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MOLECULAR AND TRANSLATIONAL MEDICINE
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UNIVERSITY OF MILANO-BICOCCA
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SIMILARITIES AND DIFFERENCES OF INNATE IMMUNE RESPONSES ELICITED BY SMOOTH AND ROUGH LPS

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To my parents.

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Chapter 1.

Introduction

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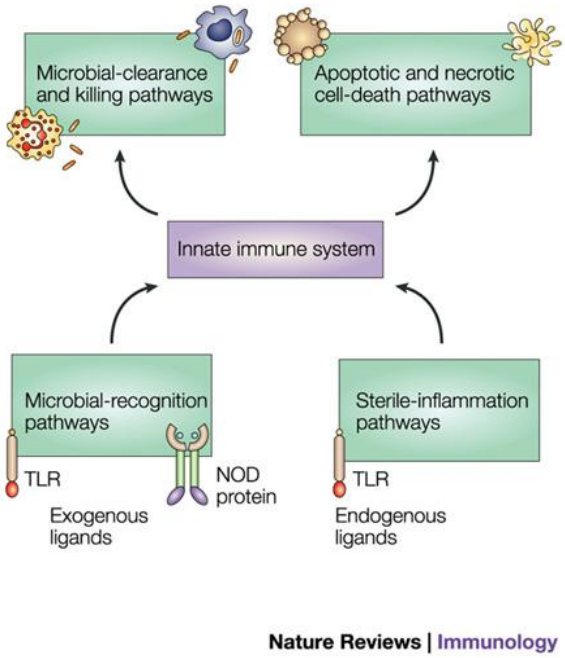
1. Introduction to the innate immune system

Over the years, the human immune system has evolved under selective pressure imposed by infectious microorganisms. Hence, all multi-cellular organisms have developed various defence mechanisms which have the capacity to be triggered by infection and to protect the host organism by destroying the invading microbes and neutralizing their virulence factors. The immune system is composed of two major components; the innate and the adaptive specific immune system. The innate immune system plays the role of being our first line of defence against invading organisms while the adaptive immune system acts as a second line of defence which gives protection against re-exposure to the same pathogen. Each of the major subdivisions of the immune system have both cellular and humoral components by which they carry out their protective function. In addition, the innate immune system also has anatomical features that function as barriers to infection. Although these two arms of the immune system have distinct functions, both the functions of these two systems overlap.

The decision to activate an immune response is made by antigen-presenting cells (APCs) that are quiescent until they encounter a foreign microorganism or inflammatory stimulus. APCs that are activated early trigger innate immune responses that represent the first line of attack against invading pathogens to limit the infections. At later times, activated APCs acquire the ability to prime antigen-specific immune responses that clear the infections and give rise to

memory. During the immune response, self-tissue damage is limited and tolerance to self is maintained through life[1.]



Nature Reviews Immunology 4, 512-520 (July 2004).
Fig. 1.The innate immune system pathways

The innate immune system consists of physical barriers, such as skin and epithelia lining the respiratory, intestinal and urogenital tract. If a pathogen crosses the epithelia, it will face different cell types like dendritic cells (DCs) and natural killer (NK) cells, macrophages and different granulocytes. There is a constant communication between cells and tissues in terms of cytokines, chemokines and cell to cell contact. One important task of the innate immune system is to differ self from non-self. One instrument to do so is through pattern recognition molecules, which includes Toll-like receptors (TLR) and the family of NOD-like receptors (NLR) (**Fig 1**). These receptors

recognise so called PAMPs (pathogen-associated molecular patterns) that are structures typical for microorganisms, often essential and not present in the host. TLR detect ligands, such as LPS (gram-negative bacteria), double stranded RNA (viruses), CpG DNA motifs, lipoteichoic acid (gram-positive bacteria), extracellularly or in the lumen of endocytic vesicles. NLR (such as Nod1, Nod2 and Ipaf), on the other hand are intracellular proteins responsible for detecting microbes in the cytosol.

Another type of innate immune defence mechanism is the complement system that upon activation results in clearance or lysis of the pathogen that has been attacked by the components of the complement system. If a pathogen enters the host, an early immune response is induced but does not lead to protective immunity unless the infectious agent is able to breech the barriers of the innate immune system. This is where the adaptive immunity comes in.

2. Cells of the Innate Immune Response

2.1 Dendritic Cells

Amongst the cells that build up the innate immune system, DCs play a central role. DCs are a special type of cell that are key regulators of the immune system and act as an APC capable of activating naive T cells and stimulating the growth and differentiation of B cells. DCs are found, for example, in the lymph nodes and spleen. As an APC, a DC can retain antigen for long periods on its surface, present the antigen to a T or B cell and hence influence their behaviour.

Most importantly, DCs are involved in the differentiation of regulatory T cells[2], they function as versatile APCs involved in the initiation of both innate and adaptive immunity[1] and also in the differentiation of regulatory T cells[2] required for the maintenance of self-tolerance.

The plasticity of these cells allows them to undergo a complete genetic reprogramming in response to external microbial stimuli, such as inflammatory cytokines, microorganisms or microbial products like LPS[2], lipoteichoic acid, bacterial DNA and double-stranded viral RNA[3].

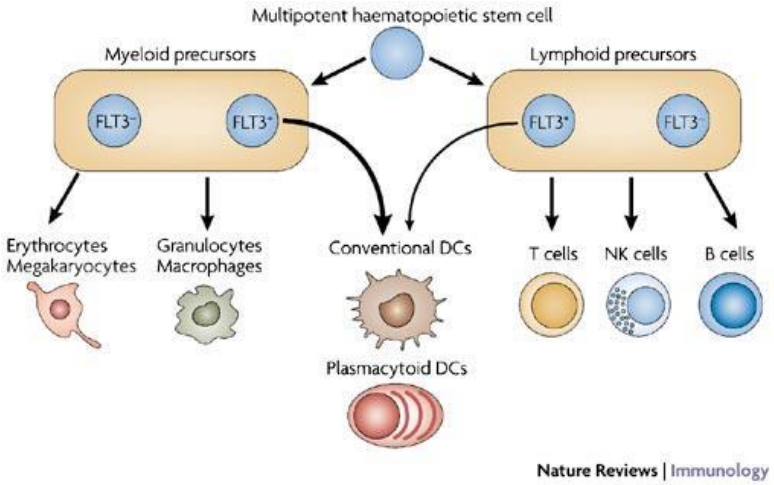
DCs vary in phenotype in their different locations, and their phenotypic variation is evident by differences in cell lineage and states of maturation. For example, it is known that specific DC lineages are located in certain tissues such that the Langerhans cells (LC) in skin are identified by specific markers like Langerin (CD207)[4].

Functional DCs resident in lymphoid tissues comprise of a heterogenous mixture of cells with few distinct markers. This diverse population represents a combination of endogenous immature DC, migrating DC, and DC in different states of differentiation and activation. Although the best characterized population represents "conventional" antigen-presenting DC (cDC), there is also evidence for a distinct lineage of plasmacytoid DC (pDC). These cells develop as distinct precursors in bone marrow and migrate through blood into lymphoid tissues including spleen. In murine spleen, cDC and pDC represent the two major DC populations[5].

Among the cDC there are two subsets distinguishable by expression of $CD8\alpha^-$, $CD11c^+$, $CD11b^-$,$CD8\alpha^+$ DC and $CD11c^+$,$CD11b^+$ and CD8α DCs[6]. These are distinct in their function of cytokine production, T helper response-generated, cross-presentation capacity, and capacity to localize in spleen[7]. Whereas cDCs function to activate effector T cells, pDC play a distinctive role in regulating function through induction of T regulatory cells and production of interferon IFNα [8].

2.1.1 Dendritic cell development

DCs are derived from hematopoietic progenitors in bone marrow (**Fig. 2**). In mouse, the nature of immediate precursors for DC in most tissue sites is unclear. A multitude of reports based on a combination of both in vitro and *in vivo* studies now describe several DC precursors. Debate arises over whether there are separate precursors for the different DC lineages and for different tissue sites. For LC in skin, the identity of precursors has been established.



Nature Reviews Immunology 7, 19.
Fig 2. Dendritic-cell development from haematopoietic precursors.

Under inflammatory conditions, monocytes differentiate into LC in the skin[9]. However, LC can also develop from endogenous progenitors that continually replenish cells in the steady state[10]. The distinct lineage of pDC is found in all lymphoid organs, and cells are characterized by the expression of CD45RA (B220 in mouse) and the production of IFN α upon stimulation[11]. Plasmacytoid-DCs develop from an immediate precursor, a CD11 p-pre DC that is found in blood[12]. DCs were from the beginning thought to have myeloid origin since DCs could be produced from BM myeloid precursors in the presence of GM-CSF. The development of pDC along with cDC has been characterized from bone marrow populations described as common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), which respond to Flt3L[13].

Today it is known that both CMP and CLP can give rise to all DC subtypes.

The ability to generate spleen DC from bone marrow and blood precursors is consistent with the existence of circulating DC precursors that continually seed spleen to replenish tissue-resident DCs. Precursors for each of the cDC and pDC lineages have been identified in both bone marrow and the blood. Further evidence in support of circulating DC precursors that lodge in spleen comes from the kinetics of DC development following transfer of bone marrow into irradiated recipients. In these mice, reconstitution of spleen cDC is rapid and sustained[14].

In the beginning of the 1990s, there was a major breakthrough in DC research when it was established that a substantial number of DC could be cultured in vitro from progenitors both in mice and humans. It was first discovered that DCs could be cultured from the mouse bone marrow and blood in the presence of GM-CSF. Cells resembling human dermal DCs could then be obtained from the human blood monocytes cultured in GM-CSF and IL-4. Addition of TGF-β gave rise to LCs[15]. For a long time, GM-CSF has played a very central role in the in vitro culturing of DCs. However, there is no direct evidence that these cells generated in vitro have an equivalent in vivo during steady state conditions. Thus, injecting mice with GM-CSF does not lead to a clear increase of $CD11c^+$ cells[16]. These DC seem to increase during inflammation, hence they have been called inflammatory DC. Injecting Flt3L on the other hand, has shown to increase cells with typically DC characteristics markedly[17], indicating that this may be a

very important cytokine when it comes to DC development in vivo during steady state conditions.

2.1.2 DC subsets

DC represent a very heterogenous cell type that can be further divided into several subsets both in mice and humans. The presence of distinct DC subsets with the quality of functional plasticity allows the DC system to cope with both maintenance of tolerance to self-antigens and protection against microbial pathogens, by eliciting distinctive types of immunity. The fact that there are also differences in the DC repertoire during steady state or inflammatory conditions further adds to the complexity. In mice, the major subtypes have been segregated according to the markers CD4 and CD8[18]. The integrin CD11b, which is a myeloid marker, 33D1 and CD205 (the multilectin domain molecule DEC-205)[19] and other markers used to describe mouse DCs. A common marker for all mature DCs is the integrin-α_x chain CD11c[20].

Conventional DCs (cDCs) can be referred to as cells having dendritic cell form and function, This catergory of D includes lymphoid-tissue resident DC and migratory DC.

Lymphoid tissue specific resident DCs include cDC resident in lymphoid tissues, such as spleen, thymus and lymph nodes. These includes lymphoid-tissue resident DC do not migrate through the lymph, but instead collect and present foreign and self-antigens in this

organ. The different cDC subsets differ in their cytokine production and presentation of antigens on MHC class I molecules.

Migratory DCs are cells that reside in the periphery, sampling antigens and carrying them to lymph nodes for presentation to T cells. LC and dermal DC belongs to this category. LC expresses high levels of langerin and have a long lifespan in the skin, but turn over rapidly once they reach the lymph nodes. In humans, they express mRNA for TLR1,2,3,5,6 and 10 enabling them to respond to viruses and gram-positive bacteria .

Inflammatory DCs are novel DC populations that are not found in the steady state appear as a consequence of infection or inflammation. These we term inflammatory DCs. One example is the DCs produced *in vivo* when pDCs are stimulated by the influenza virus. Another example is the DCs that appear after the infection of mice with *Listeria monocytogenes*. These are termed Tip DCs as they produce tumour-necrosis factor (TNF) and inducible nitric-oxide synthase (iNOS).

Irrespective of their ontogeny, the existence of multiple cDC subsets strongly suggests that distinct types of cDC have unique and divergent functionality in the immune system. This is paralleled by the existence of multiple classes of T cell, with well characterised and distinct function. This includes CD8+ cytotoxic T cells equipped for the production of perforin and granzymes and capable of initiating the death of infected host cells during a variety of pathogenic insults[21].

Exhibiting a greater diversity of potential functions, CD4+ 'helper' T cells represent a group of effector cells with shared developmental characteristics but highly divergent roles during immune responses.

Distinct CD4+ T cell subsets include Th1 and Th2 cells, producing the 'signature' cytokines IFNγ and IL-4, respectively, the characterisation of which established the paradigm of distinct helper T cell classes[22].

Initial studies proposed that T cell polarising function was strictly segregated between $CD8\alpha^+$ and $CD8\alpha^-$ cDC subsets, with $CD8\alpha^+$ cDCs yielding Th1 responses and $CD8\alpha^-$ cDCs generating Th2 responses *in vitro* and after transfer *in vivo*[23]. This differential capacity for Th1/Th2 priming was associated with a higher potential for IL-12 production by $CD8\alpha^+$ cDCs during infection[24], although $CD8\alpha^-$ cDCs can also produce IL-12 under some conditions[25].

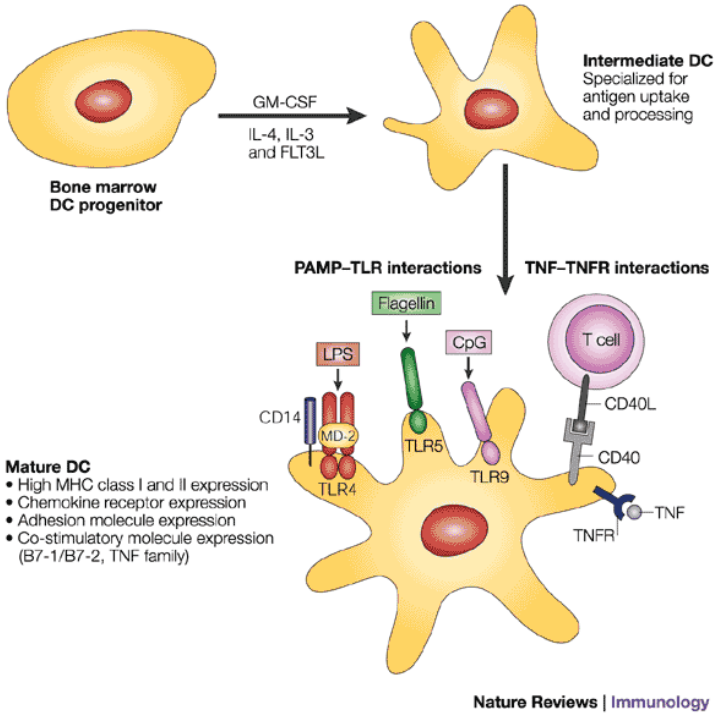
As such, the concept of distinct cDC subsets only instructing certain helper T cell classes is now thought to be an oversimplification. However, $CD8\alpha^+$ and $CD8\alpha^-$ cDC subsets possess differential antigen presentation capacity[26] and as such are thought to be functionally specialised for the activation of either CD8+ or CD4+ T cell responses, respectively.

2.1.3 DC Differentiation

Immature DCs are found as sentinels in non lymphoid organs; they are highly adapted for the uptake of antigen via receptor and non receptor

mediated mechanisms and they readily degrade antigens in endocytic vesicles to produce antigen peptides capable of binding to Human Leukocyte Antigens class II (HLA class II).

Upon maturation with pathogens such as activated T Lymphocytes and/or inflammatory signals such as TNFα, IL-1β, or LPS (Fig 3), immature DCs undergo genetic reprogramming leading to mature DCs characterized by high expression of HLA class II molecules's absence of lineage markers such as CD14 (monocytes), CD3 (T cells), CD19, CD20 (B cells), CD56 (NK cells), high level expression of costimulatory molecules CD83, CD86, CD80, CD40, and adhesion molecules such as CD11a, CD11c[27]. Mature DCs also acquire ability to migrate which is regulated by expression of chemokines and chemokines receptors CCR7[28]. These chemokines guide mature DCs to lymphatic vessels and to secondary lymphoid organs.



Nature Reviews Immunology 2, 227-238 (April 2002)

Figure 3: Multi-step pathway for dendritic-cell differentiation and activation.

2.1.4 DC activation signals and maturation

The maturation state of DCs has been suggested to be a determining factor for the induction of immune tolerance or immunity. Maturation is a terminal differentiation process that transforms DCs from cells specialized for antigen capture into cells specialized for T-cell stimulation. DC maturation is induced by components of pathogens or by host molecules associated with inflammation or tissue injury. These stimuli are often collectively referred to as "danger signals" [29].

Upon encountering a danger signal, DCs are required to mature and elicit an immune response that adequately deals with the specific

danger. However, as a wide range of microbial stimuli, tissue-derived factors and interaction with other immune cells all serve as danger signals, DCs need to be endowed with functional plasticity to cope with each specified situation. Hence, depending on the nature of the encountered activation signal, DCs are able to attain a range of immunostimulatory, immunoregulatory or tolerogenic characteristics in the mature state in response[30].

A major group of "danger signals" to activate DCs are microbial stimuli[31]. Structurally conserved pathogen associated molecular patterns (PAMP), or pathogen derived products present on or in microbial pathogens, are recognized by pattern recognition receptors (PRR) expressed by DCs and other immune cells. Four major families of receptors comprise the PRR: 1)Toll-like receptors (TLR), 2) C-type lectins (CLR), 3) RIG-I-like receptors (RLR) and 4) NOD-like receptors (NLR).

These receptor families each comprise many members which are differentially expressed by DC subsets. Consequently, DC PRR fulfil many biological functions[32]. For instance, members of the NLR family, including NALP1 and IPAF, are known to be involved in the maturation of pro-inflammatory cytokines such as pro-IL-1β 40,41. Conversely, RLR like MDA5 and LGP-2 recognize RNA viruses in most nucleated cells and in response trigger the expression of IFN-α/β[33]. Stimulatory CLR are capable of enhancing the production of pro-inflammatory cytokines, whereas inhibitory CLR hinder the activity of TLR-mediated immune complexes. Well studied CLR are dectin-1, MINCLE and CLEC9A[34].

Nevertheless, the best studied PRR in anti-cancer immunity are TLR, which stimulate cytokine secretion and antigen presentation upon stimulation. Depending on the type of receptor, the family of TLR recognize various PAMPs and elicit different responses. For example, TLR2 recognizes lipoproteins and zymosan and induces an IL-10-mediated TH2 immune profile. Contrastingly, TLR4 recognizes lipopolysaccharide (LPS) and elicits a TH1 immune response through IL-12 production. Other important TRL expressed by DC subsets are TLR7 and TRL9[35], which recognize the microbial-specific genomes single-stranded RNA and CpG oligo-deoxynucleotides, respectively 44. Interestingly, the type of pathogen also influences the response of DC to their PAMPS. Whereas LPS from *Escherichia coli* does induce a TH1 immune response, exposure to LPS from *Porphyromonas gingivalis* does not induce a TH1 response[36].

The DC system is not only adapted to respond to various microbial stimuli, but also to recognize signals from the immune microenvironment. Differential factors during the development of DC subsets, such as cellular interactions and cytokine secretion, determine their functional potential[37]. Cytokines especially are potent DC activation stimuli that have a large influence on the final phenotype of mature DC by stimulating various distinct signal transduction pathways[38]. For example, incubation with IL-15, TNF or IFN-α gives rise to immunostimulatory DC through the induction of STAT4 and IRF-8 signalling[39].

Conversely, activation of NF-κB and STAT6 signalling through incubation with TSLP, IL-10 or vitamin A yields DC that maintain tolerance[40]. Although it remains unclear whether these *in vitro* cultured DC have an *in vivo* counterpart, it is known that tissue-localized DC can be polarized by interferons and interleukins produced by other cells from the environment, such as γδ-T cells, NK cells, stromal cells, lymphocytes and mast cells. In theory, these differentially polarized DC will induce distinct responses from T cells, leading to stimulation or regulation of the immune system[41].

Apart from indirect interaction through cytokines secreted by other cells, DC also directly interact with cells from the innate and adaptive immune system. In the periphery and the secondary lymphoid organs, DC are capable of reciprocally interacting with NK, natural killer T cells (NKT) and γδ T cells[42]. The activation of NK is completely dependent on DC interaction, as mice studies suggest[43], and NKT cells and γδ T cells are also found to be activated by mature DC. Upon activation, these cells enhance their capacity to secrete IFNγ, which in turn polarizes DC to induce TH1 responses. In addition, γδ T cells secrete TNF-α and NKT cells secrete IL-4. NKT cells further acquire the capacity to kill tumour cells and to express CD40L, inducing strong DC activation[42]. DC are also known to directly interact with both T and B cells. DC are responsible for T-cell priming by inducing immune tolerance in several ways, e.g. by T cell deletion and activating Treg cells. Humoral immunity is also dependent on DC functioning by direct interaction with B cells and presentation of unprocessed antigen[44].

Maturation is also characterized by reduced phagocytic uptake, the development of cytoplasmic extensions, migration to lymphoid tissues, and enhanced T-cell activation potential. Mature DCs express a number of characteristic markers, including CD83, a cell surface molecule involved in CD4+ T-cell development and cell-cell interactions[45] and DC-LAMP, a DC-specific lysosomal protein[46]. Maturation signals act on DCs through receptors that trigger intracellular signaling, including receptors for host-derived inflammatory molecules such as CD40L, TNFα, IL-1, and IFNα . Microbial products and molecules released by damaged host tissues transmit maturation signals through TLRs, trans-membrane receptors expressed on DCs and other cell types related to Drosophila Toll protein[47].

2.1.5 Pathways to regulatory DC function

Whether inducing tolerance or immunity, a key property of DCs is their capacity to sense subtle changes in their environment. They do this by expressing a range of pattern recognition receptors (PRRs) that recognise conserved elements from a multitude of infectious organisms, also known as pathogen-associated molecular patterns.

TLRs signal through the adapter molecule MyD88, which recruits other signaling molecules in a pathway that activates NF-κB and mitogen-activated protein (MAP) kinases, inducing the transcription

of genes encoding inflammatory mediators such as TNFα, IL-1, and IL-6[48]. This process is tightly regulated, with non-functional adaptor protein variants, ubiquitin ligase-mediated degradation of signalling molecules, microRNAs and RNase enzymes all controlling levels of TLR activation.

TLR ligation can also result in anti-inflammatory cytokine production, with the preferential production of IL-10 through pathogen-induced TLR signalling first described as an immune avoidance strategy by *Yersinia pestis*, a process mediated through TLR2[49]. It is now apparent that many pathogens exploit TLR2-induced IL-10 production by APCs as a strategy to establish infection, such as *Borrelia burgdoferi*, *Aspergillus fumigatus*, *Candida albicans* and *Mycobacterium tuberculosis*[50] although the precise signalling pathways involved are not completely clear. However, the activation of extracellular signal-related kinase (ERK) appears to be a conserved component in the induction of IL-10 downstream of TLR2 in DCs, which occurs as a result of MyD88 or spleen tyrosine kinase (Syk)-dependent pathways, determined by the nature of the ligand[51]. TLR2-induced IL-10 production may also require c-Fos, as DCs deficient in this transcription factor produce less IL-10 in response to TLR2 ligation.

Alongside defined TLR-induced pathways, the activation of ERK provides a generalised mechanism by which the production of IL-10 by macrophages and DCs is enhanced. Stimuli as diverse as oxidative stress[52], and CpG DNA[53] have been shown to induce ERK phosphorylation, resulting in suppression of IL-12p40 production and

augmented production of IL-10 in APCs. In addition, FcγRI ligation induces IL-10 production in macrophages[54], as a result of ERK phosphorylation and the subsequent remodelling of chromatin, allowing enhanced Sp1 and STAT3 binding and augmented IL-10 production.

Stimulation of some TLRs can trigger additional, MyD88-independent, signaling pathways[55]. In DCs, the distinct signaling pathways triggered can influence the direction of the resulting T-cell response[56] . TLR agonists, therefore, can be used to target DC subsets to induce desired T-cell responses.
Maturation imparts on peripheral DCs the ability to migrate from the tissues to T-cell zones of lymph nodes.

Recently, it has been reported that upon activation DCs exhibit transient production of IL-2 and express IL-2Rα, a property that appears to be related to their capacity to initiate immune responses[57]. Furthermore, the ability of DCs to produce IL-2 after encountering inflammatory stimuli provides the first crucial signals for the activation of naïve T cells. The kinetics of IL-2 production by DCs are compatible with the appearance of HLA class II and class I peptides at the cell surface of DCs, so IL-2 appears to be one of the key molecules conferring unique T-cell stimulating capacity on DCs.

In addition to affecting adaptive immunity, the cytokine synthesis profiles of DCs also influence their capacity to activate cells involved in innate immunity such as NK cells[58]. Activated NK cells can kill

immature, but not mature, DCs and can stimulate DCs to induce protective CD8+ T-cell responses[59].

2.2 Macrophages

Macrophages are involved in both innate and adaptive immune responses. Depending on the types of cytokines that macrophages are exposed to, these cells are subjected to classical (Th1) or alternative (Th2) activation. Classically activated (M1) macrophages were the first to be defined as pro-inflammatory. Alternatively activated (M2) macrophages have been originally characterised in the context of Th2-type immune responses.

2.2.1 Development of classically activated macrophages

The process of differentiation of classically activated macrophages requires a priming signal in the form of IFN- γ 7 via the IFN- γ R[60]. When the primed macrophage subsequently encounters an appropriate stimulus, such as bacterial LPS, it becomes classically activated. LPS is first bound by soluble LBP and then by either soluble or membrane-bound CD14. CD14 then delivers LPS to the LPS recognition complex.

Pathogens and pathogen components are subsequently taken up by phagocytosis[61] and delivered to lysosomes where they are exposed to a variety of degradation enzymes including. Suitable antigens are processed and loaded on to MHC class II molecules in late endocytic

compartments and antigen/MHCII complexes as well as co-stimulatory B7 family members are presented to T cells[62].

These events are followed closely by a significant change in cellular morphology and a dramatic alteration in the secretory profile of the cell. A variety of chemokines including IL-8/CXCL8 and RANTES/CCL5, are released as chemoattractants for neutrophils, immature DCs , NK cells, and activated T cells[63]. Further, several pro-inflammatory cytokines are released including IL-1 beta/IL-1F2, IL-6, and TNF-alpha[64]. TNF-alpha also contributes to the pro-apoptotic activity of the classically activated macrophage.

2.2.2 Activation of Macrophages

The TCRs and CD4 molecules on the Th1 cell interact with the MHC-II molecule with bound peptide epitope on the macrophage. Co-stimulatory molecules such as CD40L on the Th1 cell then bind to CD40 on a macrophage. This triggers the Th1 cells to secrete the cytokine interferon-gamma (IFNγ). IFN γ subsequently binds to IFN γ receptors on the macrophage causing its activation. Activated NKT cells also produce large amounts of IFNγ to activate macrophages[65].

The activation of macrophages hence:

1. Increases their production of toxic oxygen radicals, nitric oxide, and hydrolytic lysosomal enzymes enabling the killing of microbes within their phagolysosomes.

2. Causes the macrophages to secrete cytokines such as TNF-alpha, IL-1, and IL-12. TNF-alpha and IL-1 promote inflammation to recruit phagocytic leukocytes. lL-12 enables naive T4-lymphocytes to differentiate into Th1 cells.
3. Increases the production of B7 co-stimulator molecules and MHC-1 molecules by macrophages for increased T-lymphocyte activation.

The IFNγ produced by Th1 cells also increases the production of opsonizing and complement activating IgG to promote enhanced attachment (opsonization) of microbes to phagocytes.

2.3 NK Cells

NK cells are lymphocytes of the innate immune system that are involved in the early defenses against foreign cells, as well as autologous cells undergoing various forms of stress, such as microbial infection or tumor transformation. They were first discovered in 1975 by Kiessling et al and Herberman et al. They discovered a lymphoid cell type able to lyse tumor cells without prior stimuli that was T-cell independent. Since then, the role of NK cells in the immune system has been continuously growing. More recently, the role of NK cells in immune homeostasis and autoimmunity is being put under the microscope.

NK cells represent a lymphoid population that has innate immune functions. Unlike T-cells, NK cells do not express a diverse set of

antigen-specific receptors. Instead, they display a heterogenous array of cell surface receptors enabling them to respond to cytokines, pathogens and to recognise the difference between stressed/transformed/infected cells and normal cells.

In humans, NK cells can be divided into two functionally distinct subsets based on the expression of CD56. The $CD56^{bright}$ NK cells have poor cytolytic capacity, but produce a lot of cytokines, especially IFNγ. The $CD56^{dim}$ NK cells on the other hand, are the main killer population but are poorer at producing cytokines.

NK cell activation is controlled by a dynamic balance between complementary and antagonistic pathways that are initiated upon interaction with potential target cells. NK cells express an array of activating cell surface receptors that can trigger cytolytic programs, as well as cytokine or chemokine secretion. Some of these activating cell surface receptors initiate protein tyrosine kinase (PTK)-dependent pathways through noncovalent associations with transmembrane signaling adaptors that harbor intracytoplasmic ITAMs (immunoreceptor tyrosine-based activation motifs[66]). Additional cell surface receptors that are not directly coupled to ITAMs also participate in NK cell activation[67].

2.3.1 NK cell development

NK cells develop primarily in the bone marrow in adults and are widely distributed in the body, but the largest population can be found

in spleen, lung, liver, bone marrow, and peripheral blood. NK cells can migrate to various tissues[68]. Intestinal NK cells are phenotypically distinct from their counterparts in the blood and resemble "helper" NK cells, which have potentially important functions both in promoting antipathogen responses and in the maintenance of intestinal epithelium[69].

2.3.2 NK cell activation

The migration and recruitment of NK cells from blood vessels to target tissues are the first steps in the cascade of events for NK cell activation. NK cells act by means of direct cytotoxic attack on their targets or by producing a large array of cytokines and chemokines. NK cells are important effector cells of the innate immune system required for the first line of defense against transformed and infected cells and play an essential role in linking innate and adaptive immunity through their ability to secrete IFN-γ[70]. At the early stage of infection, NK cells are considered as the primary source of IFN-γ, shaping the adaptive immunity through differentiation of CD4+ T cells to the Th1 subsets[71]. NK cells kill their target cells through two major pathways, both requiring close contact between NK cells and the target cells. In the first pathway, cytoplasmic granule toxins including perforins and granzymes are secreted by exocytosis and together induce apoptosis of the target cells. The second pathway involves the engagement of death receptors in target cells by their cognate ligands in NK cells, resulting in classical caspase-dependent apoptosis[72].

Previous studies[73] both in mouse models of autoimmune diseases and in humans have shown that NK cells have either a disease-promoting or -controlling role. Unlike T cells, NK cells do not express a diverse set of antigen-specific receptors, but they are unique in bearing both stimulatory and inhibitory receptors, and their function is regulated by a series of inhibiting or activating signals. When NK cell inhibitory receptors bind to MHC class I molecules, their effector functions (i.e., cytotoxicity and cytokine production) are then blocked. Lower expression of stimulatory receptors could result from specific down-regulation of the receptors in such NK cells, or from a failure of these cells to up-regulate such receptors during development. Moreover, the activation of NK cells also results from the concerted action of co-stimulatory molecules already well characterized for their function in T cells. However, evidence indicates that NK cells also regulate the innate and acquired immune responses through their secretion of soluble factors and/or cell-cell contact[74]. NK cells discriminate from myeloid immature dendritic cells, which typically underexpress MHC class I molecules, and mature dendritic cells, which upregulate MHC class I expression after antigen uptake[75]. The killing of immature DCs by NK cells has been interpreted as a control of the quality of DCs, allowing only mature DCs to migrate to the lymph nodes[76].

2.3.3 Production of Cytokines

Many of the physiologic functions of NK cells are mediated at least in part by their ability to secrete cytokines. NK cells are powerful

producers of IFN- γ and granulocyte-macrophage colony stimulating factors (GM-CSF) and have also been shown to be able to produce tumor necrosis factor-a (TNF-a), macrophage-CSF (M-CSF), IL-3, IL-5, IL-8, IL-13, and other cytokines[77].

Stimulation by cytokines such as IL-2, IL-12, IL-18, TNF-a, and IL-1 and triggering of surface receptors, such as CD16 interaction with immune complexes, are among the stimuli that, acting individually or often in synergistic combination, induce NK cells to produce cytokines[78] IL-2 and IL-12 induces NK-cell proliferation[77].

2.3.4 NK cell receptors

NK cells express many different activating and inhibitory receptors, which maintain the delicate balance of positive and negative signals to the cytolytic machinery. The ligands for these receptors are classical and nonclassical MHC I molecules. The killer cell immunoglobulin like receptor (KIR) family recognizes and binds the classical MHC I molecules. Some of these receptors mediate inhibition of NK cell cytotoxicity, while the functions and ligands of other receptors are still unknown[79]. The nonclassical MHC I molecule HLA-E is recognised by the lectin-like CD94/NKG2 receptor family. Both receptor families contain both inhibitory and stimulatory forms. An individual NK cell can simultaneously express several activating and inhibitory receptors. Most NK cell receptors are encoded by the NK-gene complex and leukocyte-receptor complex[80]. Other surface molecules, such as

CD11a/CD18, 2B4, CD2, and CD69, may also induce or modulate NK cell functions [81].

Inhibitory Receptors

Several inhibitory receptors that engage with MHC-I or MHC-I-like molecules have been identified, for example killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LIRs), killer cell C type lectin-like receptors (KLRs), such as CD94:NKG2A in humans and Ly49 in mice[82]. An important signal that inhibits NK cell activity is delivered by HLA-E molecules via CD94:NKG2A. HLA-E is an instable minor HLA antigen and dependent on expression of major HLA antigens, therefore HLA-E cannot be expressed when HLA-A, B or C alleles are down-regulated. Hence, HLA-E represents an additionally control for expression of major HLA alleles[83].

Although the inhibitory receptors belong to different families and differ in extracellular domains, these receptors share a common signal motif, the immunoreceptor tyrosine-based inhibitory motif (ITIM), and accordingly a common signal transduction pathway. When an inhibitory receptor associates with its ligand, the ITIM motif is phosphorylated resulting in recruitment of the lipid phosphatase SHIP and/or the tyrosine phosphatases SHP-1 or SHP-2. Consequently, these phosphatases dephosphorylate substrates – especially Vav-related proteins – downstream of activating NK receptors resulting in inhibition of NK cells.

Activating Receptors

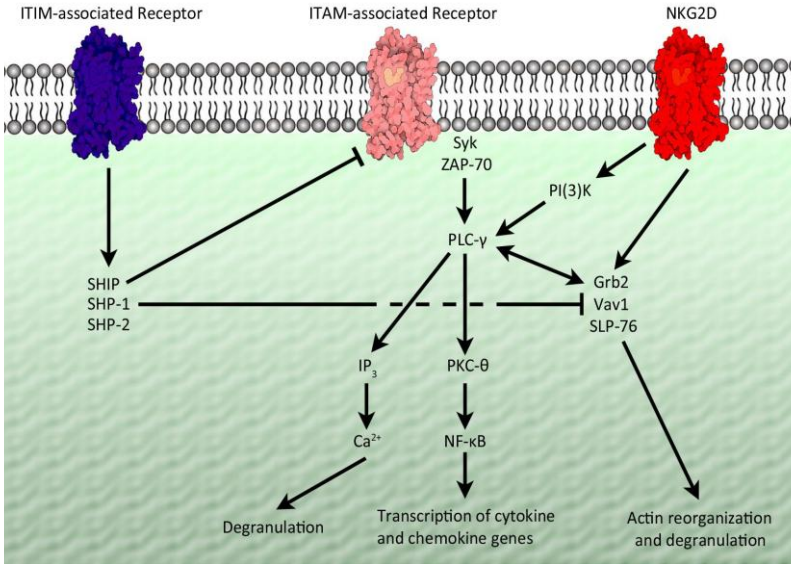
Alongside inhibitory receptors, NK cells express a divers set of activating receptors. Most of these receptors belong to the same families as the inhibitory receptors, namely the KIRs and KLRs, with the exception of the natural cytotoxicity receptor (NCR) family, which contains exclusively activating receptors. In parallel to their function as inhibitory receptors, KIRs and KLRs also recognize HLA antigens, although with much lower affinity than their inhibitory counterparts. For instance, the activating receptors CD94:NKG2C and CD94:NKG2E both bind HLA-E, just like the inhibitory receptor CD94:NKG2A. The exact function of this phenomenon remains unclear, however one could postulate that a certain level of HLA antigens must be expressed on a cell. Expression of HLA below a certain threshold results in lack of inhibitory signals to NK cells and thus permits cytolysis of the target cell. In contrast, expression of HLA antigens that exceeds the normal range – due to viral infection or transformation – also induces cytolysis, via the activating low affinity receptors [83].

The natural cytotoxicity receptor family includes the receptors NKp30, NKp44 and NKp46. Members of this family have the unique ability to recognize specific viral antigens [84].

Cross-linking of individual activating receptors by itself does not induce NK cell activation. This requires prior exposure to IL-2 or cross-linkage of multiple activating receptors at the same time. Hence,

the name co-activating receptors would be more appropriate. An exception is the activating receptor CD16, since individual cross-linking of this receptor results in NK cell cytotoxicity and cytokine production[85].

Activating receptors associate with other co-receptors than inhibiting receptors, which are involved in signal transduction pathways that induce NK cell activation. The best understood activating receptor complexes include the DAP10-associated NKG2D complexes, the ITAM-bearing co-receptor complexes and the CD244 receptor system[82] (**Fig 4**).



Cheent and Khakoo (2009).

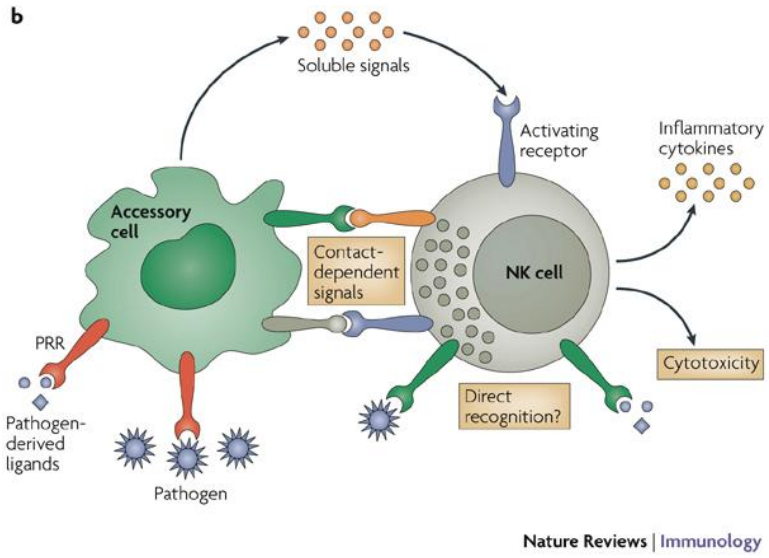
Figure 4. Schematic representation of the interaction of the activating (ITAM & NKG2D) and inhibiting (ITIM) receptor mechanisms of NK cells.

NK2GD is a type II transmembrane glycoprotein belonging to the C-type lectin-like receptor superfamily. Unlike its family members, NKG2D does not form complexes with CD94 and DAP12. But NK2GD forms homodimers via disulfide-bonds and associates with the signaling subunit DAP10[82]. NKG2D recognizes factors up-regulated by cells that are in cellular stress, such as MHC class I-like polypeptide-related sequences MICA and MICB, and UL16 binding protein 1-5 (ULBP1-5)[86]. In mice, NKG2D is expressed in two different isoforms, a long (NKG2D-L) and a short (NKG2D-S) isoform. Both isoforms bind identical ligands, namely RAE-1 family members, H60 and MULT1[87]. However, NKG2D-L exclusively associates with DAP10, while NKG2D-S can associate with both DAP12 and DAP10. Resting murine NK cells express low levels of NKG2D-S whereas activated murine NK cells express high levels of

NKG2D-S. These observations suggest different signal transduction potential for the two isoforms that may be related to activation state of the NK cell[88]. A similar mechanism has not been described in humans, but it was recently shown that prior exposure to IL-15 is necessary for cytolytic activity through NKG2D, since activity of DAP10 is regulated downstream of the IL-15R[83].

2.3.5 NK-DC cell interaction

Originally, DCs were described to capture and present antigens and prime the adaptive immune system, while the function of NK cells was to lyse tumors and virally infected cells. Today it is evident that these two cell types also have an important function in regulating the adaptive immune response by cell-cell crosstalk. Many studies have shown that this crosstalk can result in cellular maturation, activation and also death. In 1985, the first indication came that NK cells might be capable of regulating adaptive T cell responses by eliminating DCs that have interacted with antigens[89]. Later in 1999, Fernandez et al published the first evidence in vivo that DCs could trigger NK cell mediated anti-tumor effects[90]. They demonstrated how the expansion of DCs *in vivo* results in greater anti-tumor immunity, and this effect is dependent on NK cells. DCs are able to activate NK cells with both soluble signals and contact-dependent signals[91] (**Fig. 5**).



Newman and Riley (2007).

Fig 5. Accessory cell mediated NK cell activation

Several studies later demonstrated that NK cells and DCs have reciprocal effects on each other [92, 93].

DCs can activate NK cells via both cytokines and cell-to-cell contact. After encounter with a pathogen or a danger, immature DCs mature and induce resting NK cell activation. NK cells are innate cytotoxic effectors but also regulatory cells releasing cytokines involved in innate resistance and adaptive immunity. Several in vitro studies show a central role of DC-derived IL-12, IL-18, and type I IFN in the triggering of NK cell functions. IL-12 seems to be important to induce the secretion of IFNγ by NK cells in several systems, such as LPS-activated monocyte-derived DCs and splenic DCs[94], IL-18 may act in synergy with IL-12 to induce the secretion of IFNγ by NK cells but also to enhance cytotoxicity, at least when NK cells are stimulated

with human CD34+ derived DCs[95]. Type I IFNs have also been shown to enhance cytotoxicity of NK cells [96] . Although all types of DCs can secrete type I IFN, the main producer of these cytokines are pDCs, particularly when activated through TLR7 and TLR9 by virus components[97]. Nevertheless, NK cells may be activated in an IL-12-, IL-18- and type I IFN-independent manner. In fact, DCs from IL-12- and IL-18-deficient mice are able to induce IFNγ secretion by NK cells. In mice, this capability might be under the control of IL-2 secreted by bone marrow-derived DCs activated by bacterial components [98].

IL-15 produced by mature monocyte-derived DCs appears to be particularly important to stimulate NK cell proliferation. Interestingly, this effect may require the membrane-bound form of IL-15, as the proliferation is abrogated by physical separation of DCs and NK cells[99].

Despite the large mass of data showing the role of soluble factors in NK cells activation, early studies in mice suggest the involvement of cell-to-cell contact. Transwell separation of the two populations could abrogate DC-dependent NK cells cytotoxicity induction[100]. Contact through an "immunological synapse" may be necessary for the polarized secretion of IL-12 or of other cytokines by DCs toward NK cells[101] as well as for ligand-receptor interaction[102].

Likewise, it is probably through such synaptic formations that NK cells may kill DCs. Several groups have observed that NK cells

recognize and lyse monocyte-derived DCs in vitro[103] in a cell-to-cell contact dependent manner. It has been described that NK/DC ratio is a critical factor to induce NK cells-mediated DC death. Whereas a low ratio (1 : 5) leads to DCs maturation, a higher NK/DC ratio (5 : 1) causes killing of immature DCs by the autologous NK cells[103]. Interestingly, DC subsets display different susceptibilities to lysis by NK cells; human pDCs were not lysed by IL-2 activated NK cells whereas mDCs isolated directly from blood underwent only a limited lysis[94].

Moreover, mature DCs are protected from NK cell lysis by acquiring a higher expression of HLA I molecules[99]. Beside the inhibitory receptors, NK cells activating receptors play a primary role in DC targeting. The activating receptor NKp30 appears to be an important candidate during this interaction, since the single blocking of this receptor inhibits NK cell-mediated lysis of immature DCs[104].

In peripheral tissues, the bidirectional crosstalk between NK cells and DCs has been proposed to play a relevant role in the mechanisms leading to the selection of DCs with maximal capability of T cell priming[105]. In particular, distinct studies have demonstrated that human NK cells have the capability to induce DC maturation[106]. This might be important when pathogen-related molecules or inflammation are not present to drive DC maturation and, therefore, an effective antigen presentation.

The molecular mechanisms that regulate this specific part of the human NK/DC crosstalk have been also clarified. It has been found that, at low NK/DC ratio, NK-DC interactions induces cytokine production (especially, TNFα and IL-12) by DCs as well as the upregulation of a series of molecules involved in antigen presentation. This stimulating effect may depend on cell-to-cell contact as well as TNFα released by NK cells[106].

2.3.6 NK cell mediated killing of DCs

During inflammation, viral infection and tumor growth, NK cells are rapidly recruited from the blood into injured tissues[107]. At the sites of infection, NK cells encounter resident DCs already responding to signals derived from invading pathogens and proinflammatory cytokines. Given the ability of activated DCs and NK cells to influence and recruit each other, a rapid influx of both DCs and NK cells will ensue.

Piccioli et al.[108] show that the outcome between DC activation or death depends on the DC/NK cell ratio. At high NK:DC ratios (5/1), inhibition of DC functions is the dominant feature of the DC interaction with activated NK cells due to direct NK cell killing of immature DC. Indeed, both DC maturation and DC cytokine production (TNFα, IL-12), observed at low activated NK/immature DC ratios (1/5 and up to 1/40), are abrogated at high NK:DC ratios. Ferlazzo et al.[109] demonstrates (versus mature DCs) elective killing of immature DCs by activated NK cells. NK cells, after activation by IL-2 or DCs, exhibit potent killing activity against immature DCs and

secrete IFNγ. Activated NK cell lysis of immature DCs is blocked electively by anti-NKp30 Ab (and not by anti-NKp44, NKp46, NKG2D, 2B4, NKp80). In contrast, mature DCs are resistant to NK cell lysis. NK cells become capable of recognizing mature DCs in a NKp30-dependent fashion only in the presence of anti-MHC class I Ab.

These data highlight a regulatory loop whereby DC-mediated NK cell activation leads to DC killing in case of overwhelming NK cell responses.

NK cell recruitment is governed by integrated signals, which include adhesion molecules and chemotactic factors. $CD56^{low}$ $CD16^{+}$ NK cells express both $\beta 1$ and $\beta 2$ integrins[110], as well as the ligands for E- and P-selectins. In addition to these molecules, $CD56^{high}$ NK cells also express high levels of L-selectin[111], a pivotal molecule for the interaction with lymph node high endothelial venules. A crucial role in the transendothelial migration process of different leukocyte types including NK cells is also played by the DNAM-1 receptor expressed on human NK cells[112]. Various soluble factors play an important role in the early events that favor the extravasation of NK cells and the subsequent induction of their priming. These include various cytokines and chemokines that are released by resident DCs and other cell types including endothelial cells, macrophages, neutrophils, fibroblasts, mast cells and eosinophils during pathogen-induced inflammation in peripheral tissues. The mechanism of NK cell recruitment appears to involve chemokines such as CXCL8, CCL3 and CX3CL1[111]. Indeed most classical NK cells ($CD56^{low}$ $CD16^{+}$)

express CXCR1 and CX3CR1[112] while the minor $CD56^{high}$ $CD16^{-}$ NK subset express CCR7[113].

3. The inflammatory response of the innate immune system

3.1 Inflammasomes

3.1.1 Introduction

Inflammation is a host adaptational response to cell injury caused by various exogenous and endogenous stimuli. Microbial products and endogenous "danger signals" released by infected or otherwise stressed host cells are recognized by families of pattern recognition receptors (PRR) resulting in the activation of signaling pathways that initiate the inflammatory response and regulate development of adaptive immunity. TLRs and C-type lectins are PRR expressed on the cell surface or in endosomal compartments, while RIG-I-like receptor (RLR) are located in the cytosol[114]. Stimulation of these receptors results in activation of the NF-κB-, MAPK-, Syk-, and IRF-signaling pathways culminating in transcriptional induction and the secretion of a large number of cytokines, chemokines, and immunomodulatory factors. The Nod-like receptors (NLR) family is another group of cytoplasmic PRR that performs diverse immunological functions[115]. A subgroup of NLR surveys the cytoplasm for evidence of danger or infection and control activation of the inflammasome, a multiprotein complex that regulates activation of the cysteine protease caspase-1[116].

Caspase-1 itself is generated as an inactive precursor protein that contains a "caspase activation and recruitment domain" (CARD) motif in its N-terminus, which is essential for bringing two or more

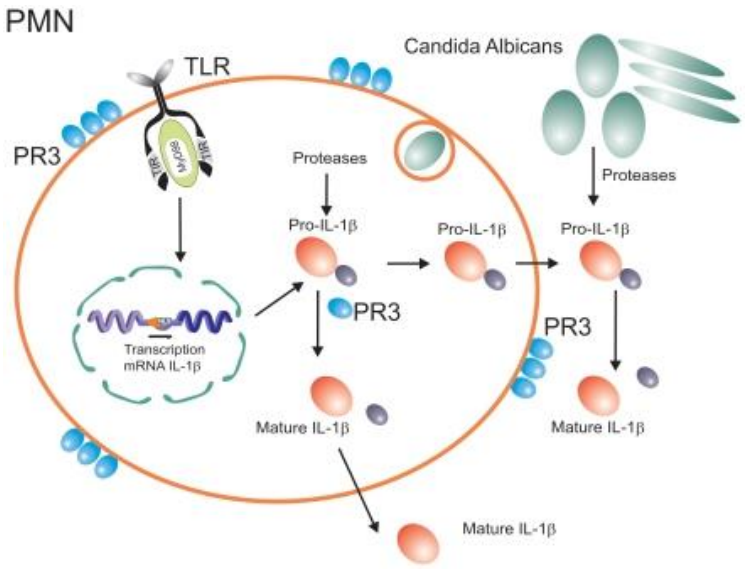
zymogens sufficiently close to induce their autocatalytic activation[117]; this process is believed to occur in inflammasomes.

Innate immune cells such as macrophages and DCs produce potent inflammatory cytokines to mount an appropriate immune response against microbial threats. The related cytokines interleukin IL-1β and IL-18 are generated as cytosolic precursors that require cleavage by the cysteine protease caspase-1 to generate biologically active IL-1β and IL-18 (**Fig.6**). Hence, mice lacking caspase-1 are defective in the maturation and secretion of IL-1β and IL-18 [118].

Several different types of inflammasomes have been identified. Inflammasomes are multiprotein complexes containing pattern recognition receptors belonging to the Nod-like receptor family or the PYHIN family and the protease caspase-1[119]. The inflammasome is an important innate immune pathway that regulates at least two host responses protective against infections:

1. secretion of the proinflammatory cytokines IL-1β and IL-18 [120] and

2. induction of pyroptosis, a form of cell death.



PLoS Pathology 6(2), (Feb 2010).

Figure 6. Inflammasome-independent processing of pro-IL-1β.(In addition to caspase-1-dependent activation, pro-IL-1β can also be processed by neutrophil-derived serine proteases, or pathogen-derived proteases)

3.1.2 Activation of Inflammasomes

Activation of caspase-1 in the context of the inflammasome is responsible for the proteolytic processing of the immature forms of IL-1β and IL-18[121]. While the production of most proinflammatory cytokines is primarily regulated at the transcriptional level, secretion of IL-1β and IL-18 requires this additional proteolytic step. Thus, IL-1β and IL-18 secretion is regulated in a two-step fashion. First, stimulation through TLR or RLR induces their synthesis as inactive precursors that lack signal peptide. Second, NLR-mediated inflammasome activation catalyzes the posttranslational processing that is required for their secretion and bioactivity. It should be noted

that although IL-1β and IL-18 processing is catalyzed most efficiently by caspase-1, other proteases can process IL-1β under particular circumstances, like during high neutrophilic inflammation[122].

In addition to regulating processing and the secretion of IL-1β and IL-18, inflammasomes also trigger pyroptosis, a form of cell death of the infected cell that is distinct from classical apoptosis or necrosis[123]. Pyroptosis, by destroying the pool of infected cells, effectively restricts intracellular bacteria growth and dissemination and, therefore, is an efficient effector mechanism to protect the host from infection[124].

3.1.3 Structure & function of the NLRP3 inflammasome

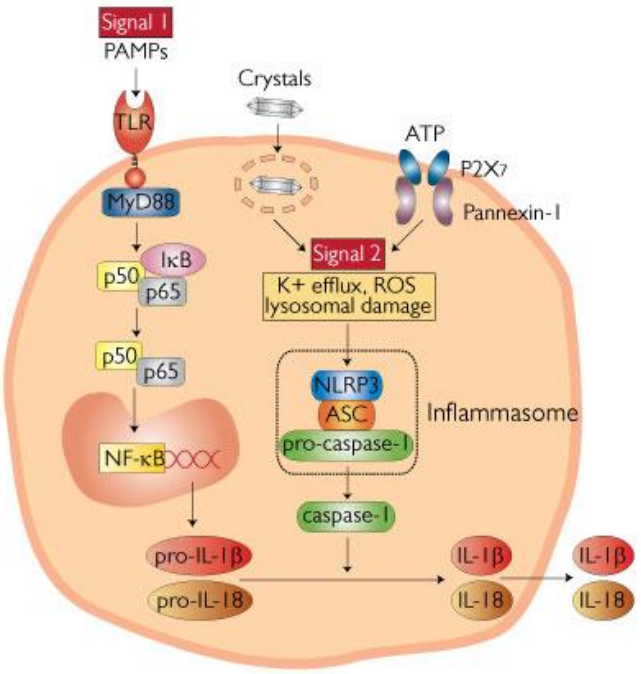
A few inflammasomes have been recently characterized, and it is likely that many more will soon be reported. The Inflammasomes so far characterized are generally composed of a PRR, the adaptor molecule ASC, and caspase-1.

There is evidence that other inflammatory caspases may be part of inflammasomes[125]. At least two families of PRR have been shown to form inflammasomes, the NLR and the PYHIN proteins.

Assembly of functional inflammasomes is believed to be driven by homophilic interaction between the PYD and CARD domains of the NLR/PYHIN receptors and the PYD and CARD domains of the adaptor molecule ASC and the CARD of caspase-1. Assembly of this

multiprotein platform, which is reminiscent of the apoptosome, leads to activation of caspase-1 by the proximity model[126].

NLRP3 and NLRC4 are the best-characterized NLR molecules. The NLRP3 inflammasome is the most studied, and yet, the logic that oversees its activation remains elusive. NLRP3 contains a PYD domain that mediates the interaction with ASC that, acting as a bridge, recruits caspase-1.



(http://www.invivogen.com/images/Inflammasome_review.jpg)

Figure 7:Activation of the NLRP3 inflammasome.

(The best characterized inflammasome is the NLRP3 inflammasome. It comprises of the NLR protein NLRP3, the adapter ASC and pro-caspase-1)

The NLRP3 inflammasome (**Fig.7**) is activated by a wide variety of particles, crystals, bacterial toxins, as well as viruses bacteria, and fungi. Because of the great variability in structure and composition of the particles and stimuli reported to activate the NLRP3 inflammasome, their direct interaction with NLRP3 seems unlikely. Rather, a more probable scenario is that NLRP3 is activated by an endogenous molecule that is generated/modified as result of the interaction of the particle/pathogen with the cell. It is possible that more than one type of NLRP3 endogenous activator exists and that different NLRP3 activators may use distinct signaling pathways to generate the same endogenous activator.

An essential requirement for NLRP3 inflammasome activation is the uptake of the particle through phagocytosis[127], as demonstrated by the fact that cytochalasins or other drugs that inhibit this process also prevent pro-IL-1β maturation in response to NLRP3-activating particles[128]. However, phagocytosis is not required for NLRP3 inflammasome activation by extracellular ATP or bacterial toxins, and conversely, phagocytosis does not always result in inflammasome activation.

3.2 Role of cytokines IL-1β and IL-18

IL-1β and IL-18 are important proinflammatory cytokines that on the one hand activate monocytes, macropages, and neutrophils, and on the other hand induce Th1 and Th17 adaptive cellular responses. They are secreted as inactive precursors, and the processing of pro-IL-1β and pro-IL-18 depends on cleavage by proteases. One of the most important of these enzymes is caspase-1, which in turn is activated by inflammasomes[129].

Upon recognition of a microorganism, proinflammatory cytokines such as tumor necrosis factor (TNF), IFNγ, IL-18, and IL-1β are secreted. These cytokines activate neutrophils and macrophages to phagocytose the invading pathogen and to release toxic oxygen and nitrogen radicals. TNF is an essential component of the host defense, as demonstrated by the important infectious complications in patients treated with anti-TNF biological agents[130]. Similarly, IFNγ activates both neutrophils and macrophages for intracellular killing of bacteria or fungi. IL-1α and IL-1β, which bind and activate the same receptor [131], activate the release of other proinflammatory cytokines such as TNF and IL-6, and induce a Th17 bias in the cellular adaptive responses[132].

3.2.1 IL-18

One of the main functions of IL-18 is to promote the production of IFNγ from T and NK cells. IL-18 also promotes the secretion of other proinflammatory cytokines like TNFα, IL-1β, IL-8, and GM-CSF and, as a consequence, enhances expansion, migration, and activation of neutrophils during infections. In addition, IL-18 enhances cytotoxic activity and proliferation of $CD8^+$ T and NK cells[133]. The protective role of IL-18 during bacterial infections is primarily related to its ability to induce IFNγ, a cytokine that activates the microbicidal activity of macrophages through induction of nitric oxide production.

3.2.2 IL-1β

IL-1β is one of the most powerful proinflammatory cytokines that exerts its protective action against infections by activating several responses including the rapid recruitment of neutrophils to inflammatory sites, activation of the endothelial adhesion molecules, induction of cytokines and chemokines[134], induction of the febrile response, and the stimulation of specific type of adaptive immunity like the Th17 response.

3.2.3 Activation of IL-1β and IL-18

The processes that generate the biologically active cytokines IL-1β and IL-18 have a similar mechanism. In contrast to most cytokines,

which are only transcriptionally induced and immediately secreted in response to inflammatory and infectious stimuli, IL-1β and IL-18 require two signals.

First, activation of TLRs, RLRs, and other PRRs that induce the inflammatory response by activation of primarily NF-κB and MAPK (mitogen-activated protein kinase) signaling pathways that collectively promote a transcription factor-mediated response, is required for the up-regulation of *IL1B* transcripts[135]. In contrast, the *IL18* transcript is constitutively produced in most cell types[136]. Transcription/translation, however, only produces the intracellular, inactive precursors, pro-IL-1β (31 kDa) and pro-IL-18 (24 kDa).

The second signal required for cytokine release causes the activation of caspase-1. Active caspase-1 proteolytically cleaves the prodomain to liberate the 17- and 18-kDa mature cytokines, which are then released by an atypical, leader peptide-independent mechanism, which is still controversial[137].

This caspase-1-dependency of IL-1β and IL-18 maturation appears to be restricted to monocytes and macrophages. Mature IL-1β and IL-18 are recognized by their receptors, IL-1RI and IL-18Rα, respectively, and cause a conformational change that allows high-affinity binding in the complex with the IL-1R accessory protein (IL-1RAc or IL-1RIII) or the IL-18Rβ, respectively[138]. Signal transduction is then mediated by the TIR (Toll/IL-1 receptor) domain, which is also present in TLRs, further emphasizing their link to innate immunity. Furthermore, both cytokines have a naturally occurring inhibitor, the IL-1R

antagonist (IL-1Ra) and the IL-18 binding protein (IL-18BP), respectively.

Despite the progress made in understanding the process of IL-1β synthesis, controversy surrounded the capacity of TLR ligands such as LPS to activate caspase-1 and cause the release of active IL-1β. By using transfected cell lines and/or NLRP3 knock-out mice, a broad panel of exogenous and endogenous stimuli have been proposed to activate the NLRP3 inflammasome[139], but purified TLR ligands such as LPS were not among these inflammasome stimuli. Therefore, based on defective responses of the monocyte-like leukemia cell line THP-1 to LPS stimulation, a concept has arisen that IL-1β production induced by LPS is due to contamination with non-LPS ligands such as peptidoglycans[140], while LPS by itself is ineffective as a stimulator of IL-1β release. A second signal, such as MDP or ATP, is required, and this would induce activation of caspase-1 followed by IL-1β processing and release [141]. However, this model is derived from data in THP-1 cells[140] and in primary mouse macrophages [142], and it is inconsistent with many studies showing abundant production and release of IL-1β from blood monocytes by TLR ligands such as purified LPS, lipopeptides, and lipoteichoic acid, as well as cytokines such as TNFα and IL-1 itself [143].

V. Signaling strategies of Toll Like Receptors

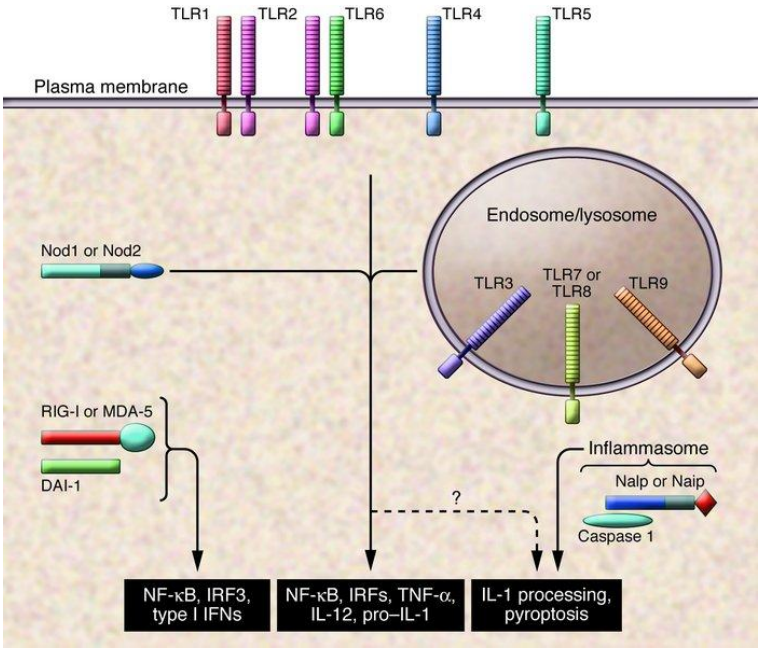
5.1 Introduction

One of the several mechanisms by which the innate immune system senses the invasion of pathogenic microorganisms is through the TLRs, which recognize specific molecular patterns that are present in microbial components. Stimulation of different TLRs induces distinct patterns of gene expression, which not only leads to the activation of innate immunity but also instructs the development of antigen-specific acquired immunity.

Cells of the innate immune system recognize and respond to pathogens by the use of TLRs. TLRs act as cell surface receptors that recognize and are stimulated by microbe associated molecular patterns[144].

Humans have 10 different TLRs that can each recognize a general type of microbe associated molecular patterns (**Fig.8**). For example, TLR4 recognizes LPS from most Gram-negative bacteria. When the TLR recognize LPS, a signaling cascade is started that leads to the activation of NF-κB. NF-κB activates genes that play a role in the adaptive immune response in addition to secreting pro-inflammatory cytokines. Stimulated TLRs activate a cascade that starts the innate immune response and alerts the adaptive immune response. Different TLR family members are found in different subcellular compartments, ranging from the plasma membrane to early, late, and recycling

endosomes. Studies have indicated that receptor delivery to endosomes also activates specific signal transduction pathways[145]

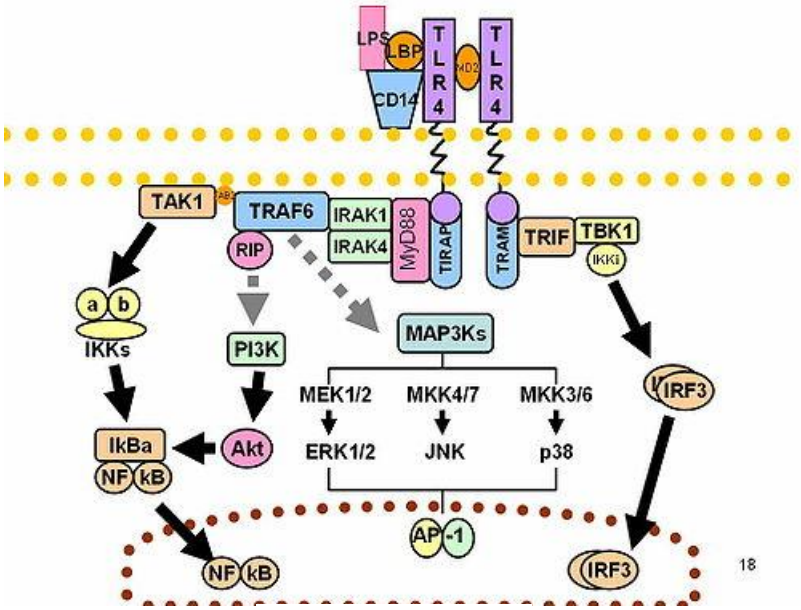


J Clin Invest. 118(2):413–420 (2008).

Figure 8: Pattern recognition by the innate immune system.

4.2 TLR4 Signaling Pathways

One of the first described examples of microbe-induced TLR transport came from studies of the LPS receptor TLR4, which induces distinct signaling pathways from two different organelles. The first signaling pathway is activated from the plasma membrane after TLR4 encounters LPS[146]. This pathway is mediated by a pair of sorting and signaling adaptor proteins called TIRAP and MyD88, respectively. These adaptors induce proinflammatory cytokine expression by linking TLR4 to downstream enzymes that activate NF-kB and AP-1[147](**Fig.9**). TLR4 is then internalized into the endosomal network where the second signaling pathway is triggered through the adaptors TRAM and TRIF. These adaptors mediate the activation of the transcription factor Interferon Regulatory Factor-3 (IRF3), which regulates Type I Interferon (IFN) expression. Thus, in the case of TLR4, the LPS-induced endocytosis of the receptor is essential for its signaling functions. While the general endocytic machinery is undoubtedly involved in internalization of plasma membrane-localized TLRs, there are no known membrane proteins that regulate TLR endocytosis specifically upon microbial recognition.



Akira and Takeda (2004).

Figure 9: TLR4 Signalling pathways.

After ligand binding, TLRs and IL-1Rs (interleukin-1 receptors) dimerize and undergo the conformational change required for the recruitment of downstream signalling molecules. These include the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), IL-1R-associated kinases (IRAKs), transforming growth factor-β(TGF-β)-activated kinase (TAK1), TAK1-binding protein 1 (TAB1), TAB2 and tumour-necrosis factor (TNF)-receptor-associated factor 6 (TRAF6)[148].

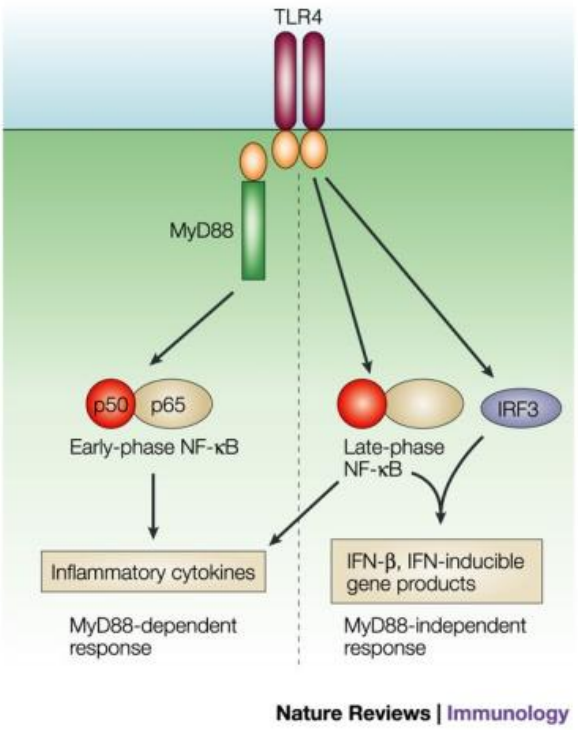
4.3 MyD88 dependent signalling pathway

MyD88 was isolated originally as a gene that is induced rapidly during the IL-6-stimulated differentiation of M1 myeloleukaemic cells into macrophages17[149]. The encoded protein has an amino (N)-

terminal death domain (DD), which is separated from its carboxy (C)-terminal TIR domain by a short linker sequence. MyD88 was subsequently cloned as an adaptor molecule that functions to recruit IRAK to the IL-1R complex following stimulation with IL-1[147]. The association between MyD88 and IRAK is mediated through a DD–DD interaction. MyD88 forms homodimers through DD–DD and TIR-domain–TIR-domain interactions and exists as a dimer when recruited to the receptor complex21[150]. Therefore, MyD88 functions as an adaptor linking TLRs/IL-1Rs with downstream signalling molecules that have DDs.

MyD88-deficient mice do not produce TNF or IL-6 when exposed to IL-1 or microbial components that are recognized by TLR2, TLR4, TLR5, TLR7 or TLR9[151].

Hence, MyD88 is essential for responses against a broad range of microbial components. However, closer study of MyD88-deficient cells has revealed the existence of MyD88-dependent and -independent pathways (**Fig.10**). For example, the activation of NF-κB in response to mycoplasmal lipopeptide, a TLR2 ligand, is completely abolished in MyD88-deficient macrophages, whereas NF-κB activation still occurs in response to LPS, a TLR4 ligand, although with delayed kinetics[152] MAPK activation is also delayed in LPS-stimulated MyD88-deficient macrophages.



Nature Reviews (2007).

Fig 10: TLR4 signalling, MyD88-dependent and -independent pathways.

Therefore, MyD88 is essential for all TLR-mediated production of inflammatory cytokines. However, stimulation of TLR3 or TLR4 results in induction of type I IFNs (IFN-α/β) in a MyD88-independent manner.

4.4 NF-κB

The NF-κB complex consists of homodimers and heterodimers of the structurally related proteins p50, p52, p65 (RelA), c-Rel, and RelB. NF-κB is typically sequestered in the cytoplasm bound by the

inhibitory molecules IκBα, IκBß, and IκB∈[153]. Activation of NF-κB involves the phosphorylation and proteolysis of the IκB proteins and the concomitant release and nuclear translocation of the NF-B factors.

Studies[154] have demonstrated a key role of NF-κB in regulating gene expression associated with the development, activation, maturation, and APC function of DCs[155]. In response to a broad range of stimuli, including LPS and CD40 engagement, the multisubunit complex IκB kinase (IKK) consisting of IKK1/IKKα, IKK2/IKKß, and IKKγ/NEMO is activated upon phosphorylation[156] .Activated IKK phosphorylates the IκB proteins, which in turn undergo polyubiquitination and subsequent degradation via the 26S proteosome[157]. The latter permits nuclear translocation of NF-κB that binds to consensus sequences and induces gene transcription. It has also been demonstrated that the immunosuppressive effect of IL-10 on DC maturation and APC function is mediated by inhibition of IKK activity and downstream NF-κB activation[158] further arguing that the NF-κB pathway is a key target for immunoregulation of DCs. In addition, IL-10–induced inhibition of DCs is dependent on suppression of the phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway. Studies have shown that NF-κB activation can be regulated by the PI3K/AKT pathway via different mechanisms[159].

NF-κB can be activated via 2 distinct signal transduction pathways. The canonical (also known as classical) NF-κB pathway requires activation of the IKK complex, consisting of the catalytic subunits IKKα and IKKβ, and the regulatory subunit NEMO/IKKγ, and

controls NF-κB activation in response to proinflammatory stimuli such as LPS, TNFα, and CD40L[160]. Activation of this pathway results predominantly in the activation, nuclear translocation, and DNA binding of the classical NF-κB dimer p50-RelA. In this pathway, IKKβ is essential for NF-κB activation, whereas IKKα is dispensable for the activation and induction of NF-κB DNA-binding activity in most cell types[161].

In contrast, the noncanonical (also known as alternative) pathway is strictly dependent on IKKα homodimers and requires neither IKKβ nor NEMO/IKKγ[162]. The target for IKKα homodimers is NF-κB2/p100, which upon activation of IKKα by NF-κB-inducing kinase (NIK) is incompletely degraded into p52, resulting in the release and nuclear translocation of mainly p52-RelB dimers. This pathway can be triggered by the activation of members of the TNF-receptor superfamily such as the lymphotoxin β receptor, B-cell activating factor belonging to the TNF family (BAFF) receptor, and CD40 (which also induce canonical NF-κB signaling), but not via pattern recognition receptors such as Toll-like receptor 4 (TLR4), the receptor for LPS[163].

Mature DCs express high levels of the NF-κB family of transcription factors[164] and signaling by members of the TNF-α receptor family, such as CD40 and RANK, results in activation of NF-κB[165]. It has been found that a small proportion of activated Rel A protein are present in the nucleus of immature D1 cells, but that a 30-min treatment with LPS induced massive translocation of the p65 molecule

to the nucleus. LPS-induced nuclear translocation of p65 was not blocked by the MEK inhibitor, indicating that NF-κB activation does not depend on the MEK/ERK pathway. This is consistent with previois findings that activation of NF-κB by TNF-α or IL-1 involves the NF-kB inducing kinase (NIK)/IKK kinase complex [166] which is independent of the ERK pathway.

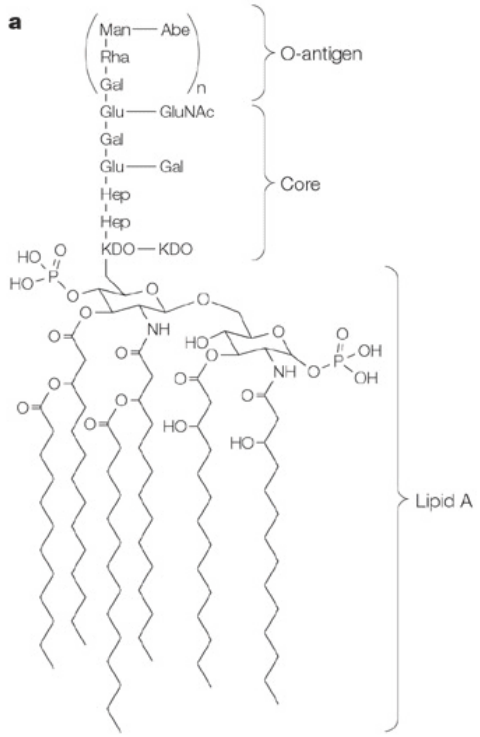
Hence, this shows that LPS induces nuclear translocation of NF-κB.

5. LPS

5.1 Structure

In 1884, Hans Christian Gram, developed a staining procedure, the Gram staining, for bacteria classification. Based on this method, almost all bacteria can be divided into two large groups depending on the structural differences of their cell wall, the Gram-positive and Gram-negative bacteria. Gram-positive bacteria retain the crystal violet dye of the Gram staining thanks to the presence of high amount of peptidoglycan in their cell wall. In contrast, Gram-negative bacteria do not retain crystal violet dye since they have a relatively thin cell wall consisting of few layers of peptidoglycan surrounded by a second lipid membrane. A major component of the outer membrane of Gram-negative bacteria is the lipopolysaccharide (LPS), a complex molecule indispensable for the maintenance of the structural and functional integrity of the membrane itself [167]. For this reason, the general structure of LPS is conserved among all Gram-negative bacteria. LPS is composed of three major parts, the lipid A, the core region and the *O*-chain [168]. The lipid A moiety is highly hydrophobic and it is largely responsible for the endotoxic activity of the whole LPS molecule. This moiety is inserted into the external face of the bacterial outer membrane. The core region is a conserved polysaccharidic structure that can be subdivided into inner and outer core. The inner core is proximal to the lipid A and it contains unusual sugars, such as Kdo and heptose, which are absolutely required for bacterial viability and therefore are well conserved among all LPS species. The outer core

concentrates the all variability of the whole region, which typically consists of common hexose sugars. (**Fig.11**)



Miller et al. Nature Reviews Microbiology 3, 36-46 (2005).
Figure 11: Chemical structure of LPS

The hydrophilic *O*-chain is the outer region of the LPS molecule. The *O*-chain is a highly variable region composed by repeating saccharidic units formed by up to eight glycosyl residues that differ between bacterial strains in terms of sugars, sequences, linkages and substitutions used. Additionally, these forming units can be repeated up to 50 times and a single organism will produce a wide range of these lengths due to incomplete synthesis of the chain. Some Gram-

negative bacteria, especially members of the *Enterobacteriaceae*, such as *E. Coli* and *Salmonella Thyphimurium*, carry mutations in the genes involved in the synthesis and attachment of the *O*-chain and do not express it at all [169]. These mutants are called "rough" because of the morphology of the colonies they form in a plate that is different from what observed for wild-type, "smooth" bacteria. Thus, the truncated form of LPS is called rough (rLPS), while the wild type form, containing the *O*-chain, is called smooth (sLPS).

5.2 Function

Although s and r LPSs share the same receptor complex (consisting of the Toll like receptor 4, TLR4, and MD2 subunits and the glycosyl-phosphtidylinositol-anchored CD14 molecule[170]) there is evidence that their mechanism of action may be different. For instance, while sLPS requires CD14 for the initiation of both MyD88-dependent and independent signal transduction pathways at least at low doses[171], rLPS leads to MyD88-dependent responses in the absence of CD14 even at low doses. This has led to the assumption that rLPS activates a broader range of cells (CD14-positive and low/negative cells) and with a higher efficiency compared to sLPS [172].

An example of a CD14 dependent signalling involving both rLPS and sLPS is its binding to TLR4-MD2 (**Fig.12**). TLR4-MD2 can bind rough but not smooth LPS without a requirement for CD14. Signaling by this complex is limited to the MyD88-dependent pathway, using the adaptors Mal and MyD88 to activate NF−κB, resulting in

transcription of TNF. TLR4-MD2 can bind both rLPS and sLPS in a CD14-dependent process. In addition to MyD88-dependent signals, these complexes also signal MyD88-independent responses via TRAM and TRIF, leading to IRF-3 activation and IFN-β transcription[173].

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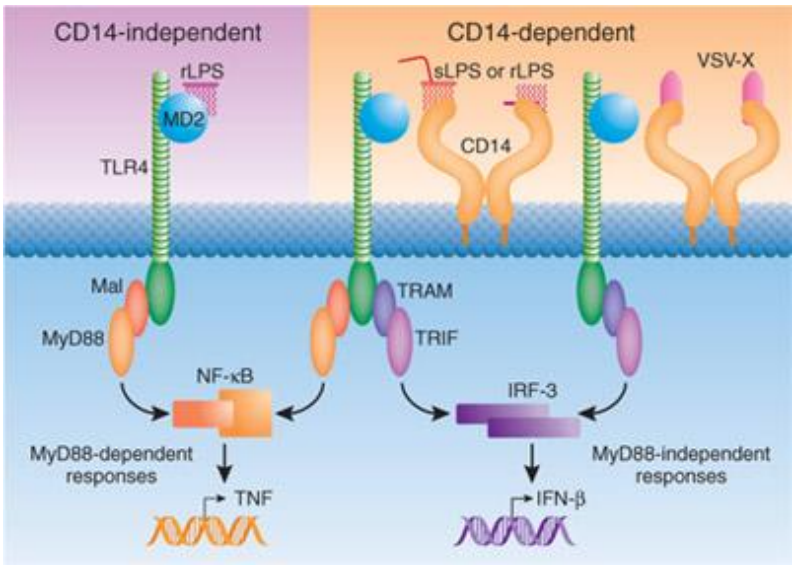

Nature Immunology 6, 544 - 546 (2005).

Figure 12. CD14-independent and CD14-dependent signaling by TLR4-MD2.

Since the two forms of LPS are used almost indistinctly to study the reactions of the innate immune system (the most common form of rLPS used is composed of the lipid A and 3 Kdo), it is important to define to what extent the consequences to s and r LPS exposure are

similar or dissimilar to avoid possible confusion between common and LPS specie-specific responses.

5. Scope of the thesis

In this thesis we provide further evidence of the diverse mechanisms through which s and rLPSs may activate pro-inflammatory innate responses.

We show that the ability of rLPS to function in a CD14-independent manner is not limited to the activation of the MyD88-dependent pathway but is also extended to the activation of the Ca^{2+}/calcineurin and NFAT pathway that leads to IL-2 production in conventional DCs.

We also show that rLPS diversely from sLPS is capable per se of activating the inflammasome and inducing IL-1β secretion by DCs. Nevertheless, though these observations could support the prediction that rLPS gave origin to more potent innate responses with respect to sLPS, an in vivo comparison revealed that the two LPS species elicit almost comparable responses, with sLPS being slightly more efficient.

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Chapter II.

Similarities and differences of innate immune responses elicited by smooth and rough LPS

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2. Materials and Methods

Chapter II.

Similarities and differences of innate immune responses elicited by smooth and rough LPS

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The lipopolysaccharide is the major component of Gram-negative bacteria outer membrane. LPS com-prises three covalently linked regions: the lipid A, the rough core oligosaccharide, and the O-antigenic side chain determining serotype specificity. Wild-type LPS (sLPS) contains the O-antigenic side chain and is referred to as smooth. Rough LPS (rLPS) does not contain the O-side chain. Most wt bacteria and especially wt Enterobacteriaceae express prevalently the sLPS form although some truncated rLPS molecules always reach the external membrane. The two sLPS and rLPS forms are used almost indistinctly to study the effects

on innate immune cells. Nevertheless, there is evidence that their mechanism of action may be different. For instance, while sLPS requires CD14 for the initiation of both MyD88-dependent and independent signal transduction pathways at least at low doses, rLPS leads to MyD88-dependent responses in the absence of CD14 even at low doses. Here we have identified additional differences in the signaling capacity of the two LPS species in the mouse. We have found that rLPS, diversely from sLPS, is capable of activating in dendritic cells (DCs) the Ca^{2+}/calcineurin and NFAT pathway in a CD14-independent manner, moreover it is also capable per se of activating the inflammasome and eliciting IL-1β secretion independent of the presence of additional stimuli required instead for sLPS. The ability of rLPS of activating the inflammasome in vitro has as a direct consequence a higher efficiency of rLPS-exposed DCs in activating natural killer (NK) cells compared to sLPS-exposed DCs. However, diversely from possible predictions, we found that the different efficiencies of the two LPS species in eliciting innate responses are almost nullified in vivo. Therefore, sLPS and rLPS induce nearly similar in vivo innate responses but with different mechanisms of signaling.

2. Materials and Methods

2.1 Ethnic Statement

All experiments were carried out in accordance with the relevant laws and institutional guidelines. The study has been approved by the "Comitato Etico" of the University of Milano-Bicocca, protocol number 0026031.

2.2 DCs and culture medium

Fresh bone marrow-derived DCs from C57BL/6 or mutant mice were cultured as previously described [1].

2.3 Mice

C57BL/6 mice were purchased from Harlan-Italy. *Cd14*$^{-/-}$ mice were purchased from CNRS d'Orléans (Orléans Cedex 2, France). *Asc*$^{-/-}$ mice were obtained from Genentech. All animals were housed under pathogen-free conditions.

2.4 Antibodies and chemicals

All the antibodies used for FACS analysis were purchased from BD Biosciences (San Diego, California). TLR4-grade LPSs (E.Coli, 055:B5, R515) were purchased from Enzo Lifesciences (Farmingdale, New York).

Indo1-AM (Molecular Probes, Leiden, The Netherlands) was dissolved in DMSO. Stock solutions were diluted in Tyrode solution (154 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM HEPES-NaOH, 5.5 mM D-glucose, adjusted to pH 7.35). The concentration of DMSO in the medium did not exceed 0.1%.

2.5 Calcium measurements

The proportion of cells displaying a Ca^{2+} response to receptor agonists was determined with a two-photon microscope, using the membrane-permeable dye indo-1. Intracellular calcium concentrations ($[Ca^{2+}]i$) were determined by a fluorimetric ratio technique. Cells were loaded with the fluorescent indicator indo-1 (Molecular Probes, Eugene, OR) by incubation at 37°C for 20 to 30 min with 2 μM indo-1. They were then washed three times with PBS to wash off the extracellular indicator and to allow for intracellular de-esterification of indo-1. Tyrode solution (Electron Microscopy, Hatfield, PA) was then added. A two-photon Ti-Sapph laser source (720 nm wavelength; Mai Tai HP, SpectraPhysics, Mountain View, CA) was used for indo-1 excitation, with the excitation beam directed into a direct optical microscope (Olympus, BX51, Tokyo) through a FV300 scanning head. The fluorescence signals emitted by indo-1-loaded cells was split by a dichroic mirror (490DCXRU, Chroma Techn., Brattelboro, VT) and acquired by two non-descanned detectors every 0.5-0.8s at 2.2Hz. The ratio of fluorescence emissions at 400 nm/bp to those at 500 nm/bp was recorded (R400/500). R400/500 was used as an index of $[Ca^{2+}]i$. Data were normalized to baseline.

This approach overcame possible problems of uncertainty related to the calibration of fluorescent Ca^{2+} indicators.

In some cases, cells were analyzed in calcium-free PBS or calcium-free PBS supplemented with thapsigargin (50 nM).

2.6 Cytokine measurements

ELISAs were performed with the DuoSet kits (R & D, Minneapolis, MN). Type I IFNs activity was measured as described [2].

2.7 NK Cell Purification

NK cells were purified from wild-type mice. NK cells were positively selected from splenocytes. 10^8 cells were stained with biotinylated anti–pan-NK cell (DX5) antibody (20 μg/ml) and washed and incubated with streptavidin MicroBeads (Miltenyi Biotech). Cells were then positively selected with MS columns, according to the manufacturer's recommendations. NK cells were used when 95% were NK1.1 positive.

2.8 NK–DC Co-cultures.

Co-culture experiments were performed with ex vivo NK cells and wt or CD14-deficient BMDCs in presence of LPS. BMDCs were re-suspended in IMDM complete medium and plated in 96-well plates ($1x10^5$cells/100μl/well), $0.5x10^5$NK cells were then added (100μ l/well). After 18h of co-culture, IFNγ concentration was measured in the supernatants.

2.9 In Vivo Activation of NK Cells.

Mice were injected i.v. with 1mg/Kg of LPSs, and after 4 h spleens were removed and analyzed for NK cell activation. Single cell suspensions were prepared and incubated with brefeldin A (10 μg/ml; Sigma-Aldrich) for 4 hr. Cells were fixed with 2% paraformaldehyde, permeabilized with PBS containing 5% FBS and 0.5% saponin, and stained with APC-labeled anti-IFNγ, PE-labeled anti-CD49b, and FITC labeled anti-CD3 mAbs. Cells were then analyzed on a FACSCalibur (Becton Dickinson), NK cells were identified as $CD49b^{+}$ $CD3^{-}$ cells.

2.10 Statistical analysis

Means were compared by paired or unpaired t- tests. Data are expressed and plotted as means ± SD values. Statistical significance was defined as $p<0.05$. Sample sizes for each experimental condition are provided in the figures and the respective legends.

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Chaper III.

Results

3. Results

3.1 Comparison of the efficiency of s and r LPSs in activating the Myd88-dependent, TRIF-independent and NFAT pathways

It is becoming more and more evident that CD14 plays a major role in the initiation of both MyD88-dependent and independent pathways, and in determining the duration and efficiency of the responses to LPS[1,2]. Moreover, we have recently shown that CD14, when engaged by sLPS, is able to initiate the $Ca^{2+/}$calcineurin and NFAT pathway in DCs in a TLR4-independent manner [3]; an observation that further emphasizes the major role of CD14 in innate immunity. Therefore, the capacity of different stimuli to operate in strict collaboration with CD14 may have a major impact in the activation of innate responses. Since it has been described that sLPS activity is more CD14-dependent compared to rLPS[4,5] we wanted to investigate the efficiency of the two LPS species in activating the MyD88-dependent, TRIF-dependent and NFAT path-ways in DCs. To this purpose bone marrow-derived DCs (BMDCs) were exposed to different doses of the two highly pure commercially available stimuli and the efficiency of the activation of the three pathways was evaluated by using three model cytokines as read out, TNFα for the MyD88-dependent pathway, Type I IFNs for the TRIF-dependent one and IL-2 for the $Ca^{2+/}$calcineurin and NFAT pathway[3]. As shown in **Fig. 1**, for the activation of the MyD88 dependent pathway rLPS was CD14-dependent only at very low concentrations (<10 ng/ml).

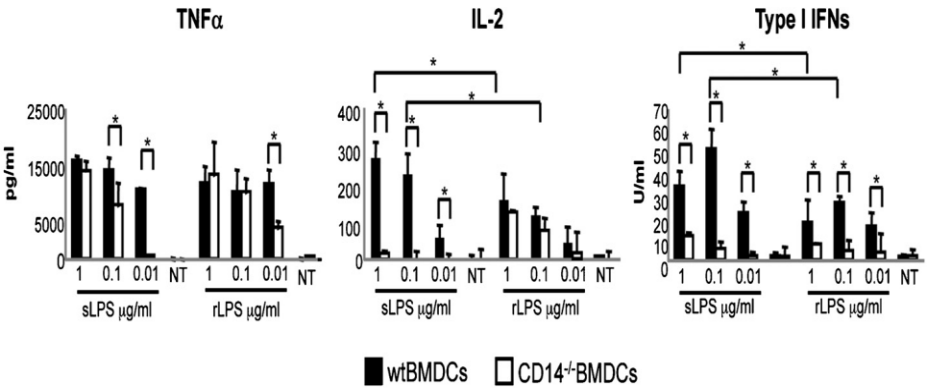

Figure 1. ***s and r LPS are equally efficient in eliciting cytokine production by BMDCs***. wt and CD14-deficient BMDCs were cultured in the presence of the indicated concentrations of s and r LPSs for 5 hours. The amounts of secreted cytokines were determined by ELISA (TNFα, and IL-2) or bioassay (Type I IFN). Data represent means and standard deviation of three independent experiments.

Diversely sLPS responses were already completely abrogated at 10 ng/ml, as previously described[4]. The two types of LPS were equally CD14-dependent for the activation of the TRIF pathway. Nevertheless, in contrast to the expectation and unlike sLPS, rLPS was able to elicit IL-2 production in DCs in a CD14-independent manner. While the two LPS species were similarly efficient in the activation of the MyD88-dependent pathway, sLPS was slightly more efficient than rLPS in activating the TRIF-dependent and NFAT pathways.

3.2 Diversely from sLPS, rLPS induces Ca^{2+} mobilization from intracellular stores and in a CD14-independent manner in DCs

We then wanted to investigate the reason why the NFAT pathway was activated in BMDCs in response to rLPS but not to sLPS in a CD14-independent manner. To address this point, since the initial step for NFAT activation is Ca^{2+} mobilization, we first measured Ca^{2+} fluxes in wt and CD14-deficient BMDCs after exposure to rLPS in comparison to sLPS. We found that rLPS was slightly more efficient than sLPS in inducing Ca^{2+} mobilization (**Fig. 2A**).

Moreover, while Ca^{2+} transients induced by sLPS in BMDCs were completely abolished in the absence of CD14 (**Fig. 2B** and [3]), Ca^{2+} fluxes induced by rLPS were preserved, although reduced, in CD14-deficient BMDCs (Fig. 2B). This observation suggests that CD14 contributes but is not necessary for the induction of Ca^{2+} mobilization in response to rLPS. Since CD14 activation by sLPS only generated extracellular Ca^{2+} influxes (**Fig. 2C** and [3]), we hypothesized that, diversely from sLPS, rLPS could also generate intracellular Ca^{2+} mobilization. We then performed Ca^{2+} fluxes analysis in Ca^{2+} free medium, that is PBS not containing Ca^{2+}. The Ca^{2+} transients measured with rLPS in the absence of extracellular Ca^{2+} (**Fig. 2C**) indicated that our prediction was indeed correct. Accordingly, Ca^{2+} transient in the presence of rLPS were totally abolished only after previous intracellular Ca^{2+} store emptying by the use of thapsigargin (**Fig. 2C**).

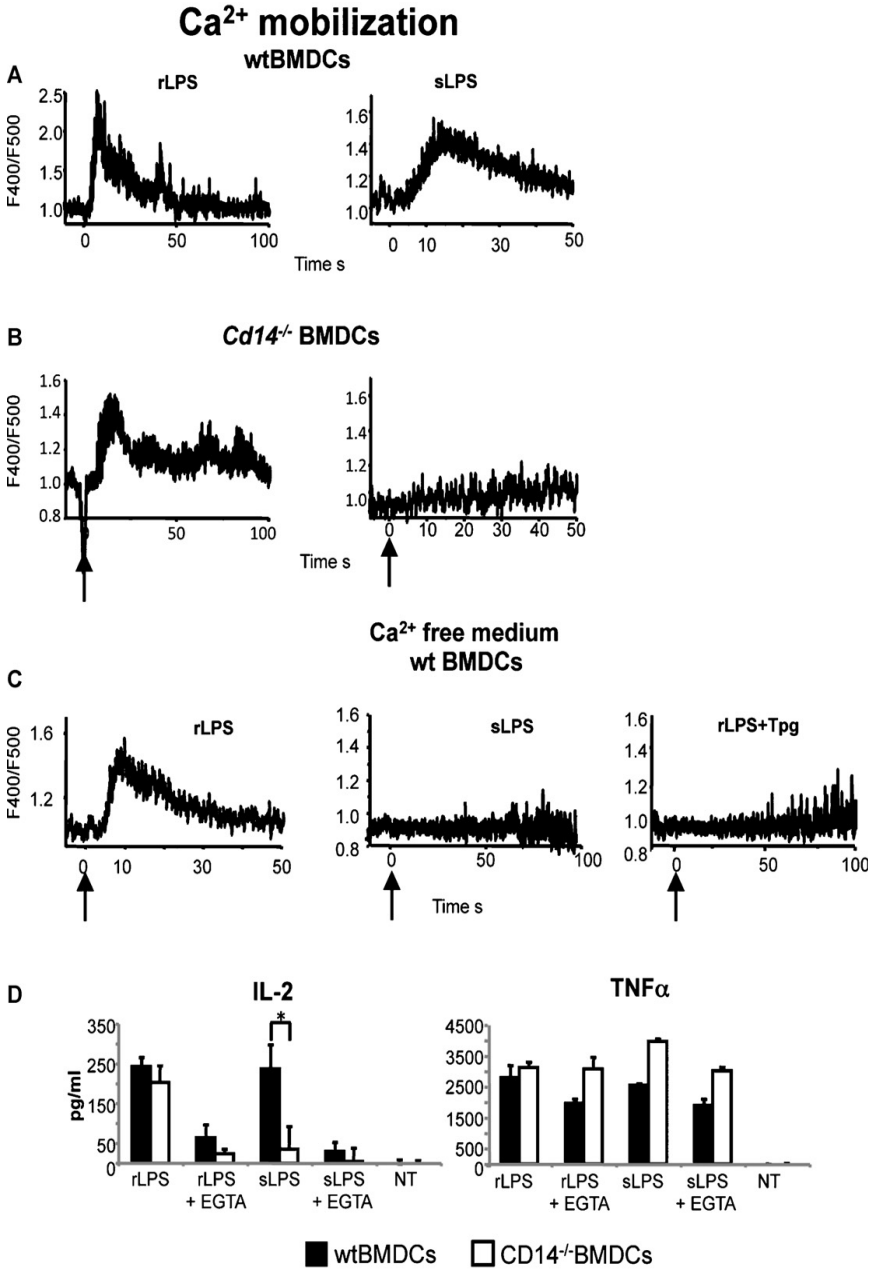

Fig. 2. ***CD14-independent Ca^{2+} mobilization and NFAT activation in BMDCs after rLPS treatment.*** (A–C) Ca^{2+} transients in wt and CD14-deficient BMDCs. In (C) Ca^{2+} transients were recorded in Ca^{2+} free medium.

Point 0 indicates the time of stimulus administration. Means and SD for a minimum of 30 cells are shown. Experiments were repeated at least three times. (D) wt and CD14-deficient BMDCs were cultured in the presence of 1 μg/ml of sLPS and rLPS for 5h and the amount of secreted cytokines determined by ELISA. Where indicated EGTA was added to the cultures. Data represent means and standard deviations of three independent experiments.

NFAT activation is induced by a sustained plateau of intracellular Ca^{2+} concentration[6]. Typically in T and B cells this plateau is obtained following a store operated Ca^{2+} entry[7]. Thus, we asked whether rLPS-mediated NFAT activation that leads to IL-2 production in DCs was due to the intracellular Ca^{2+} release or whether an extracellular Ca^{2+} influx was required. Therefore, we tested the ability of rLPS and sLPS of eliciting IL-2 production from wt and CD14-deficient BMDCs in the presence of the Ca^{2+} chelator EGTA to avoid Ca^{2+} ingress. We decided to use the Ca^{2+} chelator, since the cells do not survive in Ca^{2+} free PBS (used in the previous experiment) for 24h. As shown in **Fig. 2D**, in all cases IL-2 production was profoundly affected when EGTA was added; as control, the production of TNFα was entirely preserved. This indicated that only an extracellular Ca^{2+} influx could lead to the plateau of Ca^{2+} concentration required for NFAT activation. Diversely from sLPS, rLPS could induce Ca^{2+} entry via a CD14-independent store operated mechanism. Collectively, these data confirm and extend previous observations[1] on the capability of rLPS to signal in a CD14-independent manner, indicating that rLPS is able to induce Ca^{2+} mobilization and IL-2 production in the absence of CD14.

3.3 rLPS but not sLPS is able per se to induce IL-1β secretion via inflammasome and caspase activation

In addition to CD14- and TLR4-dependent inflammatory cytokines, LPS-induced inflammation leads to the production of inflammasome- and inflammatory caspase-dependent cytokines, such as IL-1β and IL-18, provided that additional stimuli, such as ATP, are present[8].

To further extend the comparison between sLPS and rLPS, we evaluated the ability of these two LPS species of activating the inflammasome and inducing IL-1β secretion by BMDCs.

Surprisingly, we observed that rLPS was able *per se* to induce IL-1β secretion, without the addition of any other stimulus. In contrast, sLPS did not show this capacity (**Fig. 3A**). As control, both sLPS and rLPS were equally able to induce pro-IL1β production (**Fig. 3A**). IL-1β secretion induced by rLPS was completely abolished in ASC-deficient BMDCs, confirming that IL-1β measured in the supernatants in response to rLPS was indeed due to inflammasome activation (**Fig. 3B**). In accordance with the data on IL-1β production also IL-18 was produced by BMDCs in response to rLPS and not to sLPS (**Fig. 3C**). Interestingly IL-18 production was also MyD88- and TRIF-dependent (**Fig. 3C**). It is well known that LPS renders DCs capable of activating NK cells in mice and humans[9–11]. We have recently found that, in addition to IL-2, IL-18 is one of the DC-derived molecules required for efficient NK cell activation in the presence of LPS *in vitro* and *in vivo* (manuscript in preparation).

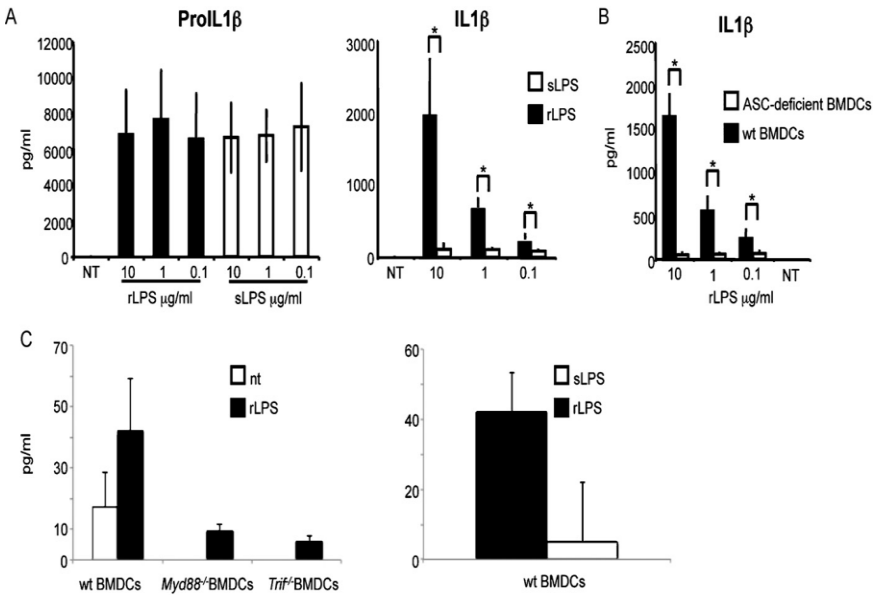

Fig. 3. Direct inflammasome activation by rLPS but not sLPS in vitro.

(A) wt BMDCs were treated with the indicated doses of sLPS and rLPS for 18h. The amounts of (left panel) intracellular proIL-1β and (right panel) secreted mature IL-1β were then evaluated by ELISA. (B) wt and ASC-deficient BMDCs were treated with the indicated doses of rLPS for 18h and the amounts of secreted IL-1β determined by ELISA. (**C**, left panel) IL-18 production by wt and mutant BMDCs induced by 1μg/ml of rLPS and measured by ELISA at 18 h. (**C**, right panel) IL-18 production induced by sLPS and rLPS (1 μg/ml) measured by ELISA after 18 h of stimuli exposure.

Therefore, we hypothesized that sLPS and rLPS differed in their efficiency of activating NK cells through DCs. This was addressed by analyzing the capacity of LPS-activated DCs of eliciting IFNγ production by NK cells. BMDCs were co-cultured with NK cells in

the presence of sLPS and rLPS and the amounts of secreted IFNγ measured 18h later. As shown in **Fig.4A**, in complete agreement with our prediction, rLPS was much more efficient compared to sLPS in rendering DCs capable of activating NK cells. Therefore, the ability of rLPS of directly activating the inflammasome strictly correlated with the efficiency of DC-dependent NK cell activation induced by this stimulus.

3.4 sLPS is more efficient than rLPS in activating innate responses in vivo

We then asked whether the same differences between sLPS and rLPS in the efficiency of NK cell activation could be observed *in vivo*. It is known that following the administration of pro-inflammatory stimuli, including LPS, DCs play a major role in NK cell activation in vivo as determined by IFNγ production[12,13]. The two species of LPS were, thus, injected iv and the percentage of IFNγ -positive NK cells measured 4h later in the spleen. In contrast to the *in vitro* results, the efficiency of sLPS and rLPS in inducing NK cell activation *in vivo* was similar, with sLPS being even more efficient (**Fig. 4B**).

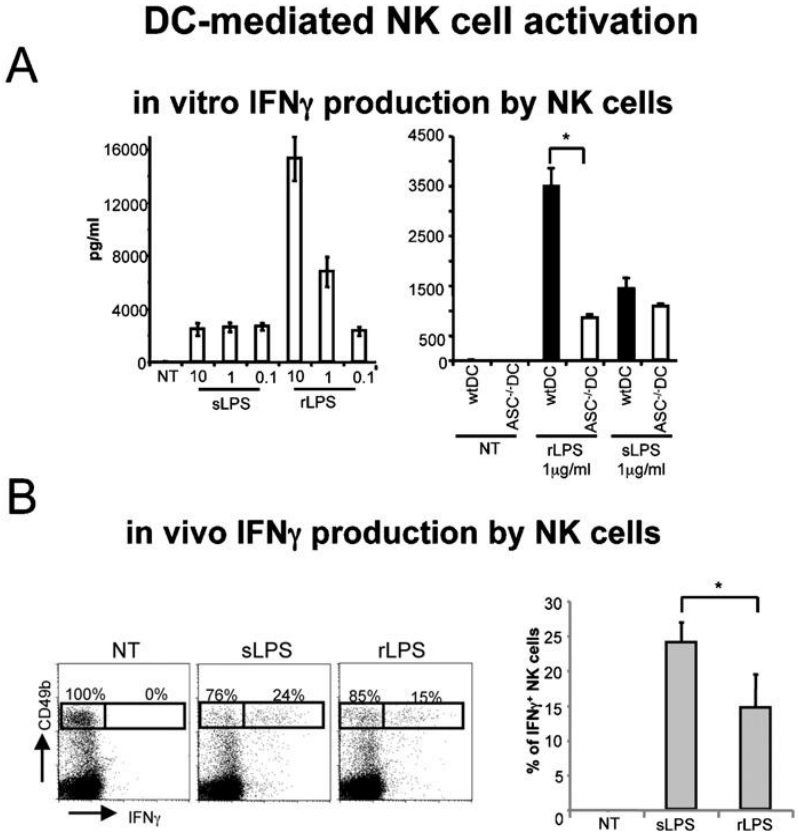

Fig. 4. sLPS and rLPS efficiency in activating NK cells via DCs in vitro and in vivo.

(A) wt and ASC-deficient BMDCs were co-cultured with syngeneic wt NK cells in the presence or not (NT) of the indicated doses of sLPS and rLPS for 18h. The amounts of IFNγ secreted in the supernatants by NK cells have been quantified by ELISA. (B) FACS analysis indicating the percent of IFNγ positive NK cells (CD49b+ cells) before and after treatment iv with 1 mg/kg of sLPS and rLPS. The indicated fraction of IFNγ -positive NK cells have been calculated as the percentage of CD49b-positive cells (100%) before and after treatment. Left panel indicates the means and standard deviations calculated on 12 animals.

Therefore, although *in vitro* studies as well as studies conducted in mutant mice[4] could lead to the conclusion that rLPS is more potent in activating innate immune responses compared to sLPS, the data on NK cells obtained *in vivo* did not confirm this prediction.

We thus compared the efficiency of s and rLPSs in activating the MyD88-dependent pathway, the TRIF-dependent pathway and the inflammasome in vivo. To this purpose we used three model cytokines as read out, TNFγ, Type I IFNs and IL-1β.

As shown in **Fig.5**, sLPS was generally slightly more efficient in signaling when compared to rLPS. Thus, the relative rLPS independence of CD14 does not reflect a higher potency compared to sLPS in activating innate responses *in vivo*.

This was true also for the inflammasome-dependent cytokines, indicating that the additional stimuli required for inflammasome activation in the presence of sLPS are, in fact, available *in vivo*.

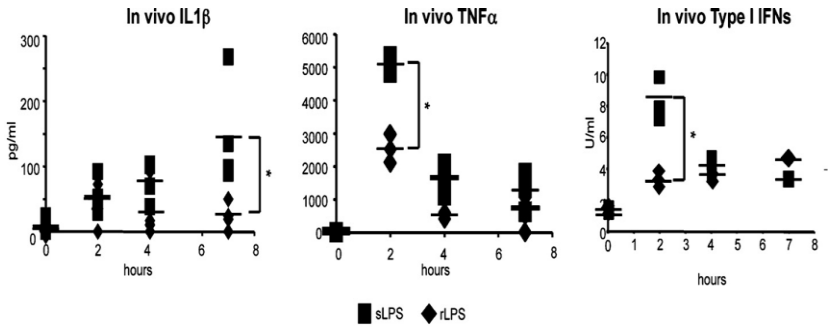

Fig. 5. sLPS is more efficient than rLPS in inducing cytokine production in vivo.

Mice were treated iv with 1 mg/kg of sLPS and rLPS and the serum concentration of the selected cytokines determined by ELISA (TNFγ, and IL-1b) or bioassay (Type I IFN) at the indicated time points.

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Chapter IV.

Discussion, Future Perspectives and Conclusions

4.1 Discussion

The sLPS and rLPS forms are used almost indistinctly *in vitro* and *in vivo* to investigate the responses of the innate and adaptive immune system to Gram-negative bacteria. Nevertheless, there is evidence that the two LPS species may signal in a different way, and in particular that rLPS may be less CD14-dependent compared to sLPS. Therefore, it has been hypothesized that rLPS induces more potent and extended responses in vivo compared to sLPS[1]. Here we identified other differences in the signaling capacity of the two LPS species. We showed that rLPS, diversely from sLPS, is capable of activating in DCs the Ca^{2+}/calcineurin and NFAT pathway in a CD14-independent manner, and is capable *per se* of activating the inflammasome independent of the presence of additional stimuli required instead for sLPS[2]. However, diversely from the expectations, we also found that sLPS is generally slightly more efficient in eliciting *in vivo* inflammatory cytokine production and innate responses activation compared to rLPS.

In respect to Ca^{2+} mobilization, while sLPS induces CD14- dependent extracellular Ca^{2+} influxes, rLPS induces both intracellular Ca^{2+} mobilization and extracellular Ca^{2+} entry. We have described that sLPS via CD14 leads to PLCγ2 activation, IP3 generation and direct opening of Ca^{2+} channels at the surface of DCs[3,4]. Presumably the IP3 receptor type 3 expressed by DCs on the plasma membrane ([4] and manuscript in preparation) are the channels responsible for Ca^{2+} current generation. In the case of rLPS, the extracellular Ca^{2+} influxes

may have a double origin: (i) CD14-dependent direct opening of IP3 receptors on the DC surface; (ii) a decline of reticular Ca^{2+} concentration leading to ICRAC generation as described for T and B lymphocytes[5,6]. The consequence of extracellular Ca^{2+} influx is the activation of NFAT transcription factors for both sLPS and rLPS[3]. Indeed, in both cases NFAT activation and IL-2 production are stunted when the extracellular Ca^{2+} influxes are blocked in the presence of the Ca^{2+} chelator EGTA.

We can assume that rLPS induces both CD14-dependent extra-cellular Ca^{2+} influxes and store operated Ca^{2+} entry. The amplitude of the Ca^{2+} current generated in response to rLPS is bigger compared to sLPS, and the amplitude of Ca^{2+} current generated in CD14-deficient BMDCs is lower compared to wt BMDCs. These observations suggest that the sum of different currents contributes to the generation of the Ca^{2+} current upon rLPS exposure. Since CD14 is only able to induce an extracellular Ca^{2+} entry, it is possible that a different unidentified receptor is required to generate the intracellular Ca^{2+} mobilization observable in response to rLPS.

The ability of rLPS to function in a CD14-independent manner does not have in any way a major relevance *in vivo*. This is probably due to the fact that other cells in addition to DCs might represent a target for LPS *in vivo*. Moreover, conventional DCs and macrophages show in mice a homogeneous CD14 expression[7]. This of course nullifies any possible advantage in rLPS signaling.

Analogously, the advantage of rLPS in activating the inflammasome is nullified *in vivo*. The most common stimuli required for inflammasome activation in addition to LPS include ATP, uric acid crystals and signals derived from cell disruption[8]. All of these stimuli can be generated in vivo following LPS administration due to its cytotoxic activity. The observation that only rLPS is capable *per se* of inducing the inflammasome activities in DCs *in vitro* demands further investigation. One possibility could be that rLPS and not sLPS induces ATP secretion which could represent the additional stimulus required for inflammasome activation.

Most wt bacteria and especially wt *Enterobacteriaceae* express both s and r forms of LPS, because some truncated LPS molecules always reach the external membrane. Therefore, the commercially available preparations of sLPS are regularly contaminated with rLPS. Nevertheless, when sLPS preparations are used the effect of the contaminating rLPS is never apparent. A possible explanation is that sLPS is much more available for signaling compared to rLPS since the hydrophilic O polysaccharides could act as water-solubilizing carriers for the active moiety represented by the lipid A[9].

The structure of the polysaccharide strongly influences also the bacterial survival capacity. It has been observed that the *Enterobacteriaceae* propagating under the pressure of the host immune system produce the s form of LPS with complex O-chains. Bacterial colonies expressing exclusively rLPS only appears after

propagation in the absence of the immune system influence, such as after proliferation *in vitro*[10].

Since the synthesis of the O-chains requires energy expenditure, Gram-negative bacteria irreversibly stop O-chain synthesis whenever the environmental conditions allow them to grow with rLPS[10]. What is the reason that only sLPS expressing *Enterobacteriaceae* can coexist with the host? A possible explanation could be that rLPS induces more potent CD14- independent immune responses that make host-microorganism coexistence difficult. Nevertheless, the results of this study seem to contradict this prediction since both sLPS and rLPS species show approximately the same efficiency of innate response activation. Perhaps the reason could be found in the fact that hydrophilic O-chain provides bacteria with an effective protection against hydrophilic antibiotics and complement proteins[11]. Moreover, the hydrophilic O-chains of wt bacteria probably allow resistance to phagocytosis[12,13].

In conclusion, although the different LPS species induce almost similar *in vivo* innate responses, they have different mechanisms of signaling that are clearly evidenced by *in vitro* studies.

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