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Defining the consequences of CD14 engagement and NFAT activation in innate immune cells: from molecular mechanisms to a preclinical model

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LIST OF ABBREVIATIONS:

DC: Dendritic cell

PRR: Pattern Recognition Receptor

TLR: Toll-like Receptor

MAMP: microorganism-associated molecular pattern

PAMP: pathogen-associated molecular pattern

LPS: lipopolysaccharide

NFAT: Nuclear factor of activated T-cells

mPGES-1: microsomal prostaglandin E synthase 1

PGE_2: Prostaglandin E_2

IP_3: Inositol trisphosphate or inositol 1,4,5-trisphosphate

IP_3R: Inositol trisphosphate Receptor

SOCE: Store-operated Ca^{2+} entry

SMOCE: second messenger-operated Ca^{2+} entry

Chapter 1: INTRODUCTION

1.1 Innate Immunity

The mammalian immune system is composed by cells and soluble mediators that interact with each other to forme a barrier against microbial infections and/or cellular and tissue stress propagation, such as tumor progression. While there is a high degree of interconnectivity between its components, the immune system can be broadly divided into two parts: the innate and the adaptive immune systems. The innate response includes soluble factors, anatomical barriers 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 germ-line-encoded pattern-recognition receptors and other cell-surface molecules quickly detect microbial constituents, thereby orchestrating inflammatory reactions [1]. By contrast, adaptive immunity, mediated by antibodies and CD4+ and CD8+ T cells, is much more specific, but takes longer to be activated. This reflects the requirement for the expansion of rare lymphocytes that express somatically rearranged immunoglobulin molecules, or T-cell receptors that are specific for either microbial-derived products or processed peptides that are presented by major histocompatibility

complex (MHC) molecules. Cells of adaptive immune system also develop immunological memory to respond more quickly and with greater specificity to future encounters with the same antigen [2].

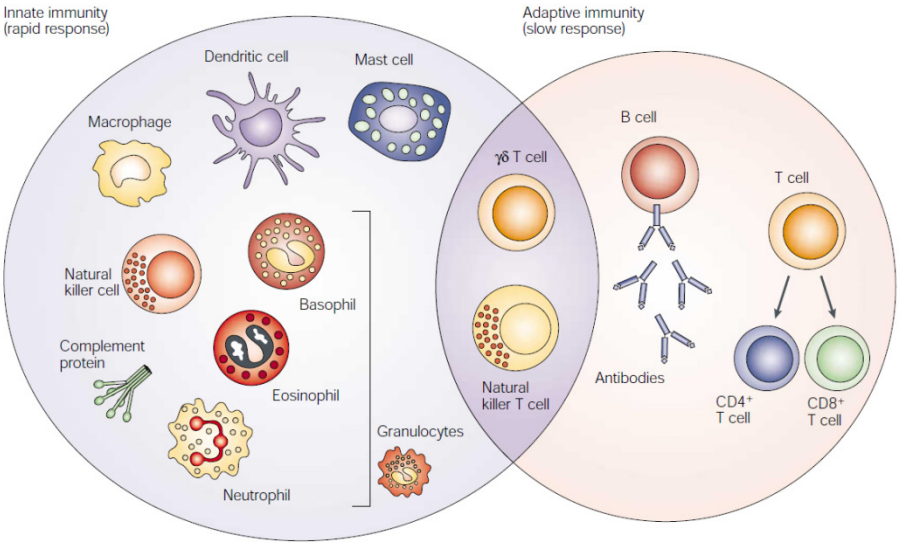

Figure 1| The innate and adaptive immune response [3]**.**

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 combination of these two components allows the immune system to recognize and eliminate invading pathogens with maximum efficiency and with minimal damage to the host. In fact, the functions of innate immunity are short-term, induced early, nonspecific and unable to develop an immunological 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. The efficiency of the whole process is ensured by the different strategies that the two systems use for the microbial recognition: the innate immunity exploits a limited pool of receptor encoded germ-line (PRRs, pattern-recognition receptors), capable to bind highly conserved structures associated to pathogens (MAMPs, microorganism-associated molecular patterns), while adaptive immunity capacity makes use of receptors generated by DNA that creates a variety of virtually unlimited antigen-specific macromolecules. This organization allows the innate immune system to direct the adaptive functions, as proposed by Charles Janeway Jr. about thirty years ago [4]: the acquired immunity cells (T and B lymphocytes), due to the stochastic formation of their receptors (TCR, T-cell receptor and BCR, B-cell receptor) cannot reliably recognize the self (endogenous antigens) from non-self (exogenous antigens, foreign) and they must be educated about the nature of antigen by a system that can determine, with high fidelity, if the identified macromolecule results

from self, infectious non-self (eg. pathogenic microorganisms) or innocuous non-self (eg. non-microbial structures). This role is played by cells of innate immunity and by the specific recognition of MAMPs, exclusively non-self elements, which induces the production of specific signals involved in the modulation of of innate and adaptive processes, which include activation, polarization, magnitude and duration of the action of the overall T and B lymphocytes [5][6]. For example, DCs are considered "sentinels" able, through a particular set of PRRs, to recognize different PAMPs and to engulf the infecting pathogen. In this way, DCs can activate their maturation and, with the production of cytokines and surface molecules (such as B7.1 and B7.2), may instruct the naïve T cells activating the clonal expansion of specific lymphocytes.

1.1.1 Pattern recognition receptors (PRRs)

The innate immune system uses a variety of pattern recognition receptors that can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids [7]. The principal functions of pattern recognition receptors include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis [5].

The targets of PRRs are sometimes referred to as pathogen-associated molecular patterns (PAMPs), although they are present on both pathogenic and non-pathogenic microorganisms (MAMPs). PAMPs are well suited to innate immune recognition for three main reasons. First, they are invariant among microorganisms of a given class. Second, they are products of pathways that are unique to microorganisms, allowing discrimination between self and non-self molecules. Third, they have essential roles in microbial physiology, limiting the ability of the microorganisms to evade innate immune recognition through adaptive evolution of these molecules [4]. Bacterial PAMPs are often components of the cell wall, such as lipopolysaccharide, peptidoglycan, lipoteichoic acids and cell-wall lipoproteins. An important fungal PAMP is β-glucan, which is a component of fungal cell walls. The detection of these structures by the innate immune system can signal the presence of microorganisms. An important aspect is that PRRs themselves do not distinguish between pathogenic microorganisms and symbiotic (non-

pathogenic) microorganisms, because the ligands of the receptors are not unique to pathogens. Yet, despite humans being colonized by trillions of symbiotic bacteria, homeostasis is somehow maintained under normal conditions. Furthermore, innate immune recognition of symbiotic microorganisms has an important role in maintaining intestinal homeostasis [8]. And dysregulation of these interactions can lead to the development of inflammatory bowel disease and other disorders [9].

Recent evidence indicates that PRRs are also responsible for recognizing endogenous molecules released from damaged cells, termed damage associated molecular patterns (DAMPs) [10]. 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 [11]. 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 an 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) [12].

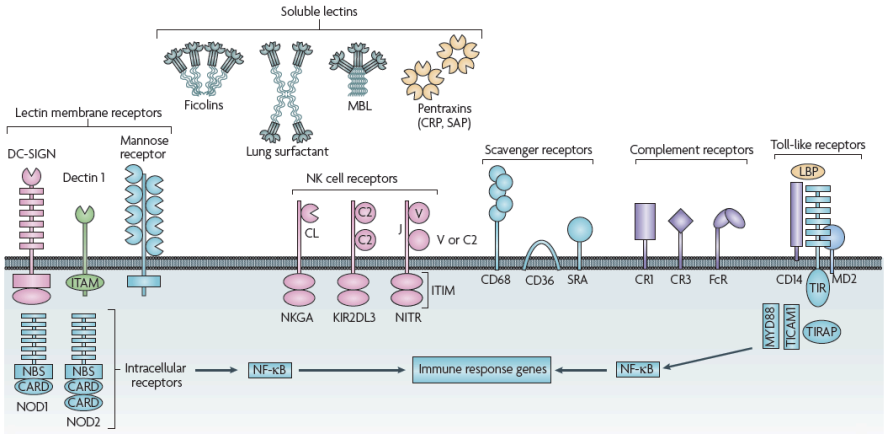

Figure 2| Pattern-recognition receptors (PRRs) [7]**.**
Pattern-recognition receptors (PRRs) include: soluble proteins, such as collectins, ficolins and pentraxins, integral membrane receptors, including Toll-like receptors, and intracellular sensors, such as oligomerization domain (NOD) receptors.

1.1.2 Toll-like receptors (TLRs)

The recognition of microorganisms is mediated by several families of innate immune receptors that collectively survey the extracellular space, endolysosomal compartments and the cytoplasm for signs of infection or tissue damage. The Toll-like receptor (TLR) family is the best characterized group of innate immune receptors in terms of known ligands, downstream signalling pathways and functional relevance [13].

Humans express ten functional TLRs (TLR1 to TLR10), whereas twelve TLRs (TLR1 to TLR9 and TLR11 to TLR13) have been identified in mice [14]. Ligands have been determined for all TLRs (ligands for mouse TLR12 [15] and mouse TLR13 [16] have been recently identified) except for human TLR10. TLR1, TLR2, TLR4, TLR5, TLR6 and 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 [17] are found intracellularly, where they mediate the recognition of nucleic acids or parasitic products (TLR11 and TLR12 [18]). Thus, the subcellular distribution of TLRs correlates, to a substantial extent, with the compartments in which their ligands are found (Table 1).

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 [19].

Table 1 | Localization and ligands of TLRs [20]

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]

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.

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 leucine-rich repeat modules located in the ectodomains of TLRs are composed of 20–30 amino acids each and contain the consensus sequence LxxLxLxxN. TLRs have different amino acid compositions within these modules, leading to variations in structural conformation that allow for ligand interaction [19]. Amino acid variations and the formation of heterodimers can only provide a limited platform for the recognition of the varied set of TLR ligands. Thus, 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 lipoteichoic acid (LTA) and diacylated lipopeptides [21]. 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 signaling [22]. For example, the endoplasmic reticulum (ER) luminal chaperones glucose-regulated protein of 94 kDa (GRP94) and protein associated with TLR4 A (PRAT4A) are responsible for the proper folding and function of TLR1, TLR2, TLR4, TLR7 and TLR9, but not TLR3 [23]. The ER membrane protein uncoordinated 93 homolog B1 (UNC93B1) is

required for the translocation of TLR3, TLR7 TLR9, TLR11, TLR12 and TLR13 to endolysosomes, where these TLRs bind to their ligands (RNA or DNA) [24] [25].

The intracellular signaling domains of TLRs have substantial sequence similarity with the interleukin-1 receptor and are termed Toll/IL-1R homology (TIR) domains. TIR containing proteins include not only receptors but also MyD88, TRIF, TIRAP, TRAM, and SARM, which are signaling-adaptor proteins [26]. 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 [27].

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 encompasses 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 [26].

TLR family members are expressed by innate immune cells such as macrophages and dendritic. 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) [28] , glucose and fat metabolism (TLR signalling pathways might contribute to the development of obesity-associated insulin resistance) [29] 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) [30].

1.2 LPS signaling

Lipopolysaccharides (LPS) is a lipid-containing carbohydrate and a major component of the outer leaflet of the outer membranes of gram-negative bacteria [31]. LPS is one of the best studied among PAMPs and it induces a potent immune response against infectious microbial agents. It also play key role in the pathogenesis of sepsis (a whole-body inflammatory state associated with a the presence or presumed presence of an infection) and ultimately septic shock, that result from a harmful or damaging host response to infection [32].

LPS is not a single molecule of well-defined chemical structure but rather a collection of molecules with extensive structural diversity [31]. Different bacterial species produce LPSs with different structures. Sometimes the same bacterial species can produce different LPS molecules under different growth condition, for example *Y. pestis* modifies its LPS structure in response to temperature [33].

LPSs are amphipathic macromolecules composed of three parts: lipid A, a core carbohydrate chain, and a highly variable O-antigen carbohydrate chain (Figure 3).

Lipid A contains multiple lipid chains attached to a diglucosamine backbone. The backbone of lipid A is composed of two glucosamines connected by a β(1–6) linkage. Typically 4∼7 lipid chains with lengths of 12∼14 carbons are connected to the glucosamine backbone by ester or amide links. Lipid A is negatively charged because phosphate

groups are often attached to the 1 and 4′ positions of the glucosamine backbone. Different bacterial species produce lipid As with significant structural variations. Both the number and structure of the lipid chains can vary, and the phosphate groups can be modified by covalent attachment of phosphate. Some of these structural differences affect the immunological activity of lipid A [34]. For example, host cells can actively modulate the LPS-induced inflammatory response by modifying the proinflammatory microbial molecule themselves. Such a mechanism has been shown in the case of an acyloxyacyl hydrolase that cleaves acyl chains from the lipid A [35]. In mice lacking this enzyme, acylated LPS persists for longer periods of time after infection with Gram-negative pathogens and elicits increased B cell proliferation and antibody production [36]. Alkaline phosphatases (AP) have also been shown to modify LPS by dephosphorylating its lipid A [37]. Lipid A, which accounts for the toxicity of LPS, contains two phosphate groups coupled to glucosamines; removal of one of the phosphate groups generates a monophosphoryl lipid A that is a 100-fold less toxic than the unmodified lipid A [38].

Lipid A is connected to the core part of the carbohydrate chain, which is relatively well-conserved among bacterial species. The core contains carbohydrate units such as Kdo (3-deoxy-D-manno-oct-2-ulosonic acid) and Hep (L-glycero-D-mannoheptose) not generally found in mammalian cells. Similar to lipid A, the core sugars are frequently modified by the addition of other chemical groups such as Kdo, Hep, glucosamines and phosphates.

The core sugar is connected to a long carbohydrate chain called the O-antigen and composed of highly variable repeating units. The number and structure of these repeating units vary in different bacterial species and strains. Repeating units are not essential for the immunological activity of LPS, but can change the triggered responses [19].

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.

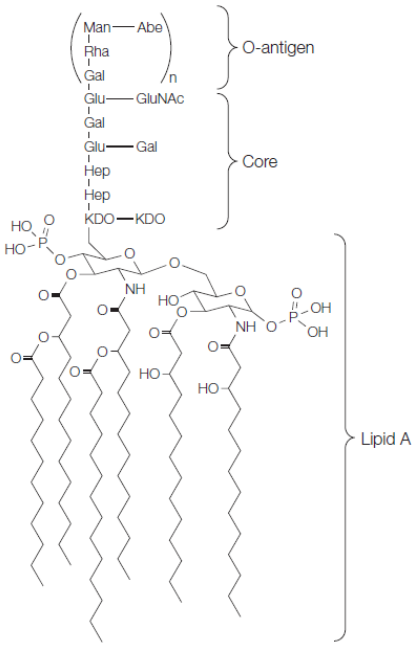

Figure 3| Chemical structure of lipopolysaccharide (LPS) [39]**.**
LPS is composed of lipid A, core oligosaccharide and O-antigen.

1.2.1 LPS recognition complex

The first indication that mammalian TLRs may function as pattern recognition receptors came with the description of a human homologue of *Drosophila* Toll, now known as TLR4 [40]. Subsequently, positional cloning analysis of the LPS-nonresponsive mouse strain, C3H/HeJ, showed that a point mutation in the TIR domain of TLR4 was responsible for the defect in LPS signal transduction [41][42]. Another mouse strain, B10.ScCR, did not respond to LPS and turned out to lack the genomic region that contains the entire tlr4 gene [41][42]. Finally, mice with a targeted deletion of the TLR4 gene were unresponsive to LPS [43].

Together, these studies demonstrated the essential role for TLR4 in recognition of a major component of gram-negative bacteria.

TLR4, however, is not the sole receptor involved in LPS recognition (Figure 4). Transport of LPS molecules in the serum is mediated by a glycoprotein called LPS-binding protein (LBP) [44]. Its main function is to extract LPS from the bacterial membrane and transfer it to the accessory protein CD14, a GPI-linked cell surface protein [44]. Careful *in vitro* studies have demonstrated that LBP can facilitate binding of LPS to CD14 and enhances the sensitivity of macrophages to LPS by 100- to 1000-fold [45][46][47].

LBP belongs to a family of lipid binding and transport proteins that includes BPI, CETP and PLTP [48]. LBP is a boomerang-shaped protein containing two domains with similar folds that consist of an extended β barrel and a long α helical backbone [49]. Both the N- and C-

terminal barrels form elongated pockets that can bind to phospholipids. The importance of these pockets for LPS binding has not been clearly defined. Mutagenesis and domain swapping experiments suggest that the N-terminal domain is primarily responsible for LPS binding while the C-terminal domain is involved in interaction with CD14 and transfer of LPS [47]. Several biophysical measurements have revealed that the LPS bound to LBP is not a monomer but an oligomeric aggregate [50].

CD14 is a leucine-rich repeat family protein that is attached to the plasma membrane by a GPI tail [44]. CD14 without the GPI link could be released into the blood stream. The main role of CD14 appears to be its capacity to monomerize LPS for efficient presentation to TLR4, indeed its critical role is underscored by the LPS hyporesponsive phenotype of CD14-deficient mice [51].

Finally, a small protein called MD-2 is also a component of the LPS-recognition complex [52]. MD-2 is a ∼14-kDa secreted glycoprotein that forms heterodimers with TLR4 (TLR4-MD-2 complex). MD-2 cannot transmit 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 [53] [54]. 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 [52].

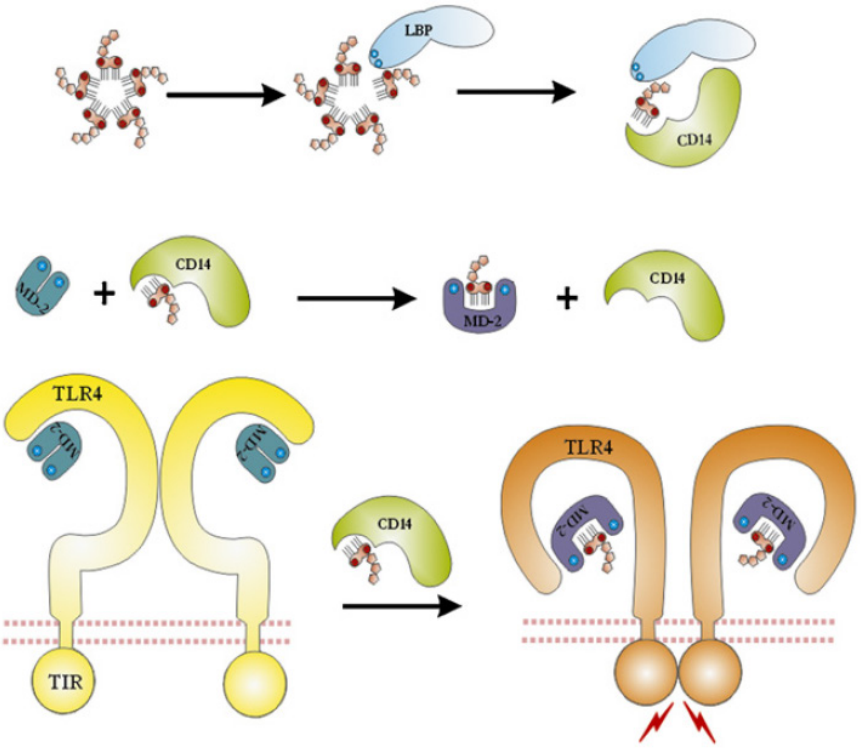

Figure 4| Schematic representation of the steps of LPS recognition [55]**.**
LBP binds to Gram-negative bacteria or aggregates of LPS, decreasing the binding energy of LPS monomers. The LPS molecule is shuttled to CD14 (activation pathway), where the acyl chain of lipid A is protected from the solvent 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 TLR4 pathway

TLR4 is unique among pathogen-recognition receptors in that it initiates two signaling pathways sequentially in different cellular locations. Binding of a bridging factor, TIRAP, allows recruitment of an adapter protein, MyD88, at the plasma membrane, which leads to the production of proinflammatory cytokines. Upon internalization, TLR4 uses a different bridging factor, TRAM, to activate a MyD88-independent pathway that results in type I interferon expression [56].

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 [57]. From this location, which is a PtdIns(4,5)P_2-rich subdomain of the plasma membrane, signaling is initiated and the receptor is endocytosed by a CD14-dependent process [58]. 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. In turn, Syk promotes phospholipase C γ2 (PLCγ2) activation that results in a drop of PtdIns(4,5)P2 concentrations, inducing membrane invagination [59] and releasing the TIRAP-MyD88 complex from the membrane. Finally, dynamin pinch off the vesicle from the plasma membrane [60], which will ultimately became an early endosome. 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 signaling

from an intracellular location, ultimately leading to the induction of the gene encoding IFN-β.

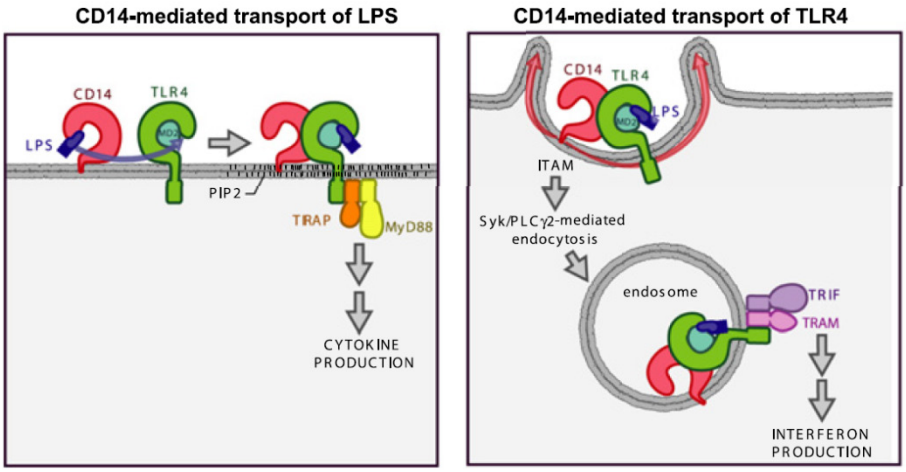

Figure 5| CD14 is involved in the transport of LPS and TLR4 [58]
CD14 first captures and transports LPS to the plasma membrane localized complex of TLR4 and MD2, which signals through the TIRAP-MyD88 adaptors to activate inflammatory cytokine expression. CD14 then transports TLR4 to endosomes by a process mediated by Syk and PLCγ2, where TRAM-TRIF signaling leads to the expression of type I IFNs.

On the plasma membrane, MyD88 recruits IRAK4 and IRAK1\2, forming a helical multiprotein complex called 'myddosome' [61]. The E3 ligase TNF receptor-associated factor 6 (TRAF6) is then recruited and activated, and synthesizes K63-linked polyubiquitin chains. Recently, IRAK2 was shown to play a central role in TRAF6 polyubiquitination [62]. 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 [63]. 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 nuclear factor-κB (NF-κB) to enter the nucleus to turn on target genes involved in immune and inflammatory responses [64].

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 [65]. In this way, RIP1 with the help of TRADD and TAK1, activate NF-kB and MAPKs to induce proinflammatory cytokines [66]. TRAF3 links TBK1 to the TRIF-dependent pathway [67], which in combination with IKKε, phosphorylates and activates IRF3 [68], 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 [69] and cancer [70].

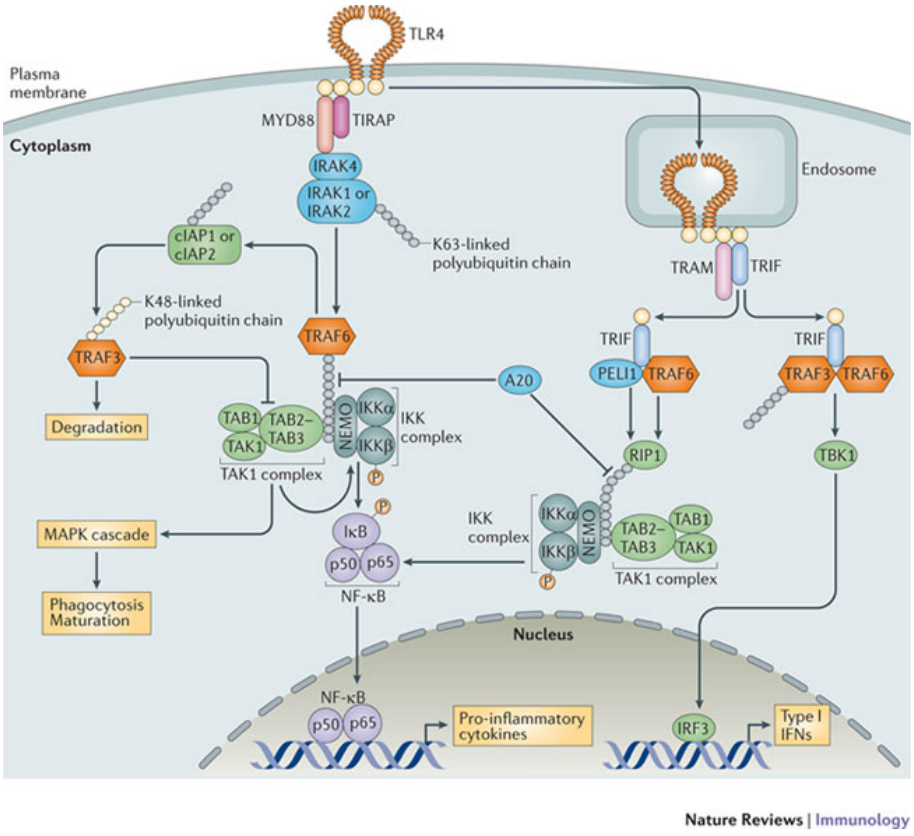

Figure 6| TLR4 pathway [64]

1.2.3 CD14-NFAT pathway

CD14 is an accessory protein that assists TRL4 in its functions. This molecule is required for LPS presentation to TLR4, thus allowing cellular responses to low doses of LPS [71] and it is also required for the recruitment of TRIF and TRAM [58] (see 1.2.2). Indeed, CD14 has shown to be absolutely required for a full response to LPS [72].

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 [73]. Activation of DCs through TLRs results in the activation of various signaling pathways and transcription factors, leading to the transcription of many cytokines. One such cytokine is interleukin-2 (IL-2) [74], a key factor that confers unique T cell [75] and NK cell [76] stimulatory capacity to DCs. Since IL-2 production by T cells is known to depend on the NFAT pathway (Figure 7), 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.

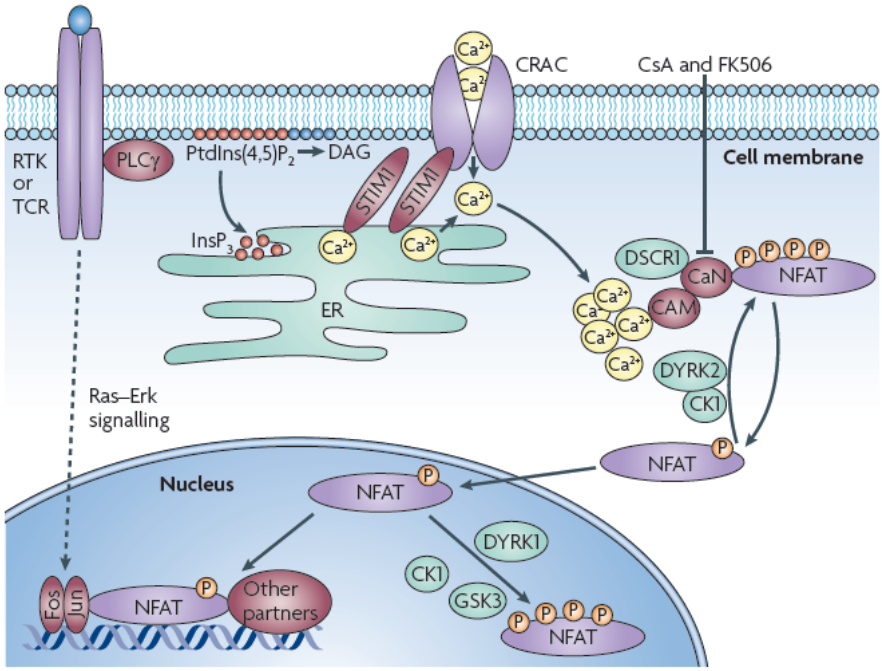

Figure 7| Calcium signaling and activation of NFAT [77]

Receptor tyrosine kinases (RTKs) and immunoreceptors such as the T cell receptor (TCR) activate phospholipase Cγ (PLCγ), which hydrolyses phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to releaseinositol-1,4,5-trisphosphate (InsP3) and diacylglycerol (DAG). InsP3 and loss of calcium binding on stromal interaction molecule 1 (STIM1) induces calcium release from the endoplasmic reticulum (ER). Calcium release-activated calcium (CRAC) channels, including ORAI1, are then opened, allowing a sustained influx of extracellular calcium. Calmodulin (CAM) binds calcium and in turn the phosphatase calcineurin (CaN). 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 nuclear factor of activated T cells (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. P, phosphorylation.

The other effector molecules of this CD14-dependent pathway has been identified and an accurate model is now available to describe the events for NFAT activation in DCs. In this model, engagement of CD14 by LPS results in Src family kinases (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. The authors tend to 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 [78] [79]. 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 [75] directly promotes NFAT activation.

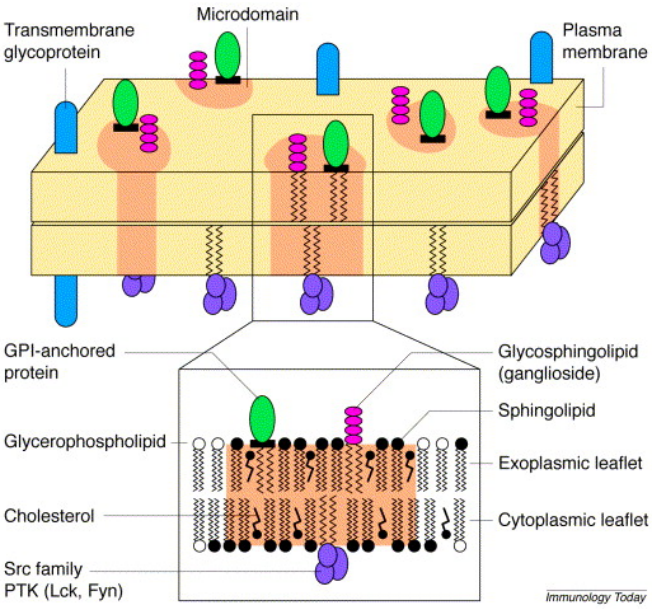

Figure 8| Organization of the plasma membrane microdomains [80]
Clusters of liquid-ordered sphingolipid and cholesterol molecules in the exoplasmic leaflet of the plasma membrane constitute 'microdomains' that are dispersed within the fluid mosaic of the membrane glycerophospholipids. Glycosylphosphatidylinositol (GPI)-anchored proteins also cluster in microdomains but most transmembrane proteins tend to be excluded. The cytoplasmic leaflet corresponding to surface microdomains remains poorly characterized. Src family protein tyrosine kinases (SFKs) are also found in microdomains. Surface crosslinking of many GPI-anchored proteins and gangliosides leads to cell stimulation following coalescence of microdomains and activation of the associated SFKs.

In turn, SFKs activate PLCγ2 by phosphorylation. This enzyme cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 then diffuses through the cytosol to bind to IP_3 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 7). 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) [81]. IP_3 binds to and opens IP_3 receptors (IP_3Rs) in the membrane of the endoplasmic reticulum (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 [82].

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 [78].

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 (Figure 9) [83].

In addition to IL-2 production, the CD14-NFAT pathway in DCs plays a key role in regulating their life cycle after LPS treatment. Indeed, DCs undergo an apoptotic process during maturation [84] 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 [73] 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 in wild-type mice [85]. 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 [86]. 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 remain 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 [87] [88], 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.

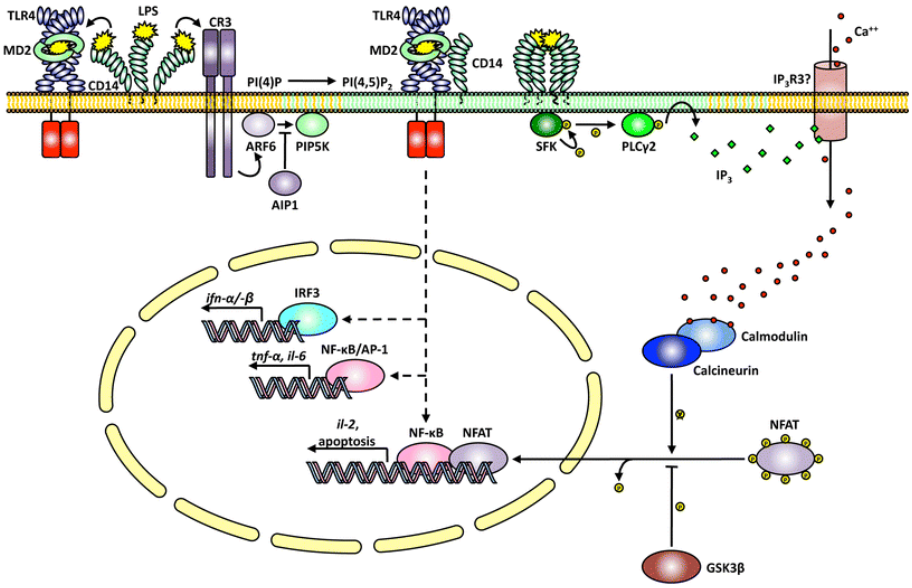

Figure 9| CD14-dependent and TLR4-independent NFAT activation in DCs [89]

In addition to its role in LPS recognition and presentation to TLR4 and CR3, CD14 has autonomous signaling functions in dendritic cells (DCs). Upon LPS-induced clusterization, CD14 transiently recruits and activates a Src family kinase (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, IP_3 triggers Ca^{2+} from external space (IP_3R3?). 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.

1.3 Dendritic cells (DCs)

Dendritic cells (DCs) are antigen presenting cells (APCs - cells that display foreign antigen complexes with major histocompatibility complex molecules to T cells) that are located throughout the body and form a sophisticated and complex network that allows them to communicate with different populations of lymphocytes, thereby forming an interface between the external environment and the adaptive immune system [90]. This function resides in the ability of DCs to couple a survey of the microenvironment, in the form of antigen uptake and responsiveness to environmental cues, to a cellular differentiation program termed “maturation” that enhances their abilities to activate immune cells [91]. To this end, DCs employ a diversity of microbial sensors and other probes, such as PRRs, that upon ligand binding initiate intracellular signaling cascades that drive both phenotypic and functional maturation.

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 Dendritic cell heterogeneity

DCs are a heterogeneous group of cells that have been divided into different subsets that differ in location, migratory pathways, detailed immunological function and dependence on infections or inflammatory stimuli for their generation. This segregation was initially based on their distinct patterns of cell-surface molecule expression (Table 2). The four major categories of DCs are conventional DCs (cDCs), which predominate in the steady state (the state of the immune system in healthy adult mice that are not subject to infections or inflammatory stimuli); Langerhans cells; plasmacytoid DCs (pDCs); and monocyte-derived DCs, which are induced in response to inflammation [92].

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.

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 in peripheral tissues, then migrating through the lymph to lymph nodes in response to danger signals; such migration to the lymph nodes also occurs, at a lower rate, in the steady state [93]. Migratory DCs are not found in the spleen and are restricted to the lymph nodes [94],

where they can be divided on the basis of peripheral tissue of origin. Migratory DCs can be broadly divided into $CD11b^+$ DCs (also known as dermal or interstitial DCs) and $CD11b^-$ DCs [95], which have more recently been shown to express CD103 [94].

The second major category of conventional DCs is the lymphoid tissue-resident DCs that are found in the major lymphoid organs, such as the lymph nodes, spleen [96] and thymus [97]. They do not migrate into lymphoid organs from the lymphatics; rather, they collect and present antigens in the lymphoid organ itself. 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). $CD8\alpha^+$ DCs are important for their capacity to cross-present antigens [98] 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, to a lesser extent, but appear to be more efficient at presenting MHC class II-associated antigens to CD4+ T cells [98]. Lymphoid tissue-resident DCs do not traffic from other tissues but develop from precursor DCs found in the lymphoid tissues themselves [99]. 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 [100].

Langerhans cells

Langerhans cells are resident in the skin and, like migratory DCs, migrate to the lymph nodes to present antigens. 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 [101].

Plasmacytoid DCs

Plasmacytoid dendritic cells (pDCs) are specialized in rapid and massive secretion of type I interferon (IFN-α/β) in response to viruses and/or virus-derived nucleic acids [102]. pDCs are rare cells (0.3–0.5% of the human peripheral blood or of murine lymphoid organs) that develop in the bone marrow and reside primarily in the lymphoid organs in the steady state, entering the lymph nodes from the blood [103]. pDCs have the round morphology of a secretory lymphocyte, turn over relatively slowly [104], and express low levels of MHC class II and costimulatory molecules. pDCs are low (mouse) or negative (human) for the integrin CD11c but positive for the B cell marker B220/CD45RA. Several relatively pDC-specific surface markers have been established, such as human blood dendritic cell antigen (BDCA)-2 and ILT7 (immunoglobulin-like transcript 7) and murine SiglecH and Bst2; other useful (albeit less specific) markers include human IL-3Rα (CD123) and BDCA-4 and murine Ly6C and Ly49Q [102].

Monocyte-derived DCs

Although monocytes were originally described as precursors to all the different subpopulations of macrophages found in the steady state and formed under inflammatory and infectious conditions [105], recent data have demonstrated conclusively that circulating blood monocytes can be rapidly mobilized and can differentiate also into DCs [106]. These monocyte-derived DCs (moDCs), newly formed during flogistic reactions, appear to fulfill an essential role in defense mechanisms against pathogens by participating in the induction of both adaptive and innate immune responses. In this regard, moDCs have the capacity to activate antigen-specific CD4+ T-cell responses and to cross-prime CD8+ T cells, during viral, bacterial, and parasitic infections [107]. Similarly to conventional DCs, monocyte-derived DCs express CD11c, MHC class II molecules, CD24 and SIRPα (also known as CD172a), and upon activation 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 LY6C [108].

Importantly, in contrast to DCs developing in the steady state, moDCs formed during inflammatory and infectious processes 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.

DC subset	DC type	CD8α	CD103	CD205	EPCAM (CD326)	CD11b	B220	DC-SIGN	Langerin (CD207)	Antigen presentation	Major cytokine produced
pDCs	Lymphoid-resident DCs	+/–	–	–	–	–	+	++	–	Poor	IFNα
CD8α+ DCs	Lymphoid-resident DCs	+	low	+	–	+	–	–	+/–	Cross-presentation on MHC class I	IL 12p70, IFNλ
CD4+ DCs	Lymphoid-resident DCs	–	–	–	–	+	–	–	–	Presentation on MHC class II	
DN DCs	Lymphoid-resident DCs	–	–	–	–	+	–	–	–	Presentation on MHC class II	
CD11b+ DCs	Migratory DCs	–	+/–	+	–	+	–	ND	–	Presentation on MHC class II	
CD103+ DCs • Lung	Migratory DCs	–	+	++	+/–	–	–	–	–	Cross-presentation on MHC class I	
• Intestine		–	+	–	–	+	–	–	+		
Langerhans cells	Migratory DCs	–	–	++	+	+	–	–	++	Presentation of self antigens for tolerance induction	IL 10
Monocyte-derived DCs	Induced by inflammation	–	–	–	–	+	–	+	–	Cross-presentation	TNF

Table 2| Phenotypic markers of DC subset [86]

1.3.2 DC maturation

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 [91]. 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 [109]. 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) [98]. 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 (Figure 10) [110] [111]. 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 [112].

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 [113]. After this process is completed, DCs can leave the inflammatory site and reach the spleen or lymph nodes to initiate the adaptive immune response [114].

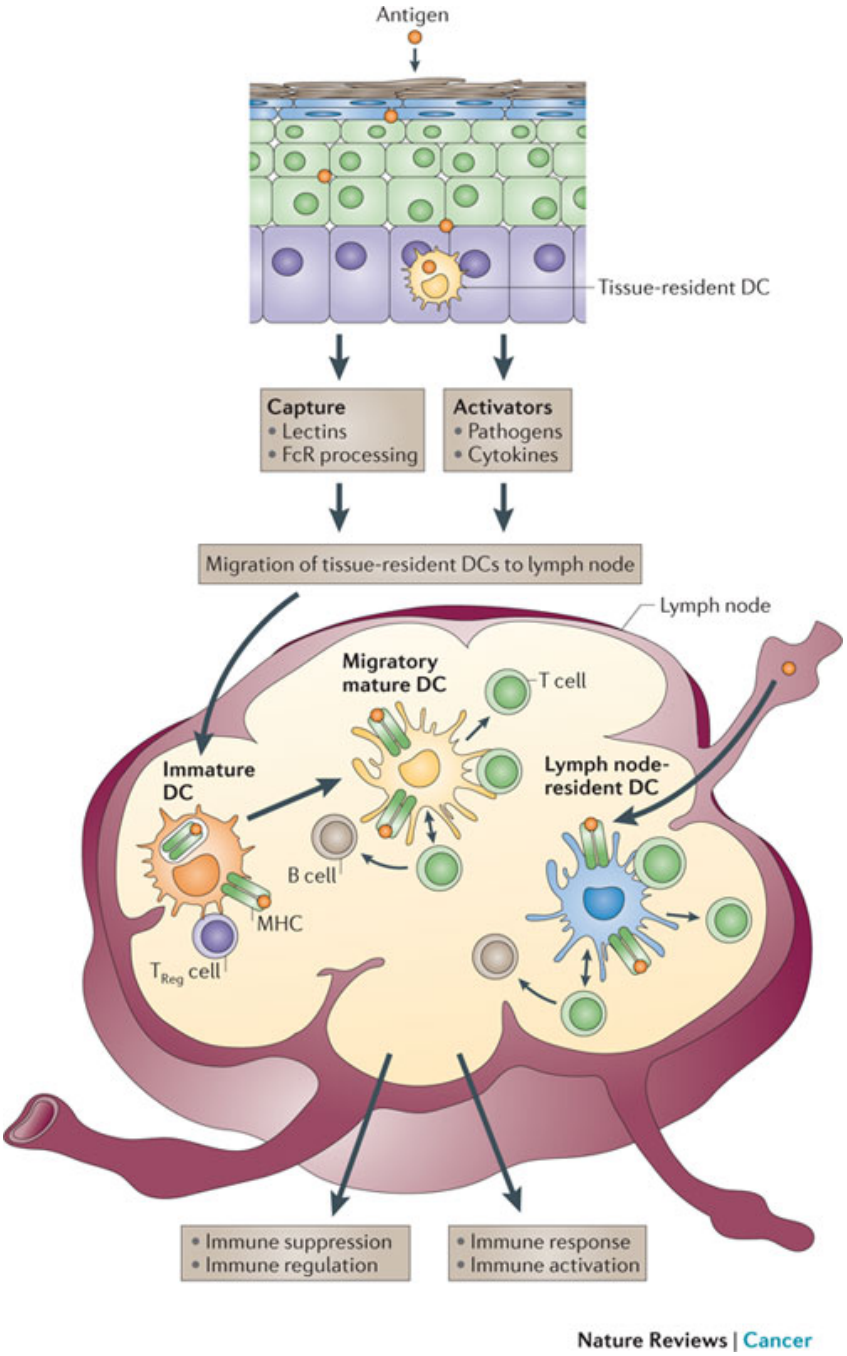

Figure 10 | Launching the immune response [115]

Antigens can reach lymph nodes through two pathways: via lymphatics, where the antigen is captured by lymph node-resident dendritic cells (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 major histocompatibility (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.

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 [116].

A significant number of investigations have linked the failure to achieve DC programmed cell death to autoimmunity [117]. 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 DC-mediated T_H-cell polarization

The different classes of specific immune responses are driven by the biased development of pathogen-specific effector CD4+ T-cell subsets, the principal and more studied are T helper 1 (T_H1) and T_H2 cells, that activate different components of cellular and humoral immunity. T_H1/T_H2-cell-mediated immunity to microbial invasion is controlled by regulatory T cells (T_{reg}), recently redefined as a diverse class of natural and adaptive regulatory T cells [118], that prevent autoimmunity and potentially lethal tissue destruction by chronic innate or adaptive immune responses, while they also ensure the development of strong immunological memory by delaying pathogen eradication.

The fate of naive T_H cells is determined by three signals that are provided by pathogen-primed DCs. The stimulatory signal 1 results from the ligation of T-cell receptors (TCRs) by pathogen-derived peptides, presented by MHC class II molecules on the cell surface of DCs, and determines the antigen-specificity of the response. The initiation of protective immunity also requires T-cell co-stimulation [119]. In the absence of this co-stimulatory signal 2, T_H cells become anergic, which might lead to tolerance. TCR stimulation and co-stimulation allow naïve T_H cells to develop into protective effector cells, normally accompanied by high level expression of selective sets of cytokines. The balance of these cytokines and the resulting class of immune response strongly depend on the conditions under which DCs are primed for the expression of the T-cell-polarizing signal 3

[120]. Indeed, adaptive immunity to pathogens is largely dependent on the nature of PAMPs and\or DAMPs, accordingly on the combinatorial PRR activation, that induce DC maturation and then the expression of T_H-cell-polarizing molecules. In this regard, it is possible to classify the structures that determine the type of signal 3 into three major groups [121]:

1. Type 1 PAMPs or TFs (tissue factor). These molecules induce DCs to produce factors that polarize T_H1 responses, such as IL-12, IL-23, IL-27 and IFN type I. To this class belong many bacterial structures, such as LPS, and endogenous factors, such as IFN-γ, IFN-α, IL-18 and CCR5L.

2. Type 2 PAMPs or FTs. The T_H2 response can be triggered by different cell types in addition to DCs, such as basophils. Generally, parasite-derived molecular structures induce DCs to activate this response.

3. Regulatory-type PAMPs or FTs. Paradoxically, pathogens that are eliminated by specific T_H1- or T_H2-cell responses might also induce some form of tolerance, for example, through the stimulation of thymus-derived $CD4^+CD25^+$ Treg [122]. Clearly, immunological tolerance will benefit the survival of the pathogen. By contrast, tolerance might

also be important for the host to prevent excessive, potentially lethal, pathology of chronic T_H1- or T_H2- cell-mediated inflammation and to ensure the formation of long-term memory.

1.4 Inflammation

Inflammation is the body's immediate response to damage to its tissues and cells by pathogens, noxious stimuli such as chemicals, or physical injury [123]. Acute inflammation is a short-term response that usually results in healing: leukocytes infiltrate the damaged region, removing the stimulus and repairing the tissue. Chronic inflammation, by contrast, is a prolonged, dysregulated and maladaptive response that involves active inflammation, tissue destruction and attempts at tissue repair. Such persistent inflammation is associated with many chronic human diseases, including allergy, atherosclerosis, cancer, arthritis and autoimmune diseases. The processes by which acute inflammation is initiated and develops are well defined, but much less is known about the causes of chronic inflammation and the associated molecular and cellular pathways.

The process of acute inflammation involves the rapid (over a period of hours) recruitment of innate immune cells and the activation of specific tissue functions that underlie the four cardinal signs of inflammation: an increase in local blood flow, accounting for the redness (*rubor*) and warmth (*calor*) of inflamed tissues; a localized leakage of plasma-protein-rich fluid (known as an exudate) into the tissue, accounting for the swelling (*tumor*) of inflamed tissues; and a localized recruitment and activation of circulating leukocytes such that they are induced to enter the infected or damaged tissues. Pain (*dolor*), the fourth cardinal sign of inflammation, is caused by

mediators released by leukocytes on C-type sensory nerve fibres. Increased blood flow augments leukocyte delivery to the site, increased leaking of plasma-protein-rich fluid creates a provisional matrix to support leukocyte entry into the tissue, and increased adhesion facilitates leukocyte capture and extravasation [124].

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 11).

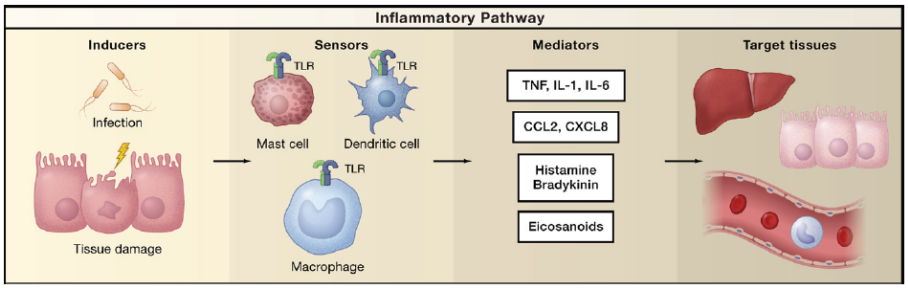

Figure 11| Inflammatory Pathway Components [125]

The inflammatory pathway consists of inducers, sensors, mediators, and target tissues. Inducers initiate the inflammatory response and are detected by sensors. Sensors, such as Toll-like receptors (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 (e.g., infection or tissue injury) 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.

Inducers are the signals that initiate the inflammatory response. They can be divided in exogenous or endogenous. Exogenous inducers are PAMPs, virulence factors or non-microbial originated molecules, such as allergens, irritants, foreign bodies and toxic compounds. Endogenous inducers are signals produced by stressed, damaged or otherwise malfunctioning tissues. One common (but not universal) theme in detecting acute tissue injury is the sensing of the desequestration of cells or molecules that are normally kept separate in intact cells and tissues.

These structures are recognized by sensors expressed on specific immune and non-immune cell types. Particularly, microbial inducers are rapidly detected by PRRs expressed by tissue-resident cells, such as macrophages, mast cells and DCs. These elements produce a inducer-specific pool of soluble mediators, which in turn alter the functionality of many tissues and organs. In addition, other mediators can be derived from plasma proteins.

Inflammatory mediators can be classified into seven groups according to their biochemical properties [126] : vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes.

1 - Vasoactive amines (histamine and serotonin) are produced by mast cells and platelets. They have complex effects on the vasculature, causing increased vascular permeability and vasodilation, or vasoconstriction, depending on the context. The immediate consequences of their release by mast cells can be highly

detrimental in sensitized organisms, resulting in vascular and respiratory collapse during anaphylactic shock.

2 - Vasoactive peptides can be stored in an active form in secretory vesicles or generated by proteolytic processing of inactive precursors in the extracellular fluid. Other vasoactive peptides are generated through proteolysis by the Hageman factor, thrombin or plasmin and cause vasodilation and pain. Pain sensation has an important physiological role in inflammation by alerting the organism to the abnormal state of the damaged tissue.

3 - Complement fragments C3a, C4a and are produced by several pathways of complement activation. C5a promotes granulocyte and monocyte recruitment and induce mast-cell degranulation, thereby affecting the vasculature.

4 - Lipid mediators (eicosanoids and platelet-activating factors) are derived from phospholipids, such as phosphatidylcholine, that are present in the inner leaflet of cellular membranes. The eicosanoid, such as prostaglandins, cause vasodilation. Platelet-activating factors are generated by the acetylation of lysophosphatidic acid and activate several processes that occur during the inflammatory response, including recruitment of leukocytes, vasodilation and vasoconstriction, increased vascular permeability and platelet activation.

5 - Inflammatory cytokines are produced by many cell types, most importantly by macrophages and mast cells. They have several roles in the inflammatory response, including activation of the endothelium and leukocytes and induction of the acute-phase response.

6 - Chemokines are produced by many cell types in response to inducers of inflammation. They control leukocyte extravasation and chemotaxis towards the affected tissues.

7 - Proteolytic enzymes (including elastin, cathepsins and matrix metalloproteinases) have diverse roles in inflammation, in part through degrading ECM and basement-membrane proteins. These proteases have important roles in many processes, including host defence, tissue remodelling and leukocyte migration.

These inflammatory mediators induce tissue modification, such as vasodilation and leukocytes recruitment, in order to remove or sequester the source of the disturbance, to allow the host to adapt to the abnormal conditions and, ultimately, to restore functionality and homeostasis to the tissue.

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. Several key

regulatory mechanisms of resolution have been identified including the switch from pro-inflammatory arachidonic acid–derived prostaglandins and leukotrienes to anti-inflammatory, resolution-inducing lipoxins. This switch, in turn, orchestrates programmed death by apoptosis of neutrophils and macrophages recruitment, leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines such as TGF-β. The anti-inflammatory program ends with the departure of macrophages through the lymphatic vessels. This transition from neutrophil to macrophages recruitment results in clearance of the dead cells and other debris and initiation of tissue repair at the affected site [127].

If the inflammatory trigger is not eliminated by the acute inflammatory response or persists for any other reason, the resolution phase may not be appropriately induced and a chronic inflammation state may ensue. This state can be caused by chronic infections, unrepaired tissue damage, persistent allergens or undigestable foreign particles. These inflammatory conditions are of particular interest because they accompany many diseases of industrialized countries, including obesity and type 2 diabetes, atherosclerosis, neurodegenerative diseases, and cancer. Interestingly, in these cases of chronic inflammation there appear to be vicious cycles and feed-forward processes connecting inflammation and the pathological process it accompanies [125].

1.4.1 Prostaglandins and PGE_2

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 prostaglandins (PG), which contain a cyclopentane ring, and thromboxanes (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 (for instance PGE_2), 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 [128].

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 [129].

Prostaglandin E_2 (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 [130].

The synthesis of PGs (Figure 12) 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 PGG_2, which is subsequently peroxidized to PGH_2. Both enzymatic reactions are catalyzed by the protein cyclooxygenase (COX), which consists of two forms [131]: 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 PGH_2 by cytosolic (cPGES) or by membrane-associated/microsomal (mPGES-1 or mPGES-2) prostaglandin E synthase [132]. 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 [133].

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 [134]. 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 [132].

PGE_2 exhibits a broad range of biological actions 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 (EP_{1-4}), each of which is encoded by distinct genes [135]. Whereas the "contractile" EP_1 receptor induces calcium mobilization by phospholipase C activation via G_q protein, "relaxant" EP_2 and EP_4 receptors are known to activate adenylyl cyclase via stimulatory G protein. On the other hand, the "inhibitory" EP_3 receptor reduces cAMP levels as it is coupled to inhibitory G proteins [128].

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 [136]. Redness and edema result from increased blood flow into the inflamed tissue through PGE_2-mediated augmentation of arterial dilatation and increased microvascular permeability [137]. In fact, PGE_2 binds to $EP_{2/4}$ on smooth muscle cells and endothelial cells (components of blood vessels), inducing a local vasodilation that results in edema formation [138]. This process is a very important event in order to orchestrate early inflammatory immune responses.

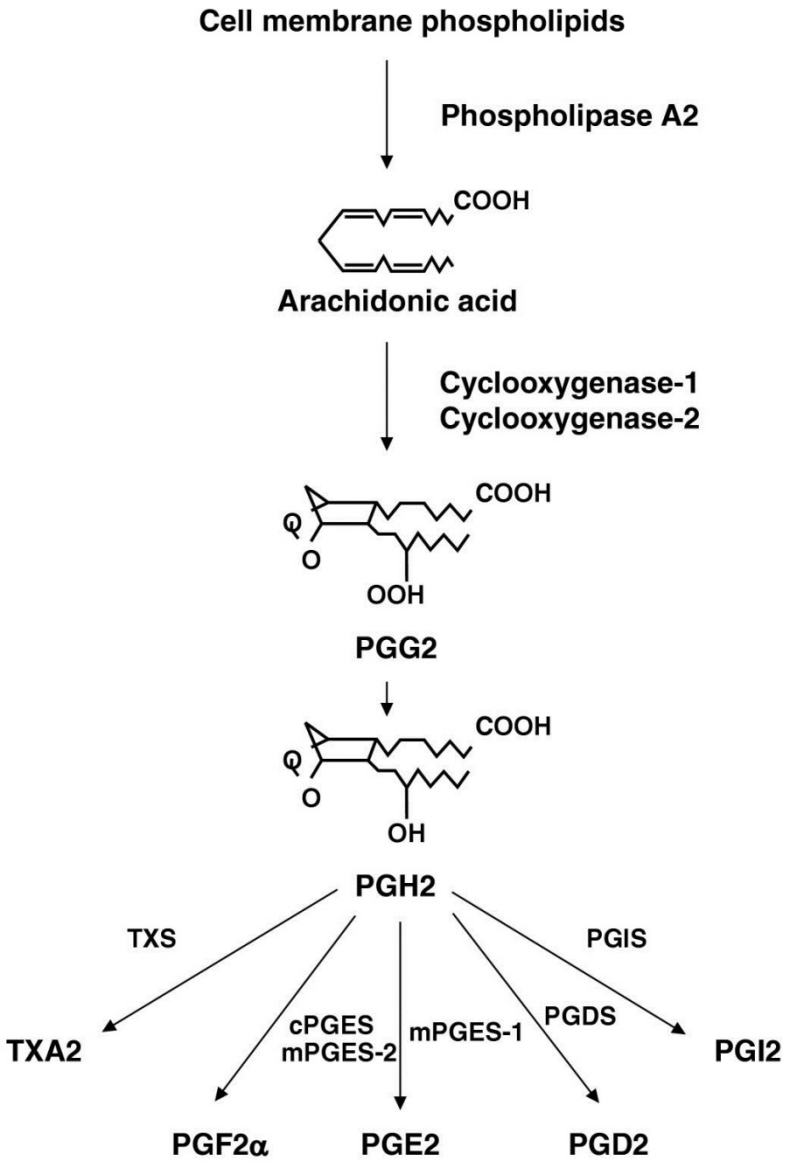

Figure 12| The prostaglandin E_2 (PGE_2) biosynthetic pathway [139]

1.5 Scope of thesis

DCs sense the presence of pathogens through germline-encoded PRRs, which 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, we have recently described that following LPS exposure different NFAT isoforms are also activated. 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 investigated the Ca^{2+} entry process in LPS-stimulated DCs, defining the molecular mechanism downstream CD14 activation that leads to Ca^{2+} mobilization.

Chapter 2: CD14 and NFAT mediate 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, PGE_2 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.

Chaper 3: Study of the role of IP_3R3 in the activation of the NFAT pathway downstream of CD14

Ca^{2+} entry is necessary to induce calcineurin activation and nuclear translocation of NFAT in electrically non-excitable cells. We demonstrated here that the plasma membrane Ca^{2+} channel IP_3R3 is part of the CD14-NFAT pathway in LPS-stimulated DCs, triggering a monophasic Ca^{2+} transient mediated by a second-messenger-operated calcium entry (SMOCE) mechanism.

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Chapter 2

CD14 and NFAT mediate lipopolysaccharide-induced skin edema formation in mice

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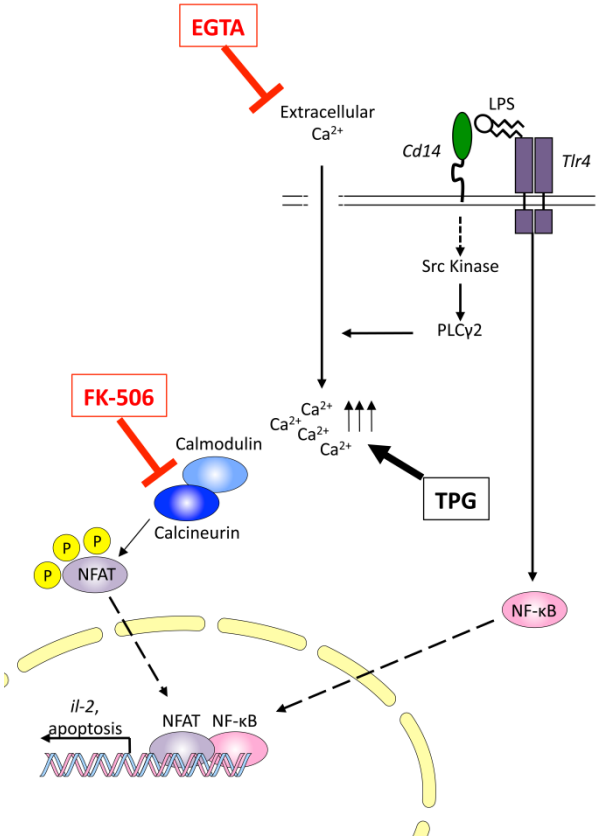
Inflammatory processes are initiated by innate immune system cells that perceive the presence of pathogens or microbial products through the expression of pattern recognition receptors (PRRs) [1]. Following the encounter with their specific ligands, PRRs initiate a signal transduction pathway, leading 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, activation protein 1 (AP-1), and IFN regulatory factors (IRFs), are activated [6].

Recently, the nuclear factor of activated T cells (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 PGG_2, which is in turn converted to PGH_2. Finally mPGES-1 converts PGH_2 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 Src family kinase (SKF) member. Active SFK then phosphorylates PLC2, which in turn catalyzes the hydrolysis of PI(4,5)P2 into the second messengers diacylglycerol (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).

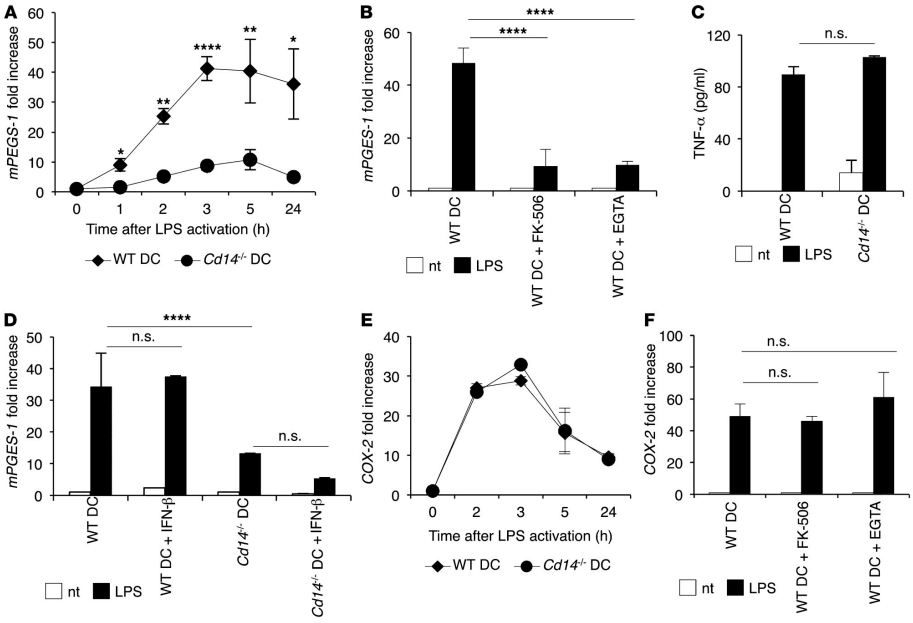
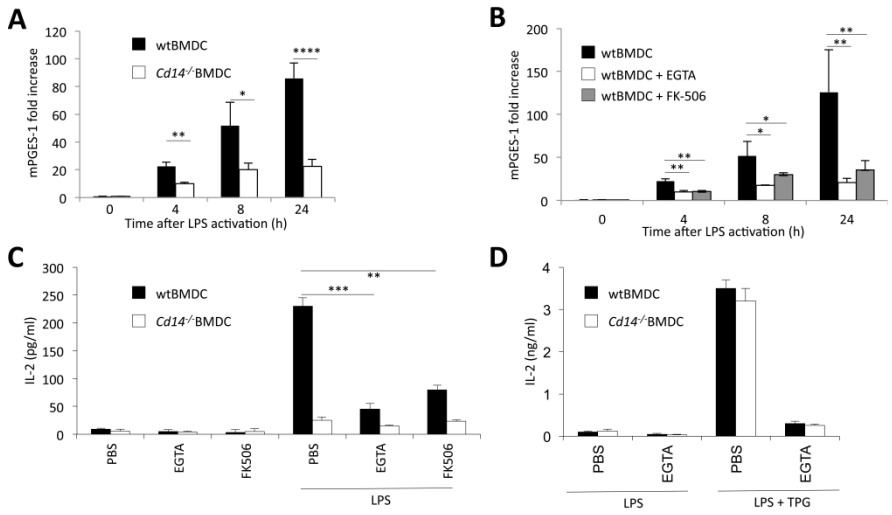


Figure 1| CD14-dependent NFAT activation induced by LPS in DCs regulates *mPGES-1* expression in vitro. (A) Real-time PCR analysis of *mPGES-1* mRNA induction kinetics in WT and CD14-deficient ex vivo DCs stimulated with LPS (1 µg/ml). (B) Upregulation of *mPGES-1* mRNA after 3 hours of LPS administration 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 thapsigargin ([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.

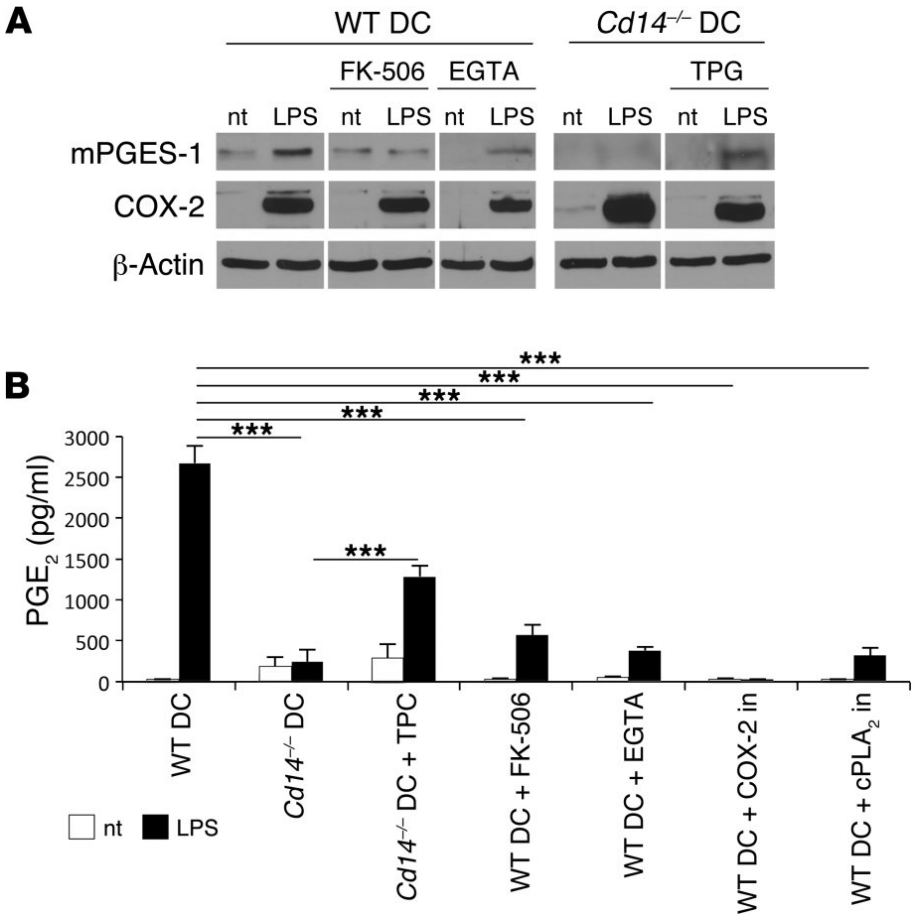


Figure 2| CD14-dependent NFAT activation induced by LPS in DCs regulates PGE_2 synthesis in vitro. (**A**) Western blot analysis of mPGES-1 and COX-2 induction in WT and CD14-deficient BMDCs 4 hours after LPS (1 μg/ml) and/or thapsigargin (TPG) (50 nM) treatment. Where indicated, the cells were pretreated 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 thapsigargin (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 $cPLA_2$ inhibitor ($cPLA_2$ in, 1 μM, 30 minutes pretreatment). Values represent means of at least 3 independent experiments performed in duplicate + SEM. ***$P < 0.0005$.

2.2 PGE2 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 thapsigargin (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.

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.

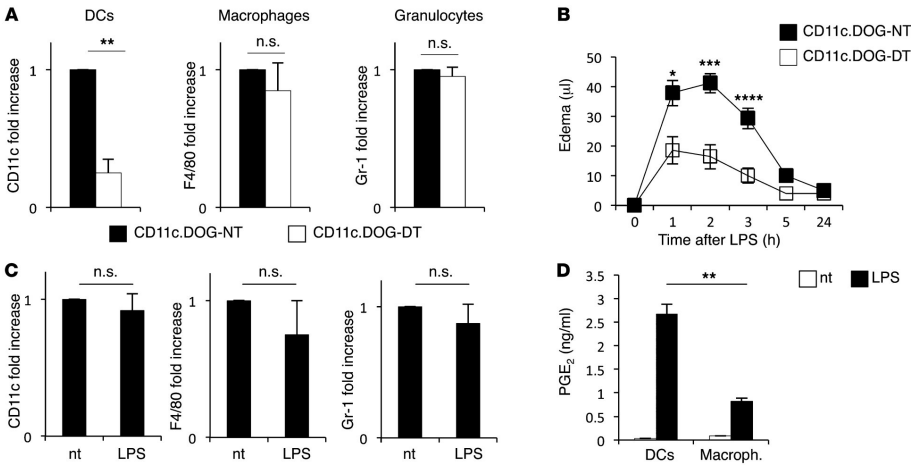
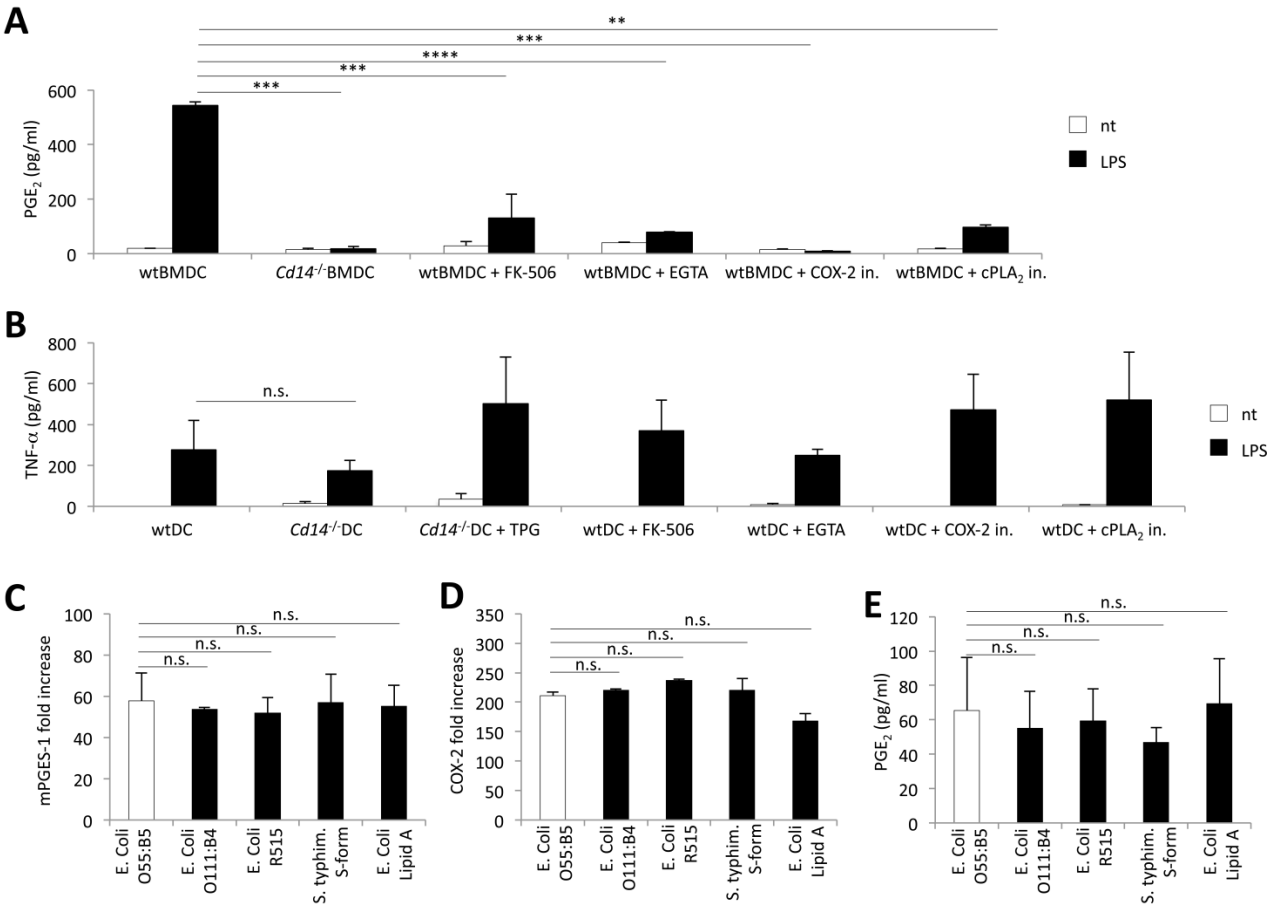


Figure 3| DCs regulate LPS-induced tissue edema formation. (**A**) Real-time PCR analysis of *CD11c*, *F4/80*, and *Gr-1* mRNA in the footpad of CD11c.DOG mice before (CD11c.DOG-NT) or after 2 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). Values represent at least 3 independent experiments with 3 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**) PGE_2 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.


A
**
***
****
***
***
600
400
200
0
PGE2 (pg/ml)
nt
LPS
wtBMDC
Cd14-/-BMDC
wtBMDC + FK-506
wtBMDC + EGTA
wtBMDC + COX-2 in.
wtBMDC + cPLA2 in.
B
800
600
400
200
0
TNF-α (pg/ml)
n.s.
nt
LPS
wtDC
Cd14-/-DC
Cd14-/-DC + TPG
wtDC + FK-506
wtDC + EGTA
wtDC + COX-2 in.
wtDC + cPLA2 in.
C
100
80
60
40
20
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mPGES-1 fold increase
n.s.
n.s.
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E. Coli O55:B5
E. Coli O111:B4
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COX-2 fold increase
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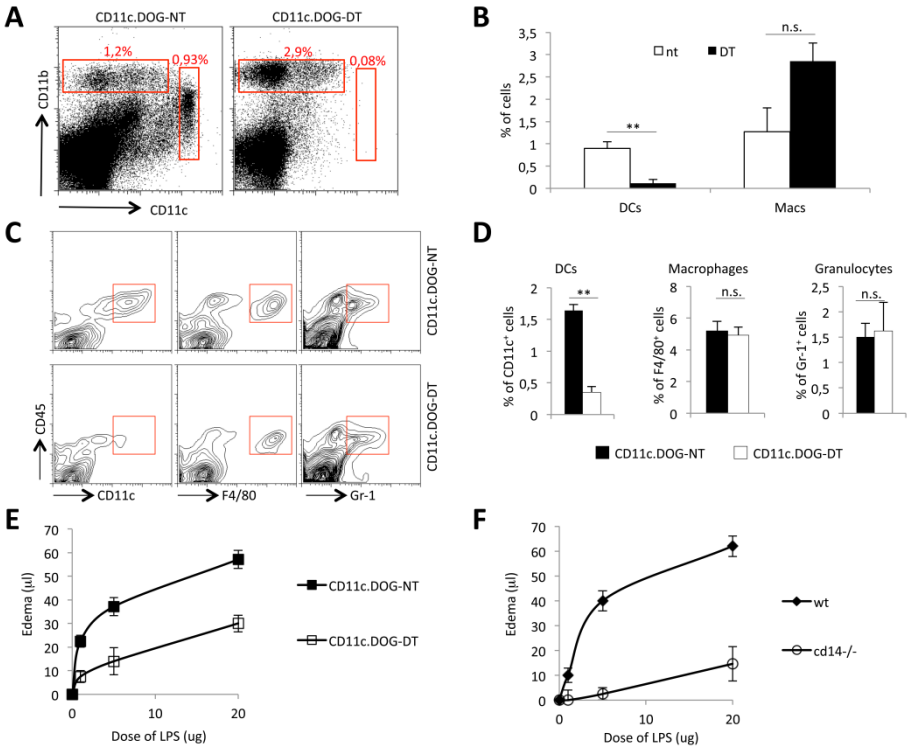
E. Coli O55:B5
E. Coli O111:B4
E. Coli R515
S. typhim. S-form
E. Coli Lipid A

Supplementary Figure 3| PGE2 production by BMDCs. (A) wt and Cd14-/- BMDCs were treated with LPS and PGE2 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 PGE_2 secretion induced by the indicated species of LPS in wt BMDCs.

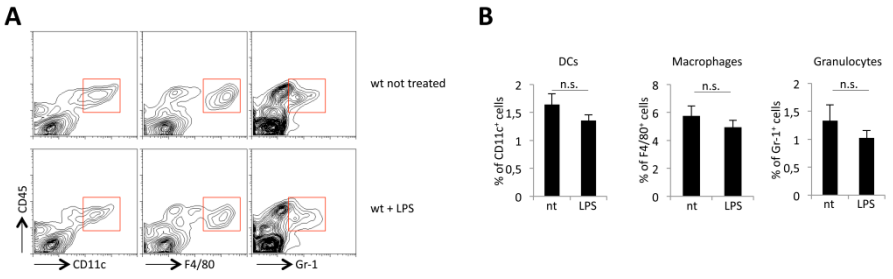


Supplementary Figure 4| DC depletion from the spleen and the skin of CD11c.DOG mice after DT treatment. (A) Representative dot plots of splenocytes from CD11c.DOG mice before (CD11c.DOG-NT) or after 2 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). $CD11c^{+}CD11b^{int}$ DC and $CD11b^{+}CD11c^{int}$ 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 $Gr\text{-}1^{+}$ (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 PGE_2 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).

2.3 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 thapsigargin, indicating a role for NFAT activation in this in vivo model of PGE2-dependent inflammation (Figure 4C). Thapsigargin 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).

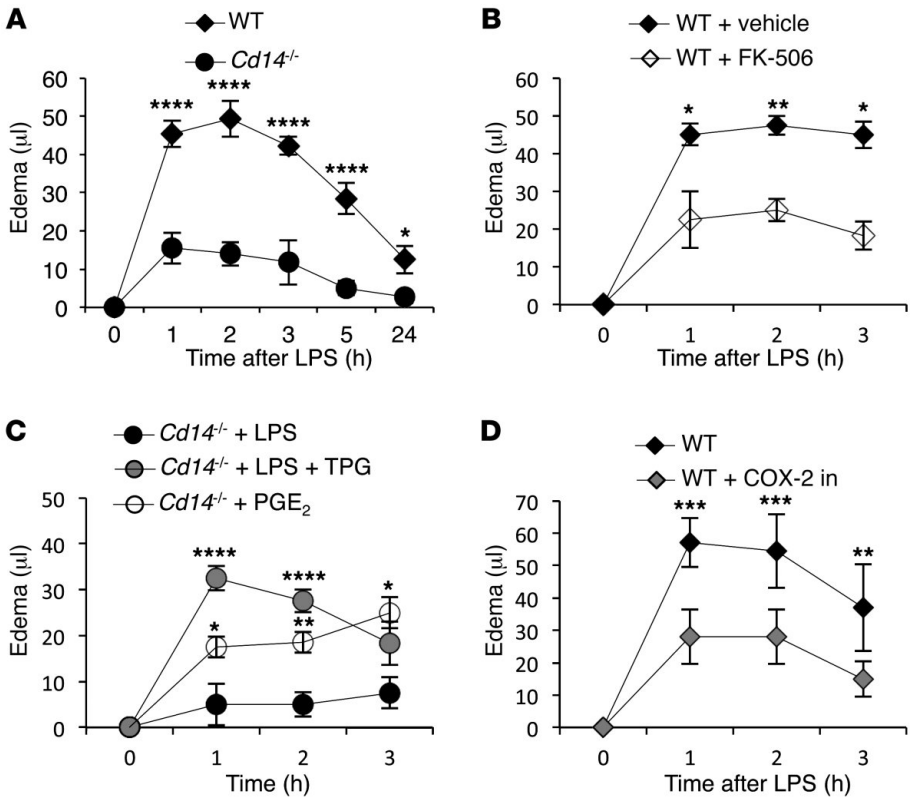
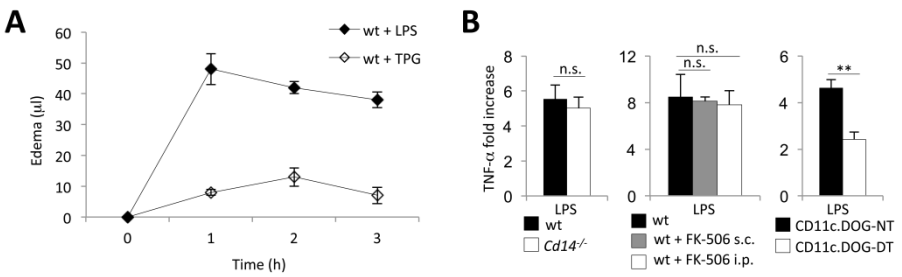


Figure 4| DCs regulate LPS-induced tissue edema formation through CD14-dependent and NFAT-dependent mPGES-1 expression. (**A**) Inflammatory swelling in the footpads of WT and *Cd14*$^{-/-}$ mice at the indicated time points after s.c. injection of LPS (20 µg/footpad). (**B**) Inflammatory swelling in 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 thapsigargin, 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 thapsigargin (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).

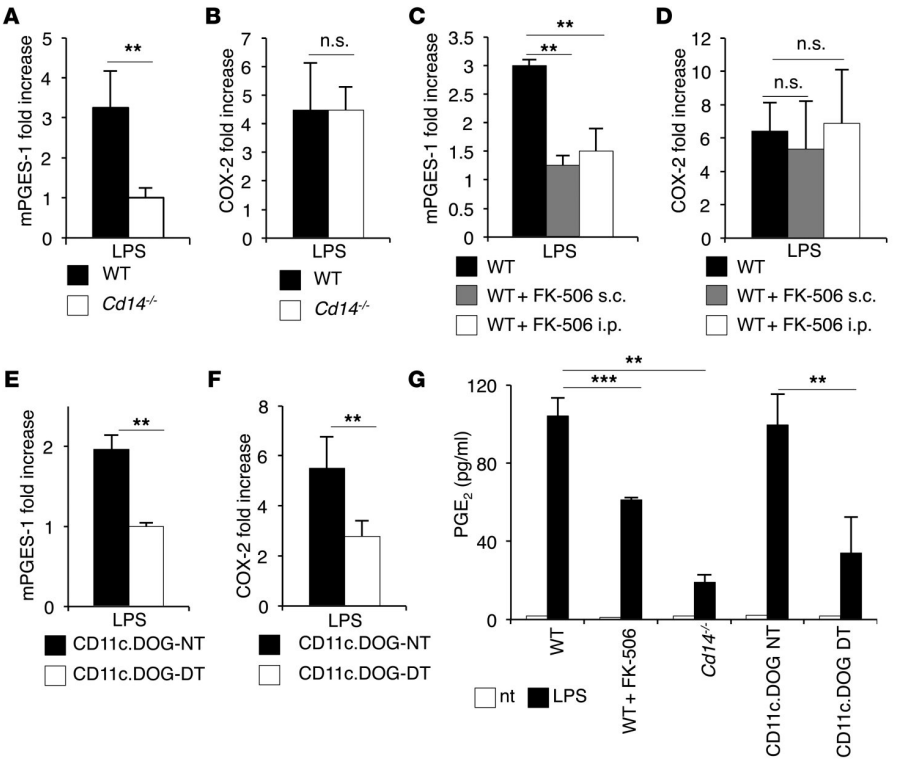


Figure 5| CD14-dependent NFAT activation induced by LPS in DCs regulates mPGES-1 expression and PGE_2 synthesis in vivo. Real-time PCR analysis of (**A**, **C**, and **E**) *mPGES-1* and (**B**, **D**, and **F**) *COX-2* mRNA induction 2 hours after LPS injection in the footpads of WT and *Cd14–/–* mice. (**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**) PGE_2 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.

2.4 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.

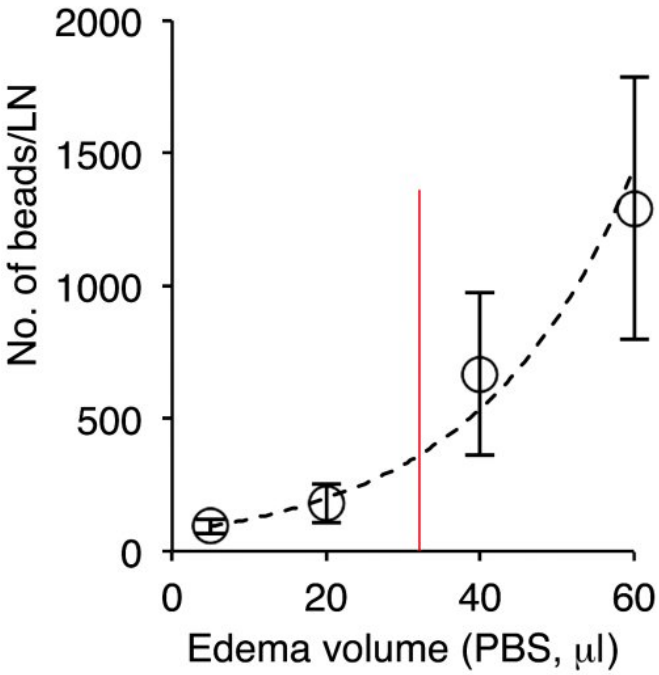
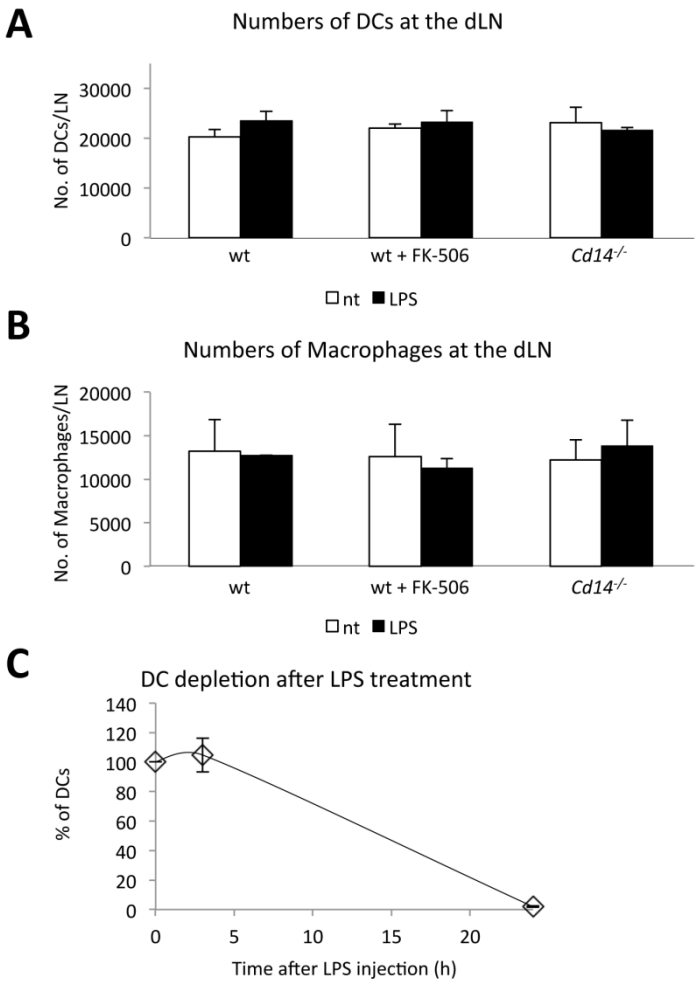


Figure 6| The efficiency of free antigen arrival at the draining lymph nodes increases with the increase of edema volume. Absolute numbers of FITC-labeled microbeads reaching the draining lymph nodes in WT mice injected in the footpad with the indicated PBS volumes. Dotted line represents an interpolated exponential curve with R^2 = 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).

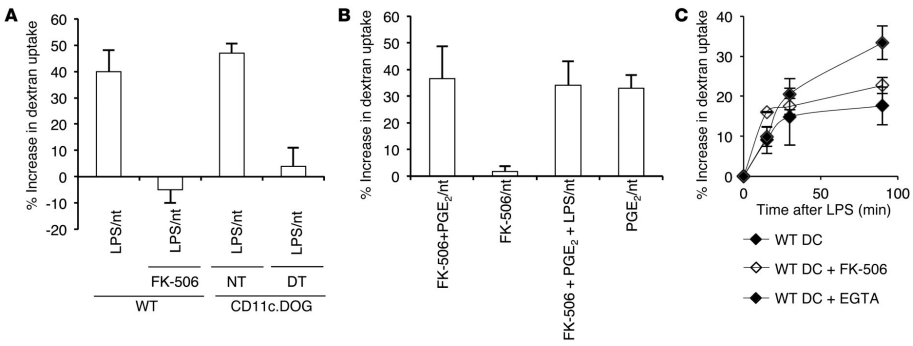


Figure 7| Edema induced by LPS increases the efficiency of dextran arrival at the draining lymph nodes. (**A**) Percentage of LPS-induced increase of dextran uptake by $CD11b^+$ cells in the lymph nodes draining the injection site. Measures were performed in WT and CD11c.DOG mice. Where indicated, WT 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 PGE_2 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).

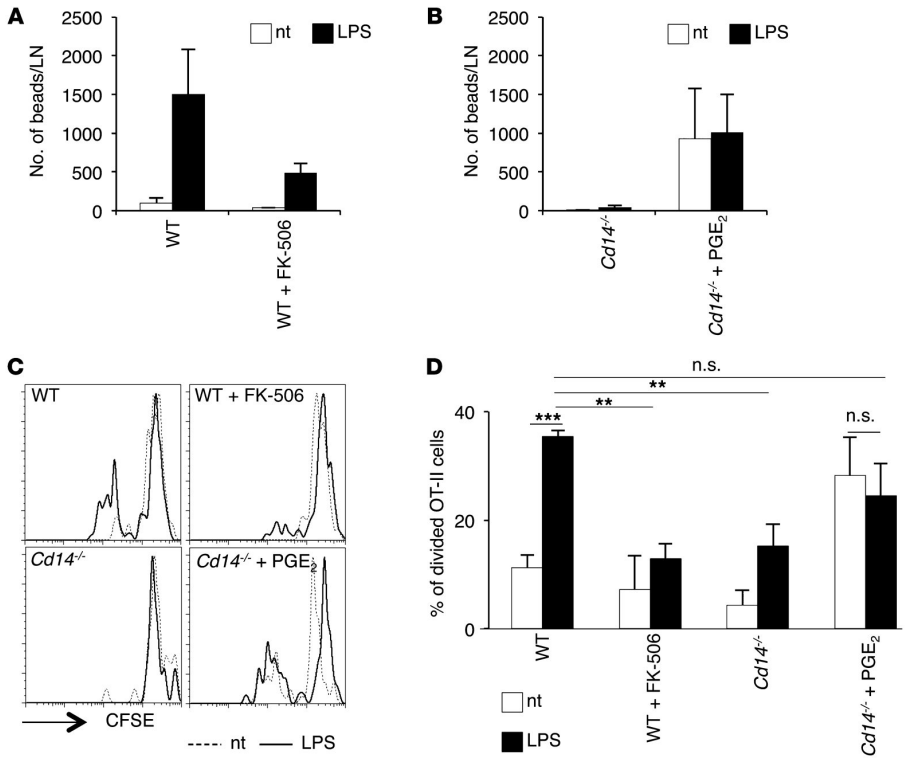


Figure 8| Edema induced by LPS increases the efficiency of bead arrival at the draining lymph nodes. (**A**) Absolute numbers of FITC-labeled microbeads reaching the draining lymph nodes in WT mice treated or not with LPS (4 hours after treatment). Where indicated, mice were pretreated s.c. with FK-506 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 PGE_2. 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.

2.5 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 EP_1 receptor expressed by naive T cells [31], while PGE2-mediated activation of the EP_2 and EP_4 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 [34]. 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] [35]. 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 [36] or the participation of different LBPs [37].

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 [38] [39]. 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 [40]. MMP-9, in turn, regulates DC migration by contributing to the degradation of the basal membrane [40]. 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] [41]. 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 [41].

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] [42]. 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.

2.6 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 (43). 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 PGE2 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 PGE2 ELISA analysis.

ELISA assays. Concentrations of IL-2 and TNF-α in supernatants were assessed by ELISA kits purchased from R&D Systems. PGE2 levels were assayed with a Monoclonal EIA Kit from Cayman Chemical.

Quantitative real-time PCR in vitro. Cells (2×10^6) 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′-ACGACATGGAGACAATCTATCCT-3′ and 5′-TGAGGACAACGAGGAAATGT-3′(mPGES-1); 5′-CCTGCTGCCCGACACCTTCAA-3′ and 5′-TCTTCCCCCAGCAACCCGGC-3′ (COX-2); and 5′-CGAAAGCATTTGCCAAGAAT-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′-TTTGTTTCTTGTCTTGGCTTCAA-3′ and 5′-TTAGTGGCTTTTATTTCCTTTGGT-3′(CD11c); 5′-CACCTTCATTTGCATCAACA-3′ and 5′-TCTGAAAAGTTGGCAAAGAGAA-3′(F4/80); and 5′-TGCTCTGGAGATAGAAGTTATTGTG-3′ and 5′-TTACCAGTGATCTCAGTATTGTCCA-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 (44). 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 thapsigargin (5 μM), and LPS plus PGE2 or PGE2 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 PGE2 (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 IP_3R3 in the activation of the NFAT pathway downstream of CD14

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Calcium (Ca^{2+}) signaling controls numerous cellular processes, including cell motility, gene transcription, exocytosis and programmed cell death [1]. In electrically non-excitable cells, the main Ca^{2+} entry pathway is regulated by intracellular Ca^{2+} stores depletion coupled with the opening of specific plasma membrane channels, in a mechanism called store-operated calcium entry (SOCE) [2] (Chapter 1.2.3). In immune cells, SOCE is initiated by antigen binding to clonotipic receptors in T and B cell or by Fc receptors, resulting in the activation of phospholipase C (PLC) and production of inositol 1,4,5-triphosphate (IP_3). This second messenger binds to IP_3 receptors (IP_3Rs) located in ER membrane and causes rapid Ca^{2+} release from ER followed by Ca^{2+} release-activated Ca^{2+} (CRAC) channels opening. This process generates a sustained intracellular Ca^{2+} concentration elevation that is necessary for transcription factor activation (in particular Ca^{2+} dependent transcription factors such as NFATc family members), gene expression and effector functions [3]. However, activation of Ca^{2+} entry into cells across the plasma membrane can occur through other two mechanism: receptor-operated calcium entry (ROCE, plasma membrane Ca^{2+} channels open in response to the binding of an extracellular ligand) and second-messenger-operated calcium entry (SMOCE, plasma membrane Ca^{2+} channels open in response to the binding of intracellular second messengers such as products of PLC-γ) [4].

IP_3Rs [5] are important for initiating Ca^{2+} signaling and are critical in orchestrating the immune responses in health and disease [6]. Three

isoforms of IP_3Rs (IP_3R1, IP_3R2, and IP_3R3) have been identified with differential expression pattern and gating capacity [7] [5].

IP_3R1 is most abundant in the nervous system [8], where it plays a relevant physiological role in cerebellum [9], but in a lesser extent it is also present in all peripheral tissues [8]. IP_3R2 and IP_3R3 are also widely distributed, with a prominent expression in particular cell types. For example, IP_3R2 prevails in cardiomyocytes and hepatocytes [10], whereas IP_3R3 is strongly expressed in the spleen [11].

Ca^{2+} signaling patterns of IP3Rs were determined using genetically engineered B cells that express either single or combined IP_3R subtypes [12]. IP_3R1 induces regular Ca^{2+} oscillations, IP_3R2 is required for robust, long lasting, and regular Ca^{2+} oscillations and IP_3R3 generates monophasic single Ca^{2+} transients. Given its gating properties, IP_3R3 is believed to play a major role in initiating Ca^{2+} signaling [13] [14].

Recently, we have describe a LPS-stimulated CD14 restricted signaling pathway in which a rapid monophasic Ca^{2+} influx is necessary to activate the downstream events that lead to NFAT translocation and the relative transcriptional program [15] (Chapter 1.2.3). This transduction cascade directly regulates the production of inflammatory mediators, such as IL-2 [15] [16] [17] and PGE_2 [18] in DCs (Chapter 2), and controls DC life cycle [15]. This process is triggered in bone-marrow derived dendritic cells (BMDCs) but not in $CD14^+$ bone-marrow derived macrophages (BMDM), which do not show Ca^{2+} fluctuations upon LPS treatment [15]. This difference in the capacity to flux calcium and, thus, to activate the NFATc

transcription family may be one of the mechanisms underlying the different behavior of these two cell types in response to LPS or gram-negative bacteria [19].

Since IP_3R3 is the major isoform expressed by DCs [20] and it has been demonstrated that IP_3R3 down-regulation in $CD4^+$ T cells results in the impairment of Ca^{2+} mobilization and NFAT activation [21], we decided to examine the possible role of IP_3R3 in the activation of the CD14-NFAT pathway and the specific mechanism of Ca^{2+} entry in DCs.

3.1 IP_3R3 is expressed on the plasma membrane of DCs

Using a confocal microscopy analysis, we evaluated the IP_3R3 expression and sub-cellular localization in a homogeneous murine dendritic cell line, D1 cells [22], and in a mouse macrophage cell line, RAW 264.7 (Figure 1A). D1 cells show a clustered distribution of IP_3R3 on the plasma membrane and on intracellular organelles (presumably ER). On the contrary, RAW 264.7 cells display an uniform IP_3R3 localization exclusively in the inner cell compartment (Figure 1C). We confirmed IP_3R3 plasma membrane localization in D1 cells by western blotting (Figure 2). This cell-specific sub-cellular distribution of IP_3R3 is conserved also by murine *ex vivo* splenic DCs compared to splenic macrophages (Figure 1B-C). In fact, only splenic DCs present a heterogeneous IP_3R3 expression on the plasma membrane. Similarly to T cells [23] [24] [25] and B cells [26] [27], these data suggest that in DCs but not in macrophages a distinct population of IP_3R3 exist on the plasma membrane.

A

B

Merge
Merge
IP3R3
IP3R3
PM
PM
Splenic DC
Splenic Macrophage

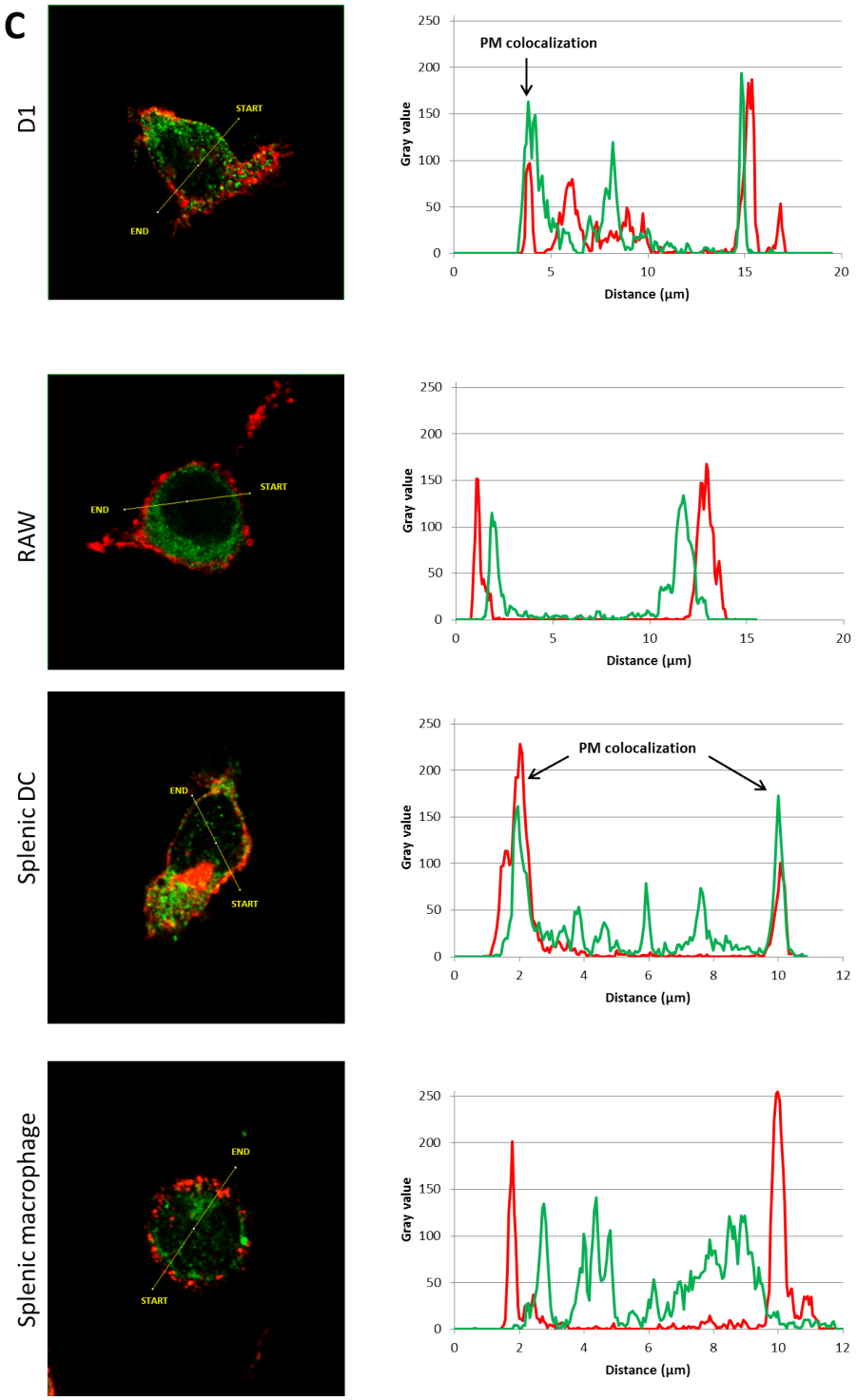

C
D1
START
END
PM colocalization
Gray value
Distance (μm)
RAW
START
END
Gray value
Distance (μm)
Splenic DC
END
START
PM colocalization
Gray value
Distance (μm)
Splenic macrophage
END
START
Gray value
Distance (μm)

Figure 1| IP_3R3 sub-cellular localization in DCs and macrophages
D1 and RAW 246.7 cell lines (A) or *ex vivo* splenic DCs and splenic macrophages (B) were seeded on glass coverslip and analyzed via confocal microscopy. Plasma membrane (PM) (red) was detected by Alexa Fluor® 555 conjugated cholera toxin subunit B, CTB or anti-F4/80 antibody (for splenic macrophages). IP_3R3 was detected by Alexa Fluor® 488 secondary antibody against anti-IP_3R3 antibody (green). Yellow signals in merge images indicate PM-IP_3R3 co-localization regions, that are present only in DCs. (C) Plots comparing the pixel intensity profiles of PM (red line) and IP_3R3 (green line) images across a line segment (yellow line). Synchronous peaks indicate co-localization (arrows). Experiments were performed in triplicate; 30–50 cells were analyzed in each experimental condition.

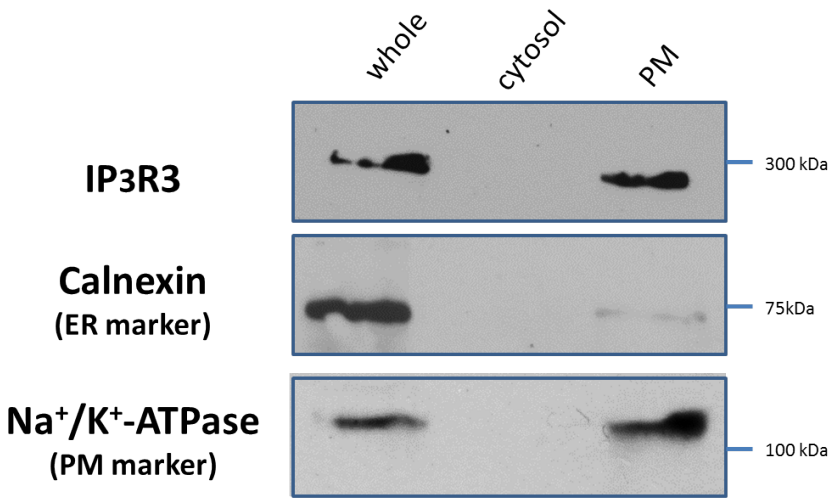


Figure 2| IP_3R3 is present on the plasma membrane of DCs
Western blot analysis of IP_3R3 in cellular sub-fractions (whole lysate, cytosolic and plasma membrane, PM, sub-fractions) of D1 cells. The same amount of protein (20 µg) was loaded in each line. The fractions purity was controlled using specific marker proteins: Calnexin for ER, Na^+/K^+ ATPase for plasma membrane.

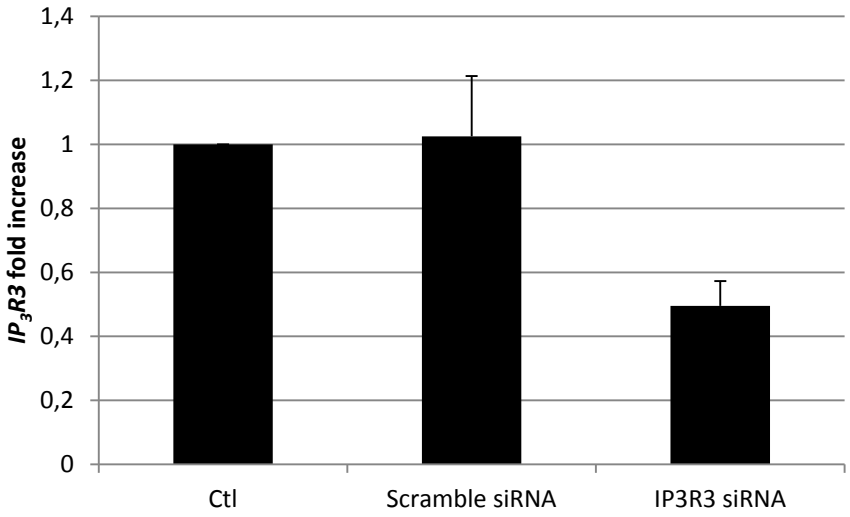
3.2 IP_3R3 regulates CD14-NFAT target genes

In order to investigate the involvement of IP_3R3 in CD14-NFAT pathway activation in DCs, the expression of IP_3R3 gene was silenced in D1 cells by the use of RNA interference. Figure 3A depicts the efficiency of gene silencing following transfection with siRNA directed against IP_3R3 or a control scramble siRNA. At 48 h after siRNA treatment the expression of the IP_3R3 was diminished by 50.2 ± 7.7%. Scramble siRNA had no effect on the expression levels of IP_3R3.

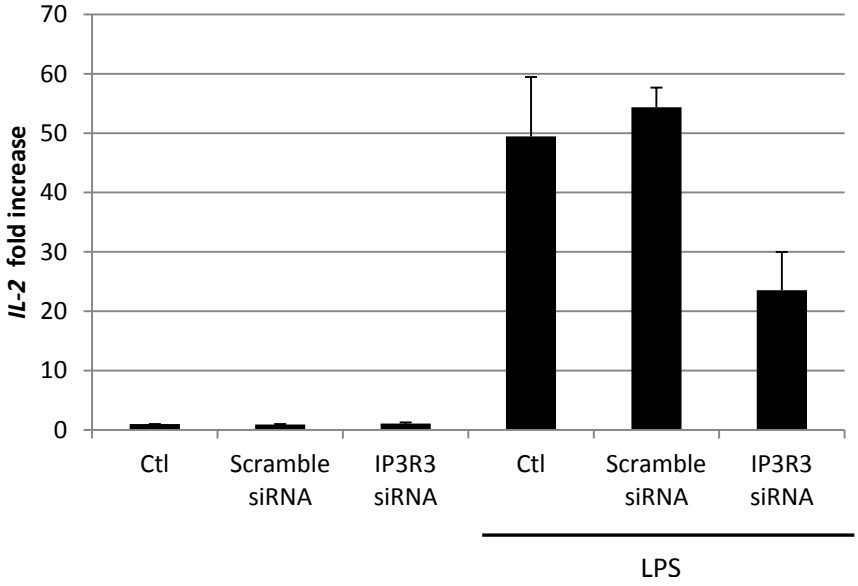
Then, we tested the effects of IP_3R3 silencing on the capacity of LPS-treated D1 cells to up-regulate a specific CD14-NFAT target gene, such as IL-2 [15]. As expected, IP_3R3 silencing strongly reduced the capacity of LPS-treated DC to up-regulate IL-2 expression (Figure 3A), leaving unaffected the induction of TNFα, a gene known to be regulatd exclusively by the TLR4-Myd88-TRIF pathway (Figure 3C). These data demonstrate that IP_3R3 plays a key role in the LPS-triggered CD14-NFAT pathway.

By using the IP_3R blocker 2-aminoethoxydiphenyl borate (2-APB) [28] [29], we evaluated in *ex vivo* splenic DCs the role of IP_3R3 in regulating the expression of another CD14-NFAT pathway target gene, such as mPGES-1 [18] (Chapter 2) (Figure 4). As previously demonstrated, we observed a strong up-regulation of mPGES-1 mRNA after LPS stimulation. This response was strongly abolished in 2-APB treated cells (Figure 4A), confirming a central role of IP_3R3 for the activation of the cD14-NFAT-dependent pathway. As expected, TNFα mRNA was normally induced both in inhibited and non-inhibited cells (Figure 4B).

A


1,4
1,2
1
0,8
0,6
0,4
0,2
0
IP3R3 fold increase
Ctl
Scramble siRNA
IP3R3 siRNA

B


70
60
50
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IL-2 fold increase
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IP3R3 siRNA
Ctl
Scramble siRNA
IP3R3 siRNA
LPS

C

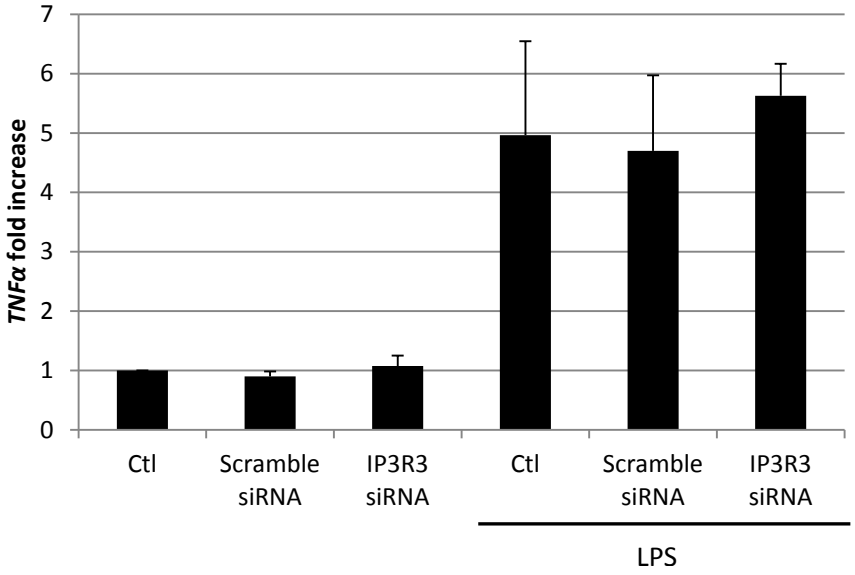


Figure 3| IP_3R3 silencing in D1 cells impairs *IL-2* up-regulation

Real-time PCR analysis of knockdown effect of scramble siRNA or IP_3R3 specific siRNA 48h after transfection in D1 cells (A). Real-time PCR analysis of *IL-2* (B) or *TNFα* (C) mRNA up-regulation after LPS (1 ug/ml) administration (4h) in 48h siRNA-treated D1 cells. Values represent at least three independent experiments performed in duplicate (+ SEM).

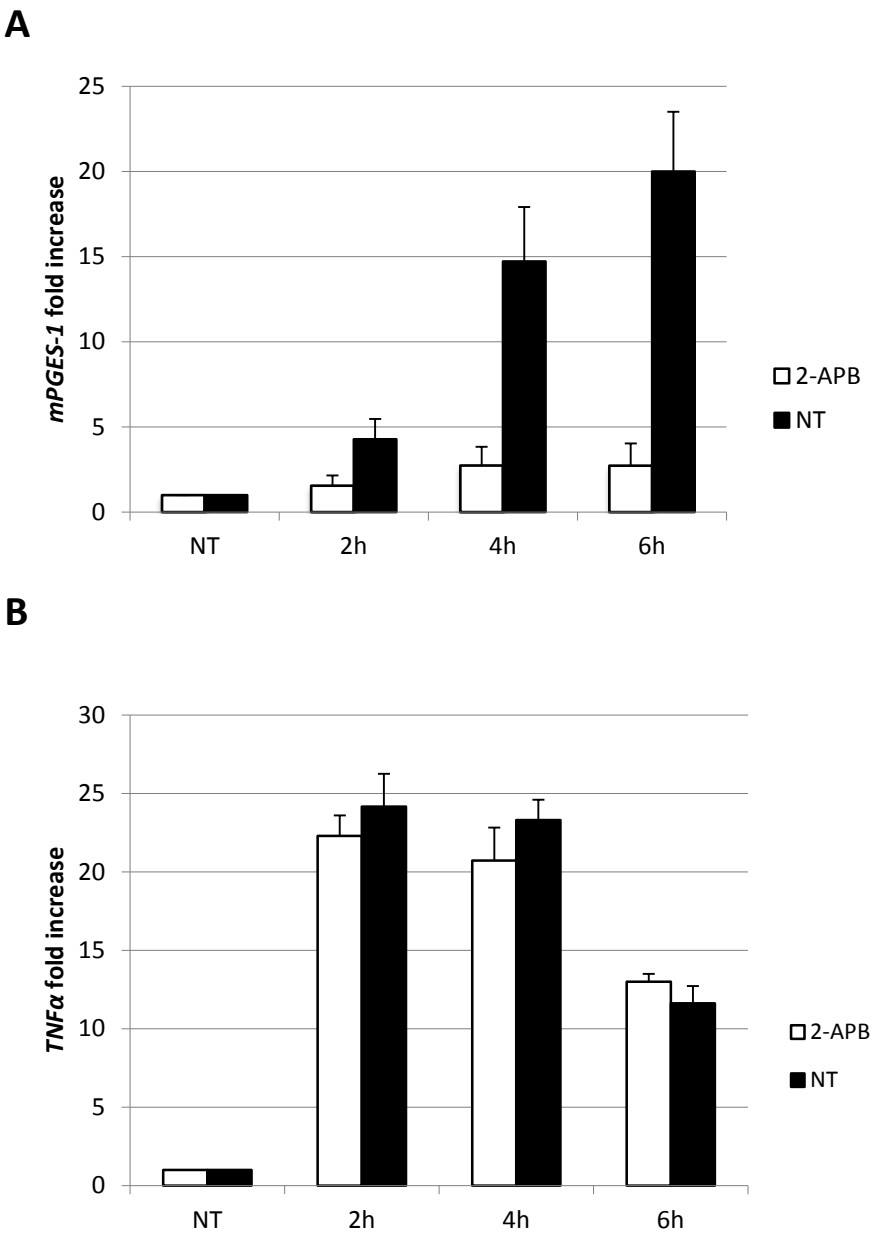


Figure 4| IP_3Rs inhibition suppress *mPGES-1* up-regulation in splenic DCs
Real-Time PCR analysis of *mPGES-1* mRNA induction in ex vivo splenic DCs stimulated with LPS (1 μg/ml). Real-Time PCR analysis of *mPGES-1* (A) or *TNFα* (B) mRNA up-regulation after LPS (1 μg/ml) administration in splenic DCs pre-treated with 2-APB (100 μM, 30 min) at the indicated time points. Values represent at least three independent experiments performed in duplicate (+ SEM).

3.3 SMOCE, not SOCE, is the Ca^{2+} entry mechanism activated in LPS-treated DCs

IP3Rs on the ER membrane are essential to induce SOCE in electrically non-excitable cells, inducing cytoplasmic Ca^{2+} transients from internal compartments that result in the opening of CRAC channels and in sustained Ca^{2+} influxes from the extracellular space [3]. Since IP_3R3 is localized also on the plasma membrane of DCs, we hypothesized, in agreement with previous reports [15] [26], that IP_3R3 may be necessary and sufficient to induce directly Ca^{2+} transients from extracellular space in LPS-treated DCs.

Using 2-APB or N-[4-3,5-bis(trifluromethyl)pyrazol-1-yl]-4-methyl-1,2,3-thiadiazole-5-carboxamide (YM-58483), a selective CRAC channel inhibitor that potently inhibits both Ca^{2+} influx through CRAC channels and NFAT-driven IL-2 production in activated T cells [30] [31] [32] [33], we evaluated the capacity of LPS-treated D1 cells to produce IL-2 or TNFα (Figure 5). Blocking IP_3Rs with 2-APB strongly affected IL-2 production. On the contrary, YM-58483 had no effect on cytokine production by DCs (Figure 5A). In addition, TNF-α production in all the tested conditions demonstrated that the NF-κB activation pathway was not influenced by these treatments (Figure 5B).

These data suggest that NFAT-triggered IL-2 production in DCs doesn't require the opening of CRAC channels and that SOCE may not be the Ca^{2+} entry mechanism activated in DCs in response to LPS.

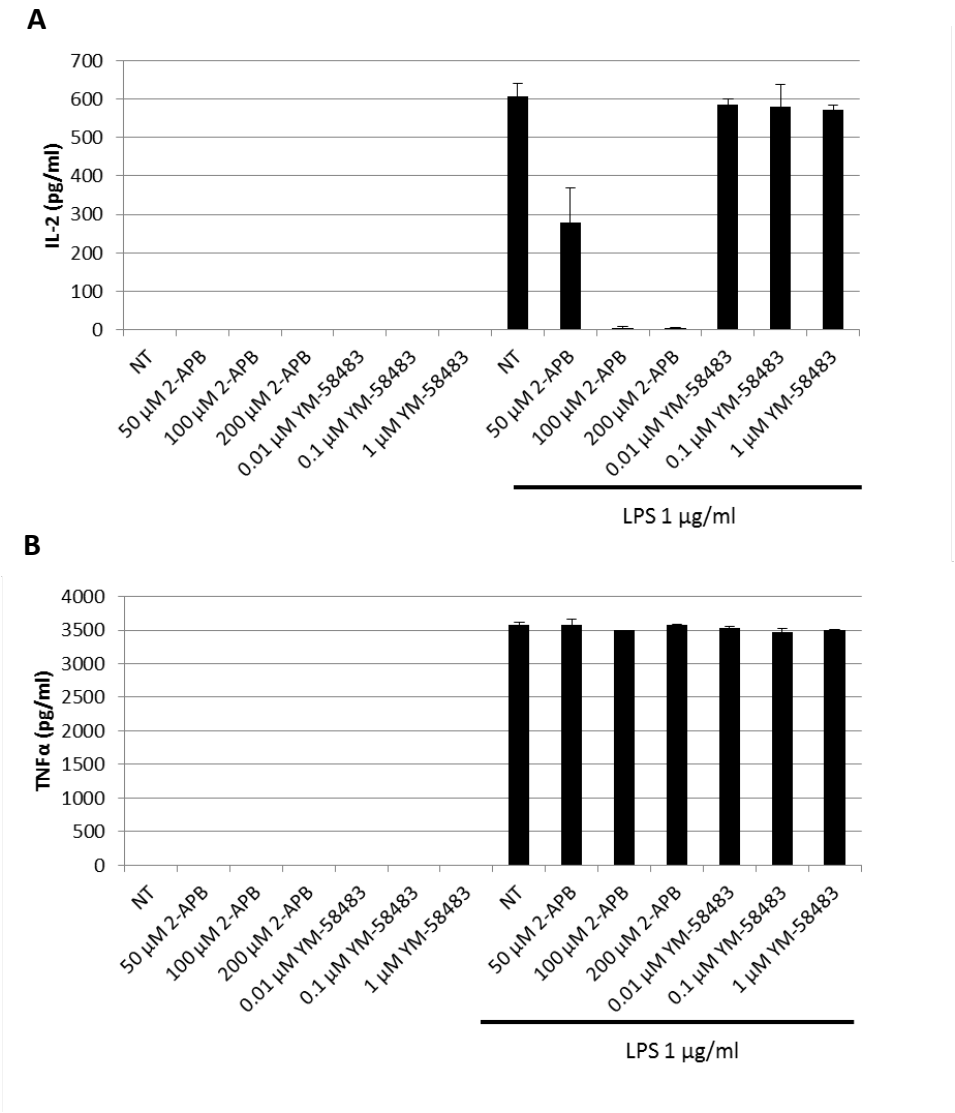


Figure 5| IP_3Rs, but not CRAC channels, inhibition impairs IL-2 production by DCs
IL-2 production (A) and TNFα production (B) by D1 cells 24 hours after LPS stimulation. D1 cells were treated with 2-APB o YM-58483 (indicated concentrations, 30 minutes pretreatment) and then stimulated with LPS (1 µg/ml). Values represent means of at least 3 independent experiments performed in duplicate (+ SEM)

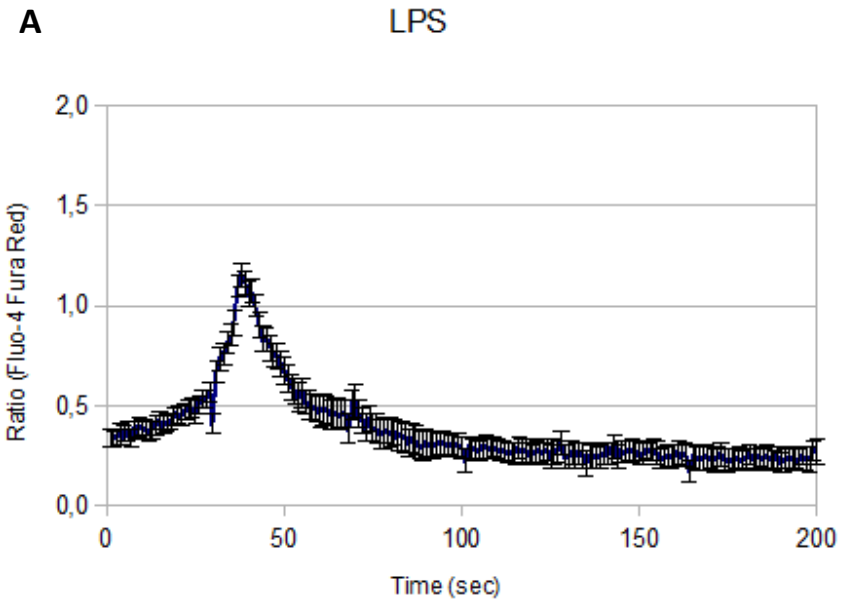
In order to better define the Ca^{2+} entry dynamics in LPS-stimulated DCs, we compared the LPS-triggered Ca^{2+} transients to a well-known SOCE process that occurs in DCs stimulated with ATP [34].

ATP and other nucleotides bind to specific plasma membrane receptors called purinergic receptors (P2Rs), subdivided into P2X, a family of ligand-gated ion channels, and G-protein–coupled P2Y receptors [34]. In DCs, ATP signaling predominantly occurs via P2Y class of receptors [35], that results in phosphatidyl inositol breakdown, release of Ca^{2+} from intracellular stores and Ca^{2+} influx across the plasma membrane through CRAC channels [6].

Laser-scanning confocal microscopy coupled with a ratiometric method was used to record intracellular Ca^{2+} signals in D1 cells (Figure 6). LPS-stimulated cells showed a rapid monophasic transient (Figure 6A) that was completely inhibited when the cells were pretreated with the Ca^{2+} chelator EGTA (inhibition of external Ca^{2+} entry) (Figure 6B) or 2-APB (Figure 6C). On the other hand, ATP induced in DCs the classic biphasic Ca^{2+} entry composed by a rapid Ca^{2+} transient (ER store depletion) followed by a CRAC-mediated long-term plateau (Figure 6D). In fact, EGTA pretreatment selectively impaired only the delayed inward Ca^{2+} influx, but not the first Ca^{2+} wave (Figure E), while 2-APB completely abolished Ca^{2+} entry in ATP-stimulated cells.

Together these results suggest that LPS induces a IP_3R-mediated Ca^{2+} transient directly across plasma membrane in DCs via a SMOCE mechanism.

A


LPS
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1,5
1,0
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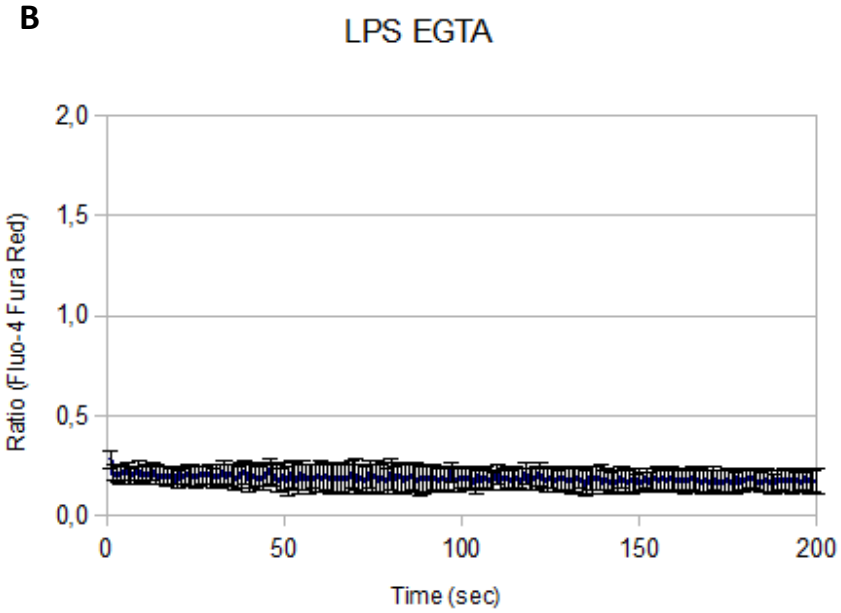
Ratio (Fluo-4 Fura Red)
0
50
100
150
200
Time (sec)

B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Ratio (Fluo-4 Fura Red)
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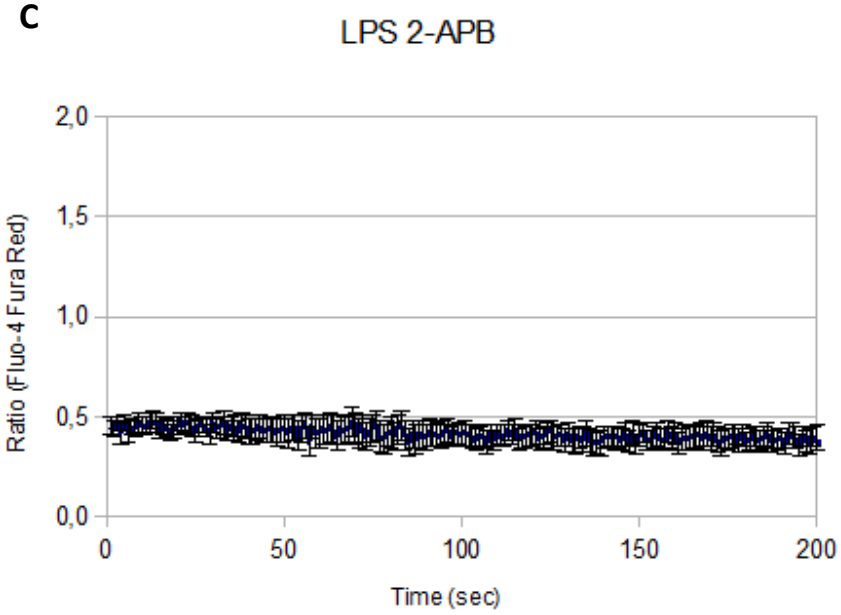
Time (sec)

C


LPS 2-APB
Ratio (Fluo-4 Fura Red)
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100
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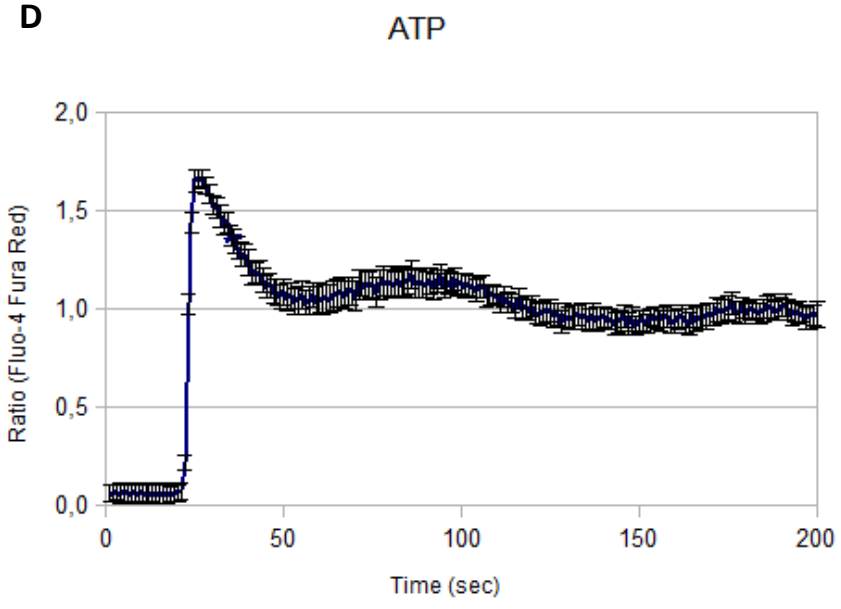
200
Time (sec)

D


ATP
Ratio (Fluo-4 Fura Red)
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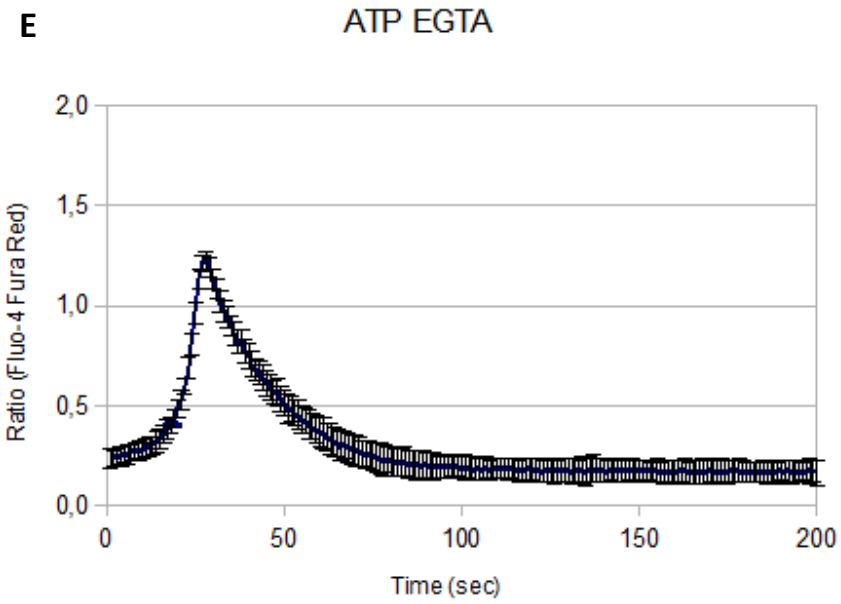
Time (sec)

E


ATP EGTA
Ratio (Fluo-4 Fura Red)
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Time (sec)

F

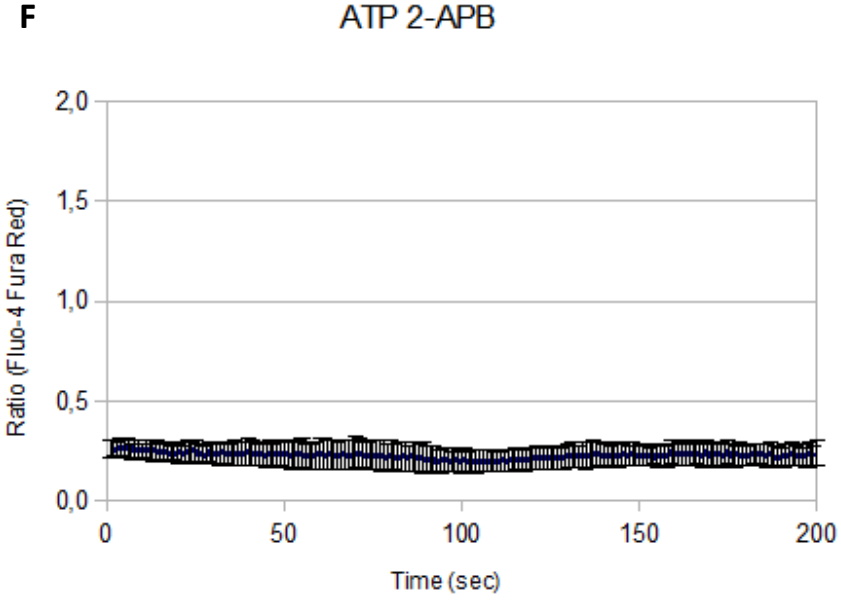
ATP 2-APB
Ratio (Fluo-4 Fura Red)
2,0
1,5
1,0
0,5
0,0
0
50
100
150
200
Time (sec)

Figure 6| Calcium influxes in DCs

D1 cells stained with paired ratiometric calcium probes Fluo-4 AM (calcium bound) and Fura Red AM (calcium free) were treated with LPS (1 μg/ml) (A-C) or ATP (100 μM) (D-F). EGTA (2mM, extracellular calcium chelator) (B,E) or 2-APB (100 μM, IP_3Rs inhibitor) (C,F) were added 10 minutes before the stimulus. $[Ca^{2+}]_i$ fluctuations were evaluated as Fluo-4/Fura Red ratio change in response to the stimuli. The trace shows the mean response (±SEM) of ten cells from the same experiment.

3.4 Conclusions

Here, we described a new Ca^{2+} entry mechanism in DCs stimulated with LPS. LPS induces the TLR4-independent CD14-NFAT pathway via SFKs activation, PLC-γ2 phosphorylation and IP_3 production (Chapter 1.2.3). The IP_3 produced triggers plasma membrane IP_3R3 local opening, inducing a transient Ca^{2+} influx from extracellular space. Here, we described that in DCs, but not in other cells that do not activate the CD14-NFAT pathway, such as macrophages, IP_3R3 are present on the plasma membrane. Furthermore, when IP_3R3 are knocked-down, we demonstrated that CD14-NFAT target genes expression was strongly impaired. Pharmacological inhibition of IP_3Rs and Ca^{2+} measurements demonstrated that in LPS-treated DCs is activated an IP_3R-dependent monophasic extracellular Ca^{2+} transient. These data confirm that IP_3R3 plasma membrane Ca^{2+} channels are necessary and sufficient to sustain the CD14-induced Ca^{2+} influx in LPS-treated DCs, thus contributing to the NFAT pathway activation.

Future experiments on $IP_3R3^{-/-}$ mice and splenic DCs will be performed to confirm the role of this Ca^{2+} channel in the physiology and immunological functions of DCs *in vivo*.

3.5 Methods

Cells. D1 cells and RAW 264.7 cells were cultured as previously described [17]. *Ex vivo* splenic DCs were purified as previously described [36]. *Ex vivo* macrophages were purified from spleen of 6 week-old C57BL/6 mice. Splenic unicellular suspensions were stained with biotinylated anti-CD11b antibodies and positively selected using MACS beads according to the manufacturer's instructions (Miltenyi Biotec).

Antibodies and chemicals. Anti-IP_3R3 antibody and purified rat anti-mouse CD16/CD32 were purchased from BD Biosciences. Anti-mouse Alexa Fluor® 488 antibody, CTB-Alexa Fluor® 555, Anti-F4/80 Alexa Fluor® 555 antibody, Fluo-4 AM, Fura red AM and water-soluble probenecid were purchased from Invitrogen. Antibody against murine calnexin and NA^+/K^+ ATPase were purchased from Abcam. LPS (E. coli, O55:B5) was purchased from Enzo Life Sciences. 2-APB, YM-58483 and EGTA were purchased from Sigma-Aldrich.

Immunocytochemistry. Cells were seeded on glass coverslip and incubate with CTB-Alexa Fluor® 555 and anti-mouse CD16/CD32 (Fc receptor block) at 4°C for 30 min. Cells were fixed in paraformaldehyde 4% and then were permeabilized with 0.2% BSA 0.1% Triton X-100 in PBS. Successively, cells were kept in blocking solution (2% BSA in PBS) for 30 min. Mouse Anti-mouse IP_3R3 and then goat anti-mouse Alexa Fluor® 488 antibodies diluted in blocking

solution were added and incubated at room temperature. Finally the samples were mounted in FluorSave™ Reagent (Calbiochem) and were imaged by Leica TCS SP2 confocal microscope. ImageJ software was used for for image analysis and processing.

Western blot. Cells were fractioned using membrane protein extraction kit according to the manufacturer's instructions (Abcam). Proteins were quantified using a BCA assay (Thermo Scientific). 20 µg cell lysate was run on a 5% 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).

siRNA transfection. D1 cells were transfected with pre-designed siRNA (Euroclone) using INTERFERin kit according to the manufacturer's instructions (Polyplus transfection)

Quantitative real-time PCR . Cells (2×10^6) 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 750 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. Housekeeping 18S mRNA was used as an internal reference for relative quantification studies.

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

Calcium measurements. $[Ca^{2+}]_i$ was determined by a fluorometric ratio technique. Cells were loaded with 3 µM Fluo-4 AM and 6 µM Fura red by incubation at 37 °C for 60 min in completed medium containing probenecid. Cells were then washed two times with completed medium plus probenecid and were incubate at 37°C for 30 min to allow intracellular de-esterification of dyes. Imaging was performed on a Leica TCS SP2 confocal microscope with a X63 oil objective. Cells were excited with a laser at 488 nm, and the intensity of the fluorescence between 505–550 nm was measured as the Fluo-4 signal, while fluorescence >635 nm was simultaneously detected as the Fura red signal. Images were acquired at 0.85 s intervals. The change in Ca^{2+} signal was determined by the change in the ratio of Fluo-4 to Fura red fluorescence.

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Chapter 4: Final considerations

4.1 Summary

Dendritic cells 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 pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), the major component of gram-negative bacteria outer membrane [2] (Chapter 1.2). The latter is recognized by a multi-receptor complex formed by LBP, CD14, and MD-2-TLR4, initiating the MyD88-dependent and TRIF-dependent pathways [3]. In addition, DCs respond to the LPS also triggering CD14-dependent signaling, that results in Src family kinases (SFKs) and PLCγ2 activation, IP_3 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 signaling 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 favors 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 the molecular events leading to Ca^{2+} entry downstream of LPS stimulation in DCs. Using a confocal microscopy approach, we demonstrated that IP_3R3 in DCs is localized not only intracellularly but also on the plasma membrane and that calcium influx after LPS treatment is due to Ca2+ mobilization from the extracellular space. On the contrary, macrophages, that do not mobilize Ca2+ after LPS treatment, express the IP_3R3 only in the ER. In addition, IP_3R3 knock-down or pharmacological inhibition of IP_3Rs severally impair Ca^{2+} influx from external space and CD14-NFAT target genes (IL-2 and mPGES-1) induction in LPS-treated DCs. These data indicate that the CD14-NFAT pathway is controlled by cell-

specific localization of IP_3R3, that, in DCs, induces a second-messenger-operated calcium entry (SMOCE) across plasma membrane.

4.2 Conclusions and future prospects

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 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 (Figure 1). 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 [11], inducing local flogistic processes and regulating T-cell differentiation [10]. Since hte CD14-NFAT pathway selectively governs PGE_2 production by DCs, drugs that inhibit elements of the CD14 signaling pathway might be used for new clinical approaches. FK-506, a drug that inhibits calcineurin and is commonly used to prevent transplant rejection, is also used for therapeutic application in inflammatory and autoimmune diseases [12] [13]. Our new data could partially explain the mechanisms used by FK-506 in all these conditions. Understanding what are the molecules involved in the CD14-NFAT pathway and the mechanisms underlying its triggering could be useful to design new drugs with reduced side effects.

We demonstrated that plasma membrane IP_3R3 Ca^{2+} channel are necessary to induce Ca^{2+} entry in LPS-stimulated DCs and to activate NFAT. This protein localization and the SMOCE mechanism involved in its functioning are DC-specific. Although we did not yet evaluated IP_3R3 role *in vivo,* using $IP_3R3^{-/-}$ mice, IP_3R3 on plasma membrane

could be a selective target to modulate CD14-NFAT pathway in DC-mediated pathologies.

The CD14-NFAT apoptotic pathway is efficiently activated in DCs, but does not occur in macrophages [4]. This is consistent with the survival of activated macrophages, which is, indeed, essential for the resolution phases of an inflammatatory process. Late-activated macrophages produce anti-inflammatory mediators, which halt the inflammatory process and initiate tissue repair [14]. 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. Pharmacological activation of NFAT in macrophages is sufficient to induce their cell death upon LPS treatment, further supporting a role for NFAT as a master regulator of the cell life cycle [4]. Despite macrophages express CD14 and TLR4-MD2 complex, they do not activate NFAT. Since macrophages do not show a rapid Ca^{2+} entry after LPS exposure and they lack of IP_3R3 on plasma membrane, we hypothesized that the difference in the IP_3R3 distribution is the basis of the different behaviour between DCs and macrophages in response to LPS encounter.

Furthermore, the cell-specific localization of IP_3R3 on the plasma membrane may be used as a novel surface marker of DCs and could be exploited as a discriminating factor between DCs and macrophages. One of the major problem in defining DCs and macrophages lies in the fact that they express common cell surface markers. For example, a widespread experimental method to

separate DCs from macrophages is based on CD11c expression. However, most, if not all, macrophages express low (or intermediate) amounts of CD11c, and this complicates the interpretation of experiments with CD11c-based cell enrichment or depletion [15]. Moreover, although F4/80 is commonly used as a macrophage marker in the mouse, there are cells classified as DCs that also express F4/80, as well as some macrophages that lack F4/80 expression [16]. Thus, the current cell surface marker-based separation strategies are not unambiguous, as demonstrated by recent published trascriptomic and proteomic approaches [17][18][19] to differentiate these two populations. In this light, IP_3R3 expression on plasma membrane could be a valid tool in order to univocally distinguish DCs and macrophages.

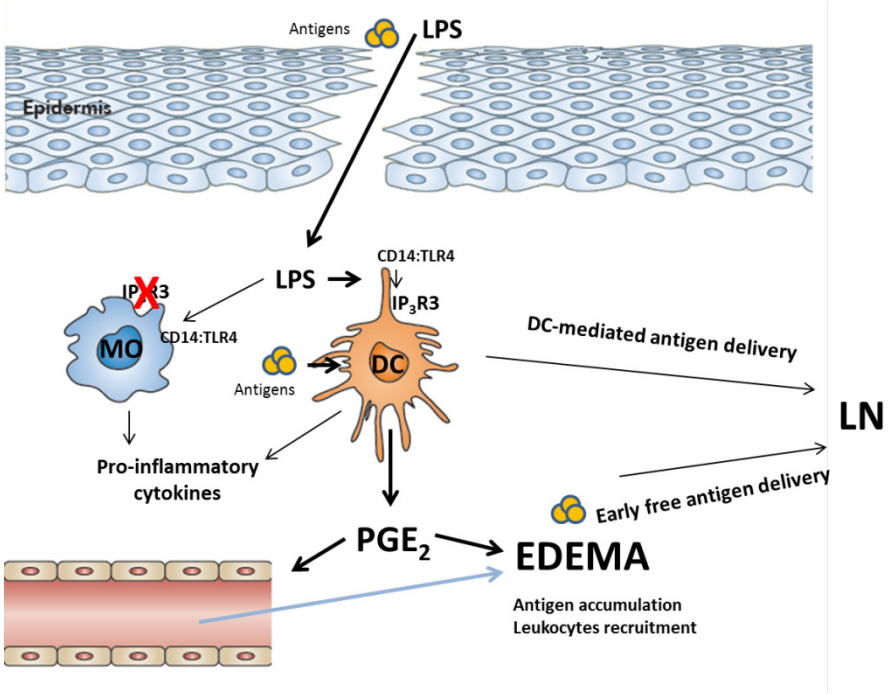

Figure 1| Model of LPS-induced skin edema formation

LPS induces PGE_2 production by DCs in a CD14-NFAT-dependent manner. Macrophages do not activate CD14 signaling because they lack of IP_3R3. Swelling is important for free antigen delivery to lymph node (LN).

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