteria that share the same ecological niche. We already discussed the case of epithelial cells in discriminating between Gram-positive and Gram-negative bacteria, now we will focus on the effect of epithelium on pDC response. The fact that only the IFNα induced by *N. meningitidis* and not this induced by *S. aureus* or *H. influenza,* was inhibited by the soluble factors from the epithelium, suggest that these bacteria activate pDC by different mechanisms. For instance, a putative receptor to *N. meningitidis* could be involved in the internalization of *N. meningitidis*, which differ from the receptors used by other bacteria. Then, the soluble factor(s) produced by the epithelium could interfere with this putative receptor inhibiting the internalization and hence the activation of pDC by *N.*

meningitidis. It would be interesting to study the internalization mechanisms of the bacteria by pDC. An important point to investigate is if other stimuli may have the same pattern than *N. meningitidis* using a broader range of bacteria. For example, we could use other strictly commensal bacteria such as *Neisseria lactamica,* a bacterium closely related with *N. meningitidis* which could explain if the differential effect produced by the epithelium on pDC bacterial response depends on the pathogenic vs. commensalism nature of the bacteria.

Commensalism vs. pathogenesis

Commensalism vs. pathogenesis of bacteria was strongly related with factors expressed by the bacteria known as PAMP. However, the results exposed here lead us to propose a model where commensal or pathogenic property of bacteria, as in the case of *N. meningitidis* will depend on the tissue where the bacteria are present rather than different PAMP exposed by the same bacteria (**Figure 12**). We showed that DC isolated from blood and tonsils have similar pro-inflammatory response when directly challenged with *N. meningitidis*, but this pro-inflammatory response was inhibited by the epithelium which is present at the mucosa. In contrast, in blood where the inhibitory effect of the epithelium is absent, an uncontrolled pro-inflammatory response can be triggered and the recruitment of more immune cells that in turn exacerbate inflammation can occurs leading to the well-known

consequences induced by *N. meningitidis* such as septicemia. Therefore, this model could explain the versatility of *N. meningitidis* as a commensal organism in the oro-nasopharynx mucosa and a high pathogenic bacterium in the bloodstream. Furthermore, this study validates the use of mucosal vaccines to prevent meningococcemia which could be improved adding appropriated soluble factors.

CONCLUSION

In this work, we studied the role of tonsil epithelium in the modulation of the immune response to *Neisseria meningitidis* through the participation of dendritic cells (DC). This was achieved using a model composed mainly by human primary cells from tonsils to mimic the conditions found during the commensalism state of *N. meningitidis*. The findings achieved in this thesis are summarized in Table 1 and highlight the importance of the crosstalk of immune cells with the epithelium during the immune response to bacteria.

Here we present the most relevant discovering of this thesis.

- Gram-negative bacteria such as *N. meningitidis* are able to activate pDC which add a new player that could potentially orchestrate the immune response to this and other bacteria.

- The epithelium play a major role in the immune response to bacterial infection since they discriminate between Gram-negative and Gram-positive bacteria and modulate the inflammatory response of DC through the production of soluble factors.

- The crosstalk between the different cellular components of mucosal tissues and the bacteria is critical for the triggering of a controlled immune response to *N. meningitidis*

- Finally, commensal vs. pathogenic versatility of *N. meningitidis* depend on the localization of the bacteria. If *N. meningitidis* is present at the oro-nasopharynx mucosa, epithelial cells will limit exacerbate inflammatory response while the absence of epithelium in blood will lead to an uncontrolled inflammation.

Table1. Most important results of this thesis

#	RESULTS
1-	*N. meningitidis* induce the activation of human tonsil myeloid dendritic cells, characterized by the secretion of IL-6, IL-10, IL-1α, IL-1β, TNFα and IL-12p70.
2-	*N. meningitidis* induce the activation of human plasmacytoid dendritic cells from tonsil and blood, characterized by the secretion of IFNα, IL-6 and TNFα and the maturation of pDC as demonstrated by the increase of CD86 expression.
3-	*N. meningitidis* trigger the secretion of IL-8, IL-6, G-CSF and GM-CSF by human tonsil epithelial cells.
4-	Human tonsil epithelial cells discriminate between Gram-negative and Gram-positive bacteria, secreting a differential pattern of cytokines.
5-	Soluble factors present during *N. meningitidis*-epithelium interaction inhibit the IL-10, TNFα and IL-12p70 produced by *N. meningitidis*-stimulated mDC.
6-	Soluble factors produced during *N. meningitidis*-epithelium interaction inhibit IFNα and TNFα production by *N. meningitidis*-stimulated pDC.
7-	The soluble factor(s) responsible for the inhibitory effect on the cytokines secreted by myeloid and plasmacytoid dendritic cells stimulated with *N. meningitidis* are yet to be determined.
8-	The inhibition of cytokines release by *N. meningitidis*-stimulated pDC by tonsil epithelium is specific for *N. meningitidis.*

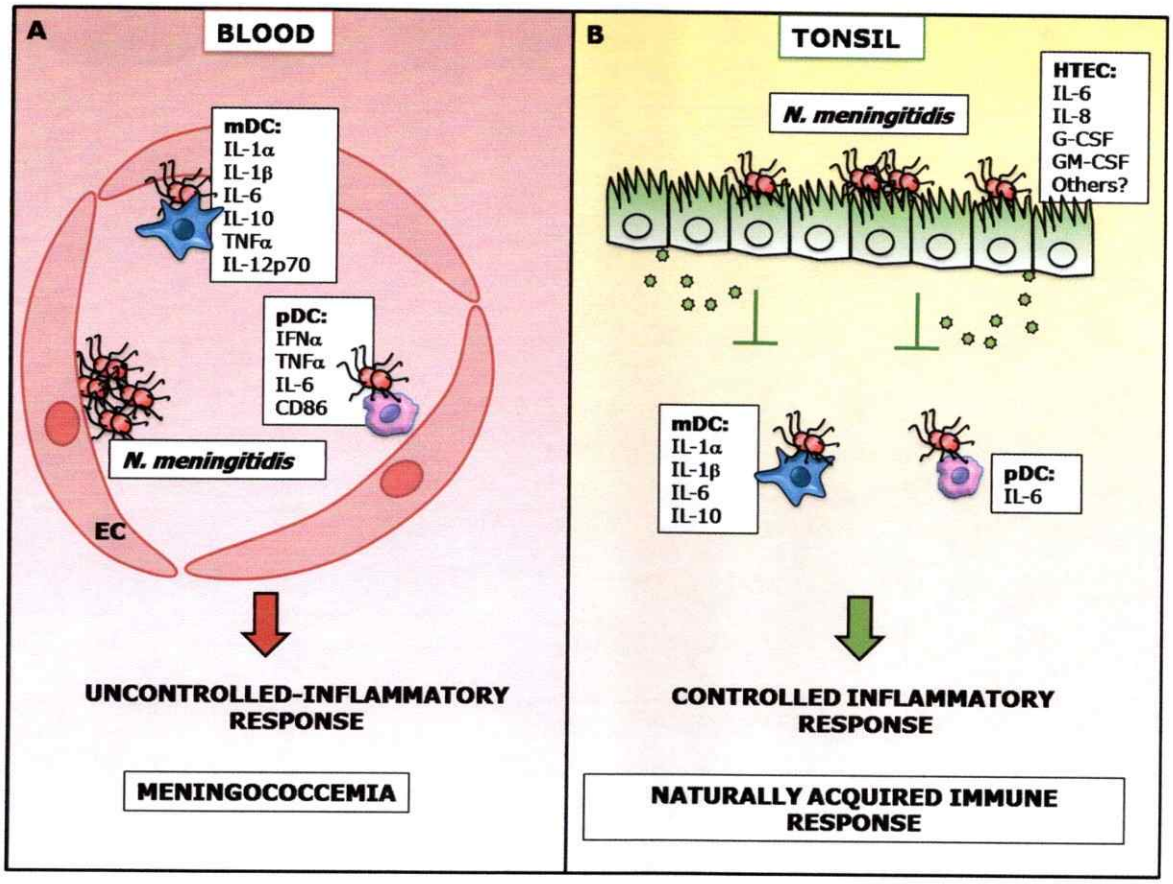


Figure 13. Model proposed.

In summary, our results show that the epithelium attenuates the inflammatory response triggered by myeloid and plasmacytoid DC during *N. meningitidis* infection at the mucosal level. **A)** When *Nm* reaches the blood it induces the activation and the secretion of IL-6, IL-10, IL-1α, IL-1β, TNFα and IL-12p70 by mDC and IFNα, IL-6 and TNFα by pDC. This leads to uncontrolled inflammatory responses and to meningococcus-associated diseases. **B)** On the other hand, when *N. meningitidis* lives in commensalism with the surface epithelium of the oro-nasopharynx mucosa, as in human tonsils, the release of soluble factors from epithelium inhibits IL-10, TNFα and IL-12p70 from *N. meningitidis*-stimulated mDC and IFNα and TNFα from *N. meningitidis*-stimulated-pDC. This leads to a controlled inflammatory response which virtually culminates in a naturally acquired immune response to *N. meningitidis*. EC: endothelial cells, HTEC: Human tonsil epithelial cells.

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