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Study of the role of NFAT pathway in the innate immune system

Coordinator: Prof. Andrea Biondi
Tutor: Prof. Francesca Granucci
Co-Tutor: Dr. Ivan Zanoni

Dr. Roberta Marzi
Matr. No. 072451

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List of Abbreviations

DC: Dendritic Cell

PRR: Pattern Recognition Receptor

LPS: Lipopolysaccharide

LTA: Lipoteichoic Acid

PAMP: Pathogen-Associated Molecular Pattern

DAMP: Damage-Associated Molecular Pattern

TLR: Toll-like Receptor

LRR: Leucine Rich Repeat

PIP_2: Phosphatidylinositol 4,5-bisphosphate

DAG: Diacylglycerol

IP_3: Inositol 1,4,5-trisphosphate

NFAT: Nuclear Factor of Activated T cells

CsA: Cyclosporin A

PG: Prostaglandin

COX: Cyclooxygenase

TPG: Thapsigargin

Chapter 1: Introduction

1.1 Innate Immunity

All living organisms, from bacteria through humans, have evolved strategies to counter act parasitic infections [1]. In higher organisms, the varied and numerous strategies involved in defence from parasitic microbes are collectively referred to as the immune system. The mammalian immune system consists of two interrelated arms: the evolutionarily ancient and immediate innate immune system, and the highly specific, but temporally delayed, adaptive immune system. In fact, the functions of innate immunity are short-term, induced early, nonspecific and is thought unable to develop an immunological memory, although recently evidence suggests that also innate immunity could developed memory. Subsequently, if the pathogen is able to overcome this initial control, highly antigen-specific responses are triggered (usually three to five days after contact with the infectious agent) which act selectively against the pathogen and generate memory cells, which may prevent subsequent infection by the same microorganism (Figure 1).

The combination of innate and adaptive immunity enables the mammalian immune system to recognize and eliminate invading pathogens with maximal efficacy and minimal damage to self, as well as to provide protection from re-infection with the same pathogen.

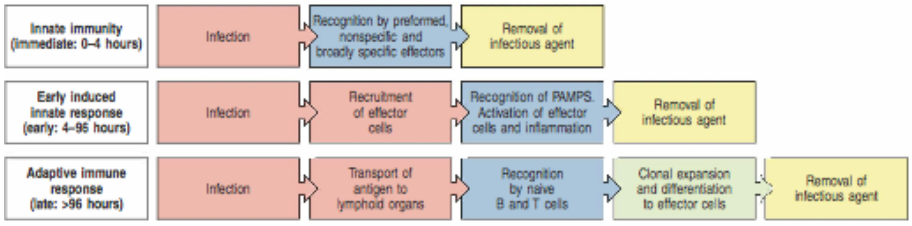


Figure 1 | The response to an initial infection occurs in three phases.

These are the innate phase, the early induced innate response, and the adaptive immune response. The first two phases rely on the recognition of pathogens by germline-encoded receptors of the innate immune system, whereas adaptive immunity uses variable antigen-specific receptors that are produced as a result of gene segment rearrangements. Adaptive immunity occurs late, because the rare B cells and T cells specific for the invading pathogen must first undergo clonal expansion before they differentiate into effector cells that migrate to the site of infection and clear the infection.

The innate and adaptive immune systems use two fundamentally different strategies to recognize microbial invaders. Specifically, the innate immune system detects infection using a limited number of germ-line encoded receptors that recognize molecular structures unique to classes of infectious microbes, while the adaptive immune system uses randomly generated, clonally expressed, highly specific receptors of seemingly limitless specificity [2]. It is the combination of these two strategies of recognition that makes the mammalian immune system highly efficient [3] (Figure 2).

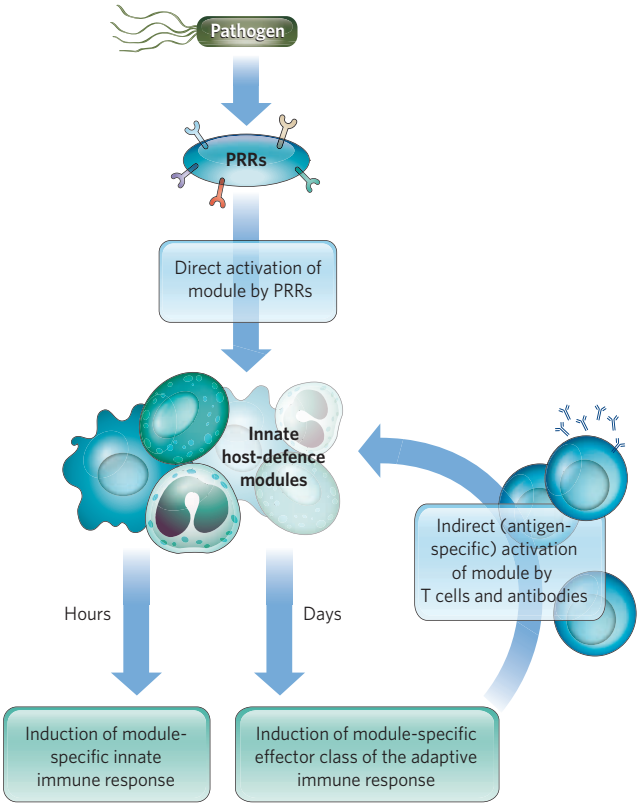


Figure 2 | Activation of the host-defence mechanisms [4].

Host-defence mechanisms can be induced directly, by engagement of PRRs, or indirectly, by T cells and/or antibodies. Each module is characterized by distinct antimicrobial defence mechanisms and can instruct the adaptive immune system to mount a response involving a module-specific effector class. After an adaptive immune response has been initiated, it results in antigen- specific activation of the same innate immune module that instructed the adaptive immune response.

The innate response includes soluble factors, such as complement proteins, and several cellular effectors, including granulocytes, mast cells, macrophages, dendritic cells (DCs) and natural killer (NK) cells. Innate immunity serves as the first line of defence against infection, as germline-encoded pattern-recognition receptors and other cell-surface

molecules quickly detect microbial constituents, thereby orchestrating inflammatory reactions [5]. By contrast, adaptive immunity, mediated by antibodies and $CD4^+$ and $CD8^+$ T cells, is slower to develop. This reflects the requirement for the expansion of rare lymphocytes that harbour somatically rearranged immunoglobulin molecules, or T-cell receptors that are specific for either microbial-derived proteins or processed peptides that are presented by Major Histocompatibility Complex (MHC) molecules [6]. NKT cells and γδ T cells are cytotoxic T lymphocytes that function at the intersection of innate and adaptive immunity [7] (Figure 3).

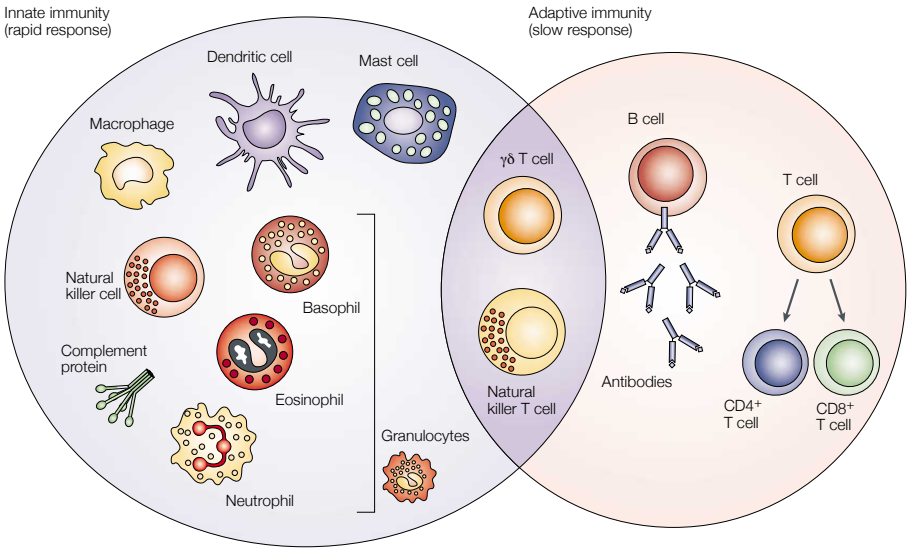


Figure 3 | The innate and adaptive immune response [7].

The innate immune response functions as the first line of defence against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. It consists of B cells, and CD4+ and CD8+ T lymphocytes. Natural killer T

cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity.

The strategy of innate immune recognition is based on the detection of constitutive and conserved products of microbial metabolism. Many metabolic pathways and individual gene products are unique to microorganisms and absent from host cells. Some of these pathways are involved in housekeeping functions and their products are conserved among microorganisms of a given class and are essential for their survival. For example, lipopolysaccharide (LPS), lipoproteins, peptidoglycan and lipoteichoic acids (LTAs) are all molecules made by bacteria, but not by eukaryotic cells. Therefore, these products can be viewed as molecular signatures of microbial invaders, and their recognition by the innate immune system can signal the presence of infection [8]. One important aspect of innate recognition is that its targets are not absolutely identical between different species of microbes. However, although there are several strain- and species-specific variations of the fine chemical structure, these are always found in the context of a common molecular pattern, which is highly conserved and invariant among microbes of a given class. For example, the lipid-A portion of LPS represents the invariant pattern found in all Gram-negative bacteria and is responsible for the pro-inflammatory effects of LPS, whereas the O-antigen portion is variable in LPS from different species of bacteria and is not recognized by the innate immune system. Because the targets of innate immune recognition are conserved molecular patterns, they are called pathogen-associated molecular patterns (PAMPs), although

they are present on both pathogenic and non-pathogenic microorganisms (MAMPs). Accordingly, the receptors of the innate immune system that recognize PAMPs are called pattern-recognition receptors (PRR) [9].

1.1.1 Pattern Recognition Receptors (PRRs)

PAMPs have three common features that make them ideal targets for innate immune recognition. First, PAMPs are produced only by microbes, and not by host cells. Therefore, recognition of PAMPs by the innate immune system allows the distinction between 'self' and 'microbial non-self'. Second, PAMPs are invariant between microorganisms of a given class. This allows a limited number of germ-line-encoded PRRs to detect the presence of any microbial infection. So, recognition of the conserved lipid-A pattern in LPS, for example, allows a single PRR to detect the presence of almost any Gram-negative bacterial infection. Third, PAMPs are essential for microbial survival. Mutations or loss of PAMPs are either lethal for that class of microorganisms, or they greatly reduce their adaptive fitness. Therefore, 'escape mutants' are not generated.

These properties of PAMPs indicate that their recognition must have emerged very early in the evolution of host-defence systems. Indeed, many PAMPs are recognized by the innate immune systems not only of mammals, but also of invertebrates and plants [9].

The innate immune system uses a variety of PRRs that can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids [10]. The principal functions of PRRs include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis [11]. Recent evidence indicates that PRRs are also responsible for recognizing endogenous

molecules released from damaged cells, termed damage associated molecular patterns (DAMPs) [12]. DAMPs include several intracellular proteins, DNA, RNA, and nucleotides. They are expressed in different cell types and play functions in normal cellular homeostasis. They are localized in the nucleus and cytoplasm (HMGB1), cytoplasm (S100 proteins), exosomes (heat shock proteins), and extracellular matrix (hyaluronic acid). On the basis of their origin and mechanism of action, the proinflammatory DAMP molecules can be classified as those that directly stimulate cells of the innate immune system and those that generate DAMPs from other extracellular molecules [13]. Because DAMPs promote the expression of cytokines, which in turn induce expression of other DAMPs, signaling events mediated by these signals provide for a feed-forward cycle of inflammatory, tissue repair, and regeneration responses.

Currently, PRRs are classified according to their ligand specificity, function, localization and/or evolutionary relationships. On the basis of function, PRRs may be divided into endocytic PRRs, that promote the attachment, engulfment and destruction of microorganisms by phagocytes, without relaying on intracellular signal (such as mannose receptors, glucan receptors and scavenger receptors) or signaling PRRs, that trigger specific transduction pathways involved in innate cell activation and in anti-microbial molecules production. This family includes transmembrane proteins such as the Toll-like receptors (TLRs), as well as cytoplasmic proteins such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Figure 4) [14].

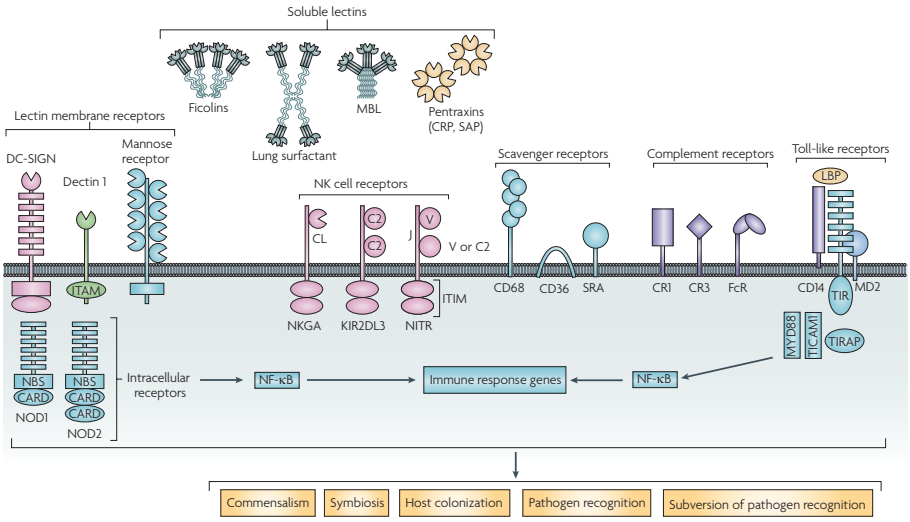


Figure 4 | Pattern-recognition receptors (PRRs)

PRRs include: soluble proteins, such as collectins, ficolins and pentraxins, integral membrane receptors, including TLRs, and intracellular sensors, such as oligomerization domain (NOD) receptors.

1.1.2 Toll-like Receptors (TLRs)

The best characterized class of PRRs are Toll-like Receptors (TLRs). In the early 1990s, several research groups identified parallels between the establishment of the dorsoventral axis by the Toll pathway in Drosophila embryos and the cytokine-induced expression of several immune genes by the interleukin-1 receptor (IL-1R)–NF-κB- signalling cascade in mammals [15]. This group noted that in both pathways, a Toll/IL-1R (TIR)-domain-containing trans-membrane receptor, Drosophila Toll or mammalian IL-1R (Figure 5), activates intracellular signalling, which culminates in the nuclear translocation of an NF-κB/NF-κB- like transcription factor. In Drosophila, the NF-κB-like factor regulated by the Toll pathway during embryonic patterning is known as Dorsal, and Dorsal regulates target genes through κB-binding motifs.

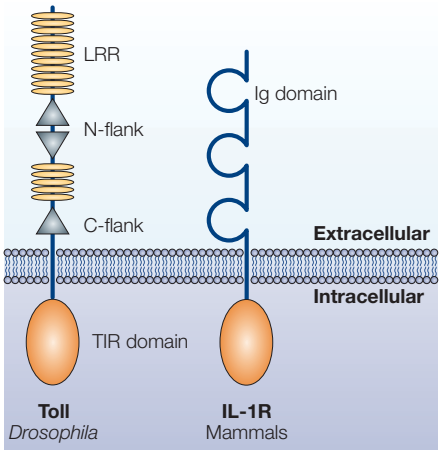


Figure 4 | Structure of the Toll and IL-1 receptors [16].
The ectodomain of Toll comprises leucine-rich repeats (LRRs) that are flanked by cysteine-rich motifs. The ectodomain of the interleukin-1 receptor (IL-1R)

comprises three immunoglobulin (Ig) domains. The intracellular Toll/IL-1R (TIR) domain of both Toll and the IL-1R interacts with TIR-domain-containing adaptor proteins [16].

The parallels between the Toll pathway and the IL-1R pathway raised the question of whether the Toll pathway, in addition to its role in dorsoventral polarity, controls the expression of antibacterial peptides in differentiated tissues. In 1993, Michael Levine and colleagues [17] reported that Dif, another member of the NF-κB family in *D. melanogaster*, translocated from the cytoplasm to the nucleus following bacterial infection or injury in the larval fat body. This study also demonstrated that Dif binds to a NF-κB-like sequence in the promoter of the gene that encodes the antimicrobial peptide cecropin. Furthermore, it was shown that Dif was activated by the constitutively active mutant of Toll. The apparent association of a Toll mutant with the Dif-dependent induction of antimicrobial peptides, together with the earlier described link between Toll and Dorsal, led Jules Hoffmann's laboratory to postulate that Toll might regulate not only developmental processes but also immune gene expression. Another important finding that they made was that fruit flies carrying mutations in the Toll pathway are highly susceptible to fungal infection (Figure 6) [18]. Defining proof of this hypothesis was provided in 1996 by Bruno Lemaitre, a member of the Hoffman laboratory. Lemaitre showed that, after microbial infection, *Drosomycin* expression was upregulated following the activation of the Toll pathway [18]. Mammalian proteins that were more similar to Toll than IL-1R1 were predicted to have TIR domains, as well as

leucine-rich repeats that are similar to those of Toll, and to differ from IL-1R1 in terms of their lack of immunoglobulin domains.

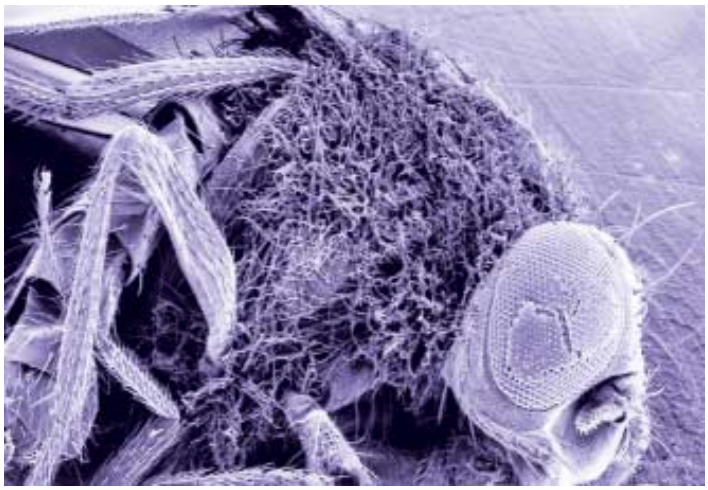


Figure 6 | *Toll* mutants are highly susceptible to fungal infection [18].
Toll-deficient fruit flies (shown), but not wild-type fruit flies, succumb rapidly to infection with the fungus *Aspergillus fumigatus*.

In 1997, one of these mammalian Toll homologues, which was termed hToll at the time, was cloned and studied by Ruslan Medzhitov and Janeway [19]. They showed that transfection of human monocytes with a CD4–hToll chimeric protein, predicted to be constitutively active in the absence of ligand, led to the activation of NF-κB and to the expression of NF-κB-dependent genes, including the gene encoding CD80. CD80 is a protein that provides co-stimulation via CD28 to T cells, and this highly important finding provided one of the first observed links between innate and adaptive immunity, as innate hToll signalling in antigen-presenting cells is associated with CD80 expression and T cell activation. This landmark discovery of the

function of hToll fulfilled the criterion that had been postulated by Janeway for the identification of PRRs [20], that they would provide an important link between innate and adaptive immunity. In 1998, five mammalian Toll homologues were described and named Toll-like receptors (TLRs), these included hToll which was renamed as TLR4 [21]. To date, more than a dozen of different TLRs have been identified (Figure 7) [22].

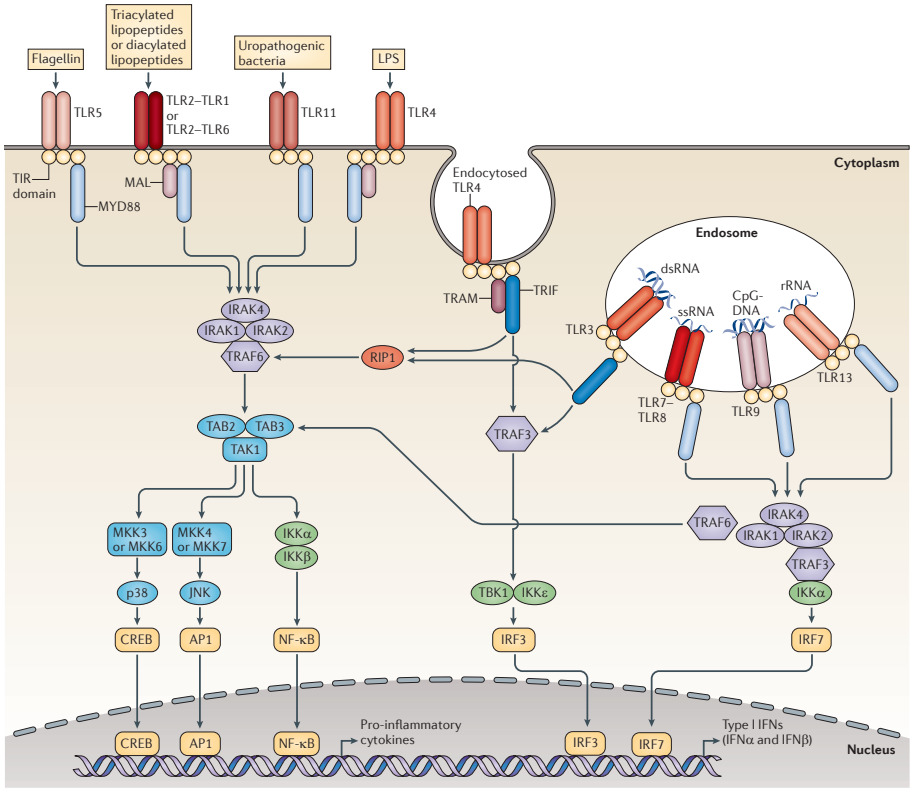


Figure 7 | Mammalian Toll-like Receptors [22].

TLR5, TLR11, TLR4, and the heterodimers of TLR2–TLR1 or TLR2–TLR6 bind to their respective ligands at the cell surface, whereas TLR3, TLR7–TLR8, TLR9 and TLR13 localize to the endosomes, where they sense microbial and host-derived nucleic acids. TLR4 localizes at both the plasma membrane and the endosomes.

Humans express ten functional TLRs (TLR1 to TLR10), whereas twelve TLRs (TLR1 to TLR9 and TLR11 to TLR13) have been identified in mice [23]. Ligands have been determined for all TLRs, except for human TLR10. TLR1, TLR2, TLR4, TLR5, TLR6 reside at the plasma membrane, where they recognize molecular components located on the surface of pathogens. By contrast, TLR3, TLR7, TLR8, TLR9, TLR11, TRL12 and TLR13 are found intracellularly, where they mediate the recognition of nucleic acids or parasitic products.

TLR	Subcellular localization	Physiological ligands
TLR1– TLR2	Plasma membrane	Triacylated lipopeptides
TLR2	Plasma membrane	Peptidoglycan, phospholipomannan, tGPI-mucins, haemagglutinin, porins, lipoarabinomannan, glucuronoxylomannan, HMGB1
TLR2– TLR6	Plasma membrane	Diacylated lipopeptides, LTA, zymosan
TLR3	Endosome	dsRNA
TLR4	Plasma membrane	LPS, VSV glycoprotein G, RSV fusion protein, MMTV envelope protein, mannan, glucuronoxylomannan, glycosylinositolphospholipids, HSP60, HSP70, fibrinogen, nickel, HMGB1
TLR4– TLR6	Plasma membrane	OxLDL, amyloid-β fibrils
TLR5	Plasma membrane	Flagellin
TLR7	Endosome	ssRNA
TLR8	Endosome	ssRNA
TLR9	Endosome	DNA, haemozoin
TLR11 (mouse)	Endosome	Profilin
TRL12 (mouse)	Endosome	Profilin [15]
TLR13 (mouse)	Endosome	23S rRNA [16]

Table 1 | Localization and ligands of TLRs [24].

dsRNA, double-stranded RNA; HMGB1, high-mobility group box 1 protein; HSP, heat-shock protein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MMTV,

mouse mammary tumour virus; oxLDL, oxidized low-density lipoprotein; RSV, respiratory syncytial virus; ssRNA, single-stranded RNA; tGPI-mucin, *Trypanosoma cruzi* glycosylphosphatidylinositol-anchored mucin-like glycoprotein; VSV, vesicular stomatitis virus.

Thus, the subcellular distribution of TLRs correlates, with the compartments in which their ligands are found (Table 1) [24].

TLRs are type I transmembrane proteins composed of an ectodomain that contains leucine-rich repeats, a single transmembrane domain and a cytoplasmic Toll/IL‐1 receptor (TIR) domain that is involved in the recruitment of signalling adaptor molecules. TLRs form heterodimers or homodimers as a means of triggering a signal. Most TLRs form homodimers, with a few exceptions . For example, TLR2 forms heterodimers with TLR1 or TLR6, which enables differential recognition of lipopeptides: TLR1–TLR2 recognizes triacylated lipopeptides, whereas TLR2–TLR6 responds to diacylated lipopeptides (Figure 8) (Table 1) [25].

Extracellular and endosomal TLRs have similar ectodomain sequences, a feature that is in sharp contrast with the diversity of the ligands that they recognize. One mode of ligand discrimination relies on the differences in the residues present in the ectodomains of distinct TLRs. The LRR modules located in the ectodomains of TLRs are composed of 20–30 amino acids each and contain the consensus sequence LxxLxLxxN (Figure 9).

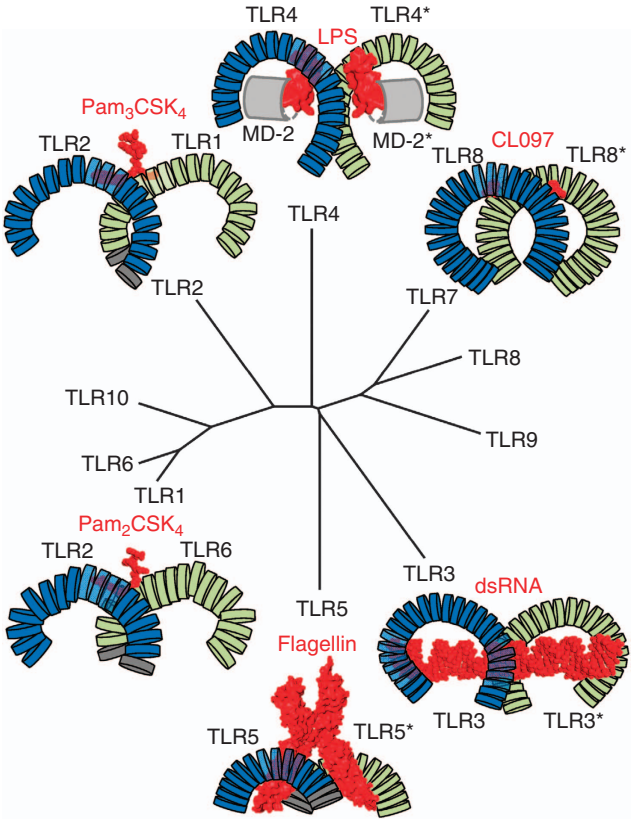


Figure 8 | Structures and a phylogenetic tree of TLRs [26].

Crystal structures of TLR4–MD-2–LPS, TLR2–TLR1–Pam_3CSK_4, TLR2– TLR6–Pam_2CSK_4, TLR5-flagellin, TLR3-dsRNA, TLR8-CL097 are shown. The ligands are colored red, and TLRs are blue and green.

TLRs have different amino acid compositions within these modules, leading to variations in structural conformation that allow for ligand interaction [25]. Amino acid variations and the formation of heterodimers can only provide a limited platform for the recognition of the varied set of TLR ligands.

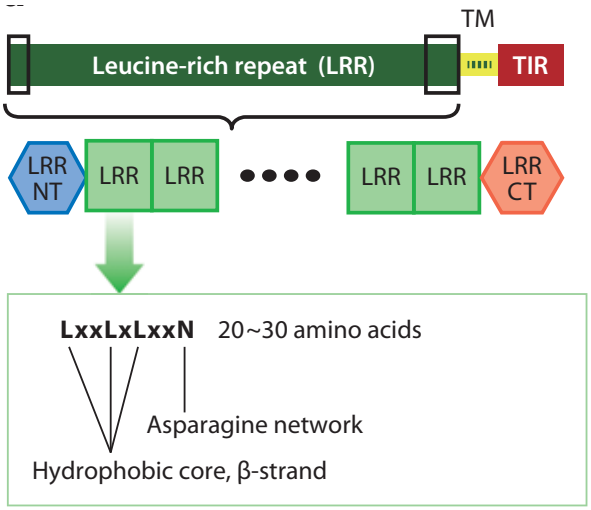


Figure 9 | Arrangement of TLR domains [25].

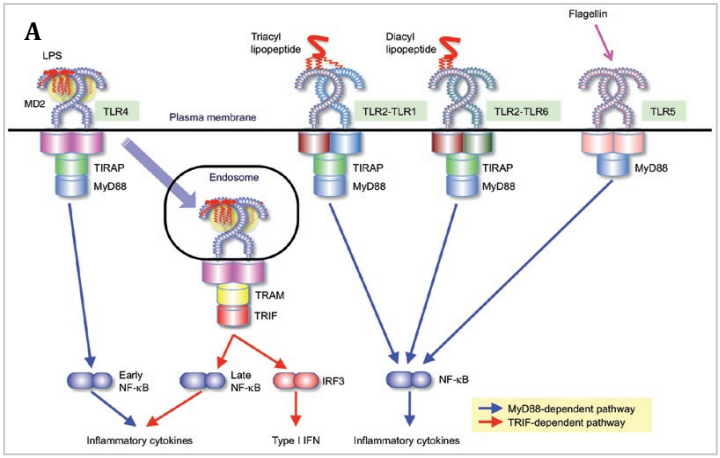
TLRs consist of an extracellular LRR domain, a transmembrane (TM) domain, and an intracellular TIR domain. The extracellular LRR domain contains 20 ~ 27 LRR modules. LRRNT and LRRCT modules cover the N and C termini of the LRR modules, respectively.

Another mechanism that reflects the complexity and diversity of TLR ligand composition is the specific association with accessory proteins or cofactors. For example, the TLR2–TLR6 heterodimer uses CD14 to respond to zymosan and both CD14 and CD36 to respond to LTA and diacylated lipopeptides [27]. These cofactors can also have roles in ensuring proper TLR folding in the endoplasmic reticulum (ER), localization to the appropriate subcellular compartment and protein processing, all of which ensure that TLRs reach their assigned subcellular compartments to bind to ligands and initiate signalling [24]. The intracellular signaling domains of TLRs have substantial sequence similarity with the interleukin-1 receptor and are termed TIR domains. TIR containing proteins include not only receptors but also

MyD88, TRIF, TIRAP, TRAM, and SARM, which are signaling-adaptor proteins [28]. After recognizing their respective PAMPs, TLRs activate signaling pathways that provide specific immunological responses tailored to the microbes expressing that PAMP. The specific response initiated by individual TLRs depends on the recruitment of these signaling adaptors to the receptor TIR domains through heterotypic TIR-TIR interactions. Aggregation of the TLRs and adaptor TIRs eventually leads to activation of transcription factors such as NF-κB, IRF3, and IRF7 through multiple signaling pathways and initiates the production and secretion of inflammatory cytokines, type I IFN, chemokines, and antimicrobial peptides [29]. The adaptor protein myeloid differentiation primary response gene 88 (MyD88) activates a family of IL-1R associated kinases (IRAKs). IRAKs in turn activate tumour necrosis factor receptor associated factor 6 (TRAF6), and elicit downstream signalling via the nuclear factor NF-kB pathway. NF-kB translocation to the nucleus activates transcription of proinflammatory genes, including tumor necrosis factor α (TNFα) and IL-6. The MyD88-dependent pathway is utilized by all TLRs, with the exception of TLR3. TLR4 signalling through both the MyD88- and the MyD88-independent pathway. The MyD88-independent pathway, engaged by TLR3 and TLR4, relies on TIR-domain-containing adaptor protein inducing interferon (TRIF). This adaptor recruits TRAF3 and the protein kinases TBK1 and IKKi, which catalyze the phosphorylation of IRF3, leading to the expression of type I IFNs. TRIF also recruits TRAF6 and TAK1 to mediate late-phase activation of NF-kB and MAP kinases. TLR2 and TLR4 use

TIRAP as an additional adaptor to recruit MyD88. TRAM acts as a bridge between TLR4 and TRIF (Figure 10) [28].

TLR family members are expressed on cells that serve as sentinels of the immune system such as DCs and macrophages. However, TLR expression is observed in a variety of other cells, including vascular endothelial cells, adipocytes, cardiac myocytes and intestinal epithelial cells. This expression pattern reflects the multifaceted role of TLRs both in disease and in healthy conditions. Indeed, TLRs can control pathogen invasion and polarization of adaptive immunity, tissue damage and remodeling (TLRs are involved in septic cardiomyopathy, viral myocarditis, atherosclerosis, ischaemia/ reperfusion injury and cardiac remodelling after myocardial infarction) [30], glucose and fat metabolism (TLR signalling pathways might contribute to the development of obesity- associated insulin resistance) [31] and the gut microbiota-host interactions (TLRs are express on intestinal epithelial cells and have a fundamental role in species variety and growth control of luminal bacteria) [32].



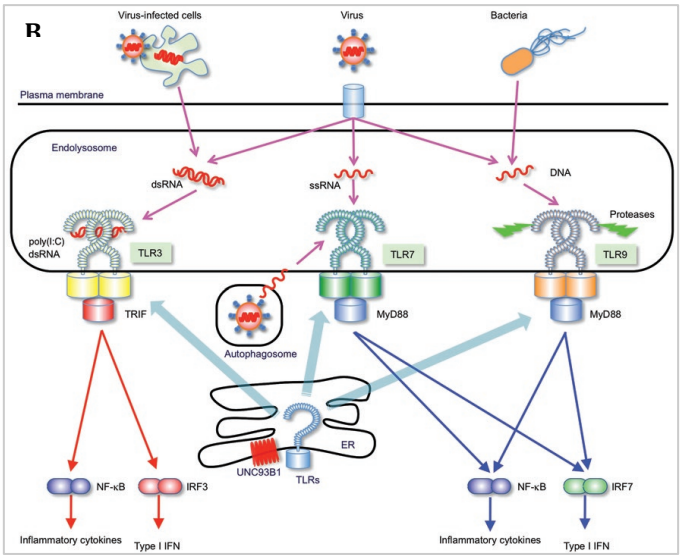


Figure 10 | PAMP recognition by cell surface and intracellular TLRs [29].

(**A**) TLR4 in complex with MD2 engages LPS. The formation of a receptor multimer composed of two copies of the TLR4-MD2-LPS complex initially transmits signals for the early-phase activation of NF-κB by recruiting the TIR domain–containing adaptors TIRAP and MyD88. The TLR4-MD2-LPS complex is then internalized and retained in the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which leads to the activation of IRF3 and late-phase. TLR2-TLR1 and TLR2-TLR6 heterodimers recognize triacylated and diacylated lipopeptide, respectively. TLR2-TLR1 and TLR2-TLR6 induce NF-κB activation through recruitment of TIRAP and MyD88. TLR5 recognizes flagellin and activates NF-κB through MyD88. (**B**) TLR3 recognizes dsRNA derived from viruses or virus-infected cells. TLR3 activates the TRIF-dependent pathway to induce type I interferon and inflammatory cytokines. In pDCs, TLR7 recognizes ssRNA derived from ssRNA viruses in endolysosomes and activates NF-κB and IRF7 via MyD88 to induce inflammatory cytokines and type I interferon, respectively. TLR9 recognizes DNA derived from both DNA viruses and bacteria. Proteolytic cleavage of TLR9 by cellular proteases is required for downstream signal transduction. TLR9 recruits MyD88 to activate NF-κB and IRF7 in pDCs. TLR3, TLR7 and TLR9 localize mainly to the ER in the steady state and traffic to the endolysosomes, where they engage with their ligands.

1.2 TLR4 Pathway

Since its discovery, TLR4 has been the focus of much attention because of its peculiar features in term of ligand recognition and signal transduction. TLR4 shows a highly orchestrated usage of co-receptors to discriminate between ligands, and this multifaceted receptor system additionally plays a role in triggering several signal transduction pathways throught the sequential recruitment of at least four adaptor proteins: TIRAP, MyD88, TRAM and TRIF (Figure 11) [33]. TLR4, together with CD14 and MD-2, forms the multireceptor complex that recognizes LPS on the cell membrane.

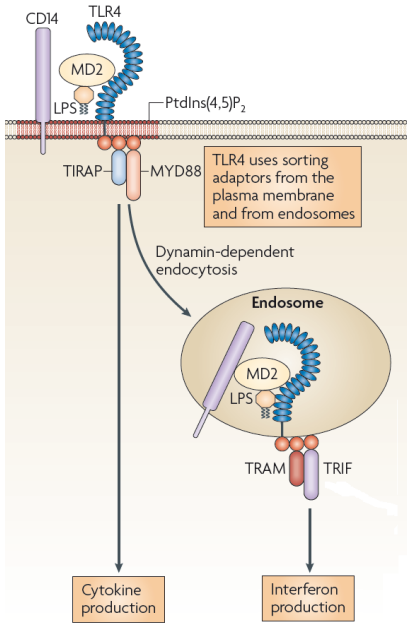


Figure 11 | TLR4 signaling pathway.

TLR4 in complex with MD2 and CD14 engages LPS. The formation of this receptor complex initially transmits signals for the early-phase activation of NF-κB by

recruiting the TIR domain–containing adaptors TIRAP and MyD88. The TLR4-MD2-CD14-LPS complex is then internalized and retained in the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which leads to the activation of IRF3 and late-phase activation of NF-κB.

1.2.1 LPS and its Receptor Complex

In 1884, Hans Christian Gram, developed 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 LPS, a complex molecule indispensable for the maintenance of the structural and functional integrity of the membrane itself [34]. For this reason, the general structure of LPS is conserved among all Gram-negative bacteria (Figure 12) [35]. LPS is a macromolecular glycolipid composed of three major parts, the lipid A, the core region and the O-chain [36]. The lipid A portion, which is responsible for most of the immunologic activity of LPS, is composed of a phosphorylated diglucosamine backbone with four to seven acyl chains attached to it. Four of the acyl groups are directly linked to the 2, 3, 2’ and 3’ positions of the glucosamine backbone, and the

remaining two are attached to the hydroxyl groups of the lipid chains. Lipid A from different bacterial species shows substantial structural diversity. The number and length of the acyl chains can vary, and the phosphate groups can be modified by other chemical groups. The carbohydrate region of LPS can be divided into two areas, the core and the O-specific chain. The core region is relatively conserved among bacterial species and contains unusual carbohydrate residues such as heptose and KDO that are not usually found in host cells. The O-specific region is composed of many copies of carbohydrate repeating units. Bacterial cells produce a highly heterogeneous set of repeating units with different structures [26]. 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. These mutants are called "rough" because of the morphology of the colonies they form in a plate that is different from that 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) [35]. LPS is recognized by TLR4 which interacts with three different extracellular proteins: LPS binding protein (LBP), CD14 and, myeloid differentiation protein 2 (MD-2), to induce a signaling cascade leading to the activation of NF-κB and the production of proinflammatory cytokines. LPS molecules, due to their amphipathic nature, form large aggregates in aqueous environments above a critical micellar concentration.

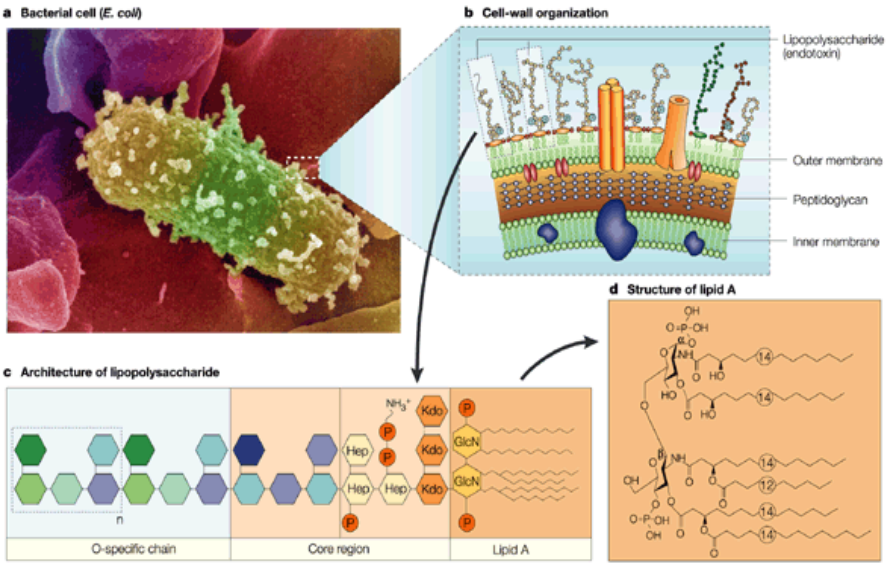


Figure 12 | LPS structure.

Lipopolysaccharide is a highly expressed component of the cell wall of all Gram-negative bacteria, and it plays a crucial role in maintaining the structural and functional integrity of the outer membrane. LPS from most Gram-negative bacteria conforms to a general architecture composed of three separate regions: the lipid A, the core, and the O-chain.

The accessory proteins, LBP and CD14, enhance the detection of LPS by the TLR4–MD-2 complex by extracting and monomerizing LPS before its presentation to TLR4–MD-2. LBP is an acutely induced plasma protein that binds avidly to LPS aggregates and delivers them to CD14 [37]. It belongs to the lipid transfer or LBP family. Other members of the family are bacterial and permeability-increasing protein (BPI), cholesterol ester transfer protein, phospholipid transfer protein and a few poorly characterized proteins. Of these proteins, the structures of BPI and cholesterol ester transfer protein have been determined; the structure of LBP has not been reported but it is

expected to share the general features of BPI because the two proteins have 48% sequence homology. BPI is a boomerang-shaped molecule composed of a central β sheet with barrel-shaped domains at its termini (Figure 13) [38]. However, the two proteins differ functionally: LBP transfers LPS to TLR4–MD-2, whereas BPI does not. Structural studies are required to account for the functional difference between the two proteins [26].

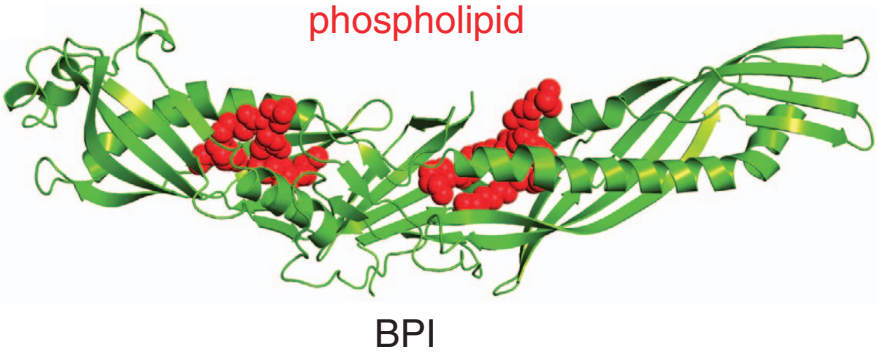


Figure 13 | The structures of accessory proteins involved in LPS recognition [26]. The crystal structure of BPI, with two phospholipid binding sites. LBP is expected to have a similar structure.

CD14 is expressed on the surface of myelomonocytic cells in the form of a glycosylphosphatidylinositol-linked glycoprotein, and as a soluble protein in the serum. Its crucial role in LPS signaling has been confirmed using knock-out mice: CD14-deficient mice are highly resistant to septic shock initiated by injection of LPS or live bacteria [39]. CD14 binds to LPS delivered by LBP and transfers the bound LPS to the TLR4–MD-2 complex. Since the presumed LPS binding

pocket of CD14 is too small for large LPS aggregates, it is likely to bind the monomeric form of LPS.

CD14 belongs to the LRR family, and has the characteristic curved solenoid structure (Figure 14) [40][41].

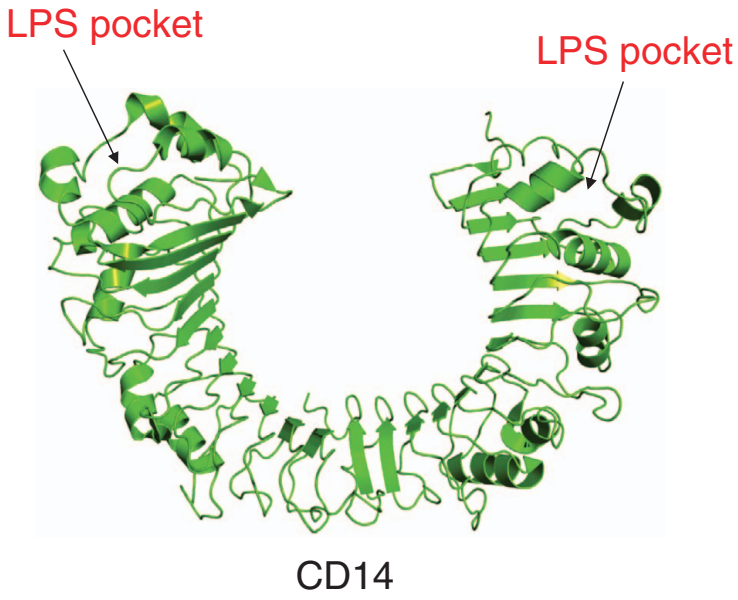


Figure 14 | The structures of accessory proteins involved in LPS recognition [26]. CD14 forms homodimers. The monomeric subunit of CD14 contains 11 LRR modules and a single LRRNT module.

LRR family proteins are composed of multiple copies of LRR modules. The individual LRR modules consist of 20–30 amino-acid residues with highly conserved 'LxxLxLxxN' motifs. The central LxL part of the module forms the core of a β strand; the two leucines point toward the interior of the protein, forming the hydrophobic core, whereas the variable x residues within the motif are exposed to solvent and some are involved in interactions with ligands.

Asparagines in the motif make stable hydrogen bonds with the backbone carbonyls of neighboring β strands throughout the entire protein, forming an extended hydrogen bonding network called an 'asparagine ladder'. As a result, the β strands are more closely packed, and assemble into a large β sheet making up the entire concave surface of the horseshoe. Variable amino acids outside the conserved β strands of each LRR module are surface exposed and some of them have important roles in ligand interactions. CD14 exists as a homodimer, the C-terminus of the LRR modules of one CD14 molecule interacts with the C-terminus of another, forming a dimer. The LPS interaction pocket of CD14 is located at the boundary of the LRRNT and the first LRR module (Figure 14). In addition to LPS, CD14 can bind other microbial products, such as peptidoglycan, LTA, lipoarabinomannan and lipoproteins. Therefore, it has broad ligand specificity and functions as a PRR by recognizing structural motifs in diverse microbial products [42]. Finally, a small protein called MD-2 is also a component of the LPS-recognition complex [43]. MD-2 is a ~ 14-kDa secreted glycoprotein that forms heterodimers with TLR4 (TLR4-MD-2 complex). MD-2 cannot transduce signals directly because it has neither a transmembrane nor an intracellular domain. Several crystal structures of complexes between the extracellular domain of TLR4 and MD-2 with and without bound ligands have been determined [44]. These show that MD-2 interacts with the concave surface of the horseshoe-like structure of TLR4. Only one-third of MD-2 is involved in TLR4 binding; the remaining part is available for interaction with LPS and other ligands. MD-2 is required for cellular

responsiveness to LPS, as demonstrated by both transfection studies and an analysis of a CHO cell line with a mutated MD-2 gene [43].

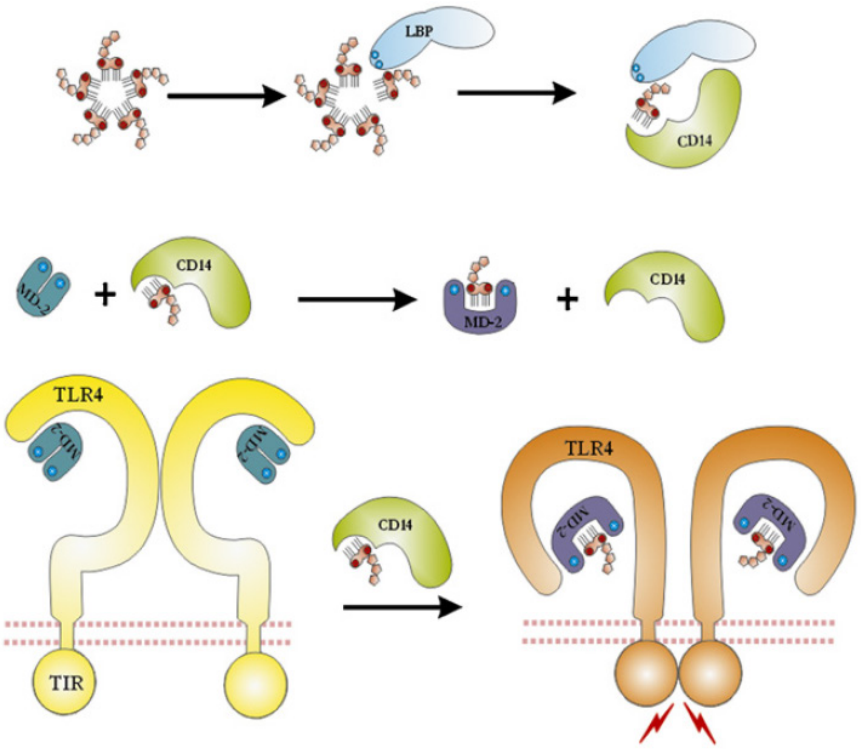


Figure 15 | Schematic representation of the steps of LPS recognition [45].
LBP binds to Gram-negative bacteria or aggregates of LPS, decreasing the binding energy of LPS monomers. The LPS molecule is shuttled to CD14 (activation pathway), where the acyl chain of lipid A is protected from the solvent in the hydrophobic binding pocket of CD14. Interaction between LBP and CD14 is important for this transfer. CD14 transfers the LPS to MD-2, which employs both electrostatic interactions with the polar head group of the lipid A and hydrophobic interactions. Binding of lipid A to MD-2 causes the rearrangement of TLR4, leading to the productive association of its intracellular TIR domains and allowing the recruitment of adapter proteins.

1.2.2 CD14 and its role in TLR4 pathway

The first role described for CD14 in LPS recognition was the enhancement of the sensitivity of innate immune cells to this inflammatory stimulus. CD14 is capable of binding LPS at picomolar concentrations and presenting and transferring it to the TLR4-MD2 complex for the initiation of the transduction pathway [46]. CD14-deficient macrophages display a markedly reduced sensitivity to low concentrations of LPS compared to wild-type cells [47]. Morover CD14-deficient mice do not develop septic shock after LPS or Gram-negative bacteria exposure, while wild-type mice do [39].

TLR4 is unique because it engages all four adaptors, TIRAP, MyD88, TRAM and TRIF, and thus is the only TLR capable of activating both the TIRAP-MyD88-dependent pathway and the TRAM-TRIF-dependent pathway leading to the secretion of type-I-interferons (IFNs) (Figure 11) [48].

LPS induces assembly of the ligand-binding complex consisting of CD14, MD-2 and TLR4 at the plasma membrane. It is at this initial site of ligand binding that the TIRAP-MyD88 complex interacts with the TIR domain of TLR4 [49]. From this location, which is a PtdIns(4,5)P2- rich subdomain of the plasma membrane, signaling is initiated and the receptor is endocytosed by a CD14-dependent process (Figure 16) [50]. In fact, it has been proposed that CD14 may recruit an immunoreceptor tyrosine-based activation motif (ITAM)-containing transmembrane adaptor to activate Syk tyrosine kinase.

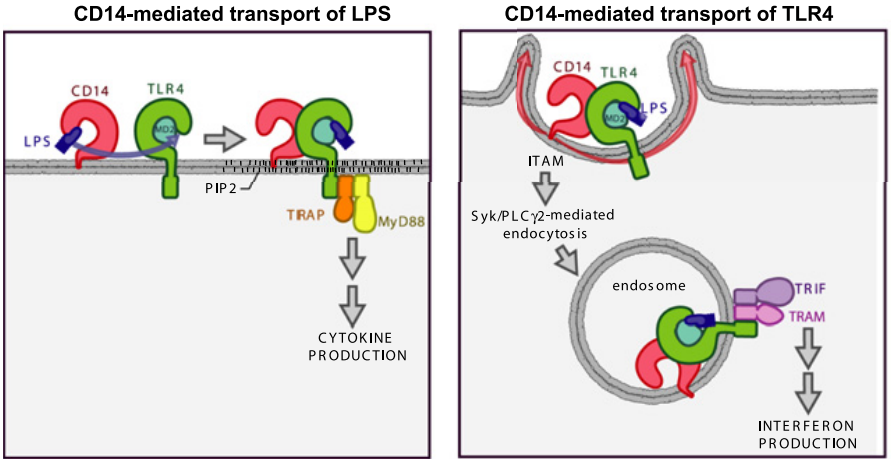


Figure 16 | CD14 is involved in the transport of LPS and TLR4 [50].

CD14 first captures and transports LPS to the plasma membrane localized complex of TLR4 and MD2, which signals through the TIRAP-MyD88 adaptors to activate inflammatory cytokine expression. CD14 then transports TLR4 to endosomes by a process mediated by Syk and PLCγ2, where TRAM-TRIF signaling leads to the expression of type I IFNs.

In turn, Syk promotes phospholipase C γ2 (PLCγ2) activation that results in a drop of PtdIns(4,5)P2 concentrations, inducing membrane invagination [51] and releasing the TIRAP-MyD88 complex from the membrane[52]. Loss of the TIRAP-MyD88 complex allows the TRAM-TRIF complex to engage the TIR domain of TLR4 on early endosomes and induce the second phase of signalling from an intracellular location, ultimately leading to the induction of the gene encoding IFN-β (Figure 17). On the plasma membrane, MyD88 recruits IRAK4 and IRAK1\2, forming a helical multiprotein complex called 'myddosome' [53].

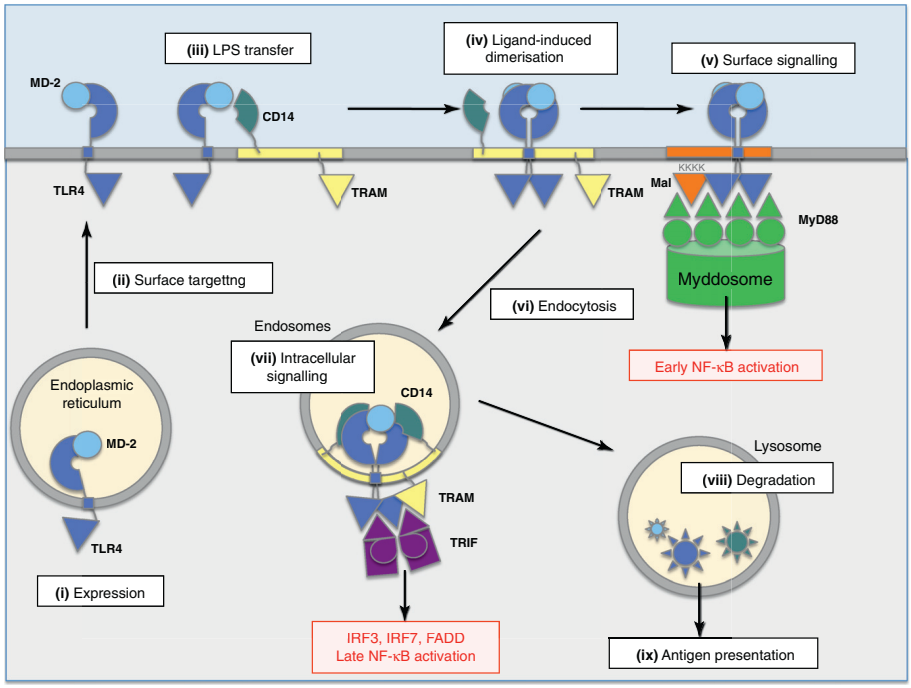


Figure 17 | TLR4-CD14 cellular targeting and signalling [54].

(i) TLR4 is expressed in the endoplasmic reticulum. (ii) It relies on MD-2 among other protein partners for surface targeting. (iii) LPS is transferred from CD14 to TLR4-MD-2. (iv) Ligand binding triggers receptor dimerisation. (v) Mal has a phosphatidylinositol 4,5- bisphosphate (PIP2) binding motif (depicted as KKKK) that targets the receptor complex to a membrane microdomain. PIP2 is a minor phospholipid exclusively located at the plasma membrane. Surface signaling involves the myddosome and leads to early NF-kB activation. (vi) Ligand binding is also required for endocytosis in a dynamin- dependent mechanism. CD14 and TRAM have lipid-raft localization signals and are engulfed along the TLR4-MD-2-LPS complex. Mal is not translocated out of the membrane. Upon endosomal acidification, TLR4 undergoes a conformational change that brings its transmembrane domains closer together. In addition, the TIR domains might arrange slightly differently under the curved membrane of the endosomes, which would lead to a different stacking of the TIR domains that would allow TRAM recruitment. (vii) Endosomal signaling results in the recruitment of TRAM and TRIF in the case of LPS and MPLA (recruitment of TRAM but not TRIF for glycoprotein G from

vesicular stomatitis virus VSV), and triggers a delayed NF-kB response. TRIF recruitment leads to the activation of IRF3, IRF7, NF-kB and FADD, respectively. (viii) In the lysosome, all endocytosed complexes are targeted for degradation and (ix) antigen presentation.

The E3 ligase TRAF6 is then recruited and activated, and synthesizes K63-linked polyubiquitin chains. Recently, IRAK2 was shown to play a central role in TRAF6 polyubiquitination [55]. These polyubiquitin chains recruit kinase complexes containing TGFβ-activated kinase 1 (TAK1) or IκB kinase (IKK) through their ubiquitin-binding subunits, TAK1-associated binding protein 2 (TAB2), TAB3 and NF-κB essential modulator (NEMO), respectively. Binding of K63-linked polyubiquitin to TAB2 and TAB3 leads to TAK1 activation, which in turn activates the mitogen-activated protein kinase (MAPK) cascade [56]. Binding of K63-linked polyubiquitin to both the IKK and TAK1 complexes facilitates the phosphorylation of IKKβ by TAK1, leading to IKK activation. IKK phosphorylates NF-κB inhibitor (IκB) proteins and targets them for polyubiquitylation by the $SCF^{\beta TrCP}$ ubiquitin E3 ligase complex. The polyubiquitylated IκB proteins are degraded by the proteasome, allowing NF-κB to enter the nucleus to turn on target genes involved in immune and inflammatory responses [57]. After internalization, the adaptor protein TRAM recruits TRIF to endocytosed TLR4. TRIF associates with TRAF3 and TRAF6, as well as receptor-interacting proteins 1 and 3 (RIP1 and RIP3). TRAF6 joins Pellino 1 (Peli1) as a E3 ubiquitin ligase. Peli1-TRAF6 interacted with adaptor kinase RIP1, and mediated RIP1 polyubiquitination [58]. In this way, RIP1 with the help of TRADD and TAK1, activate NF-kB and MAPKs to induce proinflammatory

cytokines [59]. TRAF3 links TBK1 to the TRIF-dependent pathway [60], which in combination with IKKε, phosphorylates and activates IRF3 , leading to IFNβ production. Based on specific tissue or cellular expression of TLR4 and its accessory proteins, in addition to playing a key role in triggering immune responses against gram-negative bacteria and inflammation, this pathway has been shown to be important in many other processes, including obesity, insulin resistance [61] and cancer [62].

1.2.3 CD14-NFAT Pathway

CD14, as said before, is the accessory protein that assists TLR4 in its functions. This molecule is required for LPS presentation to TLR4, thus allowing cellular responses to low doses of LPS and it is also required for the recruitment of TRIF and TRAM [50]. Indeed, CD14 was shown to be absolutely required for a full response to LPS. Recently, it has been described a new signaling cascade induced by LPS that exclusively relies on CD14 for activation of NFAT (nuclear factor of activated T cells) pathway in DCs (Figure 18) [63]. Activation of DCs through TLRs results in the activation of various signaling pathways and transcription factors, leading to the transcription of many cytokines. One of such cytokines is interleukin-2 (IL-2) [64], a key factor that confers unique T cell [65] and NK cell [66] stimulatory capacity to DCs. Since IL-2 production by T cells is known to depend on the NFAT pathway, it has been investigated whether LPS stimulation also in DCs is able to induce activation of this transcription factor. By analogy with the events after T-cell receptor engagement leading to IL-2 production, it was discovered that LPS induces a rapid and transient influx of Ca^{2+} ions in DCs. The consequent increase in the cytosolic Ca^{2+} concentration triggers the activation of calcineurin, a phosphatase that removes phosphate groups from cytosolic inactive NFAT, thereby promoting its nuclear translocation. Activation of the NFAT pathway by LPS is intact in DCs that are deficient for TLR4 or any of its signaling adaptor molecules. By contrast, the NFAT pathway is not activated in LPS-stimulated CD14-deficient DCs, and these cells do not produce IL-2.

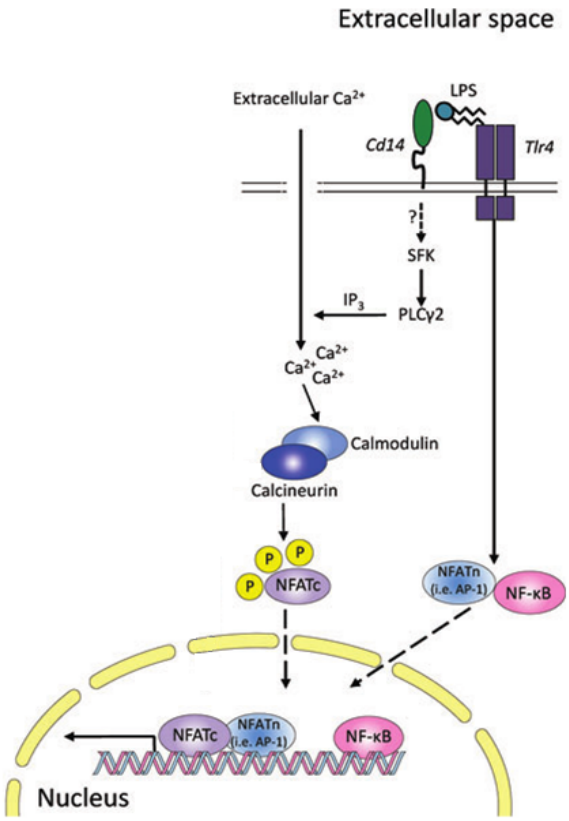


Figure 18 | CD14 signaling capacity [67].

Upon LPS engagement, CD14 transiently recruits and activates Src family kinase (SFK) members. The molecular mechanism of this process is currently unknown. Active SFKs then phosphorylate PLC-γ2, which in turn catalyzes the hydrolysis of PIP2 into DAG and IP3. IP3 directly triggers Ca^{2+} influx by acting on an as yet unidentified plasma membrane IP3 receptor with subsequent Cn activation and NFATc nuclear translocation. In the nucleus NFATc family members interact with accessory partner molecules (NFATn, usually activated via distinct signaling pathways) to form active transcription factors.

Engagement of CD14 by LPS results in SFKs and PLCγ2 activation, IP3 production and subsequent induction of Ca^{2+} influx and NFAT activation. Since CD14 is a GPI-anchored protein that lacks an intracellular signaling domain, it remains unclear how CD14 may

trigger a transduction cascade to induce Ca^{2+} entry. There are two possibilities: either CD14 acts directly through interactions with lipid rafts and SFKs, or CD14 presents LPS to a third protein, by analogy with LPS presentation to TLR4, which in turn induces Ca^{2+} mobilization. Evidences favor the first of these hypotheses, as a direct role in the activation of Ca^{2+} mobilization through interactions with lipid rafts and the activation of SFKs has been demonstrated for other GPI-anchored receptors, such as CD59 [68][69]. In fact, culture of CD14-deficient DCs with soluble CD14 and LPS do not restore IL-2 production. Thus, CD14 must be located at the cell membrane, suggesting that it could induce Ca^{2+} mobilization directly without the need to present LPS to a third protein. Furthermore, disruption of lipid raft integrity with a cholesterol-depleting agent abolishes the ability of wild-type DCs to induce a Ca^{2+} response to LPS. These observations strongly support the hypothesis that membrane-anchored CD14 that resides in lipid rafts [65] directly promotes NFAT activation. In turn, SFKs activate PLCγ2 by phosphorylation. This enzyme cleaves the PIP2 into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 then diffuses through the cytosol to bind to IP3 receptors, resulting in a single wave of extracellular Ca^{2+} influx that ultimately promotes calcineurin activation, NFAT dephosphorylation, and nuclear translocation. Interestingly, this process seems to be different than the classic mechanism described in lymphocytes to activate NFAT (Figure 19).

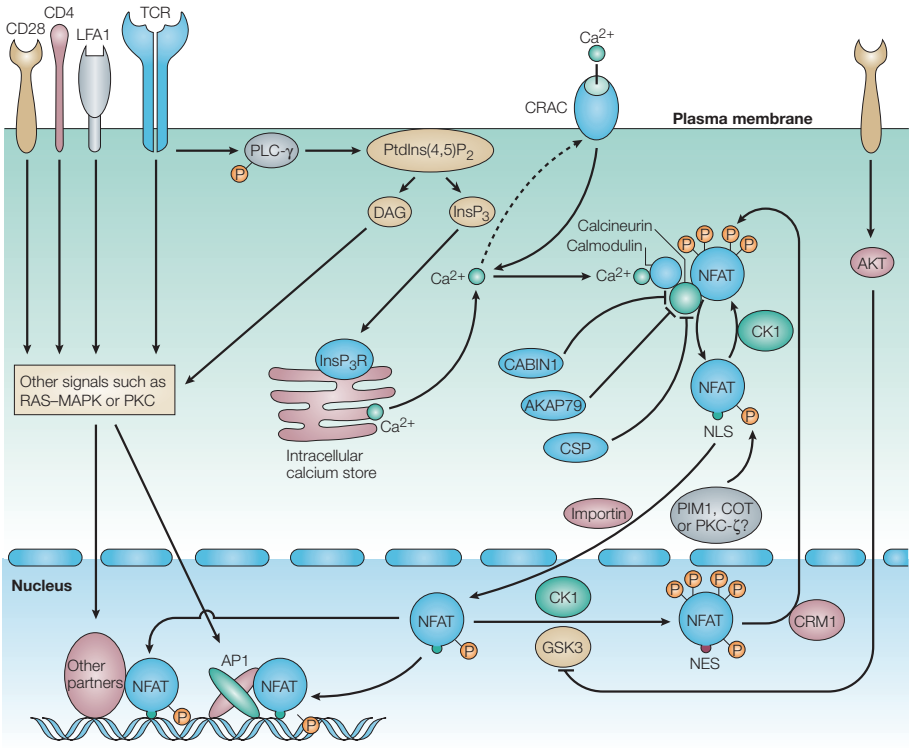


Figure 19 | Calcium signaling and activation of NFAT in lymphocytes [70].

Receptor tyrosine kinases (RTKs) and immunoreceptors such as the T cell receptor (TCR) activate PLCγ, which hydrolyses PIP2 to release IP3 and DAG. IP3 and loss of calcium binding on stromal interaction molecule 1 (STIM1) induces calcium release from the ER. Calcium release-activated calcium (CRAC) channels, including ORAI1, are then opened, allowing a sustained influx of extracellular calcium. Calmodulin binds calcium and in turn the phosphatase calcineurin. Binding of calcium to the calcineurin regulatory B subunit exposes the calmodulin-binding site on the catalytic A subunit. An autoinhibitory sequence in calcineurin is then released from the catalytic pocket, and the phosphatase can dephosphorylate cytoplasmic NFAT. Inactive NFAT is basally hyperphosphorylated; dephosphorylation promotes nuclear translocation and gene transcription. NFAT cooperates with many other transcription factors, including the activator protein 1 (AP1) complex (Fos–Jun dimers). RTK and TCR activation also stimulates signalling through the Erk pathway, leading to AP1 activation (the dashed line represents the Erk signaling pathway, for which all components are not depicted). The NFAT activation cycle is

maintained through complex mechanisms of maintenance kinases that retain cytoplasmic hyperphosphorylated NFAT, such as casein kinase 1 (CK1) and dual-specificity tyrosine phosphorylation-regulated kinase 2 (DYRK2), as well as nuclear export kinases such as CK1, DYRK1 and glycogen synthase kinase 3 (GSK3). These kinases are counteracted by negative regulators of calcineurin, such as Down syndrome candidate region 1 (DSCR1). Pharmacological antagonists of calcineurin, such as FK506 and cyclosporin A (CsA) are potent inhibitors of NFAT dephosphorylation and nuclear accumulation.

T cell receptor activation induces a sustained increase of intracellular calcium through a two-step Ca^{2+} mobilization system called store-operated Ca^{2+} entry (SOCE) [71]. IP_3 binds to and opens IP_3 receptors (IP_3Rs) in the membrane of the ER, resulting in a transient wave of Ca^{2+} obtained by release from intracellular Ca^{2+} stores. A decrease in the Ca^{2+} content of the ER is 'sensed' by stromal interaction molecule 1 (STIM1), which in turn activates calcium-release-activated calcium (CRAC) channels in the plasma membrane. Ca^{2+} influx though CRAC channels and elevated intracellular Ca^{2+} concentration activate calcineurin and thereby NFAT. In DCs, LPS induces a single and transient influx of extracellular Ca^{2+}, with no contribution from intracellular Ca^{2+} stores, which is still sufficient to activate NFAT. This suggests that LPS-induced Ca^{2+} signaling in DCs does not rely on a classical SOCE mechanism, but that IP_3 may trigger direct activation of functional plasma membrane IP_3Rs, as it has already been observed in B cells.

Notably, although NFAT activation is normally observed in TLR4-deficient DCs after LPS treatment, no appreciable gene expression occurs in these conditions, suggesting that cooperation with accessory

partner molecules (NFATn, usually activated via distinct signaling pathways) is a pre-requisite for NFAT to exert its biological function [67]. In addition to IL-2 production, the CD14-NFAT pathway in DCs plays a key role in regulating their life cycle after LPS treatment (Figure 20).

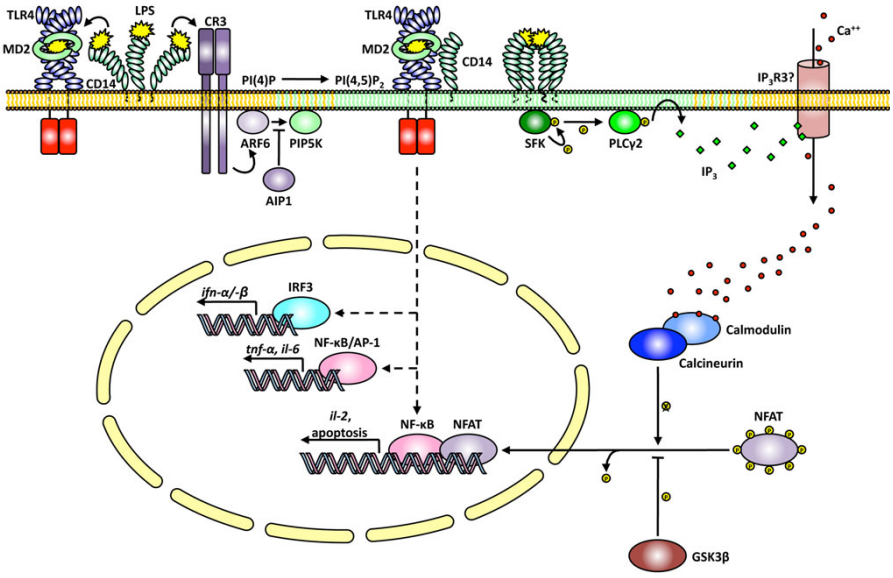


Figure 20 | CD14-dependent and TLR4-independent NFAT activation in DCs [33]. In addition to its role in LPS recognition and presentation to TLR4 and CR3, CD14 has autonomous signaling functions in DCs. Upon LPS-induced clusterization, CD14 transiently recruits and activates a SKF member through an ill-defined mechanism that relies on the CD14 GPI anchor and on its residency in lipid rafts. Active SFK then phosphorylates PLCγ2, which in turn catalyzes the hydrolysis of PI(4,5)P2 into the second messengers diacylglycerol (DAG) and IP3. Whereas the biological role of DAG in this system has not been investigated, it is likely to contribute to NF-κB activation through PKCs (not shown). On the other side, IP3 triggers Ca^{2+} from external space. The increased $[Ca^{++}]_I$ stimulates activation of calcineurin, which dephosphorylates NFAT and promotes its nuclear translocation. Active NFAT cooperates with NF-κB to drive the expression of the genes coding for

IL-2 as well as several proapoptotic proteins. It has to be noted that, although LPS-induced activation of NFAT in DCs is TLR4 independent, no change in gene expression is observed in the absence of TLR4, which is therefore required for full transcriptional activity of NFAT through activation of NF-κB.

Indeed, DCs undergo an apoptotic process during maturation [72] in order to circumscribe T cell activation in secondary lymphoid organs and to maintain self-tolerance, preventing autoimmunity in normal physiological conditions. Using a kinetic microarray analysis to identify genes modulated specifically by NFAT in LPS-treated DCs, Granucci and coworkers [63] showed that activated c2 and c3 isoforms of NFAT promote the expression of specific genes involved in programmed cell death. Among these genes, *Nur77* expression seems to be strictly regulated by NFAT in DCs following LPS stimulation. *Nur77* is an orphan nuclear receptor consisting of an N-terminal activation factor (AF)-1 domain, a DNA-binding domain containing two zinc fingers and a C-terminal ligand-binding domain. The overexpression of *Nur77* in T cells in vivo decreases the number of $CD4^+$ and $CD8^+$ T lymphocytes in the periphery to levels about 80% lower than those of wild-type mice [73]. The mechanism by which *Nur77* initiates the apoptotic pathway has not yet been completely elucidated.

This apoptotic pathway is efficiently activated in DCs, but does not occur in macrophages. This is consistent with the survival of activated macrophages, which is, indeed, essential for the resolution of inflammation. Late-activated macrophages produce anti-inflammatory mediators, which halt the inflammatory process and initiate tissue repair [74]. Thus, the different signal transduction

pathways activated in DCs and macrophages in response to LPS interaction determine the different fates of these two types of cell: apoptotic death for DCs, survival for tissue-resident macrophages. However, pharmacological activation of NFAT is sufficient to induce the cell death of macrophages upon LPS treatment, further supporting a role for NFAT as a master regulator of the cell life cycle. Macrophages express CD14 and TLR4-MD2 complex and the reasons for the lack of activation of the NFAT pathway in macrophages remains unknown. Since macrophages do not show a rapid Ca^{2+} entry after LPS exposure, there may be differences in the expression or distribution of Ca^{2+} channels, such as IP_3 receptors, involved in Ca^{2+} mobilization.

Given the involvement of CD14 in disease, including sepsis and chronic heart failure [75][76], the discovery of signal transduction pathways activated exclusively via CD14 is an important step towards the development of potential treatments involving interference with CD14 functions.

1.2.4 NFAT: Nuclear Factor of Activated T cells

NFAT was initially identified as an inducible nuclear factor that could bind the interleukin-2 (IL-2) promoter in activated T cells [77]. However, when all of the proteins of the NFAT family had been isolated and molecularly characterized, it became clear that their expression was not limited to T cells. At least one NFAT family member is expressed by almost every cell type that has been examined, including other cells of the immune system and non-immune cells [78]. The NFAT family consists of five proteins that are evolutionarily related to the REL-NF-κB family of transcription factors. All these proteins are charachterised by the same structure: (i) an ammino-terminal regulatory domain, which contains the calcineurin docking-site, the NLS and some phosphorylation site, (ii) a DNA-binding domain and (iii) a carboxy-terminal domain (Figure 21) [79].

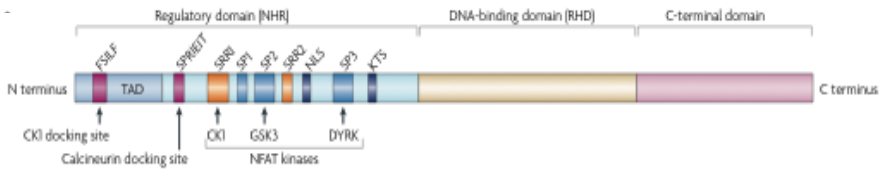


Figure 21 | General structure of NFAT transcription factors [79].

NFAT proteins consist of an amino-terminal regulatory domain (also known as an NFAT homology region (NHR)), a DNA-binding domain (also known as a REL-homology domain (RHD)) and a carboxy-terminal domain. The regulatory domain contains an N-terminal transactivation domain (TAD), as well as a docking site for for casein kinase 1 (CK1), termed FSILF, and for calcineurin, termed SPRIEIT. It also includes multiple serine-rich motifs (SRR1 , SP1, SP2, SRR2, SP3 and KTS) and a nuclear localization sequence (NLS).

They can be subdivided into two groups according to their functional domains: NFAT5 and the NFATc family. NFAT5 is the most evolutionary ancient transcription factor of the NFAT family, being present in both invertebrates and vertebrates [80]. The NFATc family is comprised of four members: (i) NFATc1, also known as NFAT2 or NFATc; (ii) NFATc2, also known as NFAT1 or NFATp; (iii) NFATc3, also known as NFAT4 or NFATx; and (iv) NFATc4 also known as NFAT3. It is thought that a recombination event occurred about 500 million years ago between an NFAT precursor and a Rel domain giving rise to the NFATc factors, which are present only in vertebrates (Figure 22) [81].

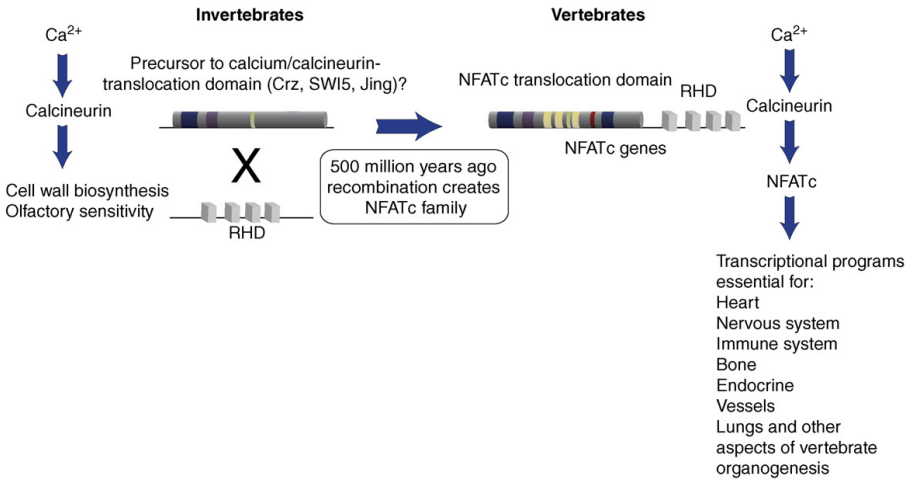


Figure 22 | Evolutionary diversification of the function of the rel domain by recombination [81]. Illustration of the proposed recombination event during evolution that juxtaposes the calcium-sensing translocation domain with the RHD to produce NFATc in vertebrates, and a new set of transcriptional programs.

The activity of the NFATc1-c4 isoforms is controlled by the Ca^{2+}/calmodulin phosphatase calcineurin (Cn), which, once activated

by an increase in intracellular Ca^{2+} concentration, de-phosphorylates the phosphorylation motifs present toward the N-terminus of NFATc, therefore exposing a nuclear import sequence and leading to nuclear import of NFATc. In the nucleus, the NFATc proteins interact with partner proteins (also termed as NFATn) to produce active NFAT transcription complexes (Table 2) [79].

Transcription partner	Interaction site	Effect
AP1 (FOS, JUN)	Many cytokine-gene promoters	Positive synergy
C/EBP	*PPAR-γ* promoter	Positive synergy
MAF	*Il-4* promoter	Positive synergy
EGR1 and EGR4	*Tnf* promoter	Positive synergy
GATA3	*Il-4* 3′ enhancer	Positive synergy
ICER	Many cytokine-gene promoters	Inhibition of NFAT activity
IRF4	*Il-4* promoter	Positive synergy
MEF2	*NUR77* promoter	Positive synergy
OCT	*IL-3* enhancer	Positive synergy
$p21^{SNFT}$	*IL-2* promoter	Inhibition of NFAT activity
PPAR-γ	*IL-2* promoter	Inhibition of NFAT activity
T-bet	*Ifn-γ* 5′ enhancer	Positive synergy

Table 2 | NFAT transcriptional partners [70].

AP1, activator protein 1; C/EBP, CCAAT/enhancer-binding protein; EGR, early growth response; GATA3, GATA-binding protein 3; ICER, inducible cyclic AMP early repressor; Ifn-γ, interferon-γ ; Il, interleukin; IRF4, IFN-regulatory factor 4; MEF2, myocyte-enhancer factor 2; NFAT, nuclear factor of activated T cells; NUR77, orphan nuclear receptor 77; OCT, octamer-binding transcription factor;

p21SNFT, 21-kDa small nuclear factor isolated from T cells; PPAR-γ, peroxisome-proliferator-activated receptor-γ; Tnf, tumour-necrosis factor.

Usually, NFATc and the partner proteins are activated via distinct signaling pathways and this provides a means for NFATc to exploit evolutionary older pathways to regulate evolutionary newer biological functions [81]. It also means that NFATc members can perform as transcription factors in the nucleus only in combination with partner transcription factors that are always activated via different signaling pathways. NFAT transcriptional activity is regulated by cycles of dephosphorylation and rephosphorylation, leading to nuclear import and export. Nuclear export mechanisms sequentially involve different kinases, such as dual-specificity tyrosine kinase 1a (Dyrk1a) that phosphorylates the N-termini of NFATc proteins for the subsequent phosphorylation by glycogen synthase kinase 3 (GSK-3) (Table 3) [82].

NFAT kinase	Kinase type	Substrate	Phosphorylation site
GSK3	Export	NFAT1	SP2
		NFAT2	SP2 and SP3
CK1	Export and maintenance	NFAT1	SRR1
DYRK1	Export	NFAT1 and NFAT2	SP3
DYRK2	Maintenance	NFAT1 and NFAT2	SP3

Table 3 | NFAT kinases [79].

CK1, casein kinase 1; DYRK, dual-specificity tyrosine-phosphorylation regulated kinase; GSK3, glycogen synthase kinase 3; NFAT, nuclear factor of activated T cells; SP, Ser-Pro-X-X repeat motif; SRR, serine-rich region.

NFAT5 differs from the NFATc members since it uses a dimerization mechanism conserved in the NF-κB proteins and is not activated following Ca^{2+} mobilization [83].

The appearance of the NFATc transcription factor family in vertebrates has presumably allowed either the development of vertebrate-specific organs and functions, such as the skeleton, lung, and adaptive immunity, or the adaptation of evolutionary older organs and functions for the characteristics of vertebrates. The transition to vertebrate life has required a progressively higher level of complexity of innate responses, and the appearance of the NFATc pathway in innate immunity may have contributed to the adaptation process.

Understanding the role of the NFATc signaling pathway in inflammatory processes may help elucidate some of the molecular mechanisms underlying innate immunity. As a consequence, understanding the effects of NFATc deregulation in innate immune cells could help elucidate the pathogenesis of inflammatory diseases [67].

1.3 Dendritic Cells (DCs)

DCs were first described by R. Steinman and Z. Cohn in 1973 [84]. They subdivided phagocytes into macrophages and DCs on the basis of their effector functions: antimicrobial and scavenging functions for macrophages and professional antigen presentation for DCs. DCs have since been characterized in great detail and the molecular basis of the regulation of their functional properties has been determined. DCs are located in lymphoid and nonlymphoid organs and are quiescent until they encounter inflammatory exogenous or endogenous stimuli. They use a repertoire of innate nonclonal receptors to perceive the different types of stimuli and to transduce this information within the cell and TLRs are the best characterized of these receptors. Following interaction with inflammatory stimuli, DCs undergo complex transcriptional reprogramming, involving the differential expression of thousands of genes and the integration of a number of signaling pathways. The active transcriptional response results in the acquisition by DCs of various functional properties relating to activation of the appropriate immune responses. In particular, after exposure to inflammatory stimuli, DCs lose their ability to take up antigens, become extremely efficient at antigen processing and acquire the ability to migrate to the T-cell areas of secondary lymphoid organs, where they present antigens to naïve T cells to initiate primary adaptive responses [85]. Finally, after achieving their effector functions, DCs undergo terminal differentiation and die by apoptosis [65]. Different subsets of DCs are located in specific tissues, where

they acquire antigens, transporting them to draining lymph nodes for T cell priming.

1.3.1 DCs Classification

DCs are highly heterogeneous, their characteristics depending on their origin and location. Two main classes of DCs have been described: conventional and pre-DCs. At the steady state, conventional DCs (cDCs) display all the typical phenotypic and functional characteristics that have been originally used to describe DCs. Indeed, they are veiled cells of myeloid origin capable of efficiently processing and presenting antigens and of priming naive T cells. By contrast, pre-DCs must undergo an additional differentiation step, induced by inflammatory stimuli (microbial and endogenous stimuli that activate TLRs) in most cases, to acquire the characteristics of DCs, including the efficient antigen-presenting capacity. Plasmacytoid DCs (pDCs) and monocytes are classified as pre-DCs, as both can further differentiate into efficient antigen-presenting cells (APCs) in the presence of microbial stimuli [86]. Conventional DCs may also be subdivided into migratory and lymphoid tissue-resident DCs. Migratory cDCs reside in nonlymphoid tissues where they continuously scan the environment to detect the presence of invading microorganism. Upon microbial encounter tissue-resident migratory cDCs migrate to the draining lymph nodes through the afferent lymphatic vessels.

Lymphoid tissue-resident cDCs are not present in the afferent lymphatic system and encounter the antigen directly inside the

lymphoid organs. Antigens can reach the lymphoid organs through the blood, by freely migrating through the lymphatics or associated to migratory cDCs. Most thymic and splenic DCs and about half the lymph node DCs are lymphoid tissue-resident cells [86]. cDCs have been in turn subdivided into different subtypes. The number of subtypes is continuously growing. Subset classification is based on tissue origin and the expression of particular markers for migratory cDCs, and on marker expression for lymphoid tissue-resident DCs [87].

1.3.1.1 Conventional DCs

Conventional DCs are specialized for antigen processing and presentation. They can be grouped into two main classes based on their localization in tissues and their migratory pathways as they circulate in the body (Figure 23). The first category of conventional DCs is generally referred to as the migratory DCs. These DCs develop from early precursors in the peripheral tissues, where they act as antigen-sampling sentinels. From the peripheral tissues, they migrate to the regional lymph nodes via afferent lymphatics, a process that is accelerated in response to danger signals, such as those that occur during pathogen infection. Migratory DCs are not found in the spleen and are restricted to the lymph nodes [88], where they constitute a variable proportion of the steady-state DC population; this proportion depends on the specific tissues that are drained by the lymph node [89] (Figure 23). Migratory DCs can be broadly divided into $CD11b^{+}$ DCs (also known as dermal or interstitial DCs) and $CD11b^{-}$ DCs, which have more recently been shown to express CD103 (also known

as integrin αE) [90]. The second major category of conventional DCs is represented by lymphoid tissue-resident DCs that are found in the major lymphoid organs, such as the lymph nodes, spleen and thymus. These DCs can be further classified by their expression of the surface markers CD4 and CD8α into $CD4^+$ DCs, $CD8\alpha^+$ DCs and $CD4^-CD8\alpha^-$ DCs (typically referred to as double-negative DCs) [91]. $CD8\alpha^+$ DCs are noted for their capacity to cross-present antigens [92] and for their major role in priming cytotoxic $CD8^+$ T cell responses. $CD4^+$ DCs and $CD4^-CD8\alpha^-$ DCs can also present MHC class I-restricted antigens in some settings, but appear to be more efficient at presenting MHC class II-associated antigens to $CD4^+$ T cells [93]. Lymphoid tissue-resident DCs do not traffic from other tissues but develop from precursor DCs found in the lymphoid tissues themselves. In the absence of infection, they exist in an immature state (which is characterized by a high endocytic capacity and lower MHC class II expression compared with activated DCs), and their residency in lymphoid tissues makes them ideally placed to sense antigens or pathogens that are transported in the blood [94].

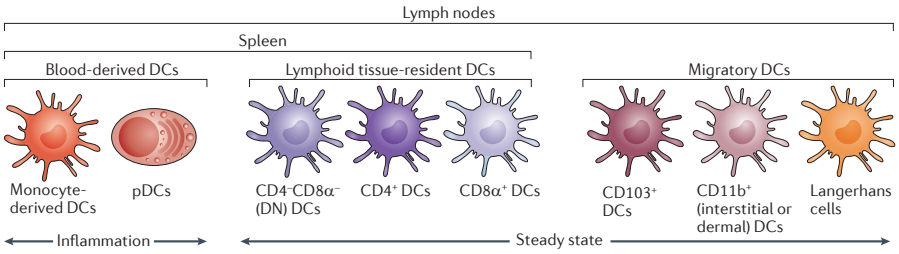


Figure 23 | The organization of the DCs network [94].

The organization of the DC network, and includes the key surface phenotype markers of different DC subsets, which are delineated on the basis of their localization in secondary lymphoid tissues.

1.3.1.2 Langerhans cells

Langerhans cells are resident in the skin and, like migratory DCs, migrate to the lymph nodes to present antigens (Figure 23). However, unlike conventional DCs, which arise from a bone marrow precursor cell, Langerhans cells are derived from a local $LY6C^{+}$ myelomonocytic precursor cell population in the skin. This precursor population originates from macrophages that are present early in embryonic development and that undergo a proliferative burst in the epidermis in the first few days after birth [95].

1.3.1.3 Plasmacytoid DCs

pDCs are quiescent cells that are broadly distributed in the body. They are characterized by their ability to rapidly produce large amounts of type I interferons (IFNs), a feature most evident during viral infection. pDCs express several characteristic markers, including sialic acid-binding immunoglobulin-like lectin H (SIGLEC-H) and bone marrow stromal antigen 2 (BST2) in mice and blood DC antigen 2 (BDCA2; also known as CLEC4C) and leukocyte immunoglobulin-like receptor, subfamily A, member 4 (LILRA4; also known as ILT7) in humans. In addition, both mouse and human pDCs express CD45RA27. pDCs have poor antigen-presenting capacity, and their precise contribution to immune responses is still unclear [96].

1.3.1.4 Monocyte-derived DCs

Under inflammatory conditions, circulating blood monocytes can be rapidly mobilized and can differentiate into cells that possess many prototypical features of DCs (Figure 23). In the steady state,

monocytes express the macrophage colony-stimulating factor receptor (M-CSFR; also known as CD115), which is essential for their development, as well as other markers, such as LY6C and CX3C-chemokine receptor 1 (CX3CR1). In response to growth factors such as granulocyte–macrophage colony-stimulating factor (GM-CSF) *in vitro* or to Toll-like receptor 4 (TLR4) ligands or bacteria *in vivo*, fully differentiated monocyte-derived DCs emerge. Similarly to conventional DCs, monocyte-derived DCs express CD11c, MHC class II molecules, CD24 and SIRPα (also known as CD172a), and they upregulate their expression of DC-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209a) but lose expression of both M-CSFR and LY6C33. Monocyte-derived DCs also express the macrophage marker MAC3 (also known as CD107b and LAMP2) [97]. In addition, these cells acquire potent antigen-presenting capacity, including the ability to cross-present antigens [98]. Thus, it is emerging that monocyte-derived DCs are a crucial reservoir of professional APCs that are recruited into immune responses to certain microorganisms and potentially have an emergency back-up role in cases of acute inflammation [94].

1.3.2 Development and Function of DCs

The life cycle of DCs is dominated by least two different maturation stages characterized by complementary properties. The first stage is defined as “immature”, the second one as “mature”.

Immature DCs (iDCs), have an unsurpassed machinery to take up antigens by constitutive macropinocytosis, receptor-mediated endocytosis and phagocytosis [99]. Efficient antigen uptake is pivotal for iDCs to fulfil their sentinel function in immunity. After internalization, most exogenous antigens are processed through an endosomal and lysosomal pathway in which proteins are cleaved into peptides and loaded onto MHC class II molecules [100]. Alternatively, exogenous antigens can be released into the cytosol, gaining access to the proteasome, the main nonlysosomal protease, that generates peptides and transfers them to the endoplasmic reticulum, where they are loaded onto MHC class I molecules (cross-presentation). Notably, the encounter between antigens and iDCs can occur in the peripheral tissues or directly at the lymph node level, where antigens are passively transported through the lymphatic flow [101]. The regulation of antigen uptake and presentation is under tight developmental control: iDCs have the highest capacity to internalize antigens but have low T-cell stimulatory activity.

Following the interaction with microorganisms or bacteria products, DCs undergo a phenotypical and functional modification and they reach the mature stage (mDCs). This activation process encompasses the downregulation of endocytic capacity, the upregulation of surface T cell co-stimulatory (CD40, CD80 and CD86) and MHC class II

molecules, the production of bioactive cytokines (for example IL-12 and TNFα), and changes in migratory behavior. In this way, mDCs control triggering events and polarization of T cells [102].

Intermediate differentiation stages have not been defined because of the lack of specific markers, and this leaves open the possibility that the transition from the immature to the mature stage is not simply a progressive itinerary (progressive loss of antigen capture ability, progressive acquisition of migration activity and progressive acquisition of T-cell activation function), but represents a sequence of precise transitional stages. It is possible that during the initial phases of activation DCs stop at the site of inflammation to maximize the antigen uptake and to recruit the cells of the innate response, important for antigen clearance and the sustenance of the inflammation. In fact, it has been shown that DCs can orchestrate the early phases of innate immune response producing of a wide variety of chemokines that attract monocyte, macrophage, neutrophil, and NK cell [103]. After this process is completed, DCs can leave the inflammatory site and reach the spleen or lymph nodes to initiate the adaptive immune response (Figure 24) [104]. Following their activation and terminal differentiation, mDCs progress toward apoptotic death. Once the DCs have presented their antigens to T cells, they are eliminated by apoptosis, to damp down the immune response and liberate the spaces they occupy after migration.

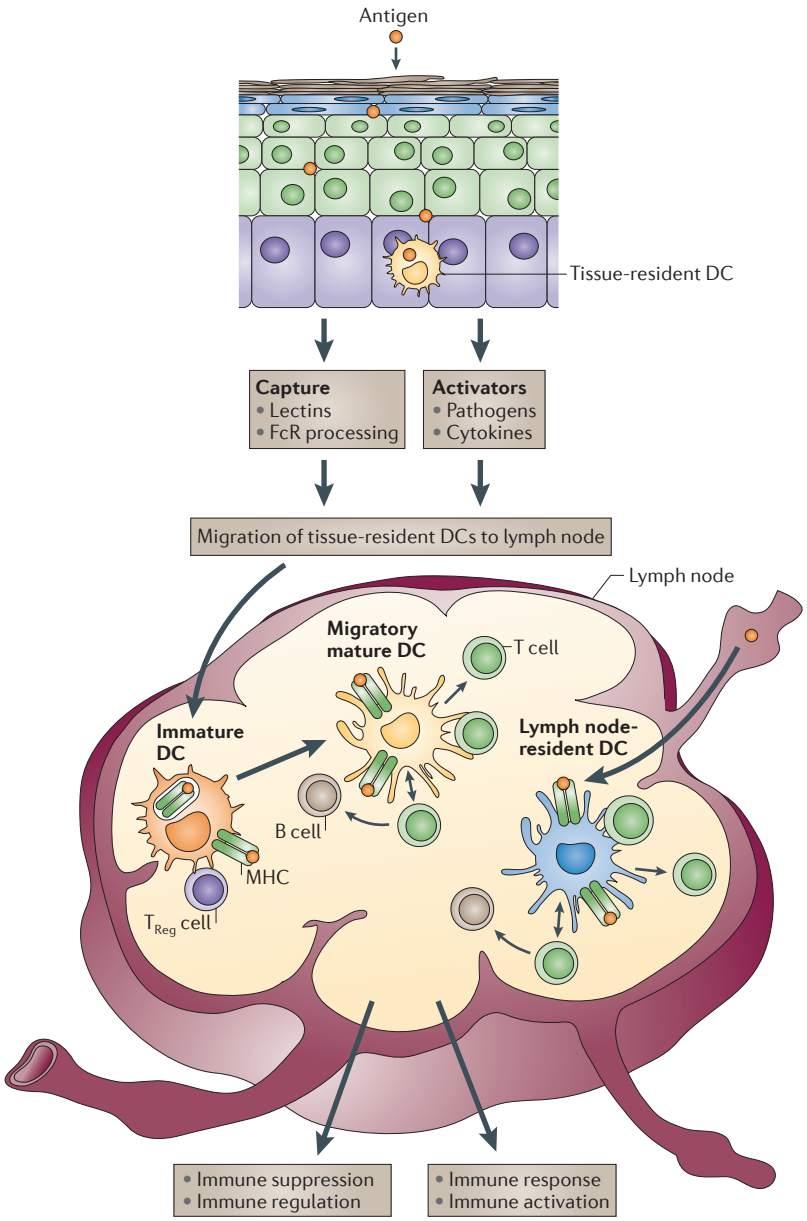


Figure 24 | Launching the immune response [105].

Antigens can reach lymph nodes through two pathways: via lymphatics, where the antigen is captured by lymph node-resident DCs, or via tissue- resident DCs. These immature DCs capture antigens, and DC activation triggers their migration towards secondary lymphoid organs and their maturation. DCs display antigens in the

context of classical MHC class I and MHC class II molecules. Activated T cells drive DCs towards their terminal maturation, which induces further expansion and differentiation of T lymphocytes into effector T cells. If DCs do not receive maturation signals, they will remain immature and antigen presentation will lead to immune regulation and/or suppression.

A significant number of investigations have linked the failure to achieve DC programmed cell death to autoimmunity [106]. This breakdown of apoptosis contributes to autoimmune phenomena, for example via the exposure of self-antigens in an prolonged inflammatory context that can initiate immune responses against them. Although defects in apoptosis propagate autoimmunity and significantly contribute to disease susceptibility, a breakdown of multiple immunoregulatory mechanisms is required for full disease penetrance.

1.3.3 Skin DC subsets

Epidermal Langerhans cells (LC) have long been regarded as the exclusive DC of the skin, taking up pathogens or allergens that penetrate the epidermis. After switching from a sessile to a mobile state, LC carry these antigens to the LN that drain cutaneous tissues (CLN) [107]. Recent studies, however, demonstrated the existence of a complex network of dermal DC (DDC) and also suggested that LC might play an indirect role in T-cell priming, for example by ferrying antigens to those DC that reside throughout their life cycle in the CLN [108]. These resident DC are generally denoted as lymphoid tissue-resident DC (LT- DC) to distinguish them from non-lymphoid tissue-derived, migratory DC (mig-DC), such as LC.

The most recent classification of skin DCs was made by Guilliams and colleagues [109] who identified 5 different DC subsets that express diverse combinations of surface markers and have specific properties (Figure 25). All 5 of the identified skin DC subsets migrate to draining LNs to transport skin-sequestered antigens. Inside cLNs, migratory DCs are distinguishable from lymphoid-resident DCs, on the basis of their expression of MHC class II and CD11c. Migratory DCs in cLNs express higher levels of MHC class II and show a variable CD11c expression from intermediate to high levels ($CD11c^{int/hi}$ $MHCII^{hi}$). Diversely lymphoid-resident DCs express high levels of CD11c and lower levels of MHC II ($CD11c^{hi}MHCII^{int}$) [109]. Migratory DCs travel from the skin in homeostatic conditions guided by the chemokine receptor CCR7 [110] and exhibit a partially activated phenotype with intermediate to high levels of CD40 and

CD86. Different specialized functions have been attributed to the skin DC subsets. For instance, in K5.mOVA mice, where a membrane form of OVA is expressed by skin keratinocytes, $CD207^+$ $CD103^+$ DDCs have been shown to be the only subset that can cross-present OVA both *in vitro* and *in vivo*, transport keratinocyte-derived antigens to cLNs, and present them to $CD4^+$ T cells [111]. Moreover, the adoptive transfer of OVA-specific naïve T cells into K5.mOVA mice results in antigen-specific pTreg-cell differentiation in cLNs. Consistently, this process is $CD207^+$ DDC dependent and is completely abrogated in CCR7-deficient mice. Since LCs are dispensable for OVA peptide transport and presentation to T cells, LCs are not necessary for pTreg-cell induction. Nevertheless, LCs are not excluded as inducers of pTreg cells [112].

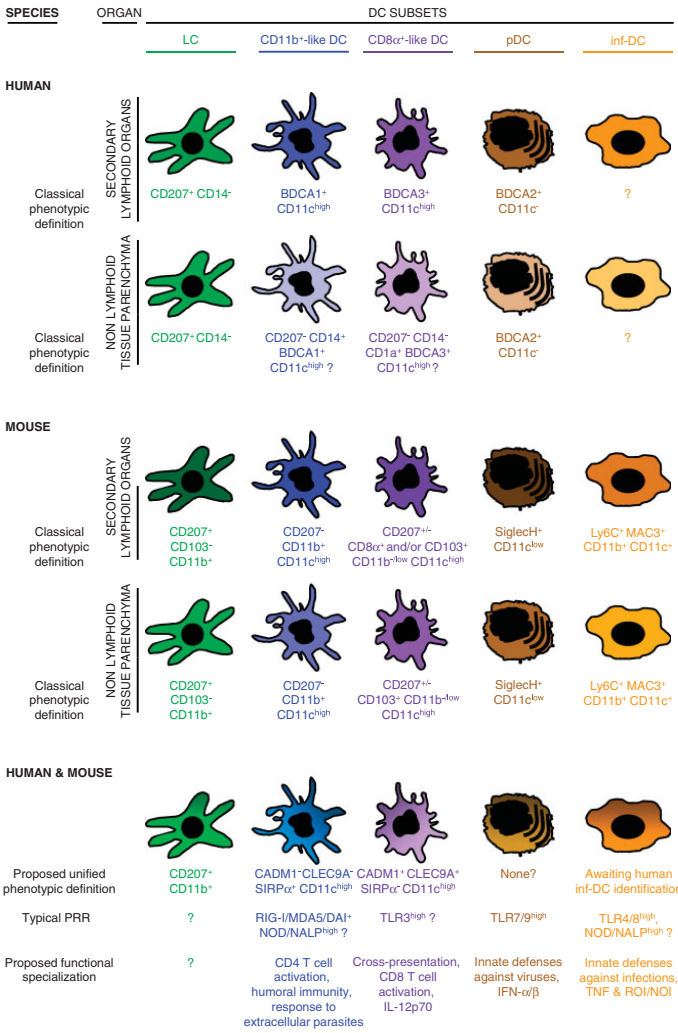


Figure 25 | A unifying model of human and mouse DC subsets [109].

Human and mouse DC subsets can be organized into five broad subsets irrespective of their primary location in secondary lymphoid organs or in the parenchyma of non-lymphoid organs. These five subsets correspond to: (i) LC (green), (ii) CD11b1 DC-like cells (blue), (iii) CD8a1 DC-like cells (violet), (iv) pDC (brown) and (v) monocyte-derived inf-DC (orange). The phenotype used to identify those subsets is specified for each condition. A general nomenclature is suggested for each DC subset (lower row, shaded colors), irrespective of their tissue and species of origin. This nomenclature is based on the unified phenotypic definition, characteristic PRR and functional specialization.

1.3.3.1 Role of skin DC in the induction of Foxp3$^+$ Treg

The migratory populations of DCs are very effective in inducing Treg-cell differentiation in the skin. Migratory DCs are particularly efficient in draining antigens from the skin into the cLNs and produce TGF-α and RA, thus favoring Treg-cell generation [113][111]. Nevertheless, the respective contribution of each subset of skin DCs to Treg-cell induction is not yet completely clear. CD207α DCs have been shown to present epidermal antigen in cLNs, causing pTreg-cell induction. Yet this population does not produce RA. Among migratory DDCs subsets, CD103$^+$ CD11b$^+$ DDCs were found to be specialized in RA production, because they express high levels of RALDH-2 activity, as revealed both by quantitative RT-PCR and with a fluorescent RALDH substrate [114]. Waiting for the direct role of these populations to be assessed *in vivo*, it can be hypothesized that RA produced by CD11b$^+$CD103$^+$ DCs can act in a trans manner to favor Treg-cell induction by other migratory DC types. Similar, to the gut where Treg cells have to reach the LP and expand to perform their function, Treg cells generated in cLNs must reach the skin and reside there for a long time to fulfil their suppressive role and maintain regulatory memory [115] (Figure 26). It remains to be determined whether a specific APC population is needed to maintain a high number of Treg cells in the skin. In this regard, some evidence indicates that both DDCs and LCs expand the number of Treg cells [116][117].

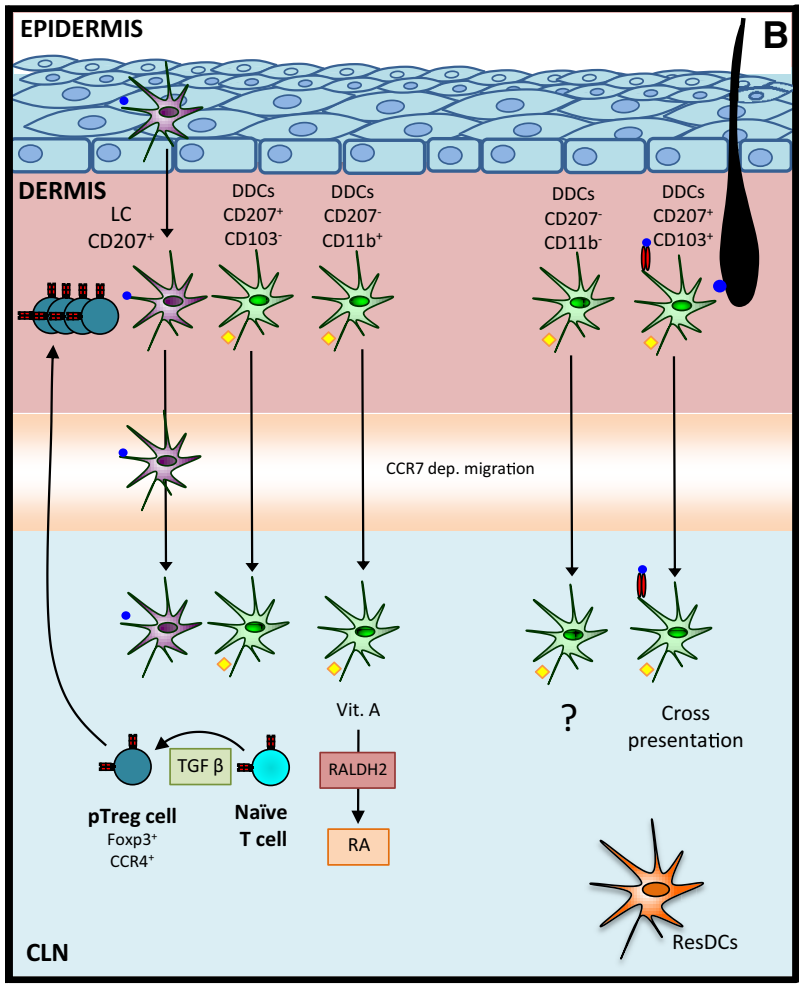


Figure 26 | mDCs in the skin [112].

LCs reside in the epithelium and migrate through the dermis and the lymph to the cLNs. DDCs migrate through the lymph to the draining cLNs and can be divided into 4 subsets: $CD207^+$ $CD103^+$ DDCs, which transport epidermal antigens to cLNs and cross-present them *in vivo*; $CD207^+$ $CD103^+$ DDCs, which can induce pTreg cells with the help of $CD207^+$ $CD11b^+$ DDCs, which produce a high amount of RA; and $CD207^+$ $CD11b^+$ DDCs, which make an unknown contribution to tolerance induction. Newly generated pTreg cells upregulate CCR4 and are directed to the skin where they can be expanded by resident LCs or DDCs.

1.4 Origin and physiological roles of inflammation

In 1794, Scottish surgeon John Hunter wrote that "Inflammation in itself is not to be considered as a disease but as a salutary operation consequent to some violence or some disease". That crucial insight emphasizes that the usual outcome of the acute inflammatory program is successful resolution and repair of tissue damage, rather than persistence of the inflammatory response, which can lead to scarring and loss of organ function [118].

Although references to inflammation can be found in ancient medical texts, apparently the first to define its clinical symptoms was the Roman doctor Cornelius Celsus in the 1st century AD. These symptoms came to be known as the four cardinal signs of inflammation: *rubor et tumor cum calore et dolore* (redness and swelling with heat and pain). Celsus mentions these signs in his treatise *De Medicina*, while describing procedures for treating chest pain, and in so doing became a medical celebrity [119]. The physiological basis of the four cardinal signs of inflammation was revealed much later by Augustus Waller in 1846 and Julius Cohnheim in 1867, who discovered leukocyte emigration from the blood vessels and other vascular changes characteristic of an acute inflammatory response. Analyzing living tissues under the microscope, Cohnheim observed vasodilation, leakage of plasma, and migration of leukocytes out of blood vessels and into the surrounding tissue [119].

The fifth cardinal sign, functio laesa (disturbance of function), was added by Rudolph Virchow in 1858. Notably, although the four cardinal signs of Celsus only apply to acute inflammation

accompanying wounds and infections, *functio laesa* is the only universal sign that accompanies all inflammatory processes. Virchow's main contribution to inflammation research was to establish the cellular basis of pathology, a dramatic departure from the traditional view of disease as an imbalance of the four humors, which had dominated medicine since the time of Hippocrates.

Another major milestone was the discovery of phagocytosis by Elie Metchnikoff and his theory of cellular immunity developed in 1892. Metchnikoff emphasized the beneficial aspects of inflammation and pointed out the key role of macrophages and microphages (neutrophils) both in host defense and in the maintenance of tissue homeostasis [120]. Meanwhile, Paul Ehrlich was developing the humoral theory of immunity following the discovery of serum therapy against diphtheria and tetanus toxins by Emil von Behring and Shibasaburo Kitasato in 1890. The role of serum components in immunity was further supported by the discovery of complement by Jules Bordet in 1896. Finally, the establishment of the germ theory of disease in the late 19th century by Robert Koch and Louis Pasteur was crucial for appreciating microbial agents as major inducers of the acute inflammatory response. Subsequent advances included the identification of different classes of inflammatory mediators, the pathways that control their production, and their mechanisms of action. Now it's known that inflammation comes in many different forms and modalities, which are governed by different mechanisms of induction, regulation, and resolution [119].

A typical inflammatory response consists of four components: inflammatory inducers, the sensors that detect them, the inflammatory

mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators (Figure 27).

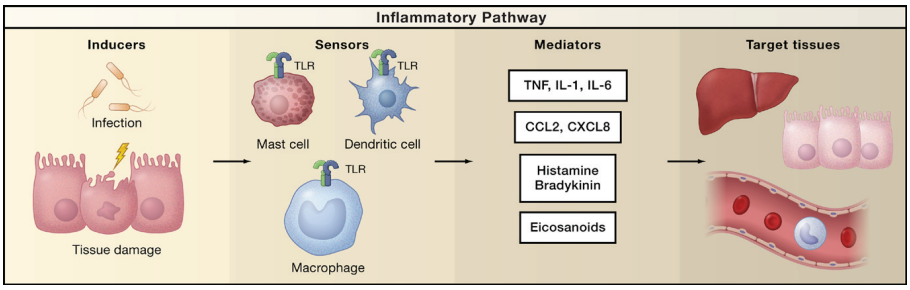


Figure 27 | Inflammatory Pathway Components [119].

The inflammatory pathway consists of inducers, sensors, mediators, and target tissues. Inducers initiate the inflammatory response and are detected by sensors. Sensors, such as TLRs, are expressed on specialized sentinel cells, such as tissue-resident macrophages, dendritic cells, and mast cells. They induce the production of mediators, including cytokines, chemokines, bioactive amines, eicosanoids, and products of proteolytic cascades, such as bradykinin. These inflammatory mediators act on various target tissues to elicit changes in their functional states that optimize adaptation to the noxious condition associated with the particular inducers that elicited the inflammatory response. The specific components shown represent only a small sample of the myriad different sensors, mediators, and target tissues involved in the inflammatory response.

Each component comes in multiple forms and their combinations function in distinct inflammatory pathways. The type of pathway induced under given conditions depends on the nature of the inflammatory trigger. Thus, bacterial pathogens are detected by receptors of the innate immune system, such as TLRs, and this induces the production of inflammatory cytokines (TNFα, IL-1, IL-6) and chemokines (CCL2 and CXCL8), as well as prostaglandins. These inflammatory mediators then act on target tissues, including local

blood vessels, to induce vasodilation, extravasation of neutrophils, and leakage of plasma into the infected tissue. In the case of sterile tissue injury in the absence of infection, acute inflammation promotes tissue repair and helps to prevent colonization of the damaged tissues by opportunistic pathogens [119]. The acute inflammatory response is normally terminated once the triggering insult is eliminated, the infection is cleared, and damaged tissue is repaired. Termination of the inflammatory response and transition to the homeostatic state is an active and highly regulated process known as the resolution of inflammation.

One of the first steps in the inflammatory process is edema formation, a fundamental event for the local accumulation of inflammatory mediators. Local swelling is also relevant for the activation of adaptive immunity since it favors free antigen transport to the draining lymph nodes. Antigens present in the inflamed tissues are delivered to the lymph nodes in two successive waves (Figure 28). In the first, antigens freely diffuse through lymphatic vessels and, in the later wave, the antigens are transported by DCs [121]. The increasing interstitial pressure due to the edema forces some of the fluid into lymphatic capillaries and favors entry of free antigen into the afferent lymphatics and the arrival of free antigen at the draining lymph nodes. Both waves of antigen transport are required for efficient activation of adaptive immune responses [121][67].

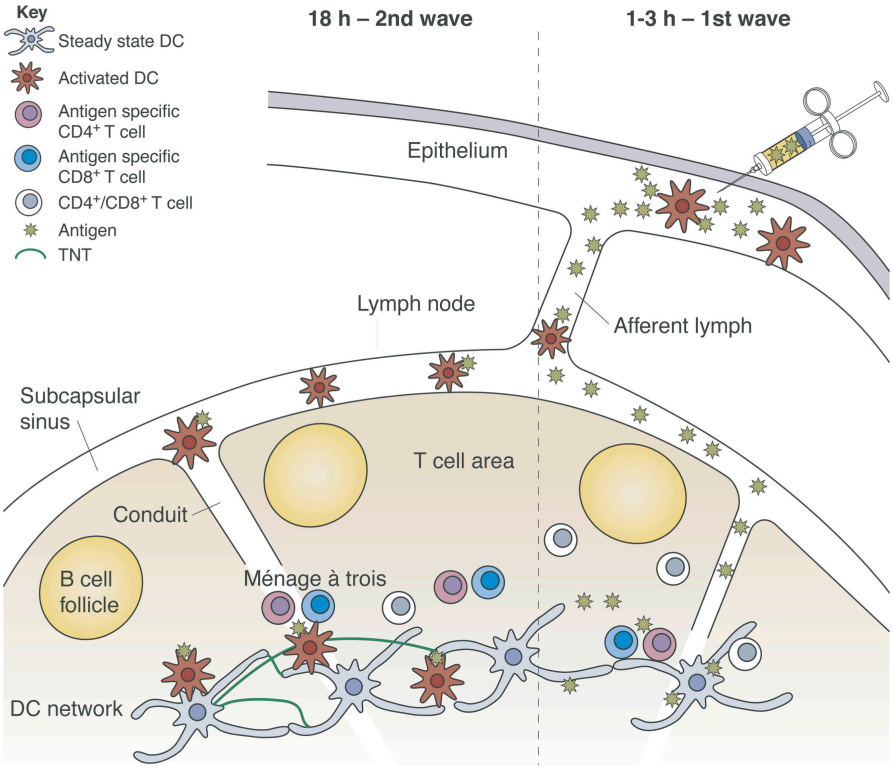


Figure 28 | Two waves of antigen delivery [121].

Antigen administered subcutaneously is delivered in two successive waves to the draining lymph node, and presented by different DCs which initiate different effector functions.

1.4.1 Prostaglandins and PGE_2

Prostaglandins (PGs) are members of the eicosanoid family and are produced by nearly all cells within the body [122]. Prostaglandins are lipid mediators that are not stored by cells; rather, they are synthesized from arachidonic acid via the actions of cyclooxygenase (COX) enzymes, either constitutively or in response to cell-specific trauma, stimuli, or signaling molecules. The most abundant prostanoid in the human body is PGE_2 [123]. Depending upon context, PGE_2 exerts homeostatic, inflammatory, or in some cases anti-inflammatory effects. Inhibition of PGE_2 synthesis has been an important anti-inflammatory strategy for more than 100 years [124].

Prostanoids are arachidonic acid metabolites and are generally accepted to play pivotal functions in inflammation, platelet aggregation, and vasoconstriction/relaxation. All prostanoids exhibit roughly the same structure as all are oxygenated fatty acids composed of 20 carbon atoms and containing a cyclic ring, a C-13→C-14 trans-double bond, and a hydroxyl group at C-15. Prostanoids can be classified into PG, which contain a cyclopentane ring, and Txs, which contain a cyclohexane ring. The first group is classified into types A to I, according to the modifications of this cyclopentane ring, in which types A, B, and C are believed not to occur naturally, but are produced during extraction procedures. Thus, naturally existing prostaglandins can be subdivided in prostaglandin D (PGD), E (PGE), F (PGF), and I (PGI). Likewise, thromboxanes are subdivided into TxA and TxB. The abbreviations are commonly followed by an index, which indicates the number of double bonds present in the various side

chains attached to the cyclopentane ring. Based on the number of these double bonds, prostanoids are further classified into three series (1, 2, and 3). The prostanoids in series 1, 2, and 3 are synthesized respectively from γ-homolinolenic acid, arachidonic acid, and 5,8,11,14,17-eicosapentaenoic acid. Among these precursor fatty acids, arachidonic acid is the most abundant in mammals (including humans), and as a result series 2 prostanoids are the most predominantly formed [125]. Prostanoids are rapidly synthesized in a variety of cells in response to various stimuli, such as inflammation, and act in an autocrine and paracrine fashion [126].

PGE_2, also known as dinoprostone, is the most abundant prostanoid in mammals and it is involved in regulating many different fundamental biological functions including normal physiology and pathophysiology [127].

The synthesis of PGs is initiated by the liberation of arachidonic acid, in response to various physiological and pathological stimuli, from the cell membrane by phospholipase A_2 (PLA_2). Arachidonic acid is converted to the prostanoid precursor PGG2, which is subsequently peroxidized to PGH2. Both enzymatic reactions are catalyzed by the protein COX, which consists of two forms: the constitutively expressed COX-1 is responsible for basal, and upon stimulation, for immediate PG synthesis, which also occurs at high AA concentrations. COX-2 is induced by cytokines and growth factors and primarily involved in the regulation of inflammatory responses. Following COX activity, prostanoid synthesis is completed by cell-specific synthases. In particular, PGE_2 is synthesized from PGH2 by cytosolic (cPGES) or by membrane-associated/microsomal (mPGES-1

or mPGES-2) prostaglandin E synthase [128]. Of these enzymes, cPGES and mPGES-2 are constitutively expressed and preferentially couple with COX-1, whereas mPGES-1 is mainly induced by pro-inflammatory stimuli, with a concomitant increased expression of COX-2 (Figure 29) [124].

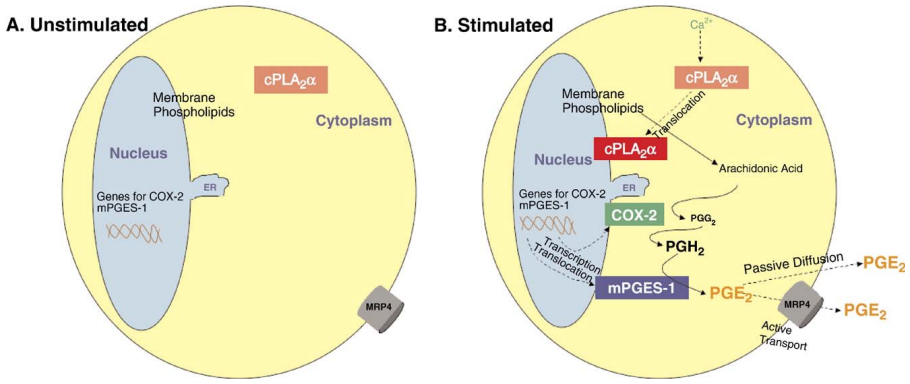


Figure 29 | Coordinate production of PGE_2 by cPLA2a, COX-2, and mPGES-1 [129]. (A) Unstimulated cell. cPLA2a is constitutively present in the cytoplasm. In unstimulated cells, COX-2 and mPGES-1 are not expressed. **(B)** Stimulated cell. Inflammatory stimulation results in calcium influx which leads to the translocation of cPLA2a from the cytosol to the nuclear membrane where it enzymatically hydrolyzes membrane phospholipids to release arachidonic acid. Inflammatory stimuli also induce the transcription and protein expression of both COX-2 and mPGES-1 at the nuclear membrane and endoplasmic reticulum. COX-2 transforms arachidonic acid to PGG2 which is subsequently converted to PGH2. mPGES-1 may then act on PGH2 to generate PGE_2. PGE_2 may exit the cell by simple diffusion, or by active transport via the MRP4 transporter.

In fact, it has been shown that mPGES-1 and COX-2 expression, is regulated in response to LPS by a TLR4/MyD88 dependent signaling pathway [130]. Notably, although the gene mPGES-1 is co-regulated

with COX-2, differences in the kinetics of the expression of the two enzymes suggest distinct regulatory mechanisms for their induction.

PGE_2 exhibits a broad range of biological activity in diverse tissues through its binding to specific receptors on plasma membrane. These receptors belong to the family of G protein-coupled receptors, and they can be divided into four subtypes (EP1-4), each of which is encoded by distinct genes. Whereas the "contractile" EP1 receptor induces calcium mobilization by phospholipase C activation via Gq protein, "relaxant" EP2 and EP4 receptors are known to activate adenylyl cyclase via stimulatory G protein. On the other hand, the "inhibitory" EP3 receptor reduces cAMP levels as it is coupled to inhibitory G proteins. In a flogistic context, PGE_2 plays a key role as an inflammatory mediator because it is involved in all processes leading to the classic signs of inflammation: redness, swelling and pain. Redness and edema result from increased blood flow into the inflamed tissue through PGE_2-mediated augmentation of arterial dilation and increased microvascular permeability. In fact, PGE_2 binds to EP2/4 on smooth muscle cells and endothelial cells (components of blood vessels), inducing a local vasodilation that results in edema formation. This process is a very important event in order to orchestrate early inflammatory immune responses [125].

1.5 Transplantation Tolerance

Earl C. Padgett first described the phenomenon of allograft rejection in 1932. He used nonrelated skin allografts to cover severely burned patients and reported that none of the skin allografts survived permanently. However, he observed that skin grafts from relatives seemed to survive longer than those from unrelated donors [131]. In 1943, Gibson and Medawar developed the first scientific explanation of the phenomenon of allorejection. They observed that patients who received autografts (tissue from the same individual transplanted to a different part of the body) accepted the tissue with no complications unlike patients that had received a sibling's skin allograft (tissue from a different individual belonging to the same species) who eventually rejected the allograft. In addition, they observed that a second skin transplant with skin from the same donor resulted in more rapid rejection compared with the first skin transplantation. The observation of the accelerated rejection of the second graft from the same donor was convincing evidence that supported the involvement of an immunological process during allograft rejection [132].

In 1948, Medawar and colleagues excluded an important role of antibodies in allograft rejection and designed an experiment to assess whether cellular components of the immune system are responsible for transplant rejection. They injected cells from the allograft-draining lymph node from transplanted mice into mice recently transplanted with skin from the same donor. They observed that mice rejected the allograft as similar to mice transplanted for a second time, indicating

that cellular components of the immune system are responsible for the generation of the immune response against the allograft [133].

Advances achieved in surgical techniques in parallel with improvements in knowledge of the immune mechanisms mediating allograft rejection allowed the first kidney transplant in 1963 [133]. Joseph E. Murray and his colleagues at Peter Bent Brigham Hospital in Boston performed the first successful kidney transplant from one twin to another. It was a great advance in medicine, demonstrating that it was possible to perform successful organ transplants in humans, but it was still necessary to solve the problem of rejection between unrelated donors [134]. Since then, different pharmacological treatments have been developed in order to induce an immunosuppressive state that allows the acceptance of an allograft transplant between unrelated donors.

The immunosuppressive effects of CsA were discovered in Switzerland in 1972. Some trials to compare CsA versus azathioprine and steroids were developed and the promising results led to clinical approval for the use of CsA in human transplants in 1980. The introduction of CsA contributed substantially to the improvement of allograft and patient survival [135]. The massive development of immunosuppressive drugs opened the door to organ transplantation, extending to other organs such as the liver, lungs, and heart. In parallel with the increased number of organ transplants, several investigators are currently working on developing new immunosuppressive drug protocols that will further improve the outcome and reduce tissue toxicity in transplanted patients. However, despite these efforts, currently all immunosuppressive drugs have

serious side effects including nephrotoxicity, development of malignancies, and susceptibility to infections by opportunistic pathogens. For this reason, immunologists face a new challenge in developing strategies to reduce or eliminate the use of immunosuppressive drugs in organ transplants. These efforts are being focused on reeducating the immune system or inducing allograft-specific tolerance mechanisms.

1.5.1 Mechanisms of Allograft Rejection

Despite the advances in transplantation tolerance, the mechanisms that mediate allograft rejection have not yet been fully described. Clinical rejection may occur at any time following transplantation and therefore is classified according to the time in which it occurs after the transplant.

1.5.1.1 Hyperacute Rejection

Hyperacute rejection may occur within a few minutes to hours after transplantation. It is due to preformed alloantibodies by the recipient, mainly against MHC antigens, which become deposited in the allograft and induce complement activation and recruitment of inflammatory cells that trigger platelet aggregation, with consequent capillary obstruction and tissue necrosis. This type of rejection is not very common nowadays because it is easily prevented by blood typing and crossmatching prior to transplantation [134].

1.5.1.2 Acute Rejection

Acute rejection occurs days to months after the transplant. It consists of a tissue injury process mediated by alloantibodies and alloreactive T cells, mainly in response to MHC antigens. Acute cellular rejection is due to alloreactive cytotoxic $CD8^+$ T cells that recognize the alloantigens present in the transplanted tissue and carry out its destruction. The lesion occurs mostly in the endothelial cells, which in response to the injury develop a microvascular endothelialitis and arteritis. Antibody-mediated rejection, on the other hand, is

characterized by alloantibodies that induce complement activation, neutrophil recruitment, and the consequent inflammation and coagulation activation that results in thrombotic ischemia of the transplanted tissue. This type of rejection was a critical obstacle to overcome in the early steps of organ transplantation; however, today it is well managed by the employment of immunosuppressive drugs [134].

1.5.1.3 Chronic Rejection

Chronic rejection is today the main cause of allograft failure. It occurs months or years following transplantation. Organ failure occurs due to chronic inflammation that triggers the proliferation of intimal smooth muscle cells and results in vascular occlusion and ischemic damage. The pathogenesis involves the chronic secretion of cytokines by activated T lymphocytes and the production of alloantibodies that are able to activate the complement system through the classical pathway, thus generating chronic damage [136]. Despite the advances in immunosuppressive therapy, this type of rejection remains unresolved and it is necessary to develop new strategies to improve organ acceptance [134].

Alloantibodies have an important role in the different types of rejection mechanisms. These antibodies can be directed against HLA (major antigens) or non-HLA molecules (minor antigens). Therefore it is important to detect their presence in order to prevent possible events of organ rejection.

1.5.2 Immune Tolerance

One of the hallmarks of the adaptive immune system is its ability to recognize a vast number of different antigens. This ability is a consequence of the large lymphocyte repertoire, in which each cell has a different antigen receptor generated by the process of somatic recombination. This process is able to produce an estimate of 10^{15} different lymphocyte clones, each with a different antigen receptor that can hypothetically recognize any naturally occurring structure [137]. Since somatic recombination is a random process, it generates T cell clones that can recognize self-structures or self-peptides (auto-antigens). The mechanism used by the immune system in order to avoid a possible harmful immune response against an individual's own cells and tissues is known as immune tolerance and can be classified into central and peripheral tolerance (Figure 30) [134].

1.5.2.1 Central Tolerance

Central tolerance occurs in the thymus and allows the deletion of a major percentage of auto-reactive T cells. The thymus is the major site of maturation of T cells and can be anatomically and functionally separated into two zones: the thymic cortex and medulla. The cortex is the region where the process of positive selection occurs and contains densely packed immature thymocytes. The medulla contains loosely packed mature lymphocytes and is the site where the process of negative selection takes place [138].

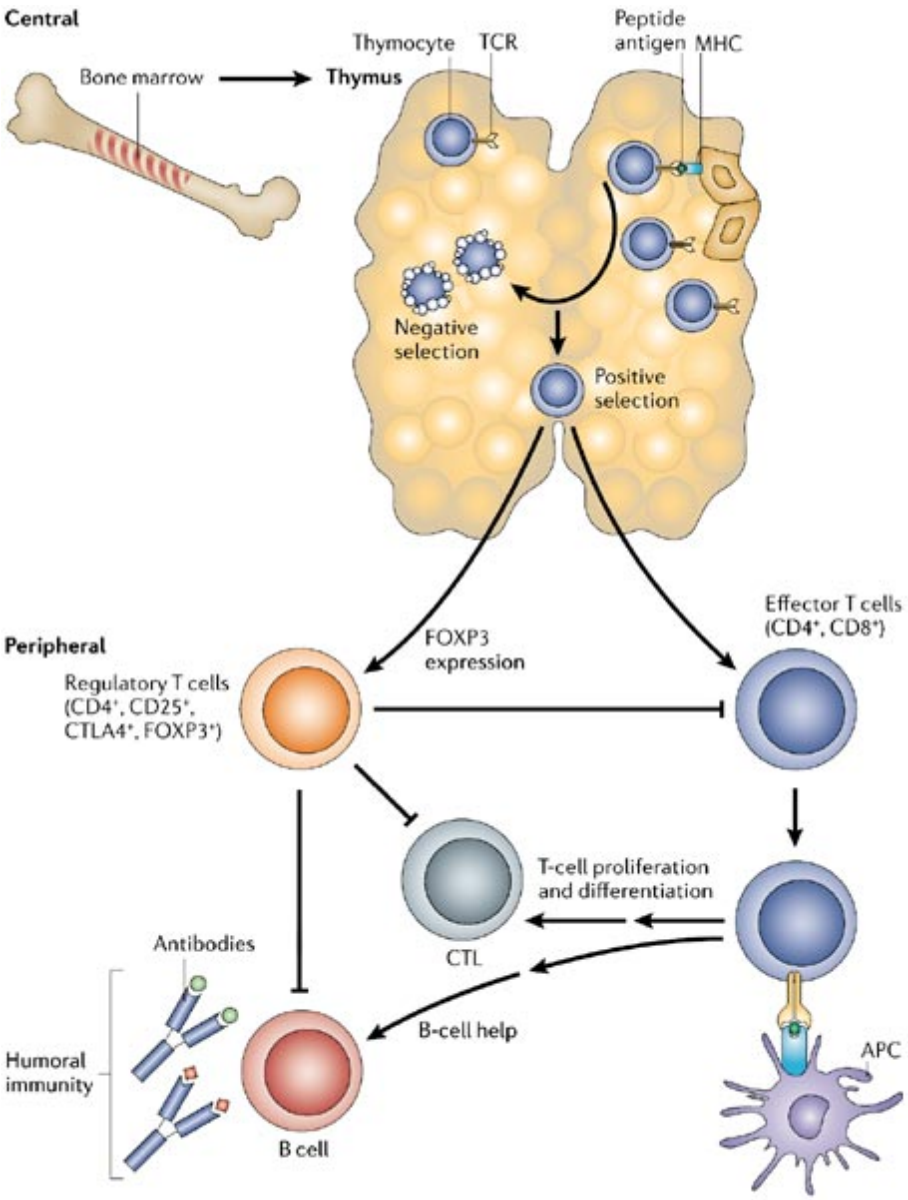


Figure 30 | Central and peripheral tolerance mechanisms [139].

The top panel depicts events involved in central tolerance, which takes place in the thymus. Thymocytes undergo a maturation and selection process in which strongly self-reactive thymocytes, as determined by interactions with MHC proteins in combination with self peptides, are deleted. Similarly, non-functional thymocytes undergo apoptosis. Only thymocytes that are activated by self peptide and MHC

below a certain threshold are positively selected and migrate into the periphery as mature T cells. Most of these thymic emigrants develop into effector $CD4^+$ and $CD8^+$ T cells, and mediate both humoral (antibody-mediated) and cellular immune responses. A small percentage of T cells that emigrate from the thymus express the transcription factor Foxp3 and develop into $CD4^+$, $CD25^+$ and $CTLA4^+$ regulatory T cells (natural Treg cells). Once in the periphery, these cells are key mediators of peripheral tolerance. The mechanism of action of Treg cells is incompletely understood, but includes actions at many levels of the effector immune response. Treg cells might inhibit T-cell activation by APCs and inhibit T-cell differentiation into cytotoxic effector cells, as well as preventing T cells from providing help to B cells in the production of antibodies. $Foxp3^+$ Treg cells can also be generated from peripheral T cells.

After originating in the bone marrow, the early precursors of T cells enter the thymus and migrate into the cortex where most of the subsequent maturation events take place. These T cell precursors do not express the TCR, CD3, ζ chains, CD4, or CD8 coreceptors and therefore are called $CD4^-CD8^-$ double negative (DN) thymocytes. Within the cortex, DN cells undergo TCR rearrangement and become $CD4^+CD8^+$ double positive (DP) cells, which express the TCR α and β chains as well as both CD4 and CD8 coreceptors.

Double positive cells are programmed to undergo apoptosis by default unless they receive a "rescue signal" which is provided by cortical thymic epithelial cells (cTEC) that express self-peptide/MHC. Only thymocytes recognizing self-peptide/MHC complex with low avidity will receive the rescue signals and will continue with the maturation process. The DP clones that are rescued will continue with the process of maturation and will become single positive (SP) cells that express either the CD4 or CD8 coreceptor [140].

The acquisition of adequate chemokine receptors allows SP cells to exit the thymic cortex and to enter the medulla. It is in the medulla where they will continue with the negative selection process, which is crucial to central tolerance [140] [134]. One of the questions regarding negative selection is how autoreactive clones that recognize self-peptides that are not normally found in the thymus are controlled. Recent evidence has demonstrated that the AIRE transcription factor is involved in the promiscuous gene expression in mTEC cells that allows an increase in the repertory of auto-antigens presented by APCs during negative selection [141].

As a consequence of positive and negative selection, T cells that leave the thymus and populate peripheral lymphoid tissues are self-MHC restricted and tolerant to many auto-antigens.

1.5.2.2 Peripheral Tolerance

Although central tolerance mechanisms are efficient in deleting the auto-reactive T cell clones that recognize self-antigen/MHC complex with high affinity, some autoreactive T cells are able to bypass this control and exit the thymus [142]. In the periphery, these auto-reactive clones are able to induce autoimmune responses, generally in response to an inflammatory environment such as one triggered during infection [143]. Therefore, there is a constant threat of potential autoimmune responses due to the escape of auto-reactive T cells clones to the periphery. These potentially harmful auto-reactive cells must be effectively controlled by peripheral tolerance mechanisms.

Peripheral tolerance mechanisms involve the deletion of activated effector T cells, anergy induction, clonal exhaustion, and active

regulation of effectors T cells [144]. Tregs mediate active regulation of the immune response preventing autoimmune and inflammatory diseases and restraining responses to infections of viral, bacterial, or parasitic origin. Moreover Tregs can restrain immune responses directed towards tumors or transplanted tissue. Two different types of Tregs have been described; natural $CD4^+CD25^+Foxp3^+$ regulatory T cells (nTregs), which are generated in the thymus and regulate immune responses in the periphery, and inducible $CD4^+CD25^+Foxp3^+$ regulatory T cells (iTregs) which develop in the periphery from naive $CD4^+$ T cells after exposure to antigens in a specific cytokine microenvironment, tolerogenic APCs, or immunosuppressive drugs [134].

1.5.2.3 Tolerogenic Dendritic Cells

Dendritic cells play an important role in establishing peripheral tolerance. DCs are crucial for priming antigen-specific T cell responses, including those to alloantigens. However, they can also promote tolerogenic responses [145].

Initially, immature conventional myeloid DCs that express low levels of MHC class II and co-stimulatory molecules at their cell surface were identified as the dominant type of DC with the capacity to induce T cell tolerance. Indeed, immature DCs can promote tolerance to solid-organ allografts and bone marrow grafts [146]. For example, a single injection of immature donor-derived DCs seven days before the transplantation of an MHC-mismatched heart allograft extends the survival of the allograft or prolongs it indefinitely [147]. Moreover, the injection of donor-derived DCs prevents the rejection of

MHC-mismatched skin grafts and protects recipient mice from developing lethal acute GVHD [148][149]. The tolerogenic effects of immature DCs can be enhanced by administering the cells together with other immunomodulatory agents, such as drugs that block the CD40–CD40L co-stimulatory axis.

pDCs can also promote tolerance in transplantation [150]. In experimental models, pDCs acquired alloantigens in the allograft and then migrated to the draining lymphoid tissue, where they induced the generation of Treg cells. In mice, pre-pDCs appear to be the principal cell type that facilitates haematopoietic stem cell engraftment and the induction of donor-specific skin graft tolerance in allogeneic recipients [151].

In summary, both myeloid DCs and pDCs can promote tolerance to alloantigens, and DC maturation in itself does not appear to be the distinguishing feature that separates immunogenic DC functions from tolerogenic ones. However, despite the tolerogenic functions of DCs discussed above, the use of DCs to facilitate the induction of operational tolerance is not without risk. DCs are better known for their ability to prime the immune system. Indeed, DCs pulsed with antigens are being used clinically as vaccines to stimulate immune responses to tumour antigens. Using DCs as a cellular therapy in transplantation may therefore carry the risk of sensitizing the recipient [145].

1.6 Scope of Thesis

DCs sense and respond to a wide range of microorganism through specialized germline-encoded receptors called PRRs, which are able to recognize molecular patterns expressed by various microorganisms and endogenous stimuli. Following activation with LPS, DCs sequentially acquire the ability to produce soluble and cell surface molecules critical for the initiation and control of innate and then adaptive immune responses. The production of most of these factors is regulated by the activation of TLR4-MD2 pathway. Nevertheless, in my laboratory it has been recently demonstrated that, following LPS exposure, different NFAT isoforms are also activated [63]. The initiation of the pathway that leads to nuclear NFAT translocation is totally dependent on CD14 that, through the activation of src family kinases and PLCγ2, leads to Ca^{2+} mobilization and calcineurin activation. Nuclear NFAT translocation is required for IL-2 production and apoptotic cell death of terminally differentiated DCs.

In the present work, we analyzed the role of CD14-NFAT pathway in a preclinical model of skin edema formation and its implications in antigen delivery. In addition we propose a new NFAT inhibitor as tool for studying *in vivo* the role of the activation of CD14-NFAT pathway in DCs in a model of acute transplant rejection.

Chapter 2: CD14 and NFAT mediated lipopolysaccharide-induced skin edema formation in mice.

Edema formation is one of the first steps in the inflammatory response and it is fundamental for the local accumulation of inflammatory mediators. Here, we showed that tissue-resident DCs are the main source of PGE_2 and the main controllers of tissue edema formation in a mouse model of LPS-induced inflammation. LPS exposure induces the expression of mPGES-1, a key enzyme in PGE_2 biosynthesis, in DCs, but not in macrophages. mPGES-1 activation, PGE2 production, and edema formation required the CD14-NFAT pathway. Moreover, DCs can regulate free antigen arrival at the draining lymph nodes by controlling edema formation and interstitial fluid pressure in the presence of LPS. We therefore concluded that the CD14/NFAT/mPGES-1 pathway represents a possible target for the development of new anti-inflammatory therapies.

Chapter 3: Study of the Role of the NFAT pathway Activation in innate immune cells during Acute Transplant Rejection.

It has been demonstrated that NFAT is important for the interaction between innate immune cells and lymphocytes. In particular its known that activatory DCs produce IL-2 and CD25, both regulated by NFATc, in the first few hours after interaction with T cells. DC-delivered IL-2 is than transpresented to T cells via CD25. Since naïve T cells start to express CD25 only many hours after antigen encounter, the DC-mediated presentation of the IL-2/CD25 complex represents a

very efficient system for T cell priming *in vitro*. The study of the role of the NFATc signalling pathway *in vivo* presents several difficulties due to the redundancy of the system because of the presence of different isoforms with overlapping functions. Thus the generation of new tools allowing the inhibition of all NFATc isoforms in vivo will be necessary for the comprehension of the role of this signalling pathway in innate immune cell types. In addition to a pure mechanistic aspects, these studies have fundamental medical implications. CsA and FK506 are the most commonly used drugs in the treatment of acute transplant rejection. These drugs are used in theory to block IL-2 and other NFATc dependent cytokines production by T cells. Although highly successful, CsA and FK506 have several side effects that result from the general inhibition of the enzymatic activity of Cn, which play other roles besides NFAT activation, and they are not specific for phagocytes.

Thus, we proposed a new NFAT inhibitor specific for innate myeloid cells as treatment for acute transplantation rejection.

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Chapter 2: CD14 and NFAT mediated lipopolysaccharide-induced skin edema formation in mice.

Department of Biotechnology and Biosciences
University of Milano-Bicocca, Milan, Italy

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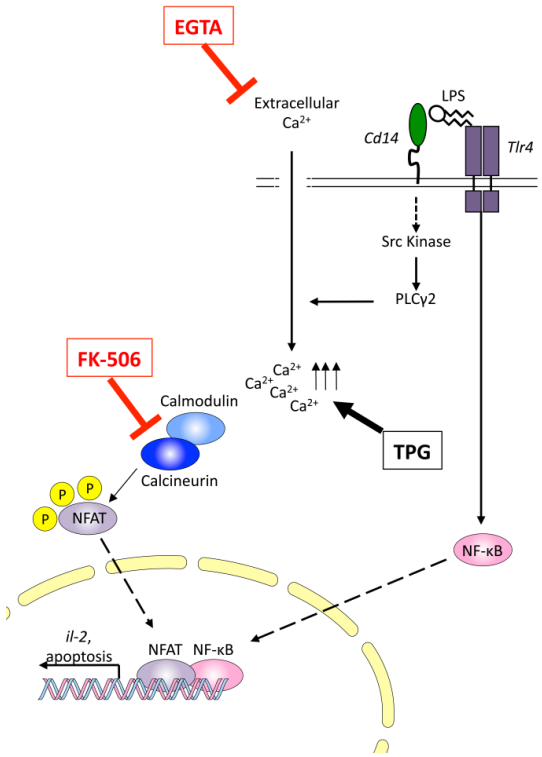
Inflammatory processes are initiated by innate immune system cells that perceive the presence of pathogens or microbial products through the expression of PRRs [1]. Following the encounter with their specific ligands, PRRs initiate a signal transduction pathway, leading to the activation of transcription factors that, in turn, regulate the expression of proinflammatory cytokines and costimulatory molecules that are important for the activation of innate and adaptive responses [2][3]. Among the PRRs, the receptor complex of the smooth form of LPS, a major constituent of the outer membrane of Gram-negative bacteria, is the best characterized. This particular receptor complex is composed of a series of proteins, including LPS-binding protein (LBP), MD2, CD14, and TLR4, required for LPS recognition, binding, and the initiation of the signaling cascade. We have recently demonstrated that CD14 is at the apex of all cellular responses to LPS [4] by controlling LPS recognition and TLR4 trafficking to the endosomal compartment with the consequent initiation of both the MyD88-dependent and TRIF- dependent pathways [5]. At the end of the signaling cascade, different transcription factors, including NF-κB, AP-1, and IRFs, are activated [6].

Recently, the NFAT isoforms have also been included among the transcription factors activated through PRR signaling, particularly in conventional DCs. NFATs translocate to the nucleus following dectin 1 activation with curdlan and CD14 engagement by LPS [7][8]. Therefore, CD14 has signal transduction capabilities as well. While NF-κB and AP-1's roles in DCs following activation have been largely defined, for instance, regulation of inflammatory cytokine production, costimulatory molecule expression, antigen uptake, and

processing and regulation of DC migration, most of the functions of NFAT remain to be elucidated. The only identified NFAT activities in activated DCs include regulation of IL-2 and IL-10 production and terminal differentiation and apoptotic death [7] [8].

In a scrutiny of data sets for the identification of genes regulated by the DC-specific CD14/NFAT signaling pathway triggered by LPS, we identified *Ptges1* as a potential transcriptional target [7]. *Ptges1* codes a protein called microsomal PGE synthase-1 (mPGES-1). This protein, together with cytosolic PLA2 (cPLA2) and COX-2, coordinates a multistep biosynthetic process leading to the release of PGE_2 [9][10][11]. In particular, following cell exposure to inflammatory stimuli, cPLA2 translocates from the cytosol to the nuclear membrane, where it hydrolyzes membrane phospholipids to form arachidonic acid. Inflammatory stimuli also induce the expression of COX-2 and mPGES-1. COX-2 acts on arachidonic acid and converts it to PGG2, which is in turn converted to PGH2. Finally mPGES-1 converts PGH2 to PGE_2. Therefore, all these 3 enzymes are required to generate PGE_2 [12], one of the most versatile prostanoids. PGE_2 is involved in the regulation of many physiological and pathophysiological responses, including local edema formation in inflammation through vasodilatation [13]. We thus hypothesized that CD14-dependent NFAT activation in DCs was required for efficient PGE_2 production and, consequently, for the local generation of edema following LPS exposure. Herein we report that this prediction was indeed correct and that local edema formation following LPS exposure is induced by tissue-resident DCs via PGE_2 production in a CD14-NFAT–dependent manner.



Supplementary Figure 1 | CD14-dependent and TLR4-independent NFAT activation in DCs.

CD14 has autonomous signaling functions. Upon LPS engagement, CD14 transiently recruits and activates a SKF member. Active SFK then phosphorylates PLC2, which in turn catalyzes the hydrolysis of PI(4,5)P2 into the second messengers DAG and IP3. IP3 directly triggers Ca^{2+} influx. The increased intracellular Ca^{2+} concentration stimulates activation of calcineurin, which dephosphorylates NFAT and promotes its nuclear translocation. EGTA and FK-506 are two inhibitors of the NFAT pathway. EGTA blocks extracellular Ca^{2+} influxes and FK-506 inhibits calcineurin activation. Diversely, thapsigargin (TPG) is an activator of the NFAT pathway. By blocking the SERCA pumps induces an increase of intracellular Ca^{2+} concentration and therefore NFAT activation.

2.1 *Ptges-1* is a transcriptional target of NFAT in DCs upon LPS stimulation.

We have recently observed that DC stimulation with LPS induces the activation of NFAT proteins [7]. In particular, LPS induces the activation of Src family kinases and PLCγ2, the influx of extracellular Ca^{2+}, the consequent calcineurin activation, and finally, calcineurin-dependent nuclear NFAT translocation. The initiation of this pathway is independent of TLR4 engagement and depends exclusively on CD14 (Supplemental Figure 1; supplemental material available online; doi: 10.1172/JCI60688DS1).

To investigate the role of NFAT in DCs following LPS exposure, we previously performed a kinetic global gene expression analysis. Immature DCs were compared with activated DCs at different time points following LPS stimulation in conditions in which NFAT nuclear translocation was either allowed or not. Ptges1 was selected among the specific NFAT targets [7].

Here, we validated this observation by quantitative RT-PCR (qRT-PCR) in mouse ex vivo and BM-derived DCs (BMDCs). We observed a strong induction of mPGES-1 mRNA in WT DCs after LPS stimulation (Figure 1A and Supplemental Figure 2A), a response that was greatly impaired in $CD14^{-/-}$ cells (Figure 1A and Supplemental Figure 2A). Blocking NFAT activation in *ex vivo* WT DCs by preincubating cells with the Ca^{2+} chelator EGTA or the calcineurin inhibitor FK-506 also resulted in reduced mPGES-1 expression (Figure 1B). The same results were obtained using BMDCs (Supplemental Figure 2B).

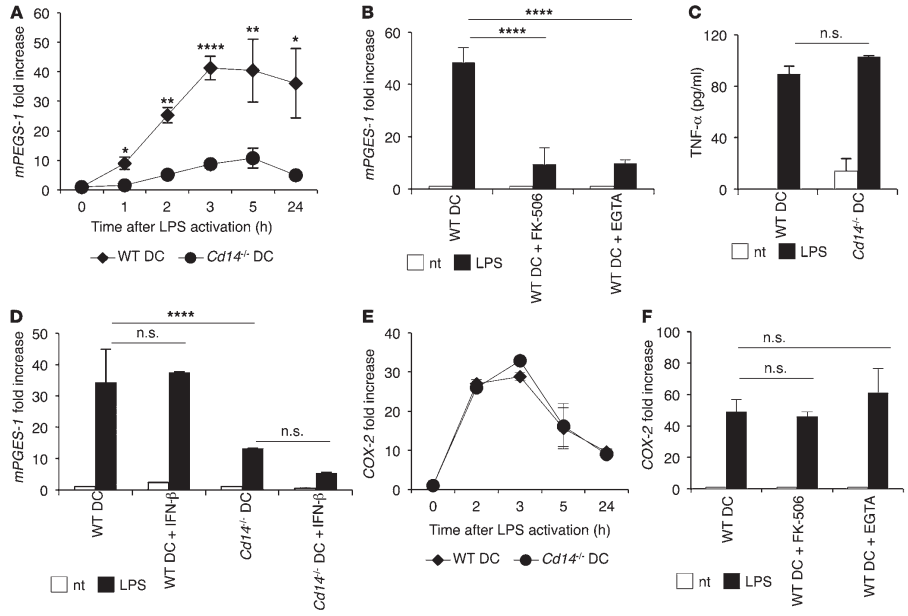
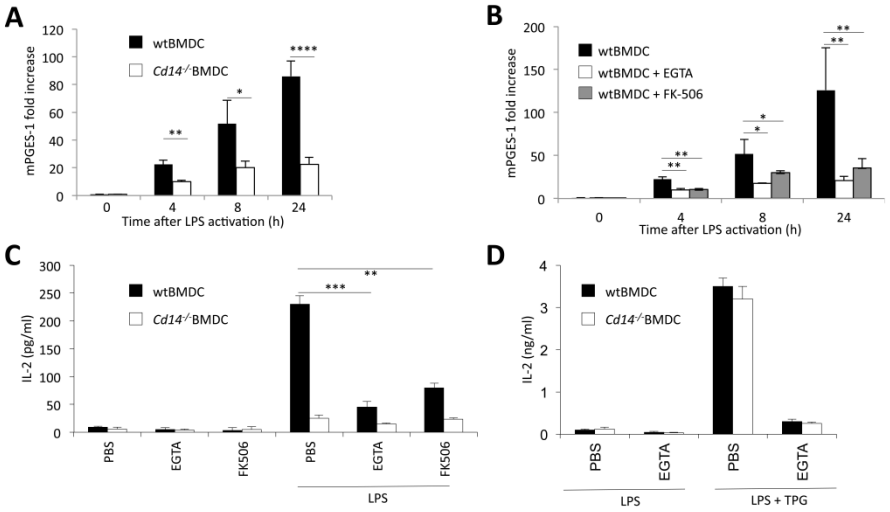


Figure 1 | CD14-dependent NFAT activation induced by LPS in DCs regulates mPGES-1 expression in vitro.

(A) Real-time PCR analysis of mPGES-1 mRNA induction kinetics in WT and CD14-deficient *ex vivo* DCs stimulated with LPS (1 μg/ml). (B) Upregulation of mPGES-1 mRNA after 3 hours of LPS administration by *ex vivo* WT DCs pretreated with PBS, FK-506 (1 μM, 90 minutes), or EGTA (2 mM, 30 minutes). (C) Production of TNF-α by *ex vivo* WT and $Cd14^{-/-}$ DCs following LPS exposure evaluated by ELISA. (D) Upregulation of mPGES-1 mRNA by ex vivo WT and $CD14^{-/-}$ DCs treated or not with IFNβ (50 U/ml) 1 hour after LPS (total LPS treatment 3 hours). (E) Real-time PCR analysis of COX-2 mRNA induction kinetics by WT and CD14-deficient *ex vivo* DCs stimulated with LPS (1 μg/ml). (F) Upregulation of COX-2 mRNA after 3 hours of LPS administration by WT *ex vivo* DCs pretreated with PBS, FK-506 (1 μM, 90 minutes pretreatment), or EGTA (2 mM, 30 minutes pretreatment). Values represent means of at least 3 independent experiments performed in duplicate + SEM. *P < 0.05; **P < 0.005; ****P < 0.00005. nt, not treated.



Supplementary Figure 2 | mPGES-1 is a potential target of CD14/NFAT signaling in BMDCs.

(A) Real-Time PCR analysis of mPGES-1 mRNA induction kinetics in wt and CD14-deficient BMDCs stimulated with LPS (1 g/ml). (B) Real-Time PCR analysis of mPGES-1 mRNA up-regulation after LPS (1 g/ml) administration in wt BMDCs pre- treated with PBS, FK-506 (1 M, 90 min) or EGTA (2 mM, 30 min) at the indicated time points. (C, D) Production of IL-2 by wt and $CD14^{-/-}$ BMDCs in the indicated conditions; TPG, thapsigargin (50 nM). Values represent at least three independent experiments performed in duplicate + s.e.m. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

We excluded that a reduced activation of NF-κB accounted for the defective mPGES-1 upregulation in $CD14^{-/-}$ DCs [14]. by using doses of LPS (1 μg/ml) that allowed direct agonist detection by TLR4 without an absolute requirement for CD14, as evidenced by the ability of $CD14^{-/-}$ DCs to normally secrete TNF-α (Figure 1C). Similarly, an impairment of CD14-dependent IRF3 activation [4][15] could not

explain our observations on mPGES-1 transcription. Coadministration of IFN-β (directly controlled by IRF3) did not restore mPGES-1 induction in LPS-treated $CD14^{-/-}$ DCs (Figure 1D). Supporting the hypothesis of NFAT being the key factor, mPGES-1 induction by LPS correlated with the production of IL-2, a bona fide marker for NFAT activation in DCs ([7] and Supplemental Figure 2C). The other key enzyme for PGE_2 production, COX-2, has been also reported to be regulated by NFAT in other experimental settings [16].

Therefore, we determined whether CD14 influenced its expression. However, COX-2 induction by LPS in *ex vivo* DCs was not affected by CD14 deficiency (Figure 1E). Analogously, blocking Ca^{2+} fluxes or NFAT activation did not alter LPS-induced COX-2 expression by DCs (Figure 1F).

A Western blot analysis confirmed the expression data. As shown in Figure 2A, LPS induced mPGES-1 synthesis in WT, but not in $CD14^{-/-}$, cells in a way dependent on Ca^{2+} fluxes and NFAT activation. Moreover, the deliberate induction of Ca^{2+} fluxes and NFAT activation by TPG ([7] and Supplemental Figure 2D) restored mPGES-1 upregulation in $CD14^{-/-}$ DCs (Figure 2A). Conversely, LPS-induced COX-2 synthesis was not influenced by CD14 expression or NFAT activation (Figure 2A). Together, these results indicate that CD14-dependent NFAT activation controls mPGES-1 but not COX-2 expression.

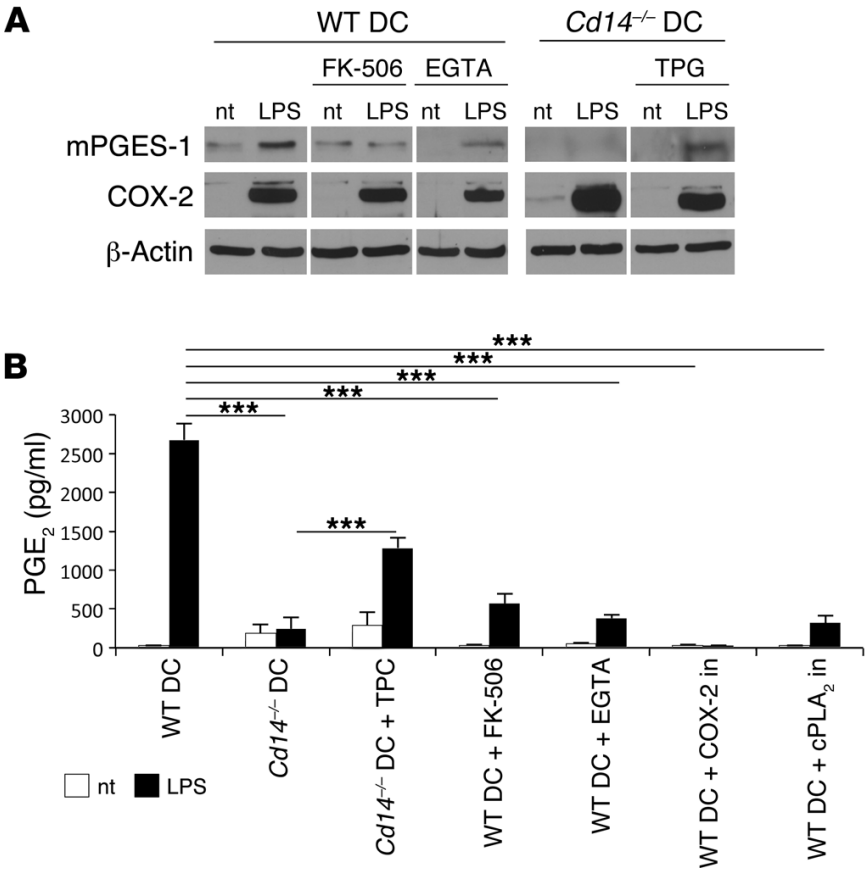


Figure 2 | CD14-dependent NFAT activation induced by LPS in DCs regulates PGE_2 synthesis *in vitro*.

(A) Western blot analysis of mPGES-1 and COX-2 induction in WT and CD14-deficient BMDCs 4 hours after LPS (1 μg/ml) and/or TPG (50 nM) treatment. Where indicated, the cells were pretreated with FK-506 or EGTA. The experiment was repeated 3 times with similar results. (B) PGE_2 production by *ex vivo* DCs 4 hours after LPS stimulation. WT and $Cd14^{-/-}$ DCs were treated with LPS or LPS plus TPG (50 nM) or TPG alone; WT DCs were also treated with LPS and/or FK506, LPS and/or EGTA, LPS and/or COX-2 inhibitor (in) (1 μM, 30 minutes pretreatment), LPS and/or cPLA2 inhibitor (cPLA2 in, 1 μM, 30 minutes pretreatment). Values represent means of at least 3 independent experiments performed in duplicate + SEM. ***P < 0.0005.

2.2 PGE_2 production by DCs following LPS stimulation depends on CD14 and NFAT.

We then measured the synthesis of PGE_2. Consistent with the mPGES-1 results, PGE_2 release *in vitro* was strongly impaired in $CD14^{-/-}$ compared with WT DCs (Figure 2B and Supplemental Figure 3A). Moreover, blocking NFAT activation by blocking Ca^{2+} influx with EGTA or blocking calcineurin by means of FK-506 strongly affected LPS- induced PGE_2 production by WT DCs (Figure 2B and Supplemental Figure 3A). We were able to restore PGE_2 production in $CD14^{-/-}$ DCs by coupling LPS stimulation with TPG (Figure 2B). As control, we confirmed the necessary role of cPLA2 and COX-2 for LPS-induced PGE_2 synthesis (Figure 2B and Supplemental Figure 3A). Moreover, the analysis of TNF-α production indicated that the tested conditions did not influence the pathway of NF-κB activation (Supplemental Figure 3B).

We have recently shown that different LPS species may elicit slightly different innate responses by initiating different signaling pathways [17]. Therefore, we evaluated whether LPS from different sources were equally able to induce mPGES-1, COX-2, and PGE_2 production. As shown in Supplemental Figure 3, C–E, all of the tested LPS species induced mPGES-1 and COX-2 upregulation and PGE_2 production with a similar efficiency.

These data indicate that PGE_2 production by DCs following LPS stimulation depends on the Ca^{2+}/calcineurin pathway activation via the

engagement of CD14. This pathway regulates mPGES-1, but not COX-2 expression.

2.3 Edema formation following LPS exposure depends on DCs.

Following interaction with TLR agonists, DCs remain at the site of infection for the time necessary to take up the antigens [18][19]. During the time of persistence at the infected tissue, DCs actively participate in the sustainment of the inflammatory process [20][21]. Subsequently, DCs acquire the ability to migrate and reach the draining lymph nodes 2 to 3 days after infection [22][23]. Moreover, PGE_2 is well known to sustain the formation of edema at the inflammatory site during the innate phase of an immune response [13]. Given the initial persistence of DCs at the site of inflammation and their ability to produce PGE_2, we investigated whether DCs could participate in edema formation. To this purpose, we used DOG mice, an animal model that expresses the diphtheria toxin receptor (DTR) under the control of the CD11c promoter. In these animals, an efficient conditional ablation of DCs can be induced by DT injections [24]. By performing consecutive DT injections, we were able to conditionally ablate DCs in lymphoid and nonlymphoid organs and tissues including the skin (Figure 3A and Supplemental Figure 4, A–D). Importantly, such a treatment did not cause any significant alteration in either macrophage or granulocyte populations in the footpad (Figure 3A and Supplemental Figure 4, C and D). The quantitative analysis of cell population distribution in the selected peripheral tissue was performed by qRT-PCR of cell-specific mRNAs, as previously described [25], and by flow cytometry.

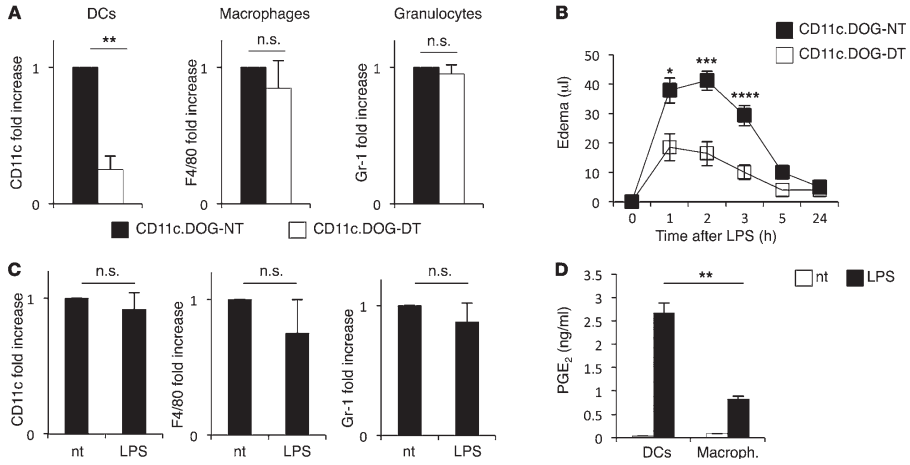
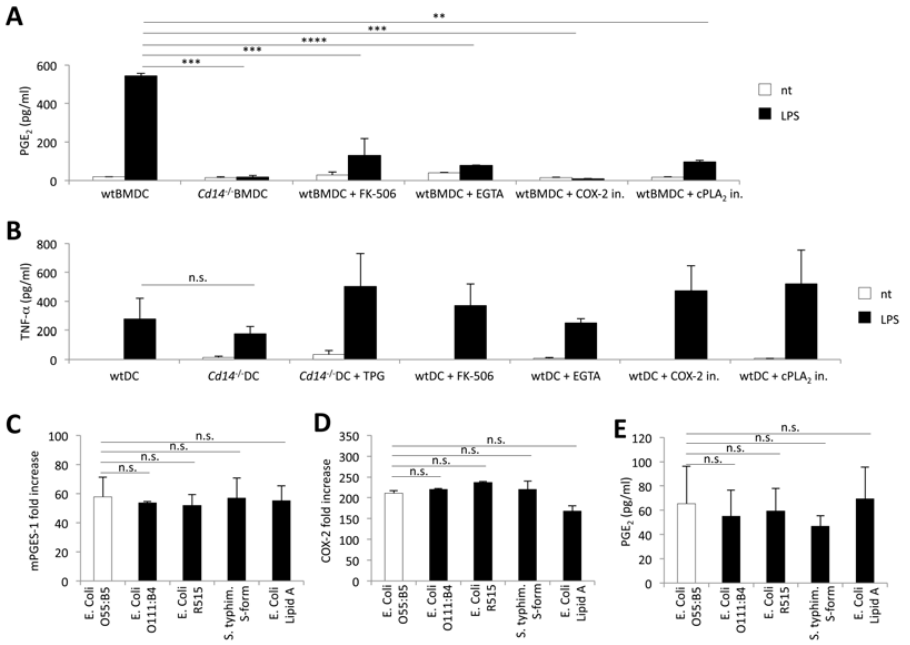


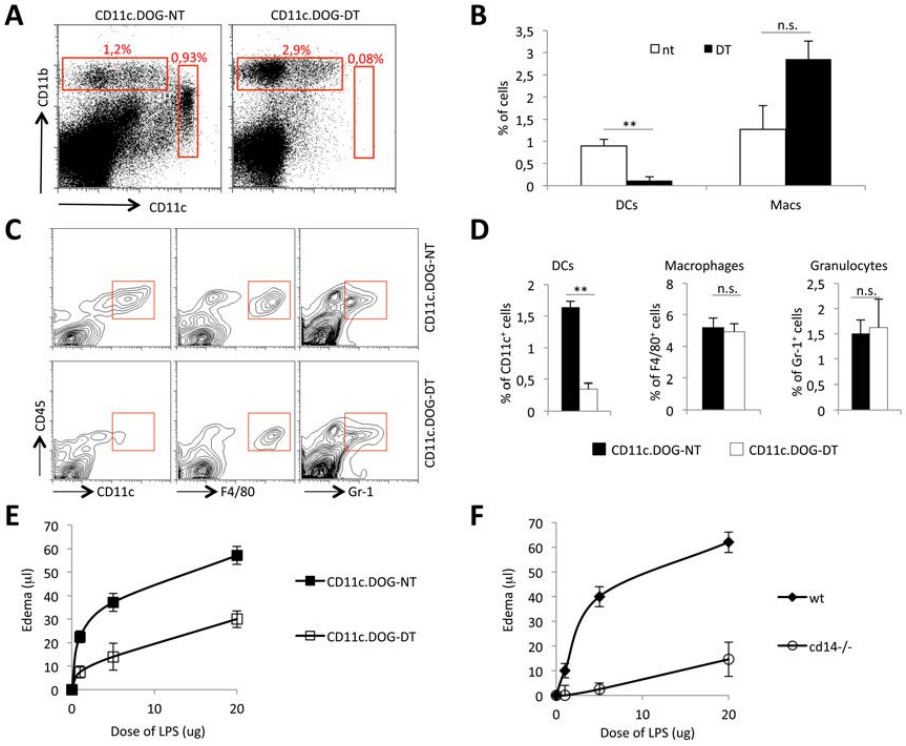
Figure 3 | DCs regulate LPS-induced tissue edema formation.

(A) Real-time PCR analysis of CD11c, F4/80, and Gr-1 mRNA in the footpad of CD11c.DOG mice before (CD11c.DOG-NT) or after 2 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). Values represent at least 3 independent experiments with 3 mice per group + SEM. (B) Inflammatory swelling in the footpad of CD11c.DOG-NT and CD11c.DOG-DT mice measured at the indicated time points after s.c. injection of LPS (20 µg/footpad). Values represent means of at least 3 independent experiments with at least 3 mice per group + SEM. (C) Real-time PCR analysis of CD11c, F4/80, and Gr-1 mRNA in the footpad of CD11c.DOG mice before and after 2 hours of s.c. LPS injection (20 µg/footpad). Values represent means of at least 3 independent experiments with 2 mice per group + SEM. (D) PGE2 production in vitro by *ex vivo* DCs and macrophages (macroph.) (F4/80+) after LPS stimulation. *P < 0.05; **P < 0.005; ***P < 0.0005; ****P < 0.00005.



Supplementary Figure 3 | PGE_2 production by BMDCs.

(A) wt and $Cd14^{-/-}$ BMDCs were treated with LPS and PGE_2 production measured in the supernatants four hours later. Where indicated wt BMDCs were pretreated with FK-506 (90 min, 1 μM), EGTA (30 min, 2 mM), COX-2 inhibitor (COX-2 in, 1 μM, 30 min) or cPLA2 inhibitor (cPLA2 in, 1 μM, 30 min). (B) TNF-α production by *ex vivo* wt or CD14-deficient DCs treated with LPS and the indicated stimuli/inhibitors; TPG, thapsigargin; COX-2 in, COX-2 inhibitor; cPLA2 in, cPLA2 inhibitor. Values represent at least three independent experiments performed in duplicate + s.e.m. ** $P < 0.005$, *** $P < 0.0005$, **** $P < 0.00005$. (C, D, E) mPGES-1 and COX-2 mRNA upregulation and PGE2 secretion induced by the indicated species of LPS in wt BMDCs.

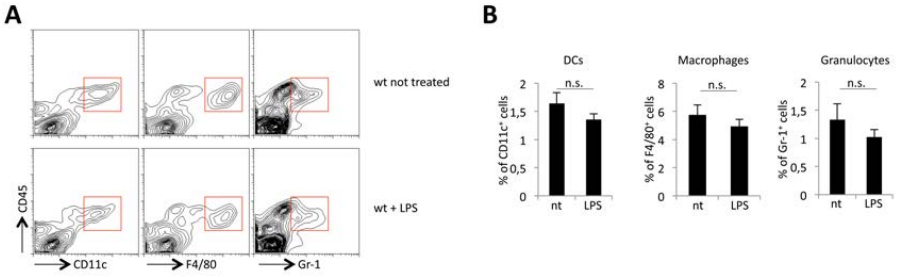


Supplementary Figure 4 | DC depletion from the spleen and the skin of CD11c.DOG mice after DT treatment.

(A) Representative dot plots of splenocytes from CD11c.DOG mice before (CD11c.DOG-NT) or after 2 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). $CD11c^{+}CD11b^{int}$ DC and $CD11b^{+}CD11c^{int}$ macrophages populations are shown. (B) Quantification and statistical analysis of the percent of DCs and macrophages in the spleen of CD11c.DOG mice before (nt) and after (DT) DT treatment. Data represent men and s.e.m. of 5 mice; ** $P < 0.005$. (C) Representative contour plots of $CD11c^{+}$ (DCs), F4/80+ (macrophages) and Gr-1+ (granulocytes) cells in the skin of CD11c.DOG mice before (CD11c.DOG-NT) or after 2 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). (D) Quantification and statistical analysis of the percent of DCs, macrophages and granulocytes in the skin of CD11c.DOG mice before (CD11c.DOG-NT) and after (CD11c.DOG-DT) DT treatment. ** $P < 0.005$. (E, F) Inflammatory footpad swelling induced by different doses of LPS three hours after treatment in (E) CD11c.DOG mice treated or not with DT and (F) wt and CD14-deficient mice. Data represent men and s.e.m. of 5 mice.

We compared paw edema formation after a single injection of LPS into the footpads of CD11c.DOG mice that were previously administered DT (CD11c.DOG-DT) or PBS (CD11c.DOG-NT). Notably, DC depletion had a strong impact on tissue edema formation (Figure 3B), and the effect was also apparent with different LPS doses (Supplemental Figure 4E). This indicated that DCs play a major role in the generation of edema. Inflammatory swelling was mainly induced by tissue-resident DCs, since no local recruitment of DCs, macrophages, or granulocytes was observed early after LPS administration (Figure 3C and Supplemental Figure 5).

The transitoriness of edema formation correlated with the kinetics of COX-2 expression by DCs (Figure 1E and Figure 3B), suggesting that edema shutoff was dictated by COX-2 and not by mPGES-1. The predominant role of DCs in tissue edema formation is also supported by the observation that LPS-stimulated ex vivo DCs secrete much higher levels of PGE2 compared with ex vivo macrophages (Figure 3D). Nevertheless, a minor role for macrophages in vivo cannot be completely excluded.



Supplementary Figure 5 | LPS injection in the footpad does not induce early inflammatory cell recruitment in the skin.

(A) Representative contour plots of $CD45^+CD11c^+$ (DCs), $CD45^+F4/80^+$ (macrophages) and $CD45^+Gr\text{-}1^+$ (granulocytes) skin cell populations before and 1 hour after LPS treatment. (B) Quantification and statistical analysis of the percent of DCs, macrophages and granulocytes in the skin of wt mice before (nt) and 1 hour after LPS treatment (LPS).

2.4 Edema formation following LPS exposure is controlled by DCs and the CD14/NFAT pathway.

DCs produce large amounts of PGE_2 after LPS exposure *in vitro* thanks to NFAT-regulated mPGES-1 expression. Moreover, tissue-resident DCs play a major role in edema formation *in vivo* at the inflammatory site generated by LPS injection. Therefore, we hypothesized that tissue-resident DCs could promote edema formation via the activation of the CD14/NFAT pathway and the consequent mPGES-1-mediated efficient PGE_2 production following LPS exposure.

We thus predicted that alterations in the PGE_2 biosynthetic pathway of DCs should recapitulate the LPS-unresponsive phenotype in terms of tissue swelling of DC-depleted mice. To this purpose, we compared LPS-induced paw edema in conditions that allow or do not allow NFAT activation in DCs. In particular, we analyzed WT, $CD14^{-/-}$, and FK-506-treated mice for the development of paw edema after LPS administration. As shown in Figure 4, A and B, and Supplemental Figure 4F, significant swells developed in WT but not in $CD14^{-/-}$ and FK-506-treated mice. The phenotype could be restored by cotreating $CD14^{-/-}$ mice with LPS and TPG, indicating a role for NFAT activation in this in vivo model of PGE_2-dependent inflammation (Figure 4C). TPG alone did not trigger a detectable inflammatory response in the paw (Supplemental Figure 6A). As a control, PGE_2 administration also induced edema formation in $CD14^{-/-}$ animals

(Figure 4C), and COX-2 inhibition affected edema formation in LPS-treated WT mice (Figure 4D).

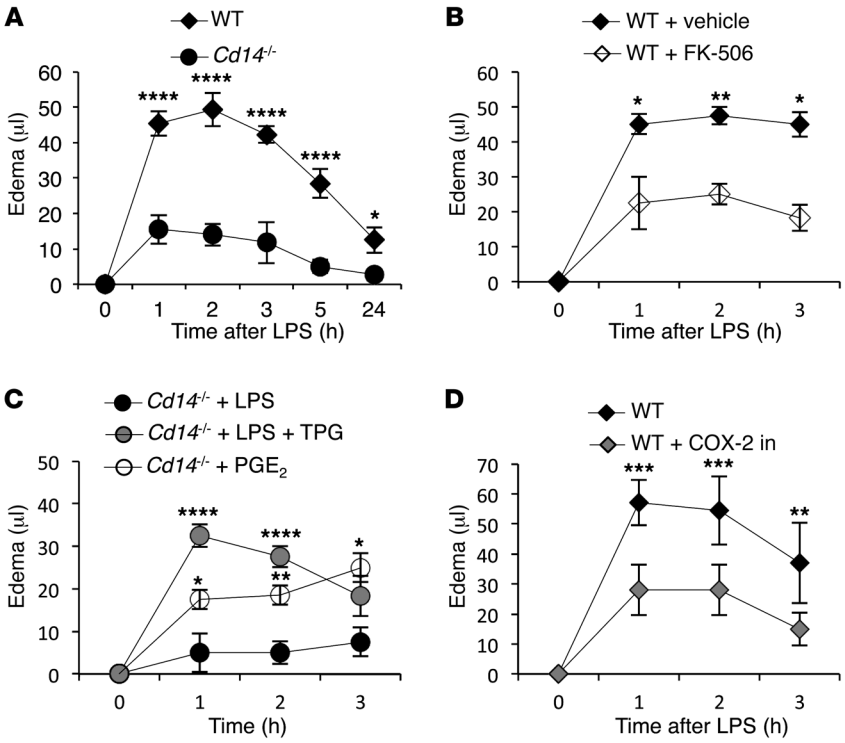
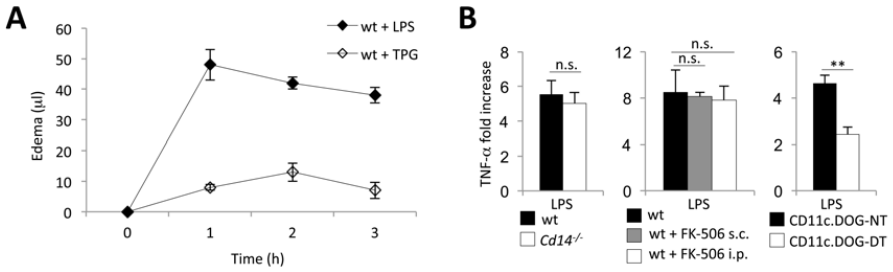


Figure 4 | DCs regulate LPS-induced tissue edema formation through CD14-dependent and NFAT-dependent mPGES-1 expression.

(A) Inflammatory swelling in the footpads of WT and CD14$^{-/-}$ mice at the indicated time points after s.c. injection of LPS (20 µg/footpad). (B) Inflammatory swelling in the footpads of WT mice treated with LPS and pretreated or not with FK-506. (C) Inflammatory swelling in the footpads of CD14-deficient mice induced by LPS, LPS plus TPG, or PGE_2 alone (10 nM). (D) Inflammatory footpad swelling induced by LPS in mice pretreated or not with the COX-2 inhibitor. Data represent 2 independent experiments with 5 mice per group. Means and SEM are shown. *P < 0.05; **P < 0.005; ***P < 0.0005; ****P < 0.00005.



Supplementary Figure 6

(A) Inflammatory swelling induced by LPS or TPG alone. (B), left and middle panels) Real-Time PCR analysis of TNF-α mRNA induction in the footpad skin of wild type and $CD14^{-/-}$ mice 2 hours after subcutaneous injection of LPS; where indicated wt mice were injected 18 hours before LPS administration with FK-506 sub-cute (s.c.) or intra-peritoneum (i.p.). (B, right panel) Real-Time PCR analysis of TNF-α mRNA induction by LPS in the footpad of CD11c.DOG mice treated (-DT) or not (-NT) with DT. Values represent at least two independent experiments (n=5) +s.e.m. ** $P < 0.005$, n.s. not significant.

To further substantiate the role of DC-derived PGE_2 in edema formation following LPS exposure, we conducted an in vivo analysis of mPGES-1 and COX-2 mRNA expression in the footpads of WT, $CD14^{-/-}$, FK-506-treated, and DC-depleted mice. A global 3-fold transcriptional induction of mPGES-1 upon LPS treatment was observed in WT mice (Figure 5A), while it was completely lost in $CD14^{-/-}$ and FK-506-treated mice (Figure 5, A and C). In contrast, COX-2 expression was not affected by the inhibition of the CD14/NFAT pathway (Figure 5, B and D).

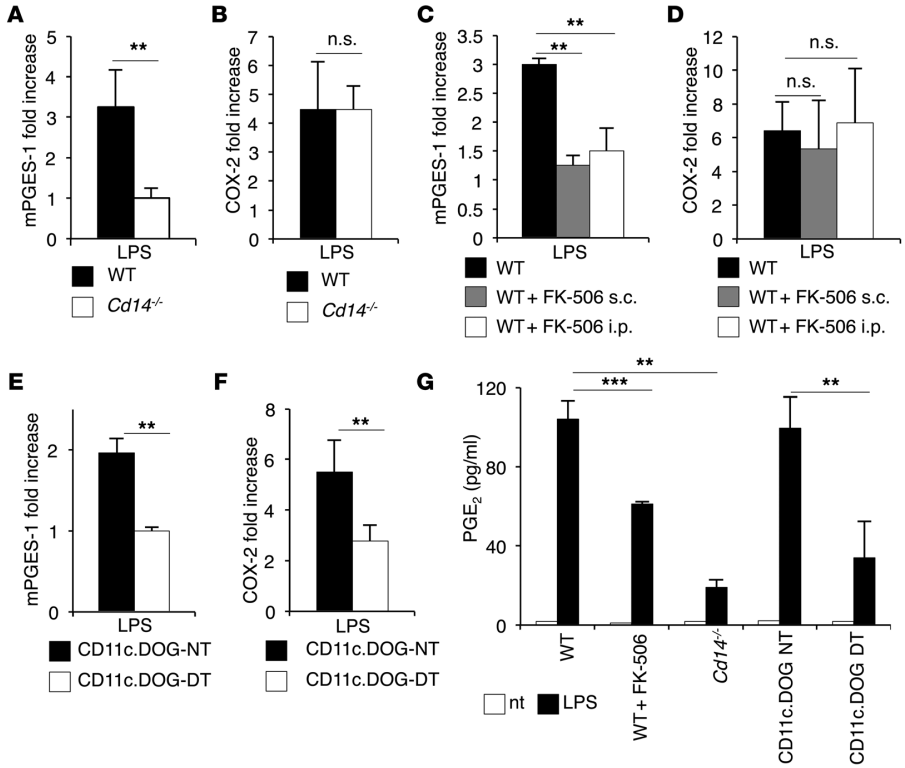


Figure 5 | CD14-dependent NFAT activation induced by LPS in DCs regulates mPGES-1 expression and PGE_2 synthesis *in vivo*.

Real-time PCR analysis of (A, C, and E) mPGES-1 and (B, D, and F) COX-2 mRNA induction 2 hours after LPS injection in the footpads of WT and $CD14^{-/-}$ mice. (C and D) WT mice were pretreated with FK-506 s.c. or i.p. (E and F) CD11c.DOG mice treated or not with DT. Values represent at least 2 independent experiments (n = 5) + SEM. (G) PGE2 production in vivo induced by LPS in WT, CD14-deficient, and CD11c.DOG mice treated or not with DT. Measurement was performed 3 hours after LPS administration. Where indicated, WT mice were pretreated for 18 hours with FK-506 (s.c.). Data represent 3 independent experiments with 3 animals per group + SEM. **P < 0.005; ***P < 0.0005.

We also measured TNF-α mRNA in the whole tissue under the same conditions as in controls. We observed a similar upregulation in WT, $CD14^{-/-}$, and FK-506-treated mice (Supplemental Figure 6B), indicating that there was not a defect in LPS sensing.

Interestingly, depletion of DCs not only affected mPGES-1 mRNA upregulation (Figure 5E), but also the local induction of COX-2 and TNF-α mRNAs (Figure 5F and Supplemental Figure 6B). This observation and the capacity of DCs to regulate edema generation strongly reinforce the idea that DCs are crucial innate immune players that directly regulate the onset of inflammation.

Finally, we measured the amounts of PGE_2 secreted *in vivo* in the footpads in response to LPS. In complete agreement with the data on mPGES-1 expression, PGE_2 production was strongly affected in $CD14^{-/-}$, NFAT-inhibited, and DC-depleted mice (Figure 5G).

Together, these data indicate that the reduction in paw edema observed in mice in which DCs were impeded in their CD14/NFAT signaling pathway was due to defective mPGES-1 upregulation.

2.5 DC-mediated edema formation controls free antigen arrival at the draining lymph nodes.

Exogenous antigens present in the inflamed skin or administered s.c. are delivered at the lymph nodes in 2 successive waves. In the first wave, antigens freely diffuse through lymphatic vessels, and in the late wave, they are transported by DCs [26][27], including $CD14^+$ dermal DCs [28]. It is thought that one of the consequences of edema formation is the increase in the efficiency of free antigen arrival at the draining lymph nodes, since the rise of the interstitial pressure would force some of the fluid into lymphatic capillaries. To determine whether this is indeed the case, local edema was artificially generated by injecting increasing amounts of PBS into the footpad. FITC-labeled microbeads were also administered. As shown in Figure 6, the efficiency of bead arrival to the draining lymph node increased with a gain in edema volume. Interestingly, a minimum threshold of edema size was required to see the effect of antigen delivery. Therefore, we predicted that the ability of DCs to control tissue swelling in the presence of LPS could have as a consequence the control of the first wave of antigen arrival to the lymph nodes. To investigate this question, we evaluated FITC-dextran delivery and FITC-coupled bead delivery.

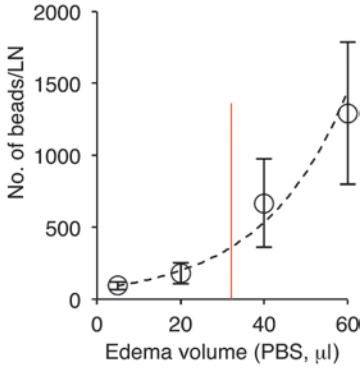
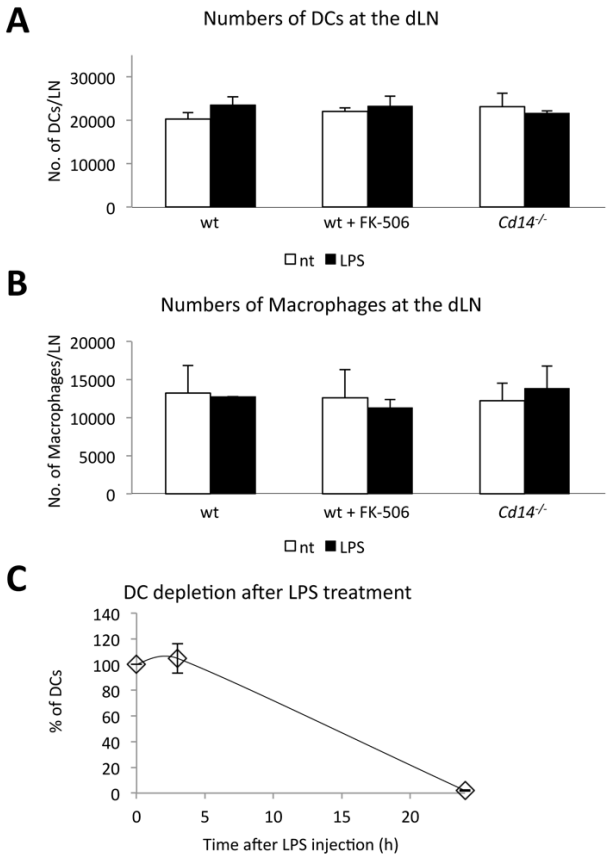


Figure 6 | The efficiency of free antigen arrival at the draining lymph nodes increases with the increase of edema volume.

Absolute numbers of FITC-labeled microbeads reaching the draining lymph nodes in WT mice injected in the footpad with the indicated PBS volumes. Dotted line represents an interpolated exponential curve with R2 = 0.98. Red line represents the putative threshold of edema volume required to observe an effect on antigen delivery. Data are expressed and plotted as mean ± SEM values.

We first performed s.c. injections of dextran in conditions either permitting or not permitting edema formation, and we analyzed the efficiency of dextran uptake by $CD11b^{+}$ phagocytes in the draining lymph nodes 2 hours after treatment. As a control, we verified that LPS treatment and NFAT inhibition did not affect DC and macrophage absolute numbers in the draining lymph nodes during the first 3 hours after LPS injection (Supplemental Figure 7, A and B). We compared mice treated with LPS and dextran with mice treated exclusively with dextran, and mice treated with dextran plus LPS plus FK-506 (to inhibit the NFAT pathway) with mice treated with dextran plus FK-506. As shown in Figure 7A, a clear increase in the efficiency of dextran lymph node arrival was measurable in the presence of LPS. This increase was completely abrogated by FK-506 treatment.

Moreover, the LPS-mediated increase in dextran lymph node arrival was also nullified when the mice were deprived of DCs (Figure 7A) and therefore were deprived of the capacity to form paw edema in response to LPS (Figure 3B).



Supplementary Figure 7

(A, B) Absolute numbers of DCs ($CD11c^{+}CD11b^{int}$) and macrophages ($F4/80^{+}$) in the draining lymph nodes of wt and CD14-deficient mice before (nt) and after LPS (three hours) treatment. Where indicated the mice were pretreated with FK-506 18 hours before LPS administration. (C) Percentage of $CD11c^{+}$ cells in draining lymph nodes after s.c LPS administration (20 μg) at the indicated time points. Data are representative of two independent experiments (four mice per group).

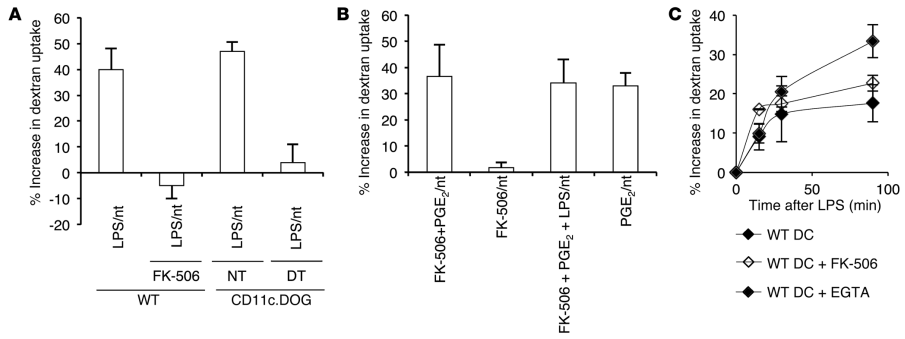


Figure 7 | Edema induced by LPS increases the efficiency of dextran arrival at the draining lymph nodes.

(A) Percentage of LPS-induced increase of dextran uptake by $CD11b^+$ cells in the lymph nodes draining the injection site. Measures were performed in WT and CD11c.DOG mice. Where indicated, WT mice were pretreated for 18 hours with FK-506. CD11c.DOG mice were treated or not with DT. Data have been calculated as percentage of uptake increase at the indicated conditions, considering as 100% the dextran uptake in the absence of any other stimulus. LPS/nt, percentage of increase of dextran uptake in mice treated with LPS plus dextran compared with dextran-treated mice. (B) Percentage of PGE_2-induced increase of dextran uptake by $CD11b^+$ cells in the lymph nodes draining the site of injection at the indicated conditions. FK-506 plus PGE_2/nt, percentage of increase of dextran uptake in mice pretreated with FK-506 and treated with PGE_2 plus dextran compared with dextran-treated mice; FK-506/nt, percentage of increase of dextran uptake in mice pretreated with FK-506 and treated with dextran compared with dextran-treated mice; FK-506 plus PGE_2 plus LPS/nt, percentage of increase of dextran uptake in mice pretreated with FK-506 and treated with PGE2 plus LPS plus dextran compared with dextran-treated mice; PGE_2/nt, percentage of increase of dextran uptake in mice treated with PGE_2 plus dextran compared with dextran- treated mice. Experiments were repeated twice with 3 mice per group each time. Means ± SEM are shown. (C) Increase in the efficiency of dextran uptake (1 mg/ml) by BMDCs treated in vitro with LPS for the times indicated. Where specified, cells were pretreated with FK-506 and EGTA.

To exclude that the treatment with FK-506 could have influenced the intrinsic efficiency of phagocyte uptake, we repeated the experiment by directly administering PGE_2 to deliberately induce edema formation (Figure 7B). When PGE_2 was added in combination with LPS and FK-506, a clear increase in phagocyte dextran uptake was observed compared with that in the untreated (dextran only) mice. The increase in uptake was also observable in the animals treated with PGE_2 and FK-506 compared with the untreated animals (dextran only), indicating that FK-506 treatment does not influence antigen uptake capacity of $CD11b^+$ cells, but only the capacity of antigen arrival at the lymph nodes by inhibiting edema formation. To further prove that the inhibition of the Ca^{2+}/NFAT pathway did not affect the antigen uptake capacity of phagocytic cells, we measured the increase of dextran uptake of DCs (Figure 7C) and macrophages (data not shown) after LPS stimulation in the presence of FK-506 or EGTA. The uptake efficiency was not reduced by these treatments (Figure 7C), confirming our hypothesis.

The described approach did not allow us to directly investigate the involvement of CD14 in controlling the amount of antigen that arrives at the lymph nodes as a consequence of edema formation. We have, indeed, recently shown that CD14 influences the efficiency of antigen uptake [4]. Therefore, we used the second method. FITC-labeled microbeads were injected in the footpads of WT and $CD14^{-/-}$ animals in the presence or absence of LPS and the numbers of microbeads reaching the draining lymph node enumerated 3 hour later, a time point compatible with free antigen arrival and not with DC migration [22]. While in WT animals, the efficiency of bead trafficking was

strongly increased by LPS (Figure 8A), in CD14$^{-/-}$ mice, LPS treatment did not influenced the capacity of microbead arrival at the lymph nodes (Figure 8B). A clear increase in the numbers of microbeads in the lymph nodes was instead observed in CD14$^{-/-}$ mice treated with PGE_2 to deliberately induce edema formation (Figure 8B). As previously observed, the treatment of WT animals with FK-506 nullified the LPS-mediated increase of free antigen arrival at the draining lymph nodes (Figure 7A).

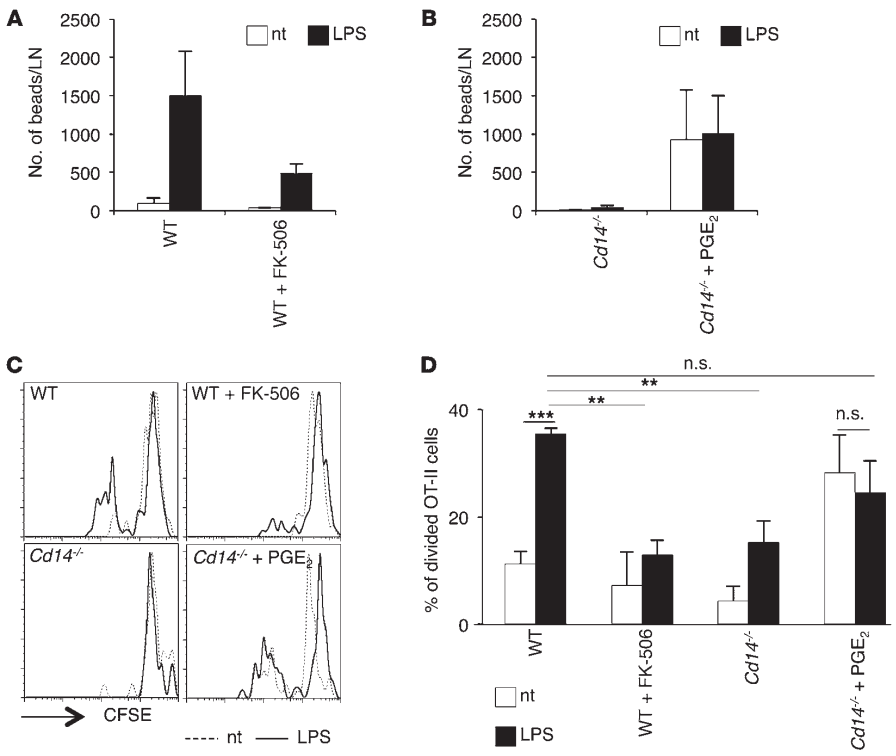


Figure 8 | Edema induced by LPS increases the efficiency of bead arrival at the draining lymph nodes.

(A) Absolute numbers of FITC-labeled microbeads reaching the draining lymph nodes in WT mice treated or not with LPS (4 hours after treatment). Where

indicated, mice were pretreated s.c. with FK-506 for 18 hours. (B) Absolute numbers of FITC-labeled microbeads reaching the draining lymph nodes in CD14-deficient mice treated or not with LPS. Where indicated, mice were cotreated with PGE_2. (A and B) Data represent mean and SEM of at least 10 animals per group. (C and D) OT-II cell proliferation in response to the amount of antigen recovered from the lymph nodes of WT or CD14-deficient mice treated or not with LPS. Where indicated, the mice were cotreated with LPS and PGE_2 or pretreated s.c. with FK-506 for 18 hours. (C) FACS histograms. (D) Histogram quantification. Data represent mean and SEM of at least 6 animals per group. **P < 0.005; ***P < 0.0005.

To investigate whether the increase in the efficiency of antigen trafficking to draining lymph nodes induced by edema was sufficient to influence the efficiency of adaptive responses, OVA-coated beads were recovered from lymph nodes of WT mice treated with LPS in the presence or absence of FK-506 and from lymph nodes of $CD14^{-/-}$ mice treated with LPS in the presence or absence of PGE2. The recovered beads were then used to measure the proliferation capacity of OVA-specific OT-II cells *in vitro*. As shown in Figure 8, C and D, OT-II cells proliferated more efficiently when challenged with the amount of antigen recovered in all the conditions allowing edema formation. Therefore, the inhibition of CD14-dependent edema formation clearly has an impact on antigen arrival to the draining lymph nodes.

2.6 Discussion

DCs are involved in the regulation of many different aspects of innate and adaptive immunity. Following activation with PRR agonists, they sequentially acquire the ability to produce soluble and cell surface molecules critical for the initiation and control of innate and then adaptive immune responses. The production of these factors is regulated by the activation of NF-κB and AP1 downstream PRRs. Nevertheless, we have recently described that following smooth LPS exposure different NFAT isoforms are also activated [7]. The initiation of the pathway that leads to nuclear NFAT translocation is totally dependent on CD14 that, through the involvement of src family kinases and PLCγ2, leads to Ca^{2+} mobilization and calcineurin activation. Nuclear NFAT translocation is required for IL-2 production and apoptotic death of terminally differentiated DCs. In the present work, we show that mPGES-1 and its direct product PGE_2 are also efficiently produced by DCs upon activation of the CD14-dependent Ca^{2+}/calcineurin and NFAT pathway.

Although COX-2 expression has been reported to be NFAT dependent in some experimental settings, we did not find any NFAT signaling pathway dependence of DC-produced COX-2 in response to LPS. A possible explanation of this discrepancy can be found in the fact that in the nucleus, the NFATc1-c4 isoforms need to interact with partner proteins, generically termed NFATn, to produce active NFAT transcription complexes. Usually, NFATc and NFATn are activated via distinct signaling pathways. NFATn in innate immunity is mostly unknown. It is possible that the NFATn factors required for the

generation of the active NFATc-NFATn heterodimers capable of binding COX-2 promoter are not activated in DCs, while they are activated in other cell types. The production of PGE_2 by DCs is particularly relevant in adaptive immune responses, since this prostanoid has been shown to regulate diverse DC functions, including DC migration and polarization of T cell responses [29][30], by acting on different receptors in an autocrine or paracrine way [31]. For instance, DC-derived PGE_2 facilitates Th1 differentiation through the EP1 receptor expressed by naive T cells [31], while PGE_2-mediated activation of the EP2 and EP4 receptors promotes Th2 differentiation [32][33]. Given the importance of PGE_2 for the regulation of DC functions, this prostanoid is one of the components of the nonmicrobial stimuli cocktail used to activate DCs for *in vivo* therapies.

During the innate phase of an immune response, it is well known that PGE_2 sustains the formation of edema at the inflammatory site [13]. Consistent with this, we have observed that LPS-activated, tissue-resident DCs contribute to the formation of edema via the activation of the NFAT signaling pathway. $CD14^{-/-}$ mice are almost totally incapable of generating edema at the LPS injection site, and this function can be restored by deliberately inducing Ca^{2+} mobilization and NFAT activation. The inefficient edema formation in the absence of CD14 cannot be attributed to a reduced responsiveness of the mutant mice to the dose of LPS used in this study. $CD14^{-/-}$ mice could, indeed, produce TNF as efficiently as WT mice. Though the crucial CD14 role in the recognition of low LPS doses has been established, CD14 has been shown to be largely dispensable for the

response to high concentrations of LPS, which occurs almost normally in $CD14^{-/-}$ macrophages and DCs [4][7][34]. This observation suggests that a high dose of LPS can also be sensed in a CD14-independent way, possibly through a direct LPS recognition by TLR4:MD-2 [35] or the participation of different LBPs [36]. The absence of CD14 and the knockdown of DCs affect the formation of edema in a very similar way, suggesting that CD14 exerts its contribution to LPS-induced edema almost exclusively through DCs. We thus assume that activation of the NFAT pathway for edema formation must occur predominately/exclusively in DCs. This observation is in agreement with our previous data showing that the CD14/NFAT pathway is not active in macrophages [7].

Neutrophils do not play a major role in LPS-induced edema formation at the cutaneous level. These results are consistent with the faster kinetics of tissue edema formation (1–2 hours) as compared with immune cell, including neutrophil, recruitment.

On first analysis, the participation of DCs in edema formation could seem surprising, since DCs leave the tissue after activation. Nevertheless, DCs do not acquire the ability to migrate immediately after LPS encounters; conversely, they persist in the peripheral tissue to maximize antigen uptake [18]. As a matter of fact, antigen uptake and migration have been proposed to be two mutually exclusive DC activities [19]. Early in the course of inflammation, in addition to performing antigen uptake, DCs contribute to the generation of edema via PGE_2 production.

It is important to note that PGE_2 is also involved in the control of DC migratory activity, in addition to the regulation of edema formation

[37][38]. These two PGE_2 functions are not contradictory. DC-derived PGE_2 controls DC migration in an autocrine and indirect way by inducing the efficient production of MMP-9 following LPS encounter. PGE_2-induced MMP-9 occurs several hours after DC activation [39]. MMP-9, in turn, regulates DC migration by contributing to the degradation of the basal membrane [39]. Thus, the capacity to control edema formation and migratory activity are two DC functions regulated by the same molecule, but segregated in time. Upon challenge with LPS, PGE_2 derived from DCs initially controls edema formation; later on, it regulates DC migration by inducing the synthesis of MMP-9.

Edema formation is one of the first steps in the generation of the inflammatory process, and it is a fundamental process for the local accumulation of inflammatory mediators. We show here that local swelling is also relevant for free antigen transport to the draining lymph nodes. Antigens present in the inflamed tissues are delivered to the lymph nodes in 2 successive waves. In the first wave, antigens freely diffuse through lymphatic vessels and in the late wave are transported by DCs [26] [27]. The increase of the interstitial pressure due to edema forces some of the fluid into lymphatic capillaries and favors free antigen entry into the afferent lymphatics and free antigen arrival to the draining lymph nodes. Thus, we propose that tissue-resident DCs control not only the second wave of antigen arrival, but also the efficiency of the first wave by controlling edema formation. Both waves are then important for efficient activation of adaptive T cell responses [26][40]. Early antigen presentation by lymphoid-resident DCs is required to initiate activation and trapping of antigen-

specific T cells in the draining lymph nodes, but is not sufficient for inducing clonal T cell expansion. Efficient proliferation is instead induced by migratory DCs arriving later to the draining lymph nodes [40]. DCs are extremely versatile cells, and our data suggest that they are one of the key players in a model of LPS-induced inflammation in vivo. They exert this primary role through their peculiar ability to respond to LPS through the initiation of the CD14/NFAT pathway, leading to the formation of edema. CD14 comes out as one of the master regulators of DC biology, as already shown in previous studies [4][7][41]. We propose the concept that DCs control skin edema formation following LPS exposure via the activation of two independent pathways: (a) the CD14/NFAT pathway, which regulates mPGES-1 production, and (b) the canonical NF-κB pathway, which controls COX-2 expression. Most of the COX-2 inhibitors also inhibit COX-1 and, when used as antiinflammatory drugs, have severe toxic secondary effects, given the importance of COX-1 in tissue homeostasis. Our findings suggest that targeting the CD14/NFAT/mPGES-1 pathway in DCs may constitute a strategy to overcome such problems by selectively blocking the biosynthesis of PGE_2 in specific inflammatory settings.

2.7 Methods

Cells. BMDCs were derived from BM progenitors of WT or mutant mice as previously described [7]. *Ex vivo* DCs were purified as previously described [42]. *Ex vivo* macrophages were purified from spleen. Splenic unicellular suspensions were stained with biotinylated anti-F4/80 antibodies and positively selected using MACS beads according to the manufacturer's instructions (Miltenyi Biotec).

Mice. C57BL/6 mice and OT-II transgenic mice were purchased from Harlan. $CD14^{-/-}$ mice were purchased from CNRS, Campus d'Orléans. N. Garbi (Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany) provided CD11c.DOG mice expressing DTR under the control of the long CD11c promoter. In these mice, a specific DC ablation can be induced by diphtheria toxin injection [24]. All animals were housed under pathogen-free conditions, and all experiments were carried out in accordance with relevant laws and institutional guidelines.

Antibodies and chemicals. Antibodies were purchased from BD Biosciences. TLR4-grade smooth LPS (E. coli, O55:B5; E. coli, O111:B4; E. coli, R515 [Re]; E. coli, lipid A; Salmonella typhimurium, S-form) were purchased from Enzo Life Sciences. CFSE was from Invitrogen. EGTA, PGE2, FITC-dextran, FK-506, and thapsigargin were purchased from Sigma-Aldrich. Recombinant murine IFN-β and diphtheria toxin were purchased from R&D

Systems. Antibody against murine mPGES- 1 and COX-2, COX-2–specific inhibitor (NS-398), and cPLA2 inhibitor (pyrrophenone) were purchased from Cayman Chemical. EndoGrade ovalbumin was purchased from Hyglos Gmbh. Fluoresbrite Carboxy YG 1-μm latex beads were from Polysciences. For adsorption of ovalbumin onto latex beads, microspheres were resuspended in ovalbumin (1 mg/ml) and incubated overnight at 4°C. Latex beads were then washed 15 times in large volumes of sterile endotoxin-free PBS.

In vivo treatment with FK-506. For *in vivo* treatment, FK-506 was resuspended in 40% w/v HCO-60/ethanol. Mice were injected s.c. (10 μg/footpad) or i.p. (40 μg/mouse) with FK-506 18 hours before stimuli injection.

DC depletion. Diphtheria toxin (16 ng/g) was daily administered to CD11c.DOG mice through an i.p. injection for 2 consecutive days. Control mice were given PBS. Effective DC depletion was assessed by FACS and qRT-PCR analysis.

Ex vivo PGE_2 extraction. Paw tissue was homogenized in 500 μl of PBS using a TissueLyser (QIAGEN) (full speed for 8 minutes). Samples were then centrifuged for 90 seconds at 5,000 g. The supernatant were collected into a new Falcon tube, and 2 ml of 100% EtOH was added and incubated 5 minutes at 4°C. Samples were centrifuged for 10 minutes at 1,000 g and supernatants collected into a new Falcon tube. Then 8 ml PPS buffer (0.1 M, pH = 3) was added.

A Solid Phase Extraction (SPE) cartridge (C-18) was activated by rinsing with 5 ml 100% EtOH and then with 5 ml of water. Samples were passed through a column, which was then washed with 5 ml of water and 5 ml of exane. Samples were eluted by gravity with 5 ml ethyl acetate containing 1% methanol. The ethyl acetate was then evaporated and samples resuspended in an appropriate buffer for PGE_2 ELISA analysis.

ELISA assays. Concentrations of IL-2 and TNF-α in supernatants were assessed by ELISA kits purchased from R&D Systems. PGE_2 levels were assayed with a Monoclonal EIA Kit from Cayman Chemical.

Quantitative real-time PCR in vitro. Cells (2 × 106) were lysed with the TRIzol reagent (Applied Biosystems), and total mRNA was extracted with an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. A NanoDrop spectrophotometer (Thermo Scientific) was used to quantify mRNA and to assess its purity, and 600 ng mRNA was retrotranscribed to cDNA using a High- Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Then 10 ng cDNA was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems), and data were analyzed using the built-in software. Primer pairs used were as follows: 5 ′ - ACGACATGGAGACAATCT ATCCT- 3 ′ and 5 ′ - TGAGGACAACGAGGAAATGT-3 ′ (mPGES-1); 5 ′ -

CCTGCTGCCCGACACCTTCAA-3′ and 5′ -TCTTCCCCCA GCAACCCGGC-3′ (COX-2); and 5′ -CGAAAGCATTTGCCA AGAAT-3′ and 5′ - AGTCGGCATCGTTTATGGTC-3′ (18S). 18S mRNA was used as an internal reference for relative quantification studies.

Quantitative real-time PCR *in vivo*. Whole skin from treated or control mice was cut, briefly washed in cold PBS, and immersed in RNAlater solution (Ambion) at 4°C for 24 hours. Skin was then lysed in TRIzol and mechanically disrupted using a TissueLyser (QIAGEN) (30 shakes/s for 3 minutes). Subsequent mRNA processing was performed as described above.

Primer pairs used were as follows: 5′ -TTTGTTTCTTGTCTTGG CTTCAA-3′ and 5′ -TTAGTGGCTTTTATTTCCTTTGGT-3′ (CD11c); 5′ - CACCTTCATTTGCATCAACA-3′ and 5′ - TCTGAAA AGTTGGCAAAGAGAA- 3′ (F4/80); and 5′ - TGCTCTGGAGATAG AAGTTATTGTG-3′ and 5′ - TTACCAGTGATCTCAGTATTGT CCA-3′ (Gr-1). Primer pairs for mPGES-1, COX-2, and 18S are indicated above. Prevalidated QuantiTect primer pairs for TNF-α and HPRT1 (reference gene) were purchased from QIAGEN.

Isolation of skin cells. Cells were isolated as previously described [43]. Briefly, skin was isolated and digested for 45 minutes in a

cocktail containing collagenase XI, hyaluronidase, and DNase. Then 10% FBS was added to stop the reaction, and cells were stained to assess the percentage of different cell populations.

Tissue edema. Following anesthesia with pentobarbital (60 mg/kg), sex- and age-matched mice were injected s.c. with LPS (20 μg/20 μl), LPS plus TPG (5 μM), and LPS plus PGE_2 or PGE_2 alone (10 μM) or PBS as a control in the footpad. In some cases, mice were pretreated with COX-2 inhibitor (30 minutes, 10 mg/kg), FK-506 (18 hours), or were depleted of DCs as previously described. The paw volume of the LPS-treated as well as the PBS-treated contralateral paw was then measured by a plethysmometer (Ugo Basile) at the indicated time points. At the 1-hour time point, most of the animals had recovered from the anesthesia, and at the 2-hour time point, all animals had recovered. The volume of the contralateral paw was subtracted from the volume of the injected paw to obtain edema volume.

Antigen delivery to the lymph node. Following anesthesia, sex- and age-matched mice were injected s.c. with the described combinations of LPS (15 μg), FITC-dextran (500 μg), or FITC–latex beads conjugated or not with ovalbumin (100.000 beads/footpad) and PGE_2 (10 μM) in the footpad (20 μl/footpad). In some cases, mice were pretreated with FK506 or were depleted of DCs as previously described. Two to four hours after injection, mice were sacrificed, draining lymph nodes collected, and bead numbers and dextran uptake by $CD11b^+$ cells measured by FACS analysis.

***In vitro* antigen presentation assay.** Anti-ovalbumin $CD4^+$ T cells were purified by positive selection from spleen and lymph nodes of OT-II mice using anti-CD4–conjugated microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Cells were then CFSE labeled according to the manufacturer's instructions.

Ovalbumin-coated latex beads were recovered from draining lymph nodes of mice. In particular, axillary and brachial lymph nodes were removed 3 hours after s.c. injection of the described stimuli. Lymph nodes were dissected in water and centrifuged at 5,000 g for 2 minutes to recover latex beads. The recovered beads were added to U-bottom 96-well plate of medium with 10,000 BMDCs, 50,000 OT-II $CD4^+$ CFSE-labeled T cells, and 10 ng/ml LPS (final volume 200 μl). After 120 hours, cell division was measured using FACScalibur.

Western blot. Cells were lysed with a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP-40 supplemented with protease, and phosphatase inhibitor cocktails (Roche). Cell debris were removed by centrifugation at 16,000 g for 15 minutes (4°C), and proteins were quantified using a BCA assay (Thermo Scientific). 10 μg cell lysate was run on a 10% polyacrylamide gel, and SDS-PAGE was performed following standard procedures. After protein transfer, nitrocellulose membranes (Thermo Scientific) were incubated with the indicated antibodies and developed using an ECL substrate reagent (Thermo Scientific).

Statistics. Means were compared by 2-tailed Student's t tests, unequal variance. Data are expressed and plotted as mean ± SEM values. Statistical significance was defined as $P < 0.05$. Sample sizes for each experimental condition are provided in the figures and the respective legends.

Study approval. The experimental protocols were approved by the Italian Ministry of Health (Rome, Italy) according to the Decreto legislativo 27 gennaio 1992, n. 116 "Attuazione della Direttiva n. 86/609/CEE in materia di protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici."

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Chapter 3: Study of the Role of the NFAT Pathway Activation in innate immune cells during Acute Transplant Rejection

Department of Biotechnology and Biosciences
University of Milano-Bicocca, Milan, Italy

In the work described in chapter 2 we have demonstrated the innate immune cells to activate NFATc members and their contribution in the initiation of the inflammatory process via edema formation.

The inflammatory process is beneficial only if tightly regulated, otherwise the host tissues may be seriously damaged by the inflammatory mediators. This tight regulation has evolved together with vertebrate complexity [1], but the molecular mechanisms forming the basis of such a complex process remain to be determined. Many different mechanisms to turn off immune responses exist, including production of anti-inflammatory cytokines to switch to noninflammatory repairing conditions [2], and the death of activated innate and adaptive immune cells. The NFATc pathway has been shown to regulate DC life cycle, thus participating to the control of the inflammatory process [3].

Besides its pro-inflammatory role in edema formation and its anti-inflammatory role in regulating DCs death, NFAT can also be important for the interaction between innate immune cells and lymphocytes. DCs are the key cells deciding whether a T-cell response should be activated or suppressed. There is evidence that this dual capacity of DCs is controlled by NF-kB [4]. Nevertheless, recent observations suggest also an important role for the NFATc pathway. In humans, activatory DCs produce IL-2 and CD25, both regulated by NFATc, in the first few hours after interaction with T cells [5]. DC-derived IL-2 is transpresented to T cells at the immunological synapse via CD25. Since naïve T cells start to express CD25 only many hours after antigen encounter, the DC-mediated presentation of the IL-2/CD25 complex represents a very efficient system for T-cell priming

in vitro [5]. Similar *in vitro* results have also been obtained in the mouse system [6].

The study of the role of the NFATc signaling pathway *in vivo* presents several difficulties due to the redundancy of the system. For instance in T cells, NFAT c2 and c3 have a redundant proapoptotic role, while the c1 has a nonredundant differentiation role [7]. In DCs, NFAT c1 and c3 are implicated in IL-2 transcription while only NFAT c2 and c3 are involved in the regulation of the apoptotic death [3]. The generation of new tools allowing the inhibition of all NFATc isoforms or different isoform combinations in single or multiple innate immune cell types *in vivo* will be necessary for the comprehension of the role of this signalling pathway in the most ancient arm of the immune system. These studies will help in clarifying important aspects of the complexity of inflammation-driven immunity through the acquisition of new information concerning the regulatory networks adopted by innate immunity to control adaptive immunity and to prevent excessive tissue damage.

In addition to the pure mechanistic aspects, these studies have fundamental medical implications. CsA and FK506 are the most commonly used drugs in the treatment of acute transplant rejection. CsA and FK506 are used in theory to block IL-2 and other NFATc-dependent cytokine production by T cells. Although highly successful, CsA and FK506 have severe side effects that very likely result from the general inhibition of the enzymatic activity of Cn, which plays other physiological roles besides NFAT activation, and they are not specific for phagocytes.

Thus we created a new NFAT inhibitor specific for innate myeloid cells that can be used as a treatment for acute transplant rejection.

3.1 The NFAT inhibitor *in vitro* characterization

In order to investigate if DCs were able to internalize the newly designed NFAT inhibitor, the uptake capacity of DCs were tested.
We incubated Bone-Marrow-Derived DCs (BMDCs) with the NFAT inhibitor conjugated with FITC (30 ug/ml) alone or in combination with LPS (1 ug/ml) in order to increase the uptake capacity of DCs, and at different time points we analysed the inhibitor uptake by FACS analysis (Figure 1). DCs internalized the NFAT inhibitor already after 10 minutes of incubation, both in the presence or absence of inflammatory stimulus.

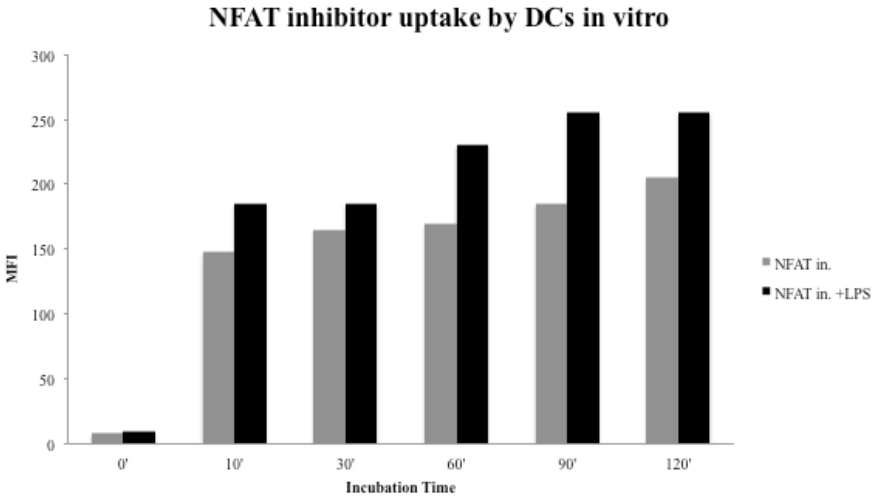


Figure 1 | NFAT inhibitor uptake by DCs in vitro.
$2*10^6$ BMDCs were incubated with FITC-NFAT in. 30 ug/ml alone or in combination with LPS for 90 minutes at 37°C and then stimulated with LPS 1 ug/ml. The MFI was measured by FACS analysis at different time points, as indicated. Experiments were performed in triplicate.

To better analyse the inhibitor uptake, we performed confocal microscopy analysis using an homogeneous murine dendritic cell line, D1 cells [8]. After 90 min. of incubation, it emerged that D1 cells efficiently internalize the NFAT inhibitor. The faint cytoplasmic staining suggests that at least part of the inhibitor escapes from the endosome compartment to the cytosol (Figure 2).

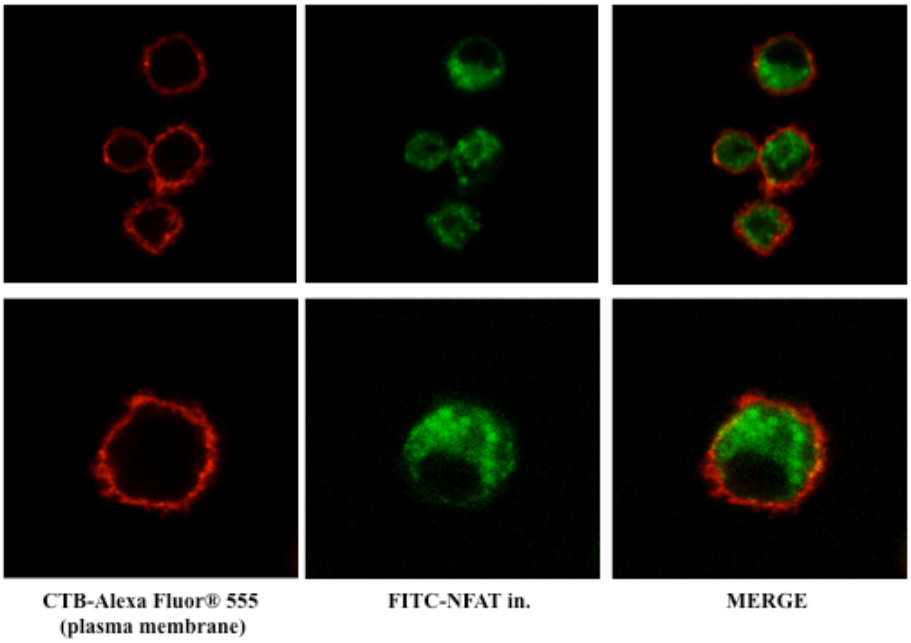


Figure 2 | Confocal analysis of NFAT inhibitor uptake.
$0.5*10^6$ D1 cells were seeded on glass coverslip and analyzed via confocal microscopy. D1 cells were incubated with FITC-NFAT in. for 90 minutes at 37°C. Plasma membrane (PM) (red) was detected by Alexa Fluor® 555 conjugated cholera toxin subunit B, CTB. Experiments were performed in triplicate.

Once determined the uptake ability, we performed functional test in vitro in order to assess the functional properties of our new NFAT inhibitor. We incubated BMDCs or D1 cells with the NFAT inhibitor

for 90 minutes at 37°C and then we stimulated them with LPS. After 12 hours we measured the production of specific cytokines, in particular IL-2 and TNFα. We chose these two particular cytokines because IL-2 production is specifically NFAT-dependent while TNFα production is NFAT-independent (Figure 3) [9].

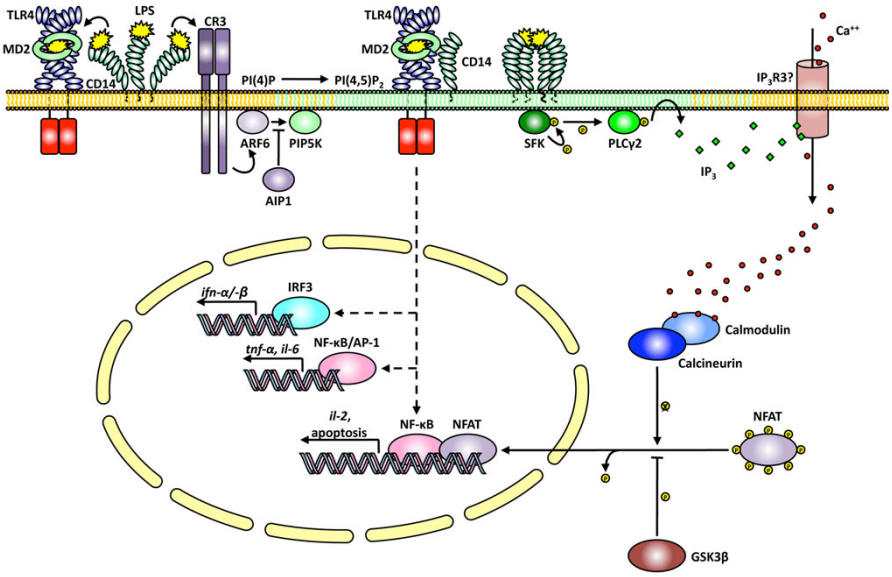


Figure 3 | NFAT-dependent IL-2 production [10].

CD14 has autonomous signaling functions in DCs. Upon LPS-induced clusterization, CD14 transiently recruits and activates a SKF member, this relies the CD14 GPI anchor and on its residency in lipid rafts. Active SFK then phosphorylates PLCγ2, which in turn catalyzes the hydrolysis of PIP2 into the second messengers DAG and IP3. IP3 triggers Ca^{2+} from external space. The increased $[Ca^{++}]_I$ stimulates activation of calcineurin, which dephosphorylates NFAT and promotes its nuclear translocation. Active NFAT cooperates with NF-κB to drive the expression of the genes coding for IL-2, as well as several proapoptotic proteins, while the production of TNFα is totally NFAT independent.

We observed a dose-response curve of IL-2 production while TNFα secretion was not affected upon LPS stimulation (Figure 4).

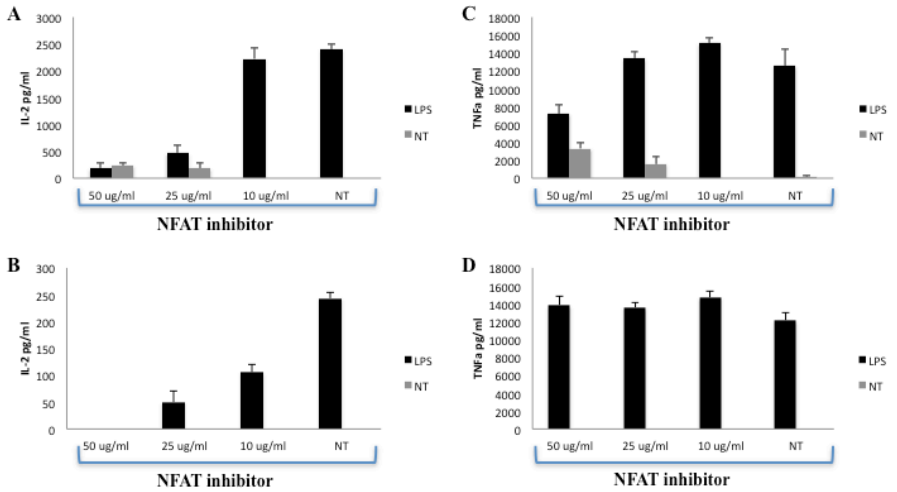


Figure 4 | NFAT inhibitor functional test *in vitro*.

$1*10^6$ BMDCs (A and C) or D1 (B and D) cells were incubated with NFAT in. for 90 minutes at 37°C at different concentrations (50 ug/ml, 25 ug/ml, 10ug/ml or not treated) and then stimulated with LPS 1 ug/ml. After 12 hours the IL-2 (A and B) and TNFα (C and D) production was measured by ELISA assay. Experiments were performed in triplicate.

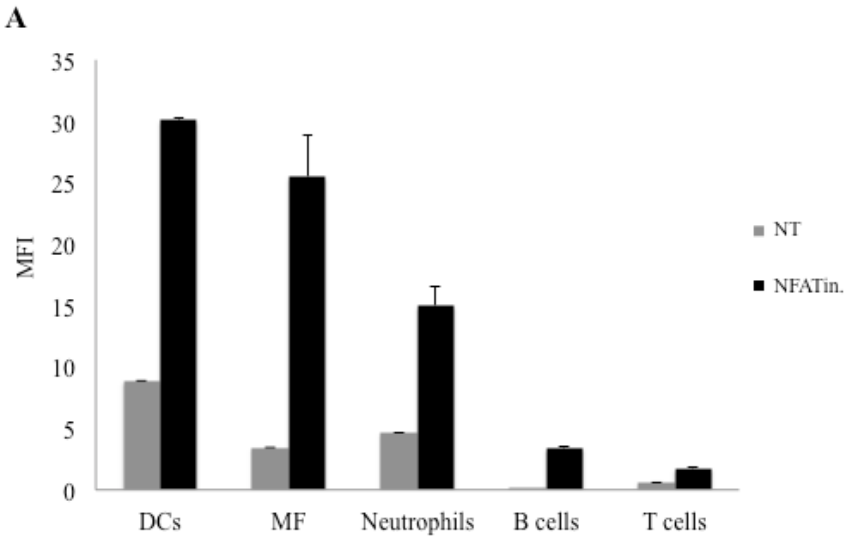
This observation let us to conclude that the treatment with our new NFAT inhibitor specifically blocks NFAT activation while does not influence the NF-kB activity, perfectly matching with our goal.

3.2 The NFAT inhibitor is specific for phagocytes

We then measured the NFAT inhibitor uptake also *in vivo*. WT mice were treated with FITC-NFAT inhibitor 200 ug i.p. and after 90 minutes we analysed different population in the spleen by FACS analysis (Figure 5). It emerged that NFAT inhibitor was picked up by DCs, macrophages and neutrophils, while B cells and T cells did not internalised the drug. In order to confirm the uptake data, we treated WT mice with FITC-NFAT inhibitor 200 ug i.p. for 2 weeks every 2 days and then analysed spleen, lymph nodes and skin (Figure 5 and data not shown).

The NFAT inhibitor was internalized mainly by DCs and less efficiently also by macrophages and neutrophils, but not by lymphocytes.

In summary, we demonstrated that the new NFAT inhibitor is capable to specifically target phagocytes *in vivo*.



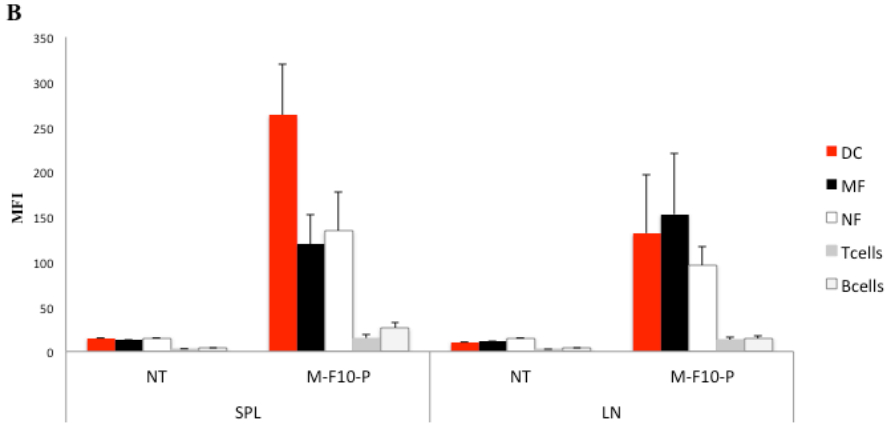


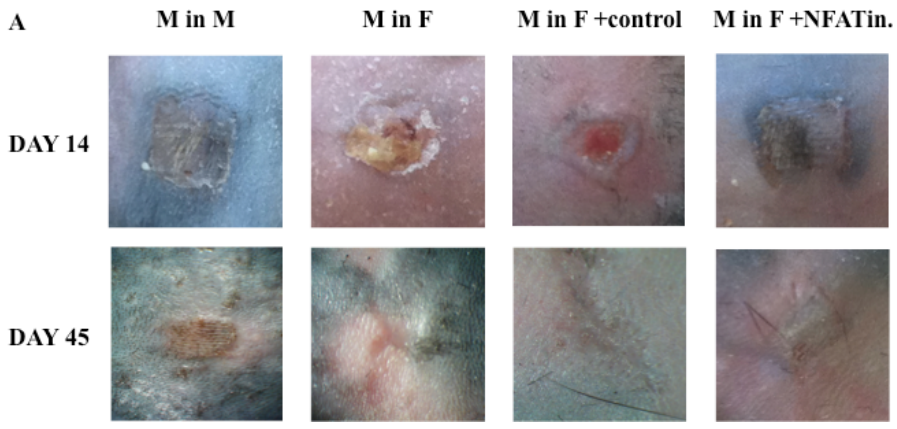
Figure 5 | NFAT inhibitor uptake *in vivo*.

C57BL/6 wild-type mice were injected i.p. with FITC-NFAT 200 ug. (A) After 90 min. mice were sacrificed and the spleen was analysed by FACS analysis for different populations: $CD11c^+$, $CD11b^+$, $Ly6G^+$, $CD19^+$ and $CD3^+$. (B) WT mice were treated for 2 weeks every 2 days and then the spleen and lymph nodes were analysed by FACS analysis for different populations: $CD11c^+$, $CD11b^+$, $Ly6G^+$, $CD19^+$ and $CD3^+$. Experiments were performed in triplicate.

3.3 NFAT inhibition in skin acute transplant rejection

In order to understand the role of NFAT activation in DCs *in vivo* during transplant rejection, we transplanted the skin of the tail of male mice into the lateral flank of female mice and we treated the recipient mice with the NFAT inhibitor 100 ug i.p. the day before the transplantation, 2 hours before the transplantation and then every 2 days for 45 days. Grafts were examined regularly after bandage removal on day 10 and until day 95 (Figure 6).

We obtained 100% of transplant success in male into male control group, and complete rejection when we transplanted male skin into female animals. Interestingly we observed that skin graft rejection decrease of 75% after treatment with NFAT inhibitor during the drug treatment period. After 45 days, we interrupted the treatment with NFAT inhibitor and followed the skin graft for other 50 days (Figure 6). At the day 95 from the transplant 55% of the treated mice showed skin transplant maintenance.



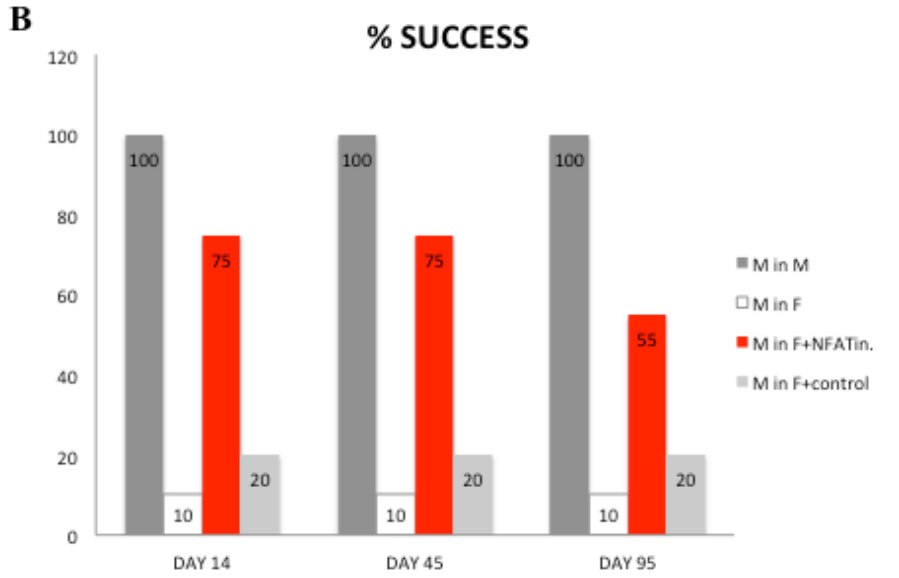


Figure 6 | NFAT inhibition in recipient mice in a model of skin transplantation. Male to female (minor-mismatched) skin grafts were performed in recipient treated with NFAT in. 100 ug i.p. or inhibitor control. Male to male and male to female were performed as control. Recipient mice were treated the day before the transplantation, 2 hours before the transplantation and so on every 2 days for 45 days; then the treatment was stopped and the fate of graft was followed till day 95. Skin graft rejection was determined macroscopically when the graft reached a necrosis of 60%. Skin grafts were performed in SPF animal facility.

The mice transplanted and treated with the drug for 45 days were also re-transplanted at day 95 in order to verify the tolerogenic level of recipient mice (Figure 7).

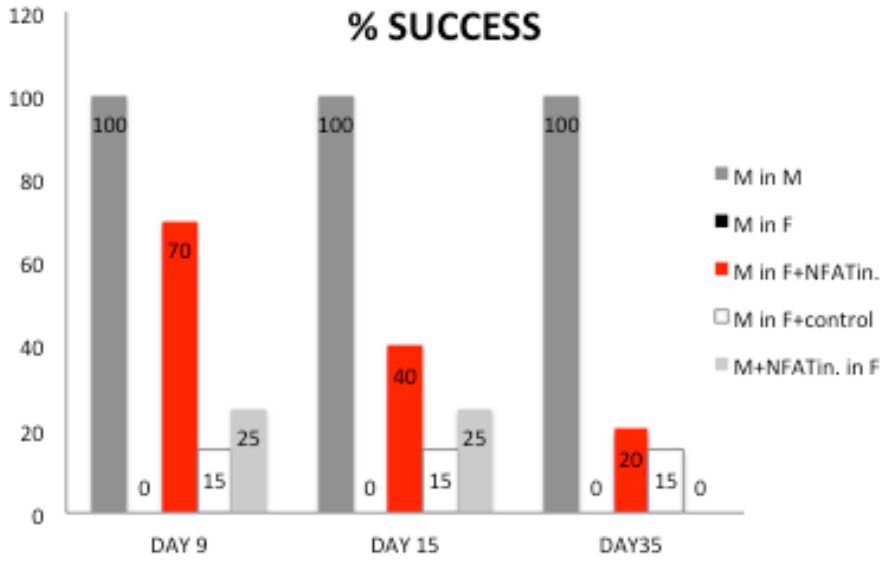


Figure 7 | Delay in graft rejection in mice pretreated with NFAT inhibitor.
Male to female (minor-mismatched) skin grafts were performed using donor male mice. The skin from the tale of male mice were retransplanted without any additional drug treatment. Recipient groups: wt male mice treated with NFAT in. for 45 days and 50 days without drug treatment; wt male mice treated with NFAT in. control for 45 days and 50 days without drug treatment; wt male mice pretransplanted with donor treated with NFAT in. for 8 days; wt male mice; wt female mice. Skin graft rejection was determined macroscopically when the graft reached a necrosis of 60%. Skin grafts were performed in SPF animal facility.

We observed a significant delay in graft rejection in the group of recipient male mice that during the first transplantation were treated with NFAT compared to control mice. This suggested that the treatment with the NFAT inhibitor induces an active state of tolerance. We also compared the treatment with our new NFAT inhibitor specific for phagocytes with FK506, one of the most commonly used

drug in clinic for the treatment of acute transplant rejection (Figure 8) [11].

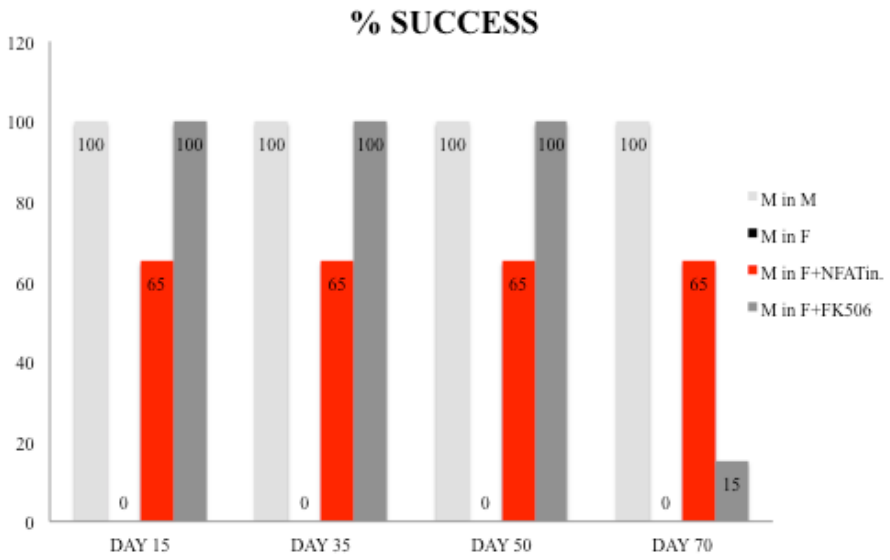


Figure 8 | Comparison between FK506 and the new NFAT inhibitor.
Male to female (minor-mismatched) skin grafts were performed in recipient treated with NFAT in. 100 ug i.p. or FK506 100 ug i.p. Male to male and male to female were performed as control. Recipient mice were treated the day before the transplantation, 2 hours before the transplantation and so on every 2 days for 50 days; then the treatment was stopped and the fate of graft was followed till day 70. Skin graft rejection was determined macroscopically when the graft reached a necrosis of 60%. Skin grafts were performed in SPF animal facility.

As expected, during the treatment with FK506 there was a complete inhibition of graft rejection. As soon as the treatment was stopped, the rejection process started in all the animals. On the contrary, the treatment with the new NFAT inhibitor allowed the transplant maintenance in 65% of the mice also in absence of pharmacological treatment.

There are two hypothesis for this induced active tolerance: T cells anergy or Treg cells induction. To test the second possibility, we set up a new experimental setting that allowed to follow the generation and the fate of specific Treg cells. We transferred in wt female recipient mice $CD4^+$ T cells purified from the spleen of DEREG OT-II female mice treated with diphtheria toxin (DT) in order to deplete endogenous $Foxp3^+$ Treg cells [12]. These mice express a DT receptor-enhanced green fluorescent protein (eGFP) fusion protein under the control of the *foxp3* gene locus, allowing selective and efficient depletion of $Foxp3^+$ Treg cells by DT injection. Female recipient mice were transplanted with the skin from the tail of K5-mOVA male mice (Figure 9). These mice express a membrane form of OVA in skin keratinocytes [13].

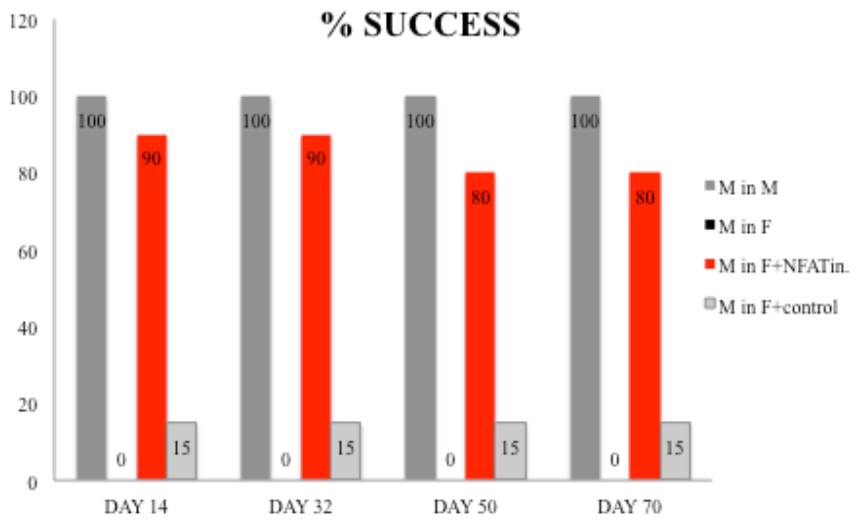


Figure 9 | NFAT inhibition in recipient mice transplanted with K5-mOVA male mice. Male K5-mOVA mice to wt female skin grafts were performed. Before transplantation, recipient mice received $CD4^+$ T cells purified from DEREG OT-II female mice pretreated for 3 days with DT 10 ug i.v. Recipient mice were treated

with NFAT in. 100 ug i.p. the day before the transplantation, 2 hours before the transplantation and so on every 2 days for 50 days; then the treatment was stopped and the fate of graft was followed till day 70. K5-mOVA male to K5-mOVA male and K5-mOVA male to wt female transplantation were performed as control. Skin graft rejection was determined macroscopically when the graft reached a necrosis of 60%. Skin grafts were performed in SPF animal facility.

We obtained higher percentage of graft success in this experimental setting. Also in this case, transplant was maintained also in the absence of drug treatment. In our on going experiments we are following the generation of $Foxp3^{+}GFP^{+}$ Treg cells in this experimental setting. We are also re-transplanting the animals 95 days after the first transplant to follow the fate of the new grafts in order to verify the tolerogenic state of the recipient mice.

Alloantigens in grafted organs are recognized in two different ways: (i) Indirect recognition of the graft involves T cells whose receptors are specific for allogeneic peptides derived from the grafted organ. Proteins from the graft are taken up and processed by the recipient's DCs and are therefore presented by self-recipient MHC class I or class II molecules. (ii) Direct recognition of a grafted organ is by T cells whose receptors have specificity for the allogeneic MHC class I or class II molecule in combination with peptide. These alloreactive T cells are stimulated by donor DCs, which express both the allogeneic MHC molecule and co-stimulatory activity.

So, also decided to investigate the role of NFAT activation in donor DCs. For this purpose we treated male donor mice with the NFAT inhibitor 100 ug i.p. every 2 days for 8 days and then transplanted the skin form these animals into female recipients (Figure 10). We

observed a full inhibition of rejection in the first 15 days which decrease till 65% during the remaining 95 days.

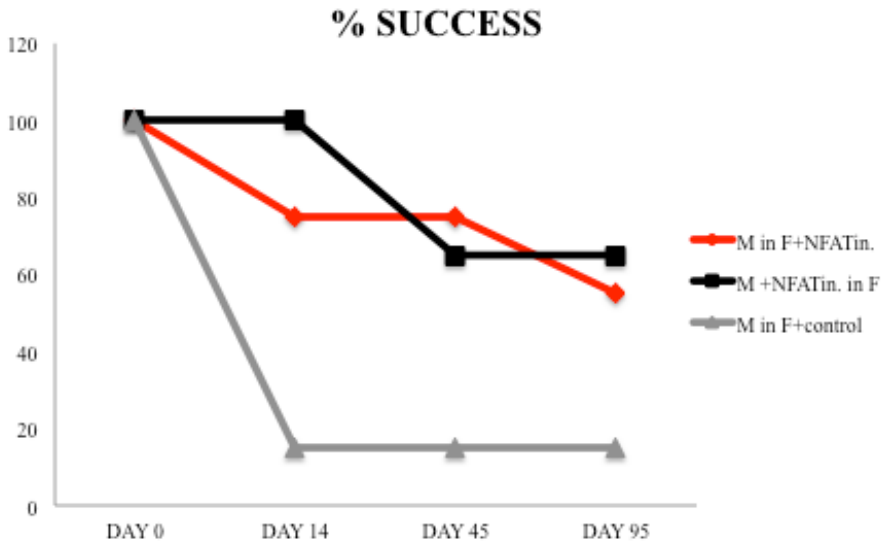


Figure 10 | NFAT inhibition in donor mice in a model of skin transplantation. Male to female (minor-mismatched) skin grafts were performed using donor male mice treated with NFAT in. 100 ug i.p. for 8 days every 2 days. Donor treatment were compared with recipient treatment with NFAT inhibitor or inhibitor control. Skin graft rejection was determined macroscopically when the graft reached a necrosis of 60%. Skin grafts were performed in SPF animal facility.

These data suggest that NFAT activation in donor DC plays a predominant role during the initial phase of transplant rejection with recipient APCs playing a major role in the later phases.

Since NFAT controls IL-2 production in LPS-stimulated DC, we also decided to investigate a possible role for IL-2 released by donor DCs. For this purpose we transplanted the skin from the tail of IL-2 ko male mice in female recipient mice and we observed a significant delay in

the rejection (Figure 11). These data confirm the importance of donor IL-2 production.

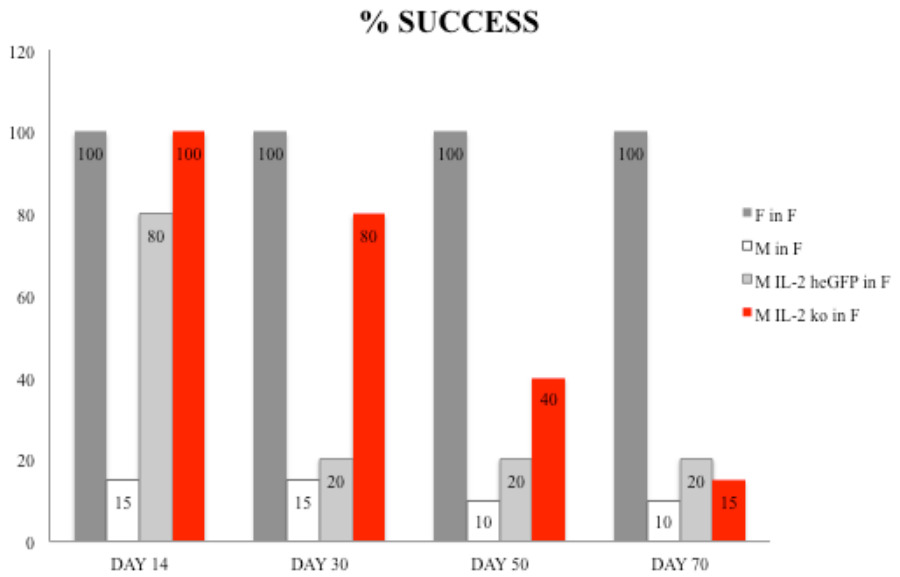


Figure 11 | Role of IL-2 donor mice in a model of skin transplantation.
Male IL-2 ko mice to wt female skin grafts were performed. Male IL-2 heGFP to wt female, wt male to wt male and wt male to wt female transplantation were performed as control. The fate of graft was followed till day 70. Skin graft rejection was determined macroscopically when the graft reached a necrosis of 60%. Skin grafts were performed in SPF animal facility.

3.4 Conclusions

NFAT was initially identified as an inducible nuclear factor that could bind IL-2 promoter in activated T cells [14]. However, its expression is not limited to T cells. At least one of the NFAT family member is expressed by almost every cell type, including other cells of the immune system, such as DCs, macrophages and neutrophils, and non-immune cells [15].

The NFAT family consist of five members and these proteins can be subdivided into two groups according to their functional domains: NFAT5 and NFATc family. NFAT5 is the most evolutionary ancient transcription factor of NFAT family, being present in both invertebrate and vertebrates, and it is activated in response of osmotic stress. The NFATc family in comprised of four members: NFATc1-4, which are present only in vertebrates and are regulated by calcium signalling [7]. The study of the NFATc signalling pathway *in vivo* presents several difficulties due to the redundancy of the system because of the presence of different isoforms with overlapping functions [7][3]. In 2001 in mouse [6] and in 2011 in human [5] it has been demonstrated with *in vitro* studies that NFATc-dependent IL-2 production and transpresentation via CD25 by DCs has a key role in T cells priming since naïve T cells start to express CD25 only many hour after antigen encounter. So we hypothesize that inhibition of NFAT activity in DCs could inhibit IL-2 production and consequent T cells priming, leading to T cells tolerace.

Here, we describe a new tool allowing the inhibition of all NFATc isoforms in innate myeloid cells *in vivo.*

We characterized the delivery of this newly design drug, showing its capacity to specifically target phagocytes. We analysed the drug uptake both *in vitro* and *in vivo* and we identify its high specificity for DCs, macrophages and neutrophils. In order to verify our hypothesis regarding a possible contribution of NFATc activation in DCs to control T cell activation, we tested the new inhibitor in a model of acute skin transplant rejection. In our model, inhibiting the NFATc activation in DCs both in recipient mice and in donor mice led to inhibition of graft rejection, even when the treatment with the drug was interrupted. This was not true when the drug FK506, one of the most commonly used drug in the treatment of acute transplant rejection, was used. Transplanting the skin from IL-2 ko mice, we also showed that donor IL-2 production plays a key role in the first weeks after transplantation. It remains now to be determined if DCs are the main source of this IL-2 production and which stimuli lead to its release. To understand if donor DCs play a major role also in our transplantation model, we are now transplanting the skin from the tail of CD11c.DOG male mice in female recipient mice. In these mice we induced DC ablation by injectin DT [16].

In the future we will also determine which type of tolerance is induced by the new NFAT inhibitor and through which mechanism.

3.5 Methods

Cells. BMDCs were derived from BM progenitors of WT mice as previously described [7]. D1 cells were cultured as previously described [6].

Isolation of skin cells. Cells were isolated as previously describe [17]. Briefly, skin was isolated and digested for 45 minutes in a cocktail containing collagenase XI, hyaluronidase, and DNase. Then 10% FBS was added to stop the reaction, and cells were stained to assess the percentage of different cell populations.

Mice. C57BL/6 mice and IL-2 ko transgenic mice were purchased from Harlan. N. Garbi (Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany) provided CD11c.DOG mice expressing DTR under the control of the long CD11c promoter. In these mice, a specific DC ablation can be induced by diphtheria toxin injection [16]. DEREG OT-II mice were purchased from Harlan. These mice express a DT receptor-enhanced green fluorescent protein fusion protein under the control of the *foxp3* gene locus, allowing selective and efficient depletion of $Foxp3^+$ Treg cells by DT injection [12], in addition in these mice $CD4^+$ T cells are specific for OVA peptide. K5-mOVA transgenic mice were purchased from Charles River, these mice express a membrane form of OVA in skin keratinocytes [13]. All animals were housed under pathogen-free conditions, and all experiments were carried out in accordance with relevant laws and institutional guidelines.

DC depletion. Diphtheria toxin (16 ng/g) was daily administered to CD11c.DOG mice through an i.p. injection for 2 consecutive days. Control mice were given PBS. Effective DC depletion was assessed by FACS and qRT-PCR analysis.

Foxp3$^+$ Treg cells depletion. Diphtheria toxin 10 ug was daily administred to DEREG OT-II mice through an i.v. injection for 3 consecutive days. Effective Foxp3$^+$ Treg cells depletion was assessed by FACS analysis.

In vivo treatment with FK-506. For *in vivo* treatment, FK-506 was resuspended in 40% w/v HCO-60/ethanol. Mice were injected i.p. with FK-506 100 ug the day before the transplantation, 2 hours before the transplantation, the day after the transplantation and so on every 2 day for 50 days.

In vivo treatment with NFAT inhibitor. For *in vivo* treatment, NFAT in. was resuspended in PBS. Mice were injected i.p. with NFAT in. 100 ug the day before the transplantation, 2 hours before the transplantation, the day after the transplantation and so on every 2 day for 50 days.

In vitro treatment with NFAT inhibitor. For *in vitro* treatment, NFAT in. was resuspended in PBS. DCs cells were incubated with NFAT in. for 90 minutes at 37°C at different concentrations (50 ug/ml, 25 ug/ml, 10ug/ml or not treated).

Antibodies and chemicals. CTB-Alexa Fluor® 555 were purchased from Invitrogen. TLR4-grade smooth LPS (E. coli, O55:B5) were purchased from Enzo Life Sciences.

ELISA assays. Concentrations of IL-2 and TNF-α in supernatants were assessed by ELISA kits purchased from R&D Systems.

Immunocytochemistry. Cells were seeded on glass coverslip and incubate with CTB-Alexa Fluor® 555 at 4°C for 30 min. Cells were fixed in paraformaldehyde 4%. The samples were mounted in FluorSaveTM Reagent (Calbiochem) and were imaged by Leica TCS SP2 confocal microscope. ImageJ software was used for image analysis and processing.

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Chapter 4: Final Considerations

4.1 Summary

DCs are specialized leukocytes that orchestrate both early inflammatory innate immune reactions and adaptive immune responses against invading pathogens [1] (Chapter 1.3). DCs are specialized for sampling the environment using a series of receptors for PAMPs. One of the most studied PAMP is LPS, the major component of gram-negative bacteria outer membrane [2] (Chapter 1.2.1). LPS recognition is mediated by a multi-receptor complex formed by LBP, CD14, and MD-2-TLR4. Upon TLR4 binding, a MyD88-dependent and a TRIF-dependent pathways are initiated [3]. In addition, DCs respond to the LPS also triggering TLR4-independent, CD14-dependent signalling, that results in SFK and PLCγ2 activation, IP3 formation and induction of Ca^{2+} entry. The consequent increase in the cytosolic Ca^{2+} concentration triggers the activation of calcineurin that stimulates nuclear NFAT translocation. Once activated, NFAT participate to the control of IL-2 production and of DC-apoptotic cell program [4].

In Chapter 2, we showed that LPS-induced NFAT activation in DCs is necessary for the efficient synthesis of PGE_2, a crucial lipid mediator regulating many proinflammatory processes, including swelling and pain. Mechanistically, CD14-NFAT signalling regulates the expression of microsomal PGE synthase-1 (mPGES-1), a key enzyme in the prostanoid biosynthetic pathway. We also reported that tissue

edema formation induced by subcutaneous administration of LPS is CD14-NFAT-dependent, and that DCs play a major role in this process. Since liquid accumulation in the tissue favours free antigen entry into the afferent lymphatics, DCs can control free antigen arrival to the lymph nodes by controlling edema formation. Exogenous antigens in the inflamed skin are delivered to the lymph nodes for the activation of adaptive T cell responses in two successive waves. In the first wave, antigens freely diffuse through lymphatic vessels and in the late wave are transported by DCs. We propose that tissue-resident DCs control not only the second wave of antigen arrival but also the efficiency of the first wave by controlling edema formation.

In Chapter 3, we described a new NFAT inhibitor that is able to specifically target *in vivo* innate myeloid cells. The use of this new drug allowed the study *in vivo* of NFATc signalling pathway contribution in this most ancient arms of the immune system.

4.2 Conclusions and future perspectives

DCs play a key role in many physiological (e.g. peripheral tolerance) and pathological (e.g. inflammation, pathogen clearance, activation of adaptive immune responses) processes [5]. We reported here a new role of DCs in early cutaneous flogistic reactions and in antigen delivery to lymph nodes, mediated by the CD14-NFAT pathway (Chapter 2). We showed that tissue-resident DCs, activated with *E. coli*-derived LPS, are the principal producers of PGE_2, a potent inflammatory mediator and vasodilator [6]. PGE_2 induces edema formation, a reaction characterized by swelling and pain, that is important to orchestrate early immune responses, such as leukocytes recruitment. Indeed, we showed that edema formation is essential to passively transport antigens from periphery to draining lymph nodes. Regulators of inflammatory responses are of wide interest, because many of the most prevalent human illnesses, such as arthritis, asthma, and atherosclerosis, involve inflammation. PGE_2 is associated with a wide range of chronic inflammatory diseases such as gram- negative-mediated folliculitis and rheumatoid arthritis [7]. Nonsteroidal anti-inflammatory drugs (NSAIDs), like aspirin, are the principal agents used in patients with these diseases. These drugs act by inhibiting COX (both COX-1 and COX-2) and the synthesis of all prostanoids. So, they not only block the formation of individual prostaglandins but also inhibit the production of other "physiological" eicosanoids that might be needed to maintain homeostasis. This can lead to severe side effects, as already described for the gastrointestinal tract [8]. Interestingly, it has been demonstrated that mPGES-1 and PGE_2 are

important players involved in the pathogenesis of arthritis, making them possible new therapeutic targets [9]. DC-derived PGE_2 supports the etiology and pathogenic progression of many inflammation-associated diseases, such as rheumatoid arthritis, inducing local flogistic processes and regulating T-cell differentiation [10]. Since the CD14-NFAT pathway selectively governs PGE_2 production by DCs, drugs that inhibit elements of the CD14 signalling pathway might be used for new clinical approaches.

In the treatment of autoimmune diseases, such as rheumatoid arthritis, and acute transplant rejection, FK506 and CsA are currently used to block IL-2 and other NFATc-dependent cytokines. Unfortunately, these drug are Cn inhibitor and this give rise several side effects due to the general inhibition of this enzyme, which beside NFAT has other substrates. The newly designed NFATc inhibitor is specifically able to block NFAT activation, in addition it is very specific for phagocytes.

It has been demonstrated that NFAT is very important for the interaction between innate immune cells and lymphocytes. In particular its known that activatory DCs produce IL-2 and CD25, both regulated by NFATc, in the first few hours after internalization with T cells. DC-delivered IL-2 is then transpresented to T cells via CD25. Since naïve T cells start to express CD25 only many hours after antigen encounter, the DC-mediated presentation of the IL-2/CD25 complex represents a very efficient system for T cells priming *in vitro* [11][12]. In the context of a skin transplant mouse model, we successfully inhibited transplant rejection using this new drug. We also demonstrated that both donor DCs and DC-derived IL-2 play important roles in this process. Moreover, we demonstrated that

transplant was maintained for long period of time after suspension of drug delivery. We will now determine which type of tolerance has been established using the new NFAT inhibitor drug and we will determine the pathways and molecules involved in this process. Beside a pure mechanistic study, this drug could be used as treatment of several chronic diseases, such as rheumatoid arthritis, and as an immunosuppressant with less side effects thanks to its specific mechanism of action.

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