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LPS-dependent NFAT activation in dendritic cells is regulated by IP_4-mediated calcium entry through plasma membrane IP_3R3

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Alla mia famiglia,
balsamo della mia vita.

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List of abbreviations

DCs: Dendritic cells

APC: Antigen presenting cell

LPS: Lipopolysaccharide

PRRs: Pattern recognition receptors

PAMPs: Pathogen-associated molecular patterns

TLR: Toll-like receptor

CLR: C-type lectin receptor

CD14: Cluster of differentiation 14

NFAT: Nuclear factor of activated T cells

Ca^{2+}: Calcium

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

mPGES-1: Microsomal prostaglandin E synthase-1

PGE2: Prostaglandin E_2

SOCE: Store-operated calcium entry

SMOCE: Second messenger-operated calcium entry

PLC: Phospholipase C

Ins(1,4,5)P3/IP_3: Inositol 1,4,5 trisphosphate

Ins(1,3,4,5)P4/IP_4: Inositol 1,3,4,5 tetrakisphosphate

InsP3Rs/IP_3Rs: Inositol 1,4,5 trisphosphate receptors

ITPKB: Inositol 1,4,5 trisphosphate 3-kinase

PtdIns: Phosphatidil inositol

Chapter 1. General introduction

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1.1 Innate immunity

The immune system refers to a complex network of cells, tissues and organs that has evolved to defend the host against the great number of harmful substances and invading microorganisms that are present in the surrounding environment. The immune system has been traditionally divided into two arms called innate immunity and adaptive immunity. The mutual interplay between these two branches is essential for an optimal immune response, whose efficiency is given by the coordinated activity of different types of cells and the molecules that they produce (Medzhitov & Janeway, 2000).

The innate immune system is an evolutionary conserved form of host defence against infections which appeared much sooner than the adaptive immune system, since some forms of innate immunity exist in all multicellular organisms while adaptive immunity appeared only in vertebrates (Beutler, 2004). The relevance of the innate immune system is suggested by the evidence that defects in innate immunity, thought very rare, are almost always lethal (Janeway & Medzhitov, 2002).

Innate immunity, also referred to as natural immunity, represents the first line of defence against intruding microbial pathogens. The anatomical barriers, which are present at the interphase between the organism and the extracellular environment, for instance the skin and the mucosal membranes, represent a first physical obstacle to keep out pathogens. The crossing of these barriers forces the pathogens to deal with innate immune cells and secreted proteins which augment the protection offered by the anatomical barriers (Janeway & Medzhitov, 2002).

Numerous cells are involved in the innate immune responses, which comprise myeloid cells such as macrophages, dendritic cells (DCs), granulocytes and mast cells but also particular lymphoid cells like natural killer cells (NK cells) and γδ T cells. Innate immune cells are able to recognize microbial pathogens and then triggering an inflammatory response aimed at limiting pathogen invasion (Janeway, 2001) (Fig.1).

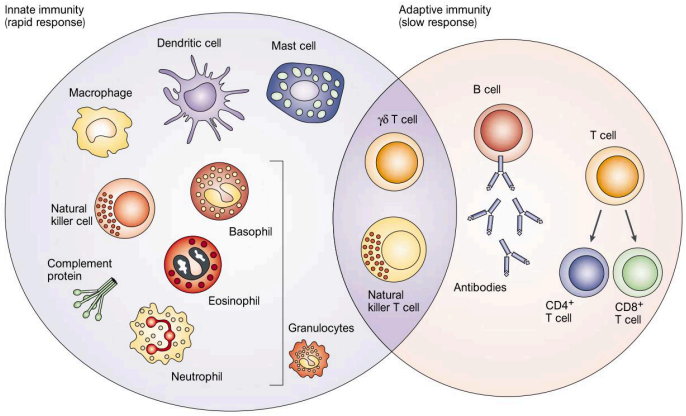

Figure.1 Innate and adaptive immune cells.
Innate immune mediates a rapid response and is considered the first line of defence against infection. Innate immunity comprises soluble factors, such as complement proteins, and various cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, DCs and natural killer cells. The adaptive immune response is delayed but is characterized by antigenic specificity and memory. It consists of antibodies, B cells, and $CD4^+$ and $CD8^+$ T cells. Natural killer T cells and γδ T cells are in between the innate and adaptive immunity. Adapted from: Dranoff, 2004.

Innate immune cells use germline-encoded receptors which detect molecular structures that are expressed and highly conserved in pathogens but not in self-tissue (Akira, 2006; Medzhitov & Janeway, 1997). Therefore, innate immunity is responsible for the discrimination

between infection non-self (exogenous antigen) and non-infection self (endogenous antigen); this capability is essential to eliminate pathogens while keeping the host tissues unharmed (Medzhitov, 2002).

Innate immunity has also a crucial role in the subsequent activation of the adaptive immunity. The adaptive immune system, also referred as acquired immunity, relies on two classes of specialized lymphoid cells, T cells and B cells and on the production of specific antibodies (Medzhitov & Janeway, 2000).

The mechanisms and the receptors used by innate and adaptive immune cells for the immune recognition represent the main difference between innate and adaptive immunity. In the adaptive immune system, the T-cell receptor and the B-cell receptor are generated by somatic recombination, during the maturation of these cells; this process generates a huge repertoire of antigen receptors with random specificities. By the end, antigen-receptors are clonally distributed, which means that each lymphocyte is endowed with a structurally unique receptor. As a consequence, differently from innate immunity, which has a restricted number of pathogen receptors, adaptive immunity can virtually recognize any antigen, being responsible for the specific recognition of all non-self-antigens (Medzhitov & Janeway, 1997; Medzhitov & Janeway, 2000). The capability of adaptive immune cells to discern between non-self and self antigens is provided by mechanisms that educate lymphocytes to the lack of response upon the encountering of self antigens. This phenomenon, known as tolerance, occurs both during and after lymphocytes maturation, and is called "central" and "peripheral" tolerance respectively (Zinkernagel, 1996).

The different recognition mechanisms used by innate and adaptive immune cells has an important implication in the kinetics of the innate versus adaptive immune response. Innate immunity is immediately active upon pathogen encounter, giving rise to a rapid response that intervenes from minutes to hours after infection. Conversely, adaptive immunity needs time for the expansion of the antigen-specific lymphocyte subset, meaning that adaptive immune response is delayed (Medzhitov & Janeway, 2000).

A pivotal role in the orchestration of the immune response and particularly in the coordination of innate with adaptive immunity is carried on by DCs (Janeway & Medzhitov, 2002).

1.1.1 Pattern recognition receptors (PRRs)

The innate immune system relies on genetically encoded receptors to sense invading pathogens. Since every organism has a limit to the number of genes it can encode in its genome, it is clear that innate immunity is equipped with a relative restricted repertoire of receptors. To overcome this limit, innate immunity has evolved an ingenious strategy. Innate immunity recognizes molecular structures that are essential for the survival of the microorganisms and, for this reason, are not subject to variability, indeed, although their alteration would render the microorganism able to elute the innate immune control it would be lethal for the microorganism itself. These molecular structures, named pathogen-associated molecular patterns (PAMPs), are conserved and shared among groups of pathogens. Accordingly, their receptors are

called pattern recognition receptors (PRRs). (Janeway, 1989; Medzhitov & Janeway, 1997).

Following Charles Janeway's pattern recognition theory, which dates back to 1989, in 1994, an alternative to self-non self-discrimination theory was proposed by Polly Matzinger's danger theory (Matzinger, 1994, 2002). It became increasingly clear that PRRs can be also activated by endogenous molecules released from damages cells, named, analogously to the previous model, damage-associated molecular patterns (DAMPs). This apparently risky phenomenon is instead useful for the organism's homeostasis, since it allows the elimination of damaged cells and the beginning of the tissue repair process. The inflammation process in this case is referred to as "sterile inflammation" (Chen & Nuñez, 2010).

PRRs are non-clonally distributed on innate immune cells, this means that identical receptors are expressed on the same type of cells. They are mostly expressed on antigen presenting cells such as DCs and macrophages, but are also present on other immune and non-immune cells (Medzhitov & Janeway, 2000). PRRs can be present in different cellular compartments. Based on their localization it is possible to distinguish: cell-surface PRRs, which include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) and cytoplasmic PRRs such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), NOD-like receptors (NLRs) and AIM2-like receptors (ALRs). (Kumar, Kawai, & Akira, 2011; Takeuchi & Akira, 2010) (Table 1).

Family	Members	Shared domains	Receptor locations
TLR	1–10 in humans, 1–9 and 11–13 mice	LRR, TIR	Cell surface, endosomal compartments
CLR	Dectin-1, Dectin-2, . . . etc.	C-type lectin	Cell surface
NLR	NOD1 (NLRC1), NOD2 (NLRC2), NLRC3–5, NLRP1–9 and 11–14, NAIP1, -2, -5, -6	Nucleotide binding, LRR	Cytoplasm, plasma, and endosomal membrane associated
RLR	RIG-I, MDA5, LGP2	DExD/H helicase	Cytoplasm
ALR	AIM2, IFI16	PYRIN, HIN-200	Cytoplasm, nucleus (IFI16)

Table 1 Pattern recognition receptors families.
Adapted from Brubaker, 2015.

The subcellular localization of PRRs influence the accessibility to different types of ligand and the response they elicit. For instance, the activation of cytosolic PRRs commonly induces cell death while this does not happen following cell surface PRRs activation (Lamkanfi & Dixit, 2014; Lei et al., 2009). PRRs compartmentalization is also important to avoid improper activation by self-molecules not associated with infection. For example, the nucleic acid-sensing TLRs are compartmentalized within the intracellular space, which limits activation by self-nucleic acids that are abundantly present in the extracellular fluids of mammals (Barbalat et al., 2011).

The engagement of PRRs immediately triggers signal-transduction pathways inside the cell leading to both transcriptional and non-transcriptional responses, such as inflammatory cytokines production and phagocytosis respectively (Brubaker et al., 2015). The immediate activation of the effector functions of innate immune cells upon PRRs engagement is responsible for the rapid kinetics of innate immune response since there is no need for cell proliferation and expansion, as required for adaptive immune cells.

1.1.2 Toll-like receptors (TLRs)

Toll-like receptors (TLRs) received their name according to their similarity to the Drosophila melanogaster Toll, a transmembrane protein that was identified as an essential component of a pathway responsible for the dorsoventral polarization in the development of Drosophila embryos (Hashimoto et al., 1988; Rock et al., 1998). Later on, it was described that Toll is also implicated in the immune response of adult Drosophila by regulating the activation of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Belvin & Anderson, 1996; Lemaitre & Hoffmann, 1996).

TLRs are the first and one of the best characterized within the PRR family and in some way considered as the prototype of PRRs. Moreover, TLRs are essential regulators of innate immune responses (O'Neill et al., 2013). TLRs are expressed on various immune cells, including macrophages, DCs, B cells and even non-immune cells such as fibroblasts and epithelial cells. Up to now, thirteen members of the TLR family have been identified in mammals, in particular ten TLRs have been described in humans (TLR1-10) while twelve in mice (TLR1-9 and TLR11-13). TLR10 is a pseudogene in mice due to an insertion of a stop codon while TLR11-13 have been lost from the human genome (Kawai & Akira, 2010; Kawasaki & Kawai, 2014).

These receptors are responsible for sensing PAMPs of various origins, which include lipids, lipoproteins, proteins and microbe-derived nucleic acids, in respect to their subcellular localization (Akira et al., 2006) (Table 2). TLRs 1, 2, 4, 5, 6,10 are classified as plasma membrane TLRs and they are involved in the recognition of molecular

structures expressed on the surface of pathogens or released in the extracellular space. On the other hand, TLRs 3, 7, 8, 9, 12, 13 are expressed at the level of the endosome. TLR11 has been reported to be both in the plasma membrane (Kawai & Akira, 2010) and in the endosome (Kawasaki & Kawai, 2014). TLR3-9 and TLR13 are responsible for the sensing of nucleic acids derived either from virus and bacteria or endogenous nucleic acids in pathogenic contexts, TLR11-12, instead, recognize microbial components (Takeuchi & Akira, 2010; Kawasaki & Kawai, 2014).

TLR	Subcellular localization	Physiological ligands	Synthetic ligands
TLR1–TLR2	Plasma membrane	Triacylated lipopeptides	Pam_3CSK_4
TLR2	Plasma membrane	Peptidoglycan, phospholipomannan, tGPI-mucins, haemagglutinin, porins, lipoarabinomannan, glucuronoxylomannan, HMGB1	ND
TLR2–TLR6	Plasma membrane	Diacylated lipopeptides, LTA, zymosan	FSL1, MALP2, Pam_2CSK_4
TLR3	Endosome	dsRNA	Polyl:C
TLR4	Plasma membrane	LPS, VSV glycoprotein G, RSV fusion protein, MMTV envelope protein, mannan, glucuronoxylomannan, glycosylinositolphospholipids, HSP60, HSP70, fibrinogen, nickel, HMGB1	ND
TLR4–TLR6	Plasma membrane	OxLDL, amyloid-β fibrils	ND
TLR5	Plasma membrane	Flagellin	ND
TLR7	Endosome	ssRNA	Imidazoquinoline compounds: imiquimod, resiquimod, loxoribine
TLR8	Endosome	ssRNA	Resiquimod
TLR9	Endosome	DNA, haemozoin	CpG-A, CpG-B and CpG-C ODNs
TLR11 (mouse)	Plasma membrane	Profilin	ND

Table 2 Localization and ligands of Toll-like receptors.
Adapted from: Lee et al., 2012.

Despite their final subcellular localization, all the TLRs are synthesized in the endoplasmic reticulum (ER) and pass through the Golgi to be then recruited to the plasma membrane or to intracellular

compartments. The proper endosomal localization of TLRs is made possible by ER membrane protein uncoordinated 93 homolog B1 (UNC93B1), a multi-pass transmembrane protein that controls the trafficking from the ER to the endosomes (Kim et al., 2008; Lee et al., 2013).

From the structural point of view, TLRs are type I transmembrane proteins characterized by: an ectodomain containing several leucine-rich-repeat (LRR) motifs, a transmembrane region and a cytoplasmic domain homologous to that of the interleukin 1 receptor (IL-1R), termed the Toll/IL-1R homology (TIR) domain (Bowie & O'Neill, 2000). The extracellular domain is responsible for ligand recognition, the LRR modules confer the typical horseshoe-shaped folding and the intracellular region represents the signalling domain (Gay & Gangloff, 2007).

Upon ligand binging, TLRs dimerize forming both homo- and hetero-dimers. The dimerization induces conformational changes that allow the recruitment of TIR-domain-containing adaptor molecules to the intracellular TIR domain of the TLR. Precise combinations of adaptor proteins account, at least partially, for the specific biological response triggered by individual TLRs. There are five TIR-domain-containing adaptors including MyD88, TRIF, TIRAP, TRAM and SARM. TIRAP and TRAM are defined "sorting adaptors" due to the lack of signalling domains. MyD88 and TRIF, instead, are defined "signalling adaptors" since they mediate the activation of two distinct pathways, the first leading to the production of proinflammatory cytokines, the latter regulating type I interferons (IFNs) release (Takeuchi & Akira, 2010).

MYD88 is engaged for the signalling of all the TLRs with the exception of TLR3. MyD88 interact with the TIR domain of TLRs and recruits IL-1R-associated kinase 4 (IRAK-4), a serine/threonine kinase that, in turn, activates other IRAK family members, IRAK-1 and IRAK-2 (Kawagoe et al., 2008). Once detached from MyD88, the IRAKs interact with tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), and trigger the activation of NF-κB signalling pathway and MAP kinases cascade. Into the nucleus, NF-κB regulates the expression of proinflammatory cytokine genes as tumour necrosis factor α (TNFα) and interleukin 6 (IL-6). MAPK pathway is responsible for the formation of another transcription factor complex, AP-1, that targets cytokines genes (Kawai & Akira, 2010). The activation of NF-κB occurs downstream all TLRs, with few differences in the upstream pathway. TLR2 and TLR4 enrol TIRAP as additional adaptor to bridge between TLR and MyD88. TLR3, the sole completely MyD88-independent TLR, relies on TRIF pathway to triggers NF-κB activation (Kawai & Akira, 2007).

The TRIF-dependent signalling pathway culminates in the activation of both IRF3 and NF-κB (Kawai & Akira, 2008). For the activation of NF-κB, TRIF recruits TRAF6 and activates the kinase TAK1, which is involved in the MYD88-dependent NF-κB activation as well. Beyond TRAF6, TRIF associates with TRAF3 that activates two IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKK-I which, in turn, phosphorylate IRF3 and IRF7. IRF3 and IRF7 dimerize and translocate into the nucleus where they induce the expression of type I IFNs (Hacker & Karin, 2006; Oganesyan et al., 2006).

Other participants in the TLRs signalling pathway have emerged to be co-receptor molecules that assist the TLRs interaction with their ligands. The function of the co-receptor molecules is not merely supporting the interaction between the receptor and its ligand, on the contrary, they have crucial roles. The co-receptors can help the presentation of the ligands to the TLRs and define the specificity or increase the affinity of this interaction, moreover, they are able to redirect the TLRs to a defined subcellular localization that is functional for the signalling pathway to happen, finally upon ligand-binging, the co-receptors can also activate transcriptional and non-transcriptional signalling pathways participating in the fulfilment of ligand-specific response (Di Gioia & Zanoni, 2015). Among these CD14 is particularly studied for its role in the LPS recognition by the TLR4, which will be discussed later.

1.1.3 C-type lectin receptors (CLRs)

C-type lectin receptors (CLRs) comprise a number of proteins originally defined for their capability to detect carbohydrate structures (Drickamer, 1988). The members of the CLR family share at least one carbohydrate recognition domain (CRD), placed at the extracellular domain, which is responsible for the binding of the receptor with carbohydrate ligands and confers also binding specificity to CLRs. CLRs exist as both transmembrane (such as Dectin-1 and Dectin-2) and soluble receptors (such as mannose binding lectin, MBL).

Transmembrane CLRs are widely expressed at the surface of myeloid cells, namely macrophages and DCs. CLRs are involved in the recognition of mannose, fucose and glucan carbohydrate structure (Geijtenbeek & Gringhuis, 2009). This capability allows CLRs to detect many classes of pathogens: mannose specificity allows the recognition of viruses, fungi and mycobacteria; fucose structures are specifically expressed by certain bacteria and helminths; glucan structures are represented on fungi and mycobacteria. CLRs engagement induces the internalization of the pathogen, its degradation and subsequent antigen presentation (Rothfuchs et al., 2007; van Kooyk & Rabinovich, 2008).

The proteins belonging to CLR family can be divided into 17 groups based on the organization of their CRDs and can be further defined as either classical or non-classical C-type lectins (Drickamer & Fadden, 2002; Zelensky & Gready, 2005). Classical C-type lectins contain conserved residues in the CRD for the binding of carbohydrate ligands and Ca^{2+} binding sites. Non-classical C-type lectins generally do not contain such residues and may bind non-carbohydrate ligands.

It is possible to identify two groups of CLRs. The first one is the Dectin-1 cluster that includes MICL, CLEC-2, CLEC9A, CLEC12B, CLEC-1, Dectin-1 and LOX-1). The second one is the Dectin-2 cluster that comprehends BDCA-2, DCAR, DCIR, Dectin-2, Clecsf8 and Mincle (Kingeter & Lin, 2012).

The general structure of CLRs comprises, besides the CRD, a stalk region, a transmembrane region and intracellular domain (Fig.2). The structure of the intracellular tail endows the CLRs with direct or indirect signal transduction abilities. Among the transcription factors regulated by CLRs there is NF-κB, resulting in the regulation of

inflammatory cytokines production. The cytoplasmic domain of Dectin-1, CLEC-2, CLEC9a and LOX-1 retains immunoreceptor tyrosine-based activation motif (ITAM) and ligation of these receptors leads to the induction of signalling pathways that regulate the activation of transcription factors. The cytoplasmic domain of MICL, CLEC12B and DCIR contains an immunoreceptor tyrosine-based inhibitory motif (ITIM), whose ligation, on the contrary, leads to the inhibition of the activity of transcription factors (Kingeter & Lin, 2012). On the other hand, the short intracellular domain of Dectin-2, BDCA-2, DCAR, Mincle and Clecsf8 is responsible for the lack of signal transduction ability of these proteins, therefore the signal transduction of these receptors relies on the association with ITAM-containing adaptor molecules, such as Fc receptor γ-chain (FcRγ) (Cao et al., 2007; Sato et al., 2006; Yamasaki et al., 2008).

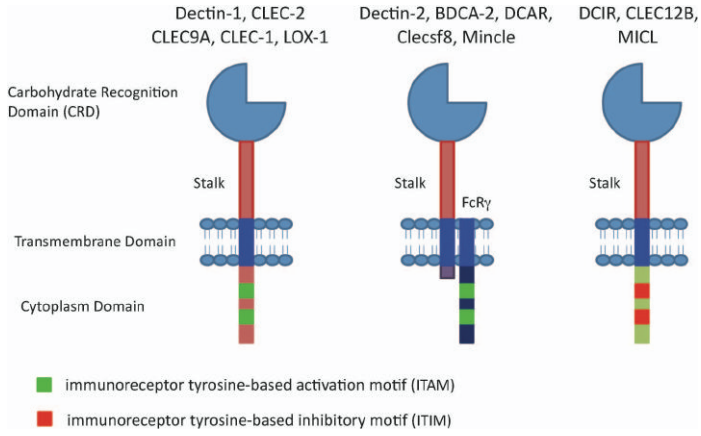

Figure 2 The structure of Dectin-1, Dectin-2 and DCIR families of C-type lectin receptor.

These C-type lectin receptors share an extracellular CRD domain, a stalk region, a transmembrane domain and a cytoplasmic one. CLRs differ their cytoplasmic domains.

Figure 2 continued on next page.

Figure 2 continued.
Dectin-1 family members have an ITAM-containing cytoplasmic domain, whereas Dectin-2 family members have a short cytoplasmic domain with its transmembrane domain associating with FcRγ that contains an ITAM. In contrast, DCIR family members have an ITIM-containing cytoplasmic domain. Adapted from: Kingeter & Lin, 2012.

In myeloid cells, the signal cascade directly activated by phosphorylated ITAM-containing CLRs or indirectly by ITAM-containing adaptor molecules leads to Syk tyrosine kinase activation, which controls various cellular outcomes, as the induction of phagocytosis, the respiratory burst and the production of cytokines and chemokines (Majeed et al., 2001), mainly through the regulation of transcription factors. The phosphorylation of ITIM-containing CLRs allows the recruitment of phosphatases that lead to down-modulation of cellular activation (Long, 1999). Nevertheless, it has been shown that some ITAMs and ITIMs can mediate the opposite effects (Barrow & Trowsdale, 2006; da Silva et al., 2008), increasing the complexity of CLR-mediated signalling.

So far, the ligand specificity of each CLR has been partially established. Dectin-1 (also known as CLEC7A) and Dectin-2 (also known as CLEC6A) are both involved in the recognition of fungi, such as *Candida albicans*, by detecting, respectively, β-glucan structures (Taylor et al., 2007) and α-mannose structures (Ishikawa et al., 2013; Saijo et al., 2010) present at the level of the cell wall of both pathogenic and opportunistic fungi.

The activation of both Dectin-1 and Dectin-2, as well as of other CLRs, culminates in the Syk-CARD9-BCL-10-MALT1–dependent induction of NF-κB transcriptional activity, thus the regulation of

inflammatory cytokines production (Bi et al., 2010; Geijtenbeek & Gringhuis, 2016; Gringhuis et al., 2009; Saijo et al., 2010). It has been shown that the stimulation of Dectin-1 by live *Candida albicans* and zymosan particles leads to the activation of the transcription factor nuclear factor of activated T cell (NFAT), both in macrophages and DCs (Goodridge at al., 2007), pointing out for the first time the activation of the transcription factor NFAT in the innate immune system, which will be further discussed in section 1.5.2.

1.2 Dendritic cells

The first description of DCs dates back to 1973, when Ralph Steinman and Zanvil Cohn shed light on a novel population identified in adherent cell preparations from mouse peripheral lymphoid organs, especially spleen, but also lymph nodes and Peyer's patch. This new cell type, stood out for its distinct morphology, characterized by a large and contorted nucleus, containing small nucleoli and a well-represented cytoplasm arranged in multiple processes of various extension (Steinman and Cohn 1973). In the following years, further researches by Steinman and co-workers elucidated that DCs express both histocompatibility complex (MHC) class I and II molecules (Nussenzweig et al. 1980; Steinman et al. 1979) and that they represent potent stimulator of T cells in primary mixed leukocytes reactions (MLR) (Steinman and Witmer 1978).

The extensive and pioneering work of Ralph Steinman in the discovery and functional characterization of DCs was honoured with the Nobel Prize in Physiology or Medicine in 2011.

Despite the initial scepticism of the scientific community in front of the biological significance of this newly discovered immune cell population, DCs are currently universally recognized as professional antigen presenting cells (APCs), able to orchestrate the immune response, being the missing link between innate immunity and adaptive immunity (Banchereau & Steinman, 1998).

The term APCs refers to a heterogeneous class of immune cells specialized in antigen capture and then presentation to lymphocytes, mainly T cells. The T-cell receptors (TCRs) recognize fragments of antigens bound to MHC on the surface of an APC. Classical APC include DCs, macrophages and B cells. Among these, DCs are the most efficient, since they require lower amount of antigen to carry on antigen presentation. This is due to the efficiency of DCs in both capturing and processing antigens (Banchereau & Steinman, 1998).

DCs employ different strategies to capture antigens, including phagocytosis (Inaba et al., 1993), that allows the engulfment of particles and microbes, micropinocytosis, which is the process of internalizing extracellular fluids by wide pinocytic vesicles and endocytosis, often mediated by receptors, such as CLRs (Sallusto et al., 1995). Then the antigen follows the endocytic pathway of the cells and is processed to be exposed in MHC molecules on the cell surface to be presented, for instance to T cells. In comparison with macrophages, DCs have MHC class II-rich compartments that allow the assembly of a large quantity

of complexes made of MHC class II with antigen-derived peptide (Nijman et al., 1995).

According to the classical paradigm, DCs can exist in two functional states, immature and mature and only mature DCs display the ability to initiate the immune response. These two maturation stages are segregated in time. Immature DCs placed in non-lymphoid tissue, such as the skin and the mucosae, are fully equipped to capture antigens and are actively involved in patrolling the environment. This characteristic is the reason why DCs are considered the "sentinels" of the immune system. Upon antigen encounter, DCs go through maturation that comprises the downregulation of antigen uptake capability and the induction of costimulatory proteins, antigen processing capability, MHC molecules expression (Fig.3).

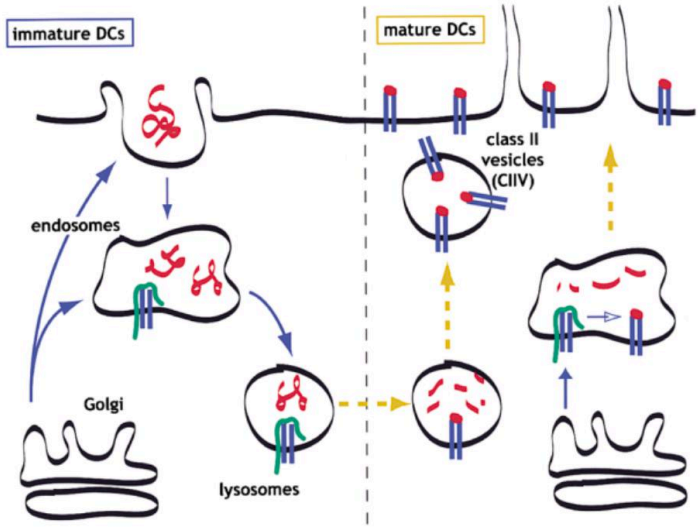

Figure 3 The two functional and phenotypic states of immature and mature dendritic cells.

Immature DCs are adept at endocytosis and express relatively low levels of surface MHC class II and costimulatory molecules.

Figure 3 continued on next page.

Figure 3 continued.
Following antigen encounter DCs convert into mature DCs characterized by a reduced ability in antigen uptake but also by an extremely efficient capability to stimulate T cells. Maturation is accompanied by cytoplasmic reorganization, redistribution of MHC class II from intracellular compartments to plasma membrane, elevated expression of co-stimulatory molecule at the cell surface and extension of dendritic processes. Adapted from: Mellman & Steinman, 2001.

Moreover, mature DCs are able to migrate to the lymphoid tissues where they can prime naïve antigen-specific T cells (Banchereau & Steinman, 1998). Therefore, antigen detection represents the actual drive that converts DCs from an antigen-sampling mode to a mature APC (Reis e Sousa, 2006).

Finally, after the achievement of their effector function, DCs dye by apoptosis. This event is extremely important to terminate the immune response without causing an over-response that could be extremely dangerous for the organism (Matsue & Takashima, 1999).

1.2.1 DCs heterogeneity

DCs constitute an extremely heterogeneous class of cells sharing the capability of antigen capture, processing and presentation to naïve T cells. So far, different DCs subtypes have been identified, in mouse models, based on their location, migratory pathways, immunological function and generation process (Shortman & Naik, 2007). It is possible to identify four main group of DCs, which are: conventional DCs (cDCs), plasmacytoid DCs (pDCs), Langerhans cells (LCs) and monocyte-derived DCs (moDCs).

cDCs and pDCs share a common origin. A bipotent progenitor in the bone marrow, named macrophage and DC precursor (MDP) is able to give rise to DCs and monocytes (Fogg et al., 2006). MDPs differentiate into common DC progenitors (CDPs) that is committed to generate pDCs and cDCs (Naik et al., 2007). The terminal differentiation of pDCs occurs in the bone marrow (Reizis, 2010), whereas cDCs stem from so called precursors of DC (pre-DCs) that exit the bone barrow and migrate to lymphoid and non-lymphoid tissues where they terminally differentiate (Liu et al., 2009; Naik et al., 2006). The MDPs also give rise to a common monocyte precursor (cMoP) which gives origin only to monocytes. Monocytes are also a source for DCs generation. Indeed, monocytes can migrate from the blood to peripheral tissues and here, depending on the milieu can differentiate into DCs, which are therefore termed monocyte-derived DCs (Schraml & Reis e Sousa, 2015).

Conventional DCs (cDCs)

The phenotypic definition of cDCs is in continuous evolution. They constitutively express CD45, MHC-II and CD11c and lack markers of any specific lineage (for instance, markers specific for: T cells, NK cells, B cells, granulocytes and erythrocytes). This rather limited definition is the reason why the discrimination of DCs from other immune cell subsets can be difficult. For example, CD11c is not specific for DCs, it can be expressed also by macrophages. Moreover, inflammatory stimuli-induced phenotypic changes make particularly complicated to discern phenotypic plasticity from distinct cDCs subsets (Merad et al., 2013).

cDCs generally have a short half-life of approximately three to six days and are constantly replenished from bone marrow precursors in a strictly dependency on the DC poietin Fms-related tyrosine kinase 3 ligand (Flt3L) (McKenna et al., 2000).

cDCs can be further grouped into either migratory or lymphoid-tissue resident DCs. Migratory DCs reflect the historical description of DCs, being the ones that uptake the antigen in periphery and migrate to lymph nodes. In this process, they progressively shut down the antigen capture ability and reach a mature phenotype characterized by the effective antigen presentation. The migration of these cells to lymph nodes has been shown to occur also in the steady state, even if at a lower rate (Huang & MacPherson, 2001).

Their counterpart is represented by lymphoid-tissue resident cDCs, which reside for their entire life within lymphoid tissues. Their function is to collect and present antigens in the lymphoid organ itself, examples of this population include most of the thymic cDCs and splenic cDCs, whereas in the lymph nodes around half of the DCs are lymph node-resident, rather than non-lymphoid tissue migratory (Shortman & Naik, 2007).

Among peripheral cDCs distinct subsets can be identified and can be broadly grouped as $CD11b^{-}CD103^{+}$ and $CD11b^{+}$ cDCs. Mouse lymphoid-tissue resident DCs can be split up into $CD8^{+}$ cDCs and $CD8^{-}$ cDCs. The $CD8\alpha^{-}$ are in turn divided into $CD4^{-}CD8^{-}$ cDCs and $CD4^{+}CD8^{-}$ cDC (Mildner & Jung, 2014; Shortman & Naik, 2007; Vremec et al., 2000).

Plasmacytoid DCs (pDCs)

Plasmacytoid DCs were firstly described in the late fifties (Lennert & Remmele, 1958). pDCs constitute a small subset of DCs that share the same origin of cDCs, deriving from CDP in the bone marrow. This type of cell stands out for its capacity to produce type I IFNs (Cella et al., 1999; Siegal et al., 1999), namely IFN-α/β and are particularly relevant for the anti-viral immune response. It was described that upon TLR7- and TLR9-dependent virus or self-nucleic acid recognition, pDCs secrete large amount of type I IFNs but also proinflammatory cytokines and chemokines (Gilliet, Cao, & Liu, 2008; Swiecki & Colonna, 2015).

As compared to cDCs, pDCs show a distinct morphology, characterized by a spherical shape resembling an antibody-secreting plasma cells. pDCs are relatively long-lived, they circulate in the blood and reach the lymph nodes mainly through high endothelial venules (HEVs) and not through afferent lymphatics (Yoneyama et al., 2004). In addition, pDC can migrate from blood towards peripheral tissues (Swiecki & Colonna, 2015).

At the steady state, pDCs can be considered pre-DC. Following the inflammatory stimuli they convert into dendritic form acquiring antigen-processing and antigen-presentation properties (Liu, 2005).

Mouse pDCs can be identified for their expression of B220, LY6C, sialic acid-binding immunoglobulin-like H (Siglec-H), bone marrow stromal antigen 2 (BST2) and intermediate expression of CD11c. Moreover, they can express CD8 and CD4.

Langerhans cells (LCs)

LCs are named after Paul Langerhans who first reported the existence of dendritic, non-pigmentary cells in the epidermis, which he considered as intraepidermal receptors for extracutaneus signals of the nervous system, also for their positivity to gold chloride stain that has affinity for nerve tissue (Langerhans, 1868). It takes long time since the actual identity of these cells was disclosed. Despite the original thought of Paul Langerhans, who hypothesized that these cells were peripheral nerve cells, as suggested by network-like distribution and dendritic-rich morphology, it is currently clear that LCs represent a specialized population of dendritic cells that reside in the epidermis of the skin, here they function as APCs and are involved in immune responses to skin infections (Kaplan, 2017; Rowden et al., 1977; Stingl et al., 1977).

LCs stand apart from cDCs concerning ontology. While cDCs arise from bone-marrow precursor, LC compartment is established before birth from fetal liver-derived monocytes that seed the skin (Hoeffel et al., 2012), resembling the ontogeny of tissue resident macrophages. Moreover, the development of LCs is independent of Flt3 and Flt3L (which is a feature of pre-DCs, cDCs and pDCs) while requiring colony-stimulating factor 1 receptor (Csf-1R), similarly to many tissue macrophages. After birth, LC population undergoes a huge expansion, assume dendritic morphology and progressively begin to express MHC-II and the C-type lectin receptor Langerin (CD207). Once formed, LCs are self-renewing and display radioresistance (Kaplan, 2017; Merad et al., 2013).

LCs migrate to the draining lymph nodes in the steady state and, at a higher rate, during inflammation. They leave the epidermis and use

the dermal lymphatic vessels to reach the T-cell area of the skin draining lymph nodes (Merad et al., 2008).

Monocyte-derived DCs (moDCs)

The classical concept about monocytes is that they are leukocytes circulating in the blood that generate different subpopulations of macrophages, during both steady state and inflammatory conditions. To date, it is clear that monocytes retain the potential to differentiate into DCs, which are therefore termed moDCs.

The first evidence for the existence of moDCs came to light from *in vitro* studies (Inaba et al., 1992; Sallusto & Lanzavecchia, 1994). Further researches confirmed the same results *in vivo*, demonstrating the capability of monocytes to differentiate into DCs in inflammatory conditions (Randolph et al., 1999), infections (León et al., 2007) and steady state (Varol et al., 2007). The current view is that monocytes give birth mainly to inflammatory DCs but are also a source of homeostatic DC populations.

Circulating monocytes originate from the bone marrow precursors MDPs and can be divided in two main subsets: $Ly6C^{hi}$ and $Ly6C^{low}$. $Ly6C^{hi}$ monocytes are $CX3CR1^{low}$, $CCR2^{+}$, $CD62L^{+}$, and $CCR5^{-}$, while $Ly6C^{low}$ monocytes are $CX3CR1^{high}$, $CCR2^{-}$, $CD62L^{-}$, and $CCR5^{+}$ (Geissmann et al., 2003). $Ly6C^{hi}$, the conventional monocytes, are considered the main source for moDCs.

Monocyte-derived DCs functions are strictly related to the environmental conditions, this is because they result from monocytes that have been exposed to multiple local mediators, thereby the

functional specificity of moDCs reflects the high degree of plasticity of their monocytic precursors (Domínguez et al., 2010).

1.2.2 Human dendritic cells

The current understanding about human DCs, comes from a number of studies that have been conducted mostly on skin and blood, being the two most available human tissues.

Human DCs are highly heterogeneous, as well as mouse DCs and they consist of multiple subtypes with unique functions. They are gathered for their constitutive expression of MHC-II (HLA-DR) and lack of typical lineage markers (CD3, CD19/20, CD56, CD14), therefore the classical description of human DCs is Lin^- and $MHC\text{-}II^+$ (Collin et al., 2013; Ziegler-Heitbrock et al., 2010).

Human DC subtypes have been historically defined based on their localization, morphology, physical properties, markers, developmental origin and functions. Three main populations have been defined that are: plasmacytoid DCs (pDCs) and two subsets of myeloid DCs, which are $CD141^+$ DCs and $CD11b^+$ DCs. (Ziegler-Heitbrock et al., 2010). Two DCs populations stand apart from this classification, monocyte-related DCs and LCs (Fig.4).

Although there is a high similarity between mouse and human DCs subsets, is not always possible to find a perfect equivalence, rather analogies.

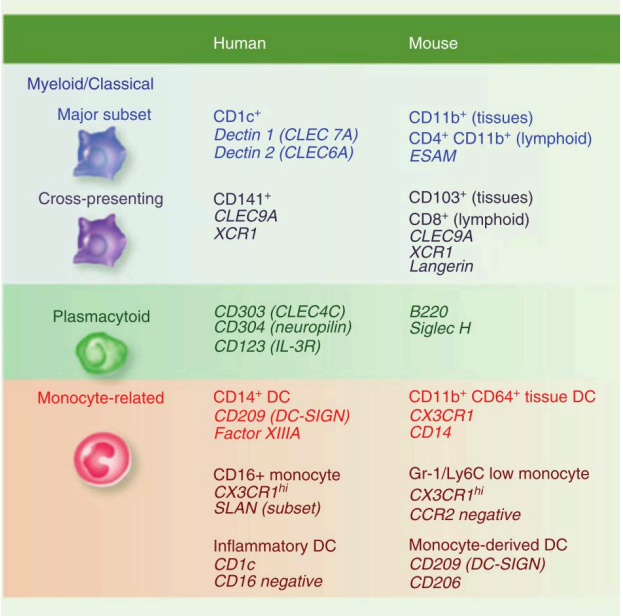

Figure 4 Dendritic cell subsets: human-mouse homology.
Adapted from Collin et al., 2013.

Myeloid DCs (mDCs)

Myeloid DCs, also named conventional DCs (cDCs) express typical myeloid antigens CD11c, CD13, CD33 and CD11b and are the human counterpart of mouse cDCs. In humans, CD11c is even less specific for DCs since it is expressed also by monocytes but, as compared with monocytes, mDCs lack CD14 or CD16 expression and they can be further divided into $CD141^+$ and $CD1c^+$ subsets. These two subsets can be considered the human counterpart of murine $CD8/CD103^+$ DCs and $CD11b^+$ DCs, respectively.

$CD1c^+$ mDCs constitute the main population of mDCs in blood, tissues and lymphoid organs. They were originally recognized in the

blood as a fraction of HLA-DR$^+$Lin$^-$ cells expressing myeloid antigens CD11b, CD11c, CD13, CD33, CD172 (SIRPa) and CD45RO (Cella et al., 1999). They are identified with BDCA-1 antibody which recognize CD1c.

In tissues, they display a more activated phenotype as compared to blood CD1c$^+$, since they have a higher expression of CD83, CD80, CD86 and CD40. They also have a higher expression of CCR7 (Angel et al., 2006; McLellan et al., 1998). It has been suggested that blood CD1c$^+$ DCs are a kind of precursor to tissue CD1c$^+$ DCs, which is supported by in vitro studies (Haniffa et al., 2012).

At the level of lymph nodes, they are represented in the T-cell area as interdigitating cells (Angel et al., 2009). Other lymphoid tissues such as tonsil and spleen also contain CD1c$^+$ DCs (Mittag et al., 2011; Summers et al., 2001).

The expression of several TLRs allow CD1c$^+$ to respond to a plethora of stimuli as LPS, R848 and poly(IC). The presence of Dectin-1 and Dectin-2 also endow CD1c$^+$ DCs with the capacity to respond to fungi. These cells have also a variable expression of DEC205 and macrophage mannose receptor (CD206). CD1c$^+$ DCs are able to stimulate naïve CD4 T cells; in comparison with CD141$^+$ DCs have a lower capability to cross present antigens to CD8 T cells (Collin et al., 2013; Haniffa et al., 2012). Following stimulation, CD1c$^+$ DCs secrete a number of cytokines as TNFα, IL8, IL-10. IL-12 can be produced upon TLR7/8 stimulation with R848. Moreover, these cells can secrete a small amount of IL-23 in response to various stimuli (Collin et al., 2013).

CD141$^+$ mDCs are commonly identified with the BDCA-3 antibody (Dzionek et al., 2000). They are present in the blood and are also found among resident DCs of lymph nodes, tonsils, spleen and bone marrow and non-lymphoid tissues, skin, lung and liver (Haniffa et al., 2012).

CD141$^+$ DCs are the human counterpart to mouse lymphoid CD8$^+$ DCs and CD103$^+$ tissue DCs. As mouse CD8$^+$ DCs, human CD141$^+$ DCs have cross-presenting ability (Bachem et al., 2010).

CD141$^+$ DCs are equipped with CLEC9A and TLR3 and TLR8 allowing them to sense dead or necrotic cells and viral nucleic acids. CD141$^+$ DCs produce TNFα, CXCL10 and interferon-λ (Collin et al., 2013).

Plasmacytoid DCs

Human pDCs are the most numerous DCs in the blood. They are negative to myeloid antigens as CD11b, CD11c, CD13 and CD33 whereas they express CD45RA, variable CD2 and CD7 and may induce T-cell receptor immunoglobulin rearrangements. The expression of the IL-3 receptor α-subunit (also known as CD123), CD303 (BDCA-2), CD304 (BDCA-4) (Dzionek et al., 2000) allow their distinction from cDCs.

pDCs are detected in both non-lymphoid tissues and lymphoid tissue, namely lymph nodes and they are rapidly recruited to both sites upon inflammatory stimuli (Cox et al., 2005).

As well as mouse pDCs, human pDCs have a great secretory capability and are known as type I IFN-producing cells (Swiecki & Colonna, 2015).

Monocyte-related DCs

Monocyte-related DCs include a population of $CD14^+$ DCs that lacks the characteristic markers of mDCs, such as CD1c or CD141, co-stimulatory molecules and CCR7, whereas express CD11c as monocytes. In addition, $CD14^+$ DCs express CD209 (DC-SIGN) and the macrophage markers FXIIIA and CD163.

$CD14^+$ DCs can be found in many non-lymphoid tissues and despite the absence of CCR7, which is relevant for DCs migration, $CD14^+$ DCs are able to migrate to lymph nodes (Angel et al., 2009; Segura et al., 2012).

Langerhans DCs

LCs are placed in suprabasal epidermis and other stratified squamous epithelia where their disposition resemble a network. Human DCs express high levels of Langerin and CD1a, a non-polymorphic class I MHC. LCs display a specialized endosomal compartment, termed Birbeck granules, dedicated to antigen capture and processing that are observable by electron microscopy and are a specific feature of both human and mouse LCs. LCs have the ability to migrate to lymph nodes (Geissmann et al., 2002). Concerning their functionality, LCs can mature into potent cross-presenting DCs (Van Rhijn et al., 2013).

Recent breakthrough in human blood DCs definition

A very recent work, by means of single-cell RNA sequencing (scRNA-seq), shed light on dendritic cell and monocyte populations in the human blood (Villani et al., 2017). The authors performed single-cell RNA sequencing of about 2400 cells isolated from healthy donors and

enriched for HLA-DR^{+}Lin^{-} cells. Following genomic classification, functional and phenotypic characterization, the authors described: six blood-DCs subsets (termed DC1-6), a conventional DC progenitor (cDCs progenitor) and four monocytes subsets (Mono1-4). This landmark study broadened the current knowledge about human blood cell populations revealing some important news.

The authors categorized human blood DCs into six clusters and pointed out some discriminative markers (Fig.5). The six DCs clusters enlightened by scRNA-seq are: DC1, closely related to the well-established CD141^{+} DCs and effectively identified by the expression of *CLEC9A*; DC2 and DC3, gathered for their common expression of CD1c; DC4 that consists of the poor characterized CD141^{-}CD1c^{-} population, accurately delineated by *FCGR3A/CD16*; DC5, a previously unknown DC population, defined by *AXL* and *SIGLEC6* markers expression and therefore named AS DC, finally, DC6 cluster that maps closely to pDCs.

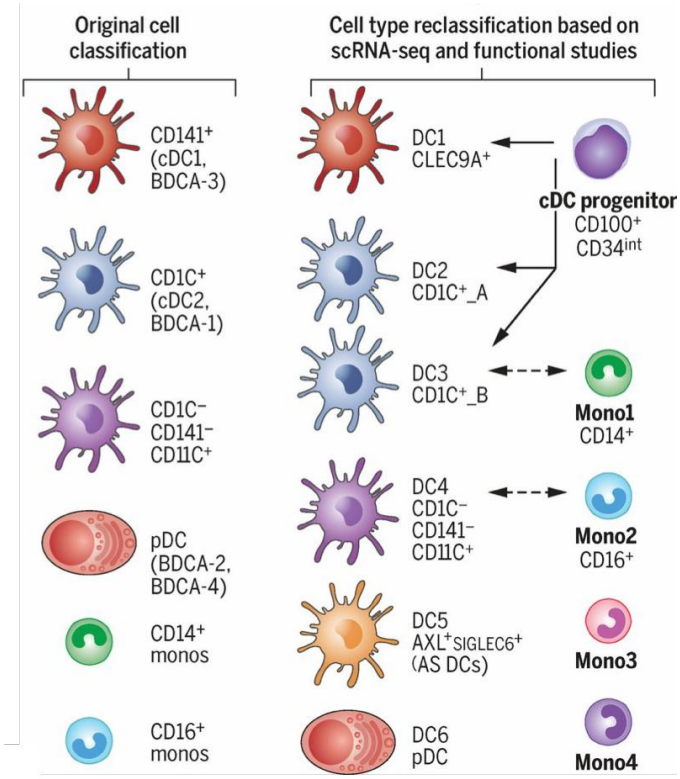

Figure 5 Atlas of human blood dendritic cells and monocytes.
Comparison between the original classification of human blood DCs and the newly described populations revealed by single-cell RNA-sequencing. Adapted from Villani et al., 2017.

AS DC subset appears to be rare within the HLA-DR$^+$Lin$^-$ population, it can be further split into two subpopulations, one showing a pDC-like signature while the other enriched for a cDC-like signature. This population was conceivable hidden within the pDC population. However, as compared to pDCs, AS DCs show a high potency in T cell activation which led the authors to propose the re-definition of pDCs as

"natural interferon-producing cells (IPCs)" with weak T cell proliferation induction ability.

A clear subdivision within the CD1c$^+$ cells was brought into focus leading to the identification of CD1C_A and CD1C_B clusters (Fig.6). CD1C_A cells (designated as DC2 population) are characterized by an increased expression of MHC-II genes and can be isolated using FCGR2B/CD32 marker. On the other hand, CD1C_B cells (designated as DC3 population) are characterized by the expression of acute and chronic inflammatory genes, such as *S100A9*, *S100A8* and also *CD14*. DC3 be effectively isolated using CD163 and CD36 markers. These two clusters are both potent stimulators of naïve T cells, consistently with the known functional properties of cDCs.

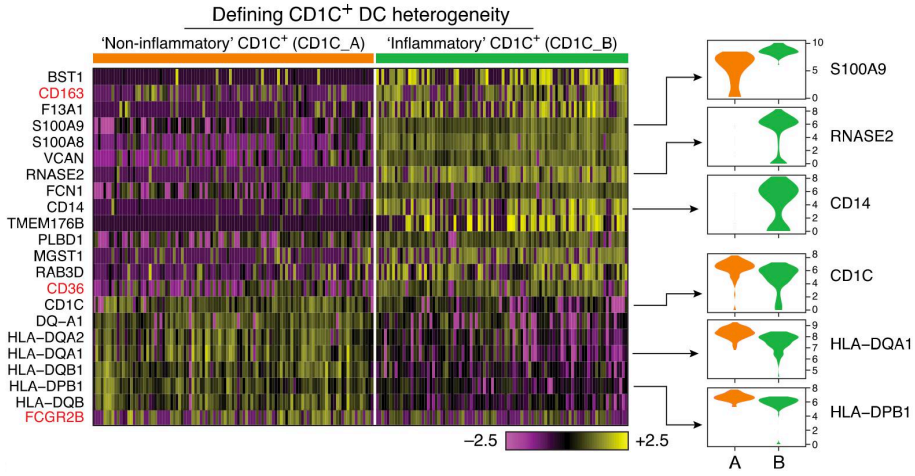

Figure 6 Single-cell RNA-sequencing unveiled the existence of two distinct clusters within the CD1c$^+$ DC population.
Heat map showing scaled expression of discriminative gene sets defining each CD1c$^+$ DC subset. Violin plots illustrate expression distribution of candidate genes across subsets on the x axis (orange for CD1C_A/DC2; green for CD1C_B/DC3). In red are indicated the three markers identified for the isolation of CD1C_A and CD1C_B clusters. Adapted from: Villani et al., 2017.

Concerning monocytes, Mono1 and Mono2 clusters consist of the $CD14^{++}CD16^{-}$ "classical" and $CD14^{+}CD16^{++}$ "non-classical" monocytes, respectively. Two additional populations were identified: Mono3 and Mono4. Mono 3 expresses a unique combination of genes that have the potential to affect cell cycle, differentiation, and trafficking (for instance, *MXD1*, *CXCR1*, *CXCR2*, *VNN2*) whereas Mono4 distinctively expressed a cytotoxic gene signature (including *PRF1*, *GNLY*, *CTSW*).

The comparative analysis between Mono1 and $CD1c^{+}$ DCs populations (DC2 and DC3) pointed out that, despite the co-expression of *CD14* and *S100A8* between DC3 and Mono1, $CD1c^{+}$ DCs express distinctive markers (which are CD1c, FCER1A, CLEC10A, CD1d, FCGR2B and CD33), moreover, they are enriched for antigen processing, MHC-II and leukocyte activation genes. In contrast, Mono1 cells were enriched for defence response, inflammatory response and chemotaxis genes.

The authors also identified the existence of a cDC progenitor in the human blood characterized by the expression of CD100+ CD34int and present with a frequency of approximately 0,02% of the HLA-DR+ Lin- fraction. It has been proved in vitro that this cDC progenitor is able to divide and give origin to CD1c+ and CLEC9A+ DCs.

1.2.3 DC-mediated T_H cell polarization

DCs play a pivotal function in the organization of the immune response. This role relies on two key attributes of DCs. The first one is that DCs can detect pathogens or tissue injury-derived antigens through a number of PRRs, this induces a maturation process that comprises the upregulation of co-stimulatory molecules. The second one is that mature DCs have the ability to prime naïve T cells.

The regulation of T cells activation is a process that occurs at the level of a complex cellular structure that forms at the interface of a T cell and an APC, mostly a DC, expressing the appropriate peptide-MHC complexes. This structure is termed immunological synapse, in analogy to the neuronal synapse and is also known as supra-molecular activation complex (SMAC). The immunological synapse is a highly organized platform that consists of three concentric rings, each containing defined group of proteins. At the central region of the SMAC (cSMAC) TCRs and one of its downstream signalling effectors, protein kinase C-θ (PKC-θ), are concentrated. In the peripheral-SMAC (p-SMAC) can be found proteins important for cell adhesion, such as lymphocyte function-associated antigen-1(LFA-1) and the cytoskeletal protein talin (Monks et al., 1998). The distal ring (dSMAC) is enriched in CD45 and CD43 molecules (Delon et al., 2001; Freiberg et al., 2002) (Fig.7).

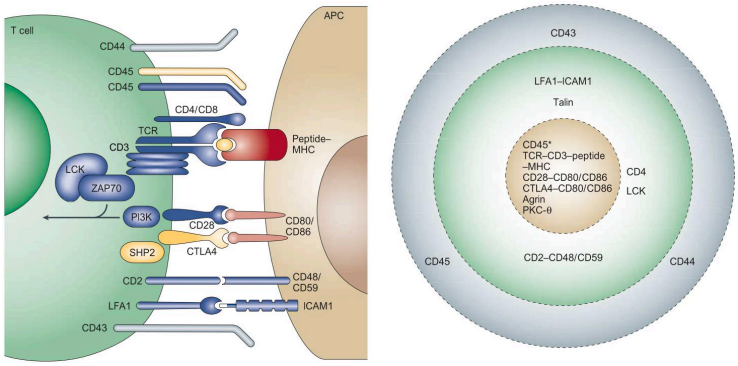

Figure 7 The architecture of a mature immunological synapse.
On the left, a profile view displaying the main ligand pairs and signalling molecules implicated in T-cell initiation. The stimulatory peptide-MHC molecule is shown in red, activating/co-stimulatory molecules are blue, inhibitory molecules are yellow and molecules that are not contributing to signalling are grey. The arrow indicates converging signals that lead to T-cell activation. On the right, a frontal view of the synapse with the characteristic "bulls-eye" zone pattern, including the cSMAC (yellow), the pSMAC (green) and dSMAC (grey) as well the molecules/ligand pairs that are found enriched within. Adapted from: Huppa & Davis, 2003.

The immunological synapse is a kind of intimate site for a bidirectional communication between a T cell and an APC. In this dialogue, DCs can be considered a carrier of pathogen-related information that is provided to T cell in a defined language consisting of three signals (Kaliński et al., 1999).

Signal 1 is antigen-specific, it is represented by the interaction of the TCR with the appropriate peptide-MHC complex on DCs and provides information about the molecular identity of the pathogen. Signal 2 is the co-stimulation that is given by the interactions of DC-expressed costimulatory molecules, as CD80 and/or CD86, with their T-cell counter-receptors, as CD28. In the absence of this co-stimulatory signal, T cell become anergic, which might lead to tolerance. Finally,

the non-contact dependent Signal 3, consisting of soluble factors, is responsible for T-cell function polarization. The nature of Signal 3 is highly dependent on the conditions under which DCs are activated (Kapsenberg, 2003) (Fig.8).

Mature DC-derived signals are extremely important for the polarization of T helper (T_H1/T_H2) cells and T regulatory cells.

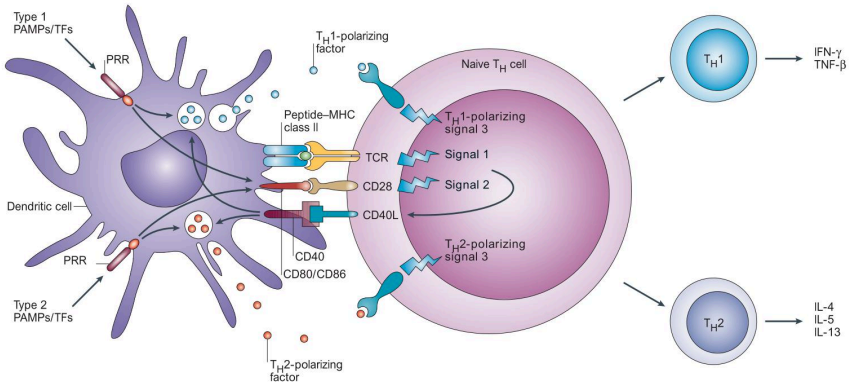

Figure 8 T cell stimulation and T helper cell polarization require three dendritic cell-derived signals.

Signal 1 is the antigen-specific signal that is mediated through TCR triggering by MHC class-II-associated peptides processed from pathogens after internalization. Signal 2 is the co-stimulatory signal, mainly mediated by triggering of CD28 by CD80 and CD86 that are expressed by mature DCs. Signal 3 is the polarizing signal that is mediated by various soluble or membrane-bound factors, such as interleukin-12 (IL-12) and CC-chemokine ligand 2 (CCL2), that promote the development of T_H1 or T_H2 cells, respectively. The nature of signal 3 depends on the activation of particular PRRs by PAMPs or tissue factors. Whereas, the profile of T-cell-polarizing factors is primed by recognition of PAMPs, optimal expression of this profile often requires feedback stimulation by CD40 ligand (CD40L) expressed by T cells after activation by signals 1 and 2. Adapted from Kapsenberg, 2003.

So far, different DC-derived molecules with T_H-cell polarization capacities have been fully described, the best characterized are those

inducing T_H1- and T_H1-cell subsets. The subset of T_H1-cell-polarizing factors include: interleukin-12 (IL-12), IL-23and IL-27 (Trinchieri, 2003), type 1 IFNs (Kadowaki et al., 2000) and cell-surface expressed intercellular adhesion molecule 1 (ICAM1) (Salomon & Bluestone, 1998). T_H1-cell-mediated responses are involved in the defence against viruses, intracellular bacteria and protozoa (Iwasaki & Medzhitov, 2015). Among T_H1-cell-polarizing factors there are: monocytic chemotactic protein 1 (MCP1, also known as CCL2) and OX40 ligand (OX40L) (Ohshima et al., 1998). T_H2-cell-mediated responses are required for helminths- and allergens-induced response (Iwasaki & Medzhitov, 2015). Finally, IL-10 and transforming-growth factor-β (TGF-β) were identified as regulatory T_{reg}-cell-polarizing factors (Groux et al., 1996; Zeller et al., 1999) (Fig.9)

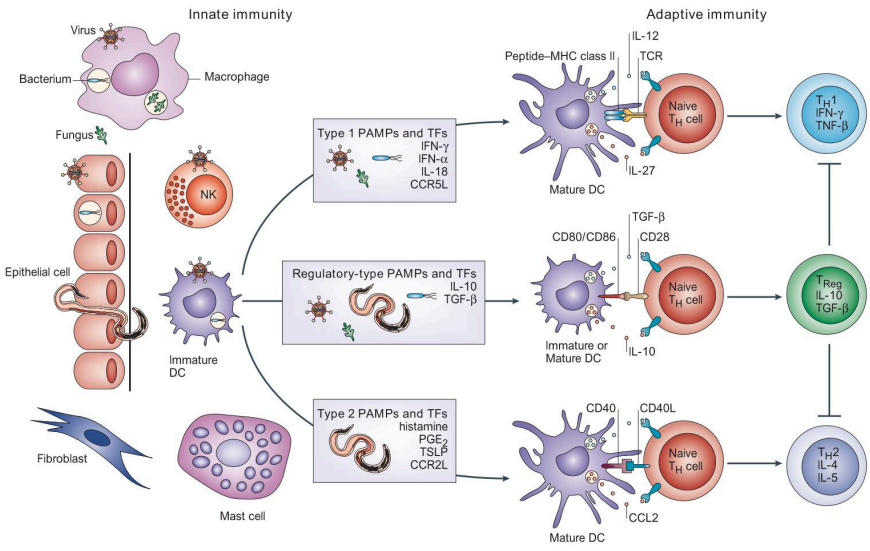

Figure 9 Dendritic cell-dependent T_H-cell polarization.
According to the type of microorganism they have recognized, mature DCs can polarize naïve T_H cell towards T_H1-, T_H2- and T_{reg}-cell.
Figure 9 continued on next page.

Figure 9 continued.
DC-polarizing tissue factors can be produced by various resident tissue cells and immune cells, including epithelial cells, natural killer (NK) cells, mast cells, macrophages, fibroblasts and many others. These cells will produce type 1, type 2 or regulatory tissue factors depending on their origin or the way they are activated. Adapted from: Kapsenberg, 2003.

1.3 Macrophages

Macrophages are myeloid cells whose distinctive feature is their enhanced ability to phagocytose. This hallmark is mirrored by their name that comes from the Greek *macros* - *phagein* meaning "big eaters", which was given by the father of cellular immunology Ilya Metchnikoff in 1883.

Macrophages are an essential component of the innate immune system and exerts multiple host-protective functions. They are also involved in several processes, such as tissue development, homeostasis and repair following damage. However, under certain circumstances, mainly represented by chronic insults, the intrinsic beneficial role of macrophages can be subverted, rendering these cells partner in crime of several pathological process, for instance chronic inflammatory diseases, cancer and neurodegeneration (Wynn et al., 2013).

Macrophages are present in all tissues and display a high degree of functional diversity. This heterogeneity, together with the distinct ontogeny that characterize different macrophage populations, makes their unified classification complicated.

An early concept about macrophage origin stated that the homeostasis of tissue-resident macrophages relies on the constant recruitment of blood monocytes, which derive from progenitors in the adult bone marrow (van Furth & Cohn, 1968). This dogma turned out to be only partially true. Although monocytes can, and actually do, differentiate into macrophages under certain circumstances and during inflammation, recent data have questioned the original dogma. In particular, it is currently known that monocytes do not contribute substantially to most tissue macrophage compartments in the steady state or during certain types of inflammation; moreover, tissue macrophages stem from embryonic precursors, either yolk-sac- or fetal liver-derived, that seed the tissues before birth; finally, tissue macrophages relies on their self-renewal capability (Ginhoux & Jung, 2014). Microglia, the brain macrophages, Kuppfer cells, specialized macrophage of the liver and lung alveolar macrophages are among the best examples of macrophages which are established in a completely bone marrow-independent fashion (Ginhoux & Guilliams, 2016).

The main expertise of macrophages is the efficient phagocytosis and neutralization of cellular debris and potential harmful agents, including pathogens. In order to properly exert this functions, tissue macrophages are endowed with a wide repertoire of receptors, such as TLRs and CLRs, which varies based on the adaptation to the hosting tissue (Akira et al., 2006).

1.3.1 Macrophages polarization

In tissues, macrophages exhibit a remarkable plasticity which means that they respond with great versatility and dynamicity to the environmental cues. Indeed, cytokines and microbial products profoundly influence the function of macrophages. It has been shown that macrophages can undergo "classical" (or M1) activation or "alternative" (or M2) activation, which mirror the T_H1-T_H2 polarization of T cells (Biswas & Mantovani, 2010; Mantovani et al., 2002). The molecular determinants of these two types of oriented activation of macrophages have been identified.

M1 phenotype is fostered by TLRs ligands, as LPS, and IFNγ, which can be released by Th1-polarized lymphocytes. M1 phenotype is characterized by production of pro-inflammatory cytokines, reactive oxygen and nitrogen species, promotion of Th1 responses and a strong microbicidal and tumoricidal activity. The goal of M1 macrophages is the elimination of the microorganism and the activation of the inflammatory process. Alternative activation, instead, is driven by IL-4 and IL-13 cytokines. M2 polarized macrophages are involved in parasitic containment and clearance, they have immunomodulatory functions, they induce tissue remodelling and dampen inflammation; on the other side, M2 macrophages can be associated with tumour progression (Mantovani et al., 2002).

More recently, it has been suggested that macrophage polarization is not represented by a merely dichotomy between M1 and M2 phenotype, rather by a spectrum of phenotypes spanning from M1 to M2. (Mantovani et al., 2004; Mosser & Edwards, 2008) (Fig.10).

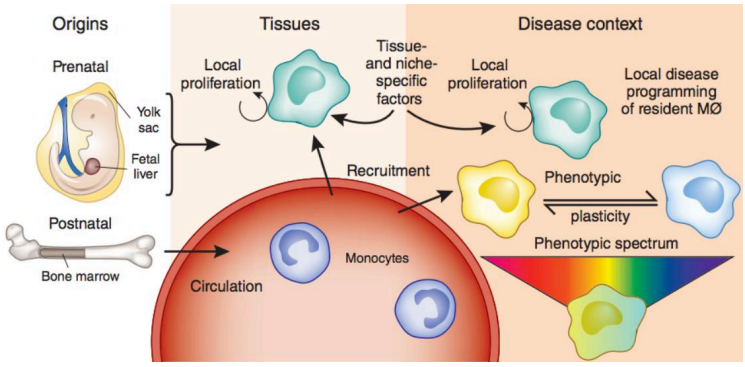

Figure 10 Macrophage origin and plasticity.
Macrophages may originate both at the prenatal stage from the yolk sac and fetal liver and during the postnatal stage from the bone marrow. In the tissues, macrophages may be either long-lived self-renewing cells or replenished from the blood monocyte pool. Tissue factors shape macrophage activation, resulting in a high degree of phenotypic plasticity. (MØ, macrophage). Adapted from: Liddiard & Taylor, 2015.

1.4 Sensing the lipopolysaccharide

The lipopolysaccharide (LPS), is the major component of the outer cell envelope of Gram-negative bacteria, such as Escherichia coli, Pseudomonas aeruguinosa and Salmonella. LPS is composed of a lipid portion (the lipid A), a core polysaccharide and an O-polysaccharide chain (the O antigen) that can have variable composition. The O-glycosylation status determines the colony morphology that could be either "smooth" or "rough". Microbial variants with long O-polysaccharide chains form smooth colonies, whereas those with short and truncated O-polysaccharide chains form rough colonies (Kelly et al., 1991).

The LPS is the most potent among the endotoxins, which are bacterial toxins released by damaged bacteria rather than actively secreted as exotoxins. LPS elicits potent immune responses and it is involved in the pathogenesis of sepsis, which is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection and can eventually culminate in septic shock (Raetz & Whitfield, 2002; Singer et al., 2016; van der Poll et al., 2017). The lipid A is mostly responsible for the endotoxic activity associated with the whole LPS (Schromm et al., 2000).

1.4.1 The LPS receptor complex

The road to the discovery of the LPS receptor begun in the early eighties and occupied the entire nineties; during these years, many scientists concentrated their research activity to this end (O'Neill et al., 2013). In 1990, it was revealed that the GPI-anchored protein CD14, works together with LPS-binding protein (LBP), a glycoprotein secreted in the serum, to trigger the LPS signalling and that CD14 dramatically augments the sensibility of cells to LPS (Wright et al., 1990).

It was just about eight years later that came out that TLR4 is the actual signalling receptor required for LPS-induced immune response (Poltorak et al., 1998). It was further demonstrated that TLR4 works together with MD2, a small glycoprotein lacking a transmembrane region whose LPS-binding capability is essential for the receptor-ligand interaction (Hoshino et al., 1999; Qureshi et al., 1999; Shimazu et al., 1999).

Currently, it is known that the LPS is sensed by a multi-receptor complex in which three proteins take part: TLR4, MD2 and CD14 (Miyake et al., 2000; Triantafilou & Triantafilou, 2002). In particular, the soluble protein LBP extracts monomers of LPS from aggregates or bacterial membranes and helps the LPS-recognition by CD14 (Schumann et al., 1990; Wright et al., 1990). In presence of LPS, CD14 and TLR4-MD2 are brought closer within lipid rafts. Afterwards, CD14 transfers LPS monomers to the TLR4-MD2 complex, thereby inducing TLR4 dimerization and signal transduction (Da Silva Correia et al., 2001).

1.4.1.1 TLR4 pathway

TLR4 has the unique property, among all the TLRs, to engage four adaptor molecules which are MyD88, TIRAP, TRAM and TRIF, thus it is the sole TLR able to activate both the TIRAP-MYD88-dependent pathway and the TRAM-TRIF-dependent pathway (Akira & Hoshino, 2003).

The TLR4-mediated MyD88-dependent signalling relies on the sorting adaptor TIRAP that, thanks to an N-terminal phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2)-binding domain is present in proximity to the membrane microdomains where TLR4 is located. TIRAP employs its TIR domain to recruit MyD88, by TIR-TIR interaction, favouring its sorting to these PtdIns(4,5)P_2-rich sites and the interaction between MyD88 and TLR4 (Horng et al., 2001; Kagan & Medzhitov, 2006). It is not excluded the possibility that

TIRAP and MyD88 are already complexed and close to the lipid rafts where the signalling takes place upon ligand-induced TLR4 movement to these regions. So, the MyD88-dependent signalling starts from the plasma membrane and leads to the first wave of NF-κB activation (Brubaker et al., 2015; Kawai et al., 2001) (Fig.11).

The TLR4 subcellular localization is critical for the initiation of the subsequent activation of the TRAM-TRIF-dependent pathway. TLR4 moves towards its second location of signalling which is represented by the endosomes. These cellular organelles are the starting site for two LPS-induced responses: a late wave of NF-κB activation and the production of type I IFNs (Yamamoto et al., 2003a; Yamamoto et al., 2003b; Kagan et al., 2008) (Fig.11).

It has been shown that CD14 orchestrates the relocation of the entire LPS-receptor complex from the plasma membrane to the endosome, thus directing the activation of the TRAM-TRIF pathway (Zanoni et al., 2011). In particular, CD14 acts by recruiting ITAM-containing transmembrane adaptors that activate the syk kinase which, in turn, triggers phospholipase C (PLC)γ activation that culminates in the endocytosis of the entire LPS-receptor complex.

It is noteworthy that while CD14 presence is not a *conditio sine qua non* for MyD88-dependent pathway while it is absolutely required for TRIF-mediated IFN expression, even at high LPS concentration, when CD14 is dispensable for LPS recognition by TLR4-MD2 complex (Jiang et al., 2005). The only case in which type I IFNs production can occur without CD14 is when LPS can directly reach the endosomes, skipping the TLR4 recognition as in the case of LPS-coated beads (Zanoni et al., 2011).

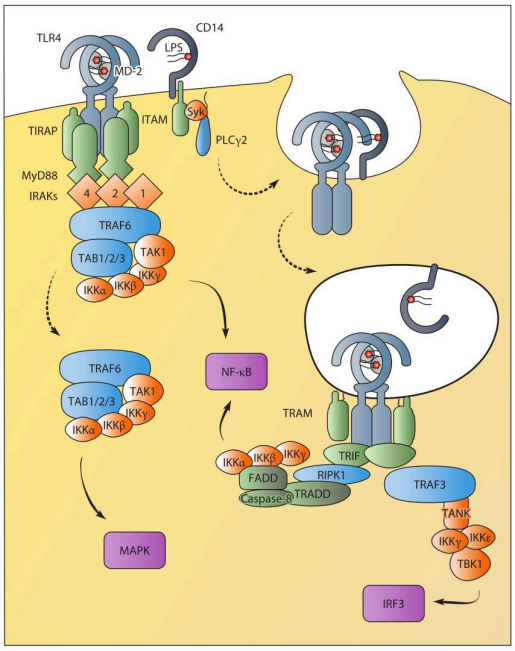

Figure 11 Toll-like receptor 4 signalling pathways.
TLR4 signals from both the plasma membrane and the endosomes. The signal from the plasma membrane requires TIRAP that allows the TIR-domain-dependent interaction between TLR4 and MyD88. The signal from the endosome requires CD14-dependent relocation of the entire receptor complex into this compartment. Adapted from Brubaker et al., 2015.

1.4.1.2 CD14

CD14 is a glycoprotein that could be present either as GPI-anchored receptor or as soluble mediator, once the phosphatidylinositol tail is cleaved (Bažil et al., 1986; Haziot et al., 1988; Simmons et al., 1989). CD14 is mainly expressed by myeloid linage cells, as monocytes, DCs, neutrophils, macrophages and microglia. Furthermore, CD14 can be detected even in non-immune cells, for instance fibroblasts, epithelial cells, adipocytes, pancreatic islet cells, smooth muscle and

spermatozoa (Jersmann, 2005). This wide expression spectrum suggests that CD14 has other functions beyond pathogen surveillance and that it could be implied in the regulation of other biological process, for instance there is evidence of its involvement in metabolism regulation (Fernández-Real et al., 2011).

CD14 resembles the horseshoe-structure typical of LRR-containing proteins. The ability to bind LPS resides in the large hydrophobic pocket placed at the N-terminal region of the protein, where a cluster of positively charged residues at the rim of the pocket allows the lipid A to settle in. The generous size of the pocket, the flexibility of its rim and the number of grooves available for ligand binding, confers promiscuity to CD14 that, actually, can bind different types of molecules orchestrating multiple TLR pathway (Kim et al., 2005). Indeed, CD14 acts as co-receptor for other TLRs besides TLR4.

The very first role described for CD14 was the enhancement of the sensitivity of innate immune cells to LPS by favouring its binding to TLR4-MD2 complex (Gioannini et al., 2004). This was corroborated by the demonstration that, in contrast to wild-type mice, CD14-deficient mice are resistant to endotoxin shock caused by LPS or Gram-negative bacterial exposure (Haziot et al., 1996) and that the absence of CD14 in macrophage causes an impaired response upon stimulation with low concentrations of LPS (Poltorak et al. 1998). On the other hand, CD14 is dispensable for the response to high concentration of LPS (Zanoni et al., 2011). Likely, at high LPS concentration, the TLR-MD2 complex has an autonomous recognition capability or takes advantage of another LPS binding protein (Poltorak et al., 2000; Triantafilou & Triantafilou, 2002).

Besides the concentration of LPS, the chemical properties of the O-antigen determine the requirement of CD14 for the initiation of the signalling. In particular, the O-glycosylated smooth (S)-form LPS induces a CD14-dependent TNFα production, whereas the immune response to rough (R)-form LPS, which lacks a typical O-antigen, do not need CD14 for the activation of the MyD88-dependent pathway (Gangloff et al., 1999; Jiang et al., 2005). This means that the chemotype discrimination relies on TLR4-MD2 rather than CD14. While the smooth LPS activity is more CD14-dependent compared to rough LPS concerning the activation of the MyD88-dependent pathway, independently of the LPS chemotype and concentration, CD14 is always needed for the activation of the TRIF-dependent pathway, which takes place after the CD14-mediated LPS-receptor complex relocation to the endosomes (Zanoni et al., 2011; Zanoni, Bodio, et al., 2012).

CD14 assumes a prominent role in innate immunity. In addition to directing the TRIF-dependent pathway, it has been shown that CD14 has a TL4-independent signalling transduction capability in myeloid cells, specifically in DCs (Zanoni et al., 2009).

1.4.2 CD14-NFAT pathway

Besides being required for complete response to LPS, CD14, independently of TLR4 engagement, has been shown to initiate defined LPS-induced responses in DCs, which involve the activity of the transcription factor NFAT (Zanoni et al., 2009).

In DCs, the stimulation of several PRRs, including TLR4, induces the activation of signalling pathways that converge on distinct transcription factors resulting in the production of a number of cytokines. One such cytokines is interleukin 2 (IL-2), whose production confers DCs crucial functions in the immune response regulation. Indeed, DC-derived IL-2 is required for complete NK cells activation (Granucci et al. 2004; Zanoni and Granucci 2013), T cell priming (Granucci et al. 2001; Wuest et al. 2011) and T_{reg} homeostasis (Kulhankova et al. 2012; Sgouroudis et al. 2011; Zanoni and Granucci 2011).

In T cells, the regulation of *Il-2* expression relies on the transcription factor NFAT (Crabtree & Olson, 2002). Similarly, Granucci and co-workers revealed that LPS stimulation of DCs activates this transcription factor that it is responsible for IL-2 production by DCs (Zanoni et al., 2009). It turned out that, following LPS treatment, a rapid and transient increase in the intracellular Ca^{2+} concentration occurs and allows the Ca^{2+}/calmodulin-dependent activation of the phosphatase calcineurin, which, in turn, fosters NFAT nuclear translocation. In the nucleus, NFAT cooperates with transcriptional partners for gene expression regulation. It was demonstrated that this process does not depend neither on TLR4 nor on molecular adaptors as MyD88 and TRIF, excluding the involvement of any other TLRs. On the contrary, the activation of NFAT in DCs counts completely on CD14, since its absence abolishes the LPS-induced increase in the intracellular Ca^{2+} concentration and consistently, NFAT-dependent IL-2 production (Zanoni et al., 2009).

The discovery of this signalling ability of CD14 raises the question of how a protein which lacks an intracellular domain, is able to transduce the signal. In the context of NFAT activation it is described that CD14 signalling capability relies on the GPI-anchor, since soluble CD14 is not functional for LPS-induced NFAT activation in DCs. Moreover, the localization of CD14 in the lipid rafts is fundamental for the signalling to happen, since the disruption of these well-organized portions of membrane abrogates LPS-induced calcium response and therefore NFAT activation. Although the involvement of another protein could not be formally excluded, it is likely that the LPS-stimulated clustering of CD14 in lipid rafts provides CD14 the possibility to recruit and activate src family kinases (SFKs), which are known to be lipid raft-associated signalling proteins (Shenoy-Scaria et al. 1994; Pike 2003). This model has already been proposed for another GPI-anchored protein as CD59 (Suzuki et al., 2007a; Suzuki et al., 2007b).

The activation of NFAT following LPS exposure plays a crucial role in the regulation of the DC life cycle (Zanoni et al., 2009). Once activated and terminally differentiated DCs undergo apoptotic death. This process is physiologically relevant to limit DCs effector functions such as antigen presentation to T cells and, therefore, prevent an excessive immune response. Indeed, a deregulation in DCs apoptosis may cause DCs accumulation and autoimmunity (Matsue & Takashima, 1999).

Granucci and co-workers showed that, following LPS exposure, CD14-dependent NFAT activation, namely NFATc2 and NFATc3, controls DCs apoptosis by transcriptional regulation of specific genes

involved in apoptotic processes. A proapoptotic role for NFATc2/c3 in T and B cells had already been reported (Serfling et al., 2006). In particular, it has been suggested *Nur77* as regulator of NFAT-mediated DCs programmed cell death. Nur77 is an orphan nuclear receptor that comprises an N-terminal activation factor (AF)-1 domain, a DNA-binding domain containing two zinc fingers and a C-terminal ligand-binding domain. Several data describe the proapoptotic role of Nur77 (Calnan et al., 1995; Weih et al., 1996), even if there is also evidence of a redundant role of this protein with other proapoptotic factors (Lee et al., 1995). The precise mechanisms underlying Nur77-induced cell death has not been completely elucidated yet.

The CD14-NFAT pathway has been shown to be active in DCs but not in macrophages. LPS-treated macrophages do not show a Ca^{2+} response, neither NFAT activation and do not go toward apoptosis (Zanoni et al., 2009). So far, it is not known which is the molecular mechanism that prevents the NFAT pathway activation in macrophages in response to LPS. Anyway, the different response to LPS of macrophages in contrast to DCs has a biological significance, indeed while the rapid DCs death is needed to damp down the immune response, macrophages survival is essential for the resolution of inflammation and the initiation of tissue repair processes, which are promoted by macrophage-derived anti-inflammatory mediators, such as TGF-β (Granucci and Zanoni 2009; Medzhitov 2008).

Last but not least, in DCs, the CD14-NFAT pathway participates in the regulation of prostaglandin (PG)E_2 (PGE_2) production (Zanoni, et al., 2012). PGs belong to the eicosanoids that are synthesized from C_{20} polyunsaturated fatty acids, such as arachidonic acid, which are

commonly incorporated within membrane phospholipids (Smith, 1989). Eicosanoids are lipid mediators of intercellular communication that act mainly through autocrine and paracrine mechanism (Paoletti et al., 2004). Because of their chemical instability, eicosanoids are synthesized, either constitutively or in response to stimuli and then released rather than stored inside the cells (Smith, 1989).

The biosynthesis of PGE_2 is a multistep process involving three enzymes, the cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenases (COX) and prostaglandin E synthases (PGES). There are three COX isoforms: COX-1, COX-2 and COX-3. COX-1 is constitutively expressed and is responsible for basal and stimuli-induced PGs synthesis. COX-2 is an inducible enzyme, primarily involved in inflammatory-induced PGs synthesis. COX-3 is a splice variant of COX-1, mainly expressed in brain and heart. Three isoforms of PGES exist as well: the microsomal PGES-1 (mPGES-1), mPGES2 and cytosolic PGES (cPGES). mPGES2 and cPGES are constitutively expressed and work together with COX-1 to maintain PGE_2 basal levels, whereas mPGES-1 is inducible, mainly by inflammatory stimuli, and is preferentially coupled with COX-2 (Murakami et al. 2000; Park and Pillinger et al. 2006). Upon inflammatory stimuli, a calcium influx leads to $cPLA_2$ translocation to the plasma membrane where it hydrolyses phospholipids to form arachidonic acid. Inflammatory stimuli also induce the expression of COX-2 and mPGES-1, COX-2 converts the arachidonic acid first into PGG_2 and in turn into PGH_2. PGH_2 is a highly instable intermediate and is rapidly converted into PGE_2 by mPGES-1 (Park and Pillinger et al. 2006) (Fig.12).

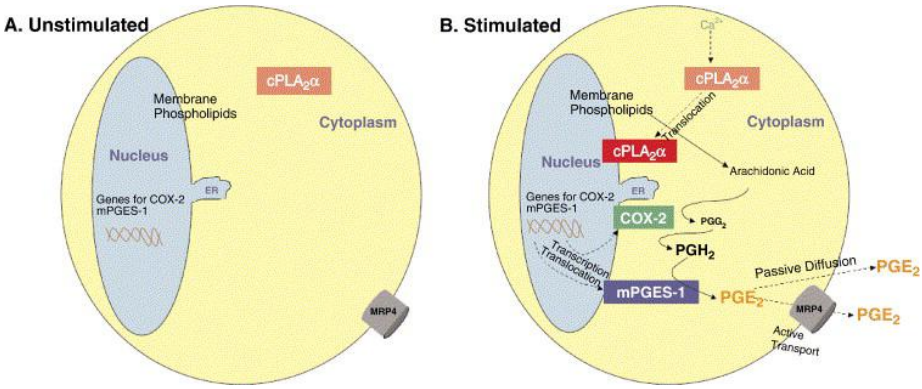

Figure 12 Biosynthesis of PGE_2 by cPLA2, COX-2, and mPGES-1.
(A) Unstimulated cell. $cPLA_2$ is constitutively present in the cytoplasm. In unstimulated cells, COX-2 and mPGES-1 are not expressed. (B) Stimulated cell. Inflammatory stimulation results in Ca^{2+} influx that leads to the translocation of $cPLA_2\alpha$ from the cytosol to the nuclear membrane where it enzymatically hydrolyses membrane phospholipids to release arachidonic acid. Inflammatory stimuli also induce the transcription and protein expression of both COX-2 and mPGES-1. COX-2 transforms arachidonic acid to PGG_2 which is subsequently converted to PGH_2. mPGES-1 may then act on PGH_2 to generate PGE2. PGE_2 may exit the cell by simple diffusion, or by active transport via the MRP4 transporter. Adapted from: Park et al., 2006.

Granucci and co-workers demonstrated that, in LPS-stimulated DCs, the CD14-NFAT pathway regulates the expression of *Ptges1*, which codes mPGES-1, a key enzyme in PGE_2 biosynthesis. Besides, the authors demonstrated that *in vivo* PGE_2 local production mediates skin edema formation and controls free antigen delivery to the draining lymph nodes (Zanoni et al., 2012).

Four different G-protein-coupled receptors have been identified, E prostanoid (EP) receptors 1 (EP1), EP2, EP3 and EP4 (Coleman et al., 1994; Narumiya et al, 1999), whose diverse signal transduction mechanisms are responsible for the wide spectrum of biological effects

of PGE2, spanning from homeostatic (Smith, 1989) to both inflammatory (Davies et al., 1984) and anti-inflammatory effects (Takayama et al., 2002). During the inflammatory response, PGE_2, mainly through EP2, promotes vasodilation and augmented microvascular permeability (Funk, 2001), which allow recruitment of leukocytes from the blood stream to the site of infection or tissue injury, thereby leading to swelling and edema formation. Moreover, PGE_2 is responsible for hyperalgesia and fever generation. Via EP1 receptors, PGE_2 decreases pain threshold by sensitizing peripheral terminals of nociceptors (Ferreira, 1972; Willis & Cornelsen, 1973) and also central nervous system nociceptors (Ferreira 1978). PGE_2-mediated fever generation is due to the activation of a group of EP3-endowed neurons that resides in the preoptic area of the hypothalamus, which is implicated in thermoregulation (Engblom et al., 2003; Lazarus et al., 2007). So far, COX inhibitors, aimed at lowering PGE_2 levels, have been widely used as anti-inflammatory drugs, anyway the high degree of adverse effects of COX-targeting drugs underlined the need for the identification of more specific drugs, possibly targeting PGES.

1.5 The Nuclear Factor of Activated T cells (NFAT) family

Nuclear factor of activated T cells (NFAT) was originally identified in 1988 in nuclear extracts of activated T lymphocytes as an inducible DNA-binding protein regulating *Il-2* promoter (Shaw et al., 1988). Great interest for NFAT came from the evidence that the immunosuppressive activity of cyclosporine A (CsA) and FK506 (also known as tacrolimus), two drugs widely used in transplant medicine, is due to their ability to block the NFAT pathway by acting on a common target, namely calcineurin, which is a crucial regulator of NFAT activation (Ho et al., 1996). The NFAT signalling is a vertebrate-specific pathway. The researches of the last 30 years have unveiled several roles of this pathway. Besides regulating T cell activation and differentiation (Hogan et al., 2003; Macian, 2005) NFAT takes on regulatory role in the development, differentiation and functions of both immune and non-immune cells (Heit et al., 2006; Horsley et al., 2008; Negishi-Koga & Takayanagi, 2009; Zanoni et al., 2009). Moreover, it has been pointed out that deregulation of NFAT signalling are associated with cancer development and progression (Robbs et al. 2008), coherently with its role in controlling cell growth, survival, migration and angiogenesis. Therefore NFAT has become a potential target for cancer therapy (Mancini & Toker, 2009).

The NFAT family comprises five proteins that are evolutionary related to the REL–NF-κB family of transcription factors (Chytil & Verdine, 1996; Graef et el., 2001) that are ubiquitously expressed in mammalian cells and tissues (Mancini & Toker, 2009). Within the

NFAT family it is possible to distinguish four members, collectively indicated as NFATc, whose common feature is their regulation by calcium signalling: NFATc1 (also known as NFAT2 and NFATc), NFATc2 (also known as NFAT1 and NFATp), NFATc3 (also known as NFAT4 andNFATx) and NFATc4 (also known as NFAT3) (Hogan et al., 2003). Finally, NFAT5, which was identified as the tonicity-responsive enhancer binding protein (TonEBP, also known as OREBP), is the most evolutionary ancient, being present in non-vertebrate as *Drosophila* (Graef et al., 2001), this member is insensitive to calcium regulation and it has been shown to be activated in response to osmotic stress (Lopez-Rodriguez et al., 1999; Miyakawa et al., 1999a).

1.5.1 NFAT structure

The general structure of the NFAT proteins is characterized by three regions: the amino (N)-terminal domain that consists in the regulatory domain called NFAT homology region (NHR), a highly conserved DNA binding domain that is the Rel-homology region (RHR), finally, the carboxyl (C)-terminal domain (Macian, 2005) (Fig.13). With the exception of NFAT5, each protein has at least two splice variants that differ in the N- and C-terminal (Imamura et al. 1998; Park et al. 1996; Chuvpilo et al. 1999).

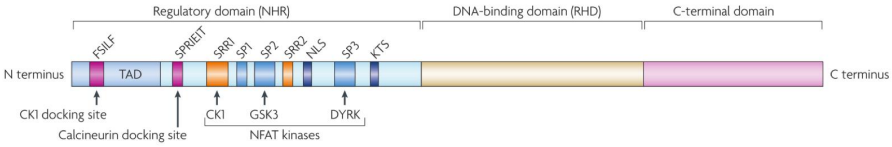

Figure 13 General structure of NFAT transcription factors.
NFAT proteins consist of a N-terminal regulatory domain (also known as an NFAT homology region (NHR)), a DNA-binding domain (also known as a REL-homology domain (RHD)) and a carboxy-terminal domain. The regulatory domain contains an N-terminal transactivation domain (TAD), as well as a docking site for casein kinase 1 (CK1), termed FSILF, and for calcineurin, termed SPRIEIT. It also includes multiple serine-rich motifs (SRR1, SP1, SP2, SRR2, SP3 and KTS) and a nuclear localization sequence (NLS). Adapted from: Müller & Rao, 2010.

Among the NFATc proteins, the RHR and the NHR domains are highly conserved, whereas NFAT5 retains only the RHR domain homology. The elevated level of similarity among the NFAT protein is likely responsible for their functional redundancy. The RHR domain is the DNA binding domain, here are located the sites for the interaction with the DNA binding domain of transcriptional partners of NFAT, such as activator protein 1 (AP1, composed of Fos-Jun complexes) (Chen et al., 1998; Jain et al., 1992), GATA4 and forkhead box P3 (FOXP3) (Crabtree & Schreiber, 2009; Wu et al., 2006). Indeed, NFAT proteins cooperate with several transcription factors by binding the DNA as homo- or hetero-dimers (Fig.13).

The NHR domain is moderately conserved among NFAT proteins. It contains a potent transactivation domain (TAD) that binds promoter elements for the transcriptional regulation. In the NHR domain also reside: the docking site for the main regulator of NFAT activation, the Ca^{2+}-calmodulin–dependent phosphatase calcineurin

and the nuclear localization signal (NLS). Moreover, the NHR contains many serine residues organized in defined motifs: serine-rich region motifs (SRR1-SRR2) and SRXX (where X stands for any amino acid) repeat motifs (SP1-SP2-SP3) (Fig.13). They have a fundamental regulatory role, since their phosphorylation modulates nuclear and cytoplasmic shuttling of NFAT, influencing its transcriptional activity (Mancini & Toker, 2009).

1.5.2 NFAT activation and regulation

The characteristics of NFATc activation by Ca^{2+} signalling have been described since time. The rise in intracellular Ca^{2+} concentration represents the triggering event and it is initiated following cell-surface receptors engagement coupled to "store-operated" calcium entry via PLCγ (Hogan et al., 2003), which is typical of antigen receptors that are expressed by T and B cells and Fc receptors. PLCγ activation leads to the formation of inositol (1,4,5)-trisphosphate ($Ins(1,4,5)P_3$) from membrane $PtdIns(4,5)P_2$. $Ins(1,4,5)P_3$ binds and opens its receptors on the ER thus inducing the release of Ca^{2+}, which triggers a process known as store-operated Ca^{2+}entry (SOCE). Within the ER, Ca^{2+} concentration is sensed by protein activation of stromal interaction molecule 1 (STIM1), a single-pass transmembrane protein mainly located at the ER, which is endowed with low affinity EF hand domains for Ca^{2+} binding. The drop in the Ca^{2+} concentration induces a conformational change in STIM1 that multimerizes and translocate to the plasma membrane (Lewis, 2007). Here STIM1 contacts and

activates ORAI1 that is the pore forming unit of the Ca^{2+}release-activated channels (CRACs) placed at the plasma membrane (Prakriya et al., 2009). The opening of ORA1 leads to a massive entrance of Ca^{2+} from the extracellular space, Ca^{2+} binds to calmodulin that, in turn, activates calcineurin, which is the crucial event for NFAT activation (Fig.14). In the basal state, NFAT is present in the cytoplasm and its NLS is masked by phosphate residues. The activation of calcineurin causes the dephosphorylation of SP motifs thereby the exposure of the NLS and the consequent nuclear import of NFAT (Feske et al., 2003; Hogan et al., 2003).

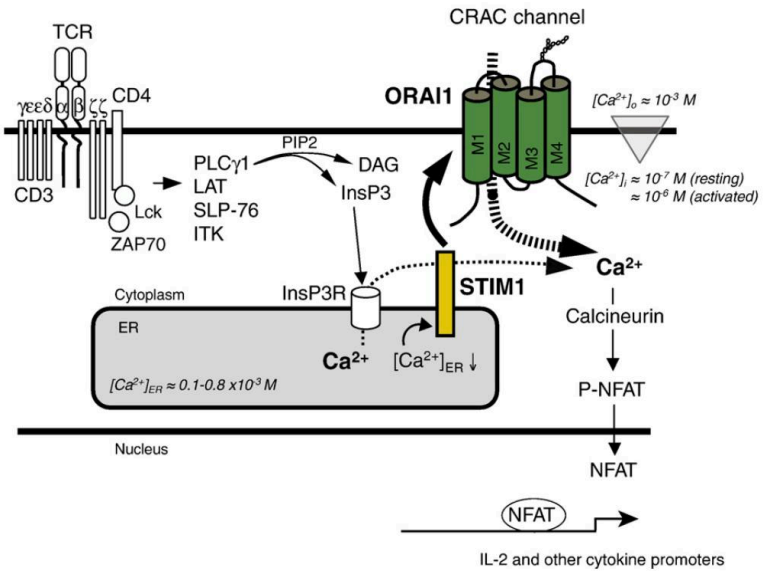

Figure 14 NFAT activation in T cells is regulated by store-operated Ca^{2+} entry.

T-cell receptor (TCR) engagement results in the activation of tyrosine kinases Lck and ZAP-70, assembly of the adaptor protein complex containing SLP76 and LAT and activation of PLCγ1. The latter hydrolyses PtdIns(4,5)P_2 (PIP_2 in the figure) to Ins(1,4,5)P_3 ($InsP_3$ in the figure) and DAG. Ins(1,4,5)P_3 binds to and opens Ins(1,4,5)P_3 receptors (InsP3Rs) in the ER, resulting in the release of Ca^{2+} from ER stores, reduction of Ca^{2+} concentration in the ER and transient increase in intracellular Ca^{2+} concentration.

Figure 14 continued on next page.

Figure 14 continued.
The decrease in Ca^{2+} concentration in the ER is sensed by STIM1 resulting in binding of STIM1 to ORAI1 and opening of the CRAC channel. Ca^{2+} influx results in increased intracellular Ca2+ concentration from ~100 nM to ~1 µM. Sustained elevation of intracellular Ca2+ concentration is required for activation of the phosphatase calcineurin, nuclear translocation of the transcription factor NFAT and cytokine gene expression. Adapted from: Feske et al., 2010.

In order to effectively remove the phosphate, calcineurin needs to dock on its binding site on NFAT, which is placed at the NHR domain at the consensus sequence PXIXIT (where X stands for any amino acid) (Aramburu et al., 1998; Garcia-Cozar et al., 1998). This motif is conserved among the NFAT family members and represents the main binding site for calcineurin on NFAT. It has also been shown that calcineurin may follows NFAT in the nucleus where it keeps its dephosphorylated state (Zhu & McKeon, 1999).

Beyond the regulation by Ca^{2+}-calmodulin binding, calcineurin activity is controlled by some endogenous inhibitor, including calcineurin-binding protein1 (CABIN1, also known as CAIN) (Sun et al., 1998), the A-kinase anchor protein AKAP79 (also known as AKAP5) (Coghlan et al., 1995; Kashishian et al., 1998) and members of the Down's syndrome critical region (DSCR)/modulatory calcineurin-interacting protein (MCIP) family of calcineurin inhibitors, which are known as calcipressins (Rothermel et al., 2000).

The permanence of NFAT in the nucleus is regulated by the activity of several kinases that have been shown to phosphorylate the serine-rich motifs in the RHR domain. Based on their mechanism of action the kinases involved in the control of NFAT activation can be divided into export and maintenance kinases, the first acting by

promoting NFAT nuclear export, the latter acting by holding its cytoplasmic localization. Each kinase shows preferential site of action and also specificity for NFAT members, moreover they often act sequentially in a way that the phosphorylation of a specific motif by the first kinase, favours the catalytic activity of the following one. The group of export kinases includes: glycogen synthase kinase 3 (GSK3) (Beals et al., 1997) and dual-specificity tyrosine-phosphorylation regulated kinase 1 (DYRK1); DYRK2, instead, is a maintenance kinase (Arron et al., 2006; Gwack et al., 2006), while casein kinase 1 (CK1) acts as both export and maintenance kinase (Okamura et al., 2004). Even if they have not been identified yet, more kinases are likely involved in the control of NFAT subcellular localization and therefore activity (Mancini & Toker, 2009).

Exclusively for NFATc1 it has been described a mechanism of autoregulation at the transcriptional level (Zhou et al., 2002). This model is isoform specific, being active only for the shortest isoform of NFATc1 that is NFATc1A. NFATc1B and NFATc1C are constitutively expressed in T cells, NFATc1A,on the other hand, is preferentially expressed in effector T cells and it is transcriptionally controlled by an NFAT-dependent promoter coupled to a more proximal polyadenylation site (Chuvpilo et al., 2002). This regulatory strategy results in the accumulation of the short NFATc1A isoform during lineage commitment, which explains why deletion of NFATc1 is generally more deleterious to development than deletion of other NFAT family members (Müller & Rao, 2010).

Besides phosphorylation, post-translational modifications such as sumoylation and ubiquitination, have also been shown to participate

in the regulation of NFAT subcellular localization (Nayak et al., 2009; Yoeli-Lerner et al., 2009). Furthermore, other mechanisms have been recently identified, including protein-based and RNA-based regulation. The cytoplasmic scaffold proteins HOMER2 and HOMER3 were reported to compete with calcineurin for NFAT binding and thus prevent NFAT dephosphorylation and activation (Huang et al., 2008). A non-coding repressor of NFAT (NRON) was identified as a specific inhibitor of NFAT nuclear trafficking (Willingham et al., 2005). Caspase 3 has been reported to regulate the expression levels of NFAT1 in non-apoptotic effector T cells (Wu et al., 2006).

In innate immune cells, NFAT has been shown to be activated by three signal transduction pathways downstream PRRs: the pathway initiated by Dectin-1 (also known as CLEC-7A) (Goodridge et al., 2007), downstream TLR9 in response to *Aspergillus fumigatus* (Herbst et al. 2015) and the signalling pathway initiated by CD14 (Zanoni et al., 2009).

The β-glucan receptor Dectin-1 belongs to the family of CLRs. The intracellular immunoreceptor tyrosine-based activation motif (ITAM)-like motif of Dectin-1 plays an essential role in the recognition of zymosan and pathogenic fungi as *Candida albicans* and *Aspergillus fumigatus* by macrophages and dendritic cells. ITAM-like motif of Dectin-1 differs from conventional ITAM which includes two YxxL (where x stands for any amino acid) sequences. On the contrary, ITAM-like motif has only one YxxL sequence, the other tyrosine motif retains an additional amino acid (YxxxL) and is non-functional. For this characteristic, the Dectin-1 ITAM has been named hemITAM (Slack et al., 2007). Following ligand binding, the ITAM-like motif undergoes

phosphorylation by SFK (Underhill et al., 2005) that allows the recruitment of syk tyrosine kinase (Rogers et al., 2005) which in turn activate PLCγ2. Consequent hydrolization of PtdIns(4,5)P_2 generates Ins(1,4,5)P_3 and DAG. Ins(1,4,5)P_3 regulates the release of Ca^{2+} from intracellular calcium store which induces calcineurin activation and therefore NFAT dephosphorylation and nuclear import (Tassi et al., 2009) (Fig.15).

As discussed in 1.4.2, after LPS stimulation of DCs, CD14 initiates a signalling pathway that culminates on NFAT activation. Granucci and co-workers demonstrated that upon LPS stimulation, CD14, at the level of lipid rafts, induces src family kinase (SFK), possibly Lyn, and then PLCγ2 activation. PLCγ2, in turn, hydrolyses its substrate PtdIns(4,5)P_2 at the plasma membrane, generating diacylglycerol (DAG) and Ins(1,4,5)P_3. This event leads to the increase in the intracellular calcium concentration culminating in calcineurin-dependent NFAT dephosphorylation and nuclear translocation (Zanoni et al., 2009). So far, the molecular mechanisms that link the activation of PLCγ2 to the augment in the intracellular calcium concentration are still undefined (Fig.15).

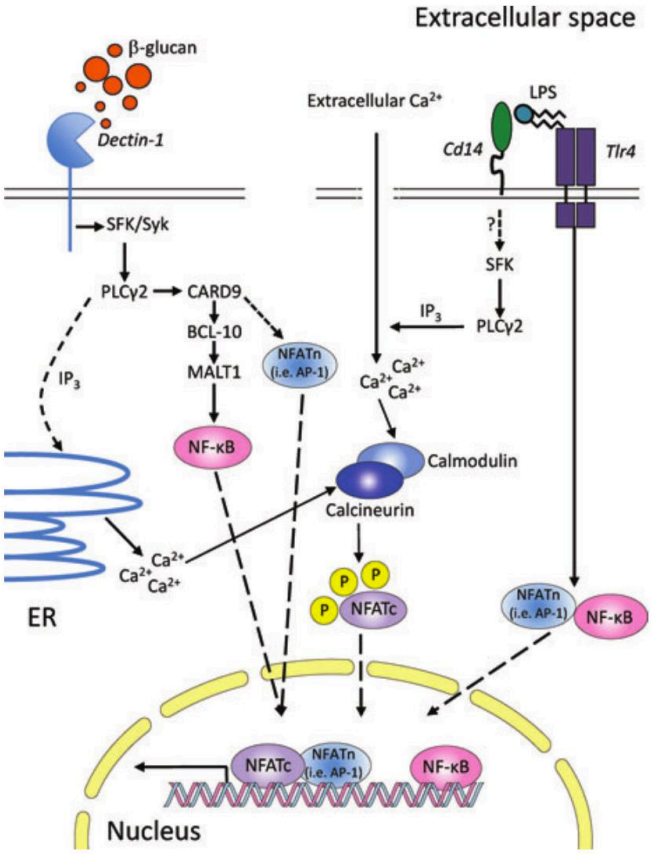

Figure 15 Mechanisms of NFAT activation in innate myeloid cells.

Dectin-1 stimulation with β-glucan leads to syk-dependent PLCγ2 activation, thus Ins(1,4,5)P_3 and DAG production. Ins(1,4,5)P_3 is then thought to induce Ca^{2+} release from the ER. The increased intracellular Ca^{2+} concentration allows calcineurin activation, therefore dephosphorylation and nuclear translocation of NFAT. Adapted from: Zanoni & Granucci, 2012.

1.6 Regulation of the intracellular calcium concentration

Ca^{2+} is a divalent cation acting as second messenger within the cell. Upon variation of environmental stimuli, a transient increase of intracellular Ca^{2+} concentration can occur, which activates different signalling pathways that, in turn, regulate many cellular functions. Indeed, Ca^{2+} is extremely important for a huge number of processes spanning from gene transcription, cell proliferation, motility to learning, fertilization and development. Nevertheless, Ca^{2+} overload is highly toxic and can lead to cell death (Berridge et al., 2000). It is therefore conceivable that the cell invests a considerable energy to control its intracellular concentration (Clapham, 2007).

Under resting conditions, the intracellular Ca^{2+} concentration is kept near 100 nanomolar despite the much higher extracellular concentration, which is about 1-2 millimolar. This great difference is achieved by the cells through three mechanisms: chelation, compartmentalization and extrusion (Fig.16). Each cell has a set of buffering proteins, pumps and exchangers with different properties to direct these processes. The cytoplasm is at the crossroad of these dynamics. At any moment, the level of intracellular calcium is the result of the balance between stimuli-induced reactions, which introduce Ca^{2+} into the cytoplasm and opposite reactions, aimed at lowering intracellular calcium concentration (Berridge et al., 2000).

The Ca^{2+} that enters the cytoplasm is rarely free, part of it binds to effector proteins, such as calmodulin, troponin C and synaptotagmin while most of it ends up with binding to buffering proteins that load

Ca^{2+} in order to limit its spatial and temporal activity, for instance parvalbumin, calbindin D-28 and calreticulin. Pumps and exchangers are also involved in the regulation of intracellular Ca^{2+} homeostasis. In particular: the Ca^{2+}-ATPase (PMCA) and the Na^{+}/Ca^{2+} exchanger (NCX) extrudes Ca^{2+} via plasma membrane; while SERCA and the mitochondrial uniporter, uptake Ca^{2+} within the ER and the mitochondria, respectively. (Berridge et al., 2003) (Fig.16).

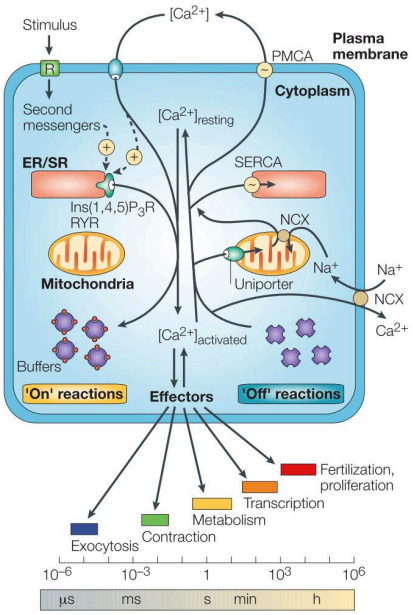

Figure 16 Ca^{2+} signalling dynamic and homeostasis.
During "on" reactions, stimuli induce both the entry of external Ca^{2+} and the release of Ca^{2+} from intracellular stores, which is mediated by a lipid-derived second messenger. Most of this Ca^{2+} is soon bound by buffering proteins or, a lower amount, by effector proteins. During the "off" reactions, Ca^{2+} leaves the effectors and buffers and is removed from the cytoplasm by pumps and exchangers. Ca^{2+} signalling directs many different functions that are precisely tuned in space and time. Adapted from: Berridge et al., 2003.

1.6.1 Calcium signalling downstream PLC activation

Ca^{2+} enters the cell through many different ways. In excitable cells, such as neurons, the opening of voltage-operated Ca^{2+} channels (VOCCs) is one of the principal mechanism of Ca^{2+} entry, which leads to very rapid increase in intracellular Ca^{2+} concentration that is crucial for a number of processes, as synaptic communication.

In non-excitable cells, Ca^{2+} entry from the extracellular space is not that frequent. There are at last three mechanisms involved in Ca^{2+} entry in these cells: the SOCE mechanism, the second-messenger-operated Ca^{2+} entry (SMOCE) mechanism and the receptor-operated Ca^{2+} entry (ROCE) mechanism (Clementi & Meldolesi, 1996). The SOCE mechanism is the most common in non-excitable cells and it requires the activity of the lipid metabolizing enzyme, phospholipase C (Patterson et al., 2002).

Phospholipases constitute a family of membrane-associated hydrolases that catalyse the hydrolysis of phospholipids. They are classified based on the position they hydrolyse on the phospholipid backbone. Four classed have been described: PLA, further divided into PLA1 and PLA2, PLB, PLC, PLD (Dennis, 2015). PLC, in particular, is able to cleave phospholipids just before the phosphate group. There are several isoforms of PLC, each activated by different mechanisms. PLCβ is activated by G-protein-coupled receptors (GPCRs), PLCγ by tyrosine-kinase-coupled receptors, PLCδ is calcium-dependent and PLCε is activated by Ras (Berridge et al., 2003). PLCζ it was shown to be the PLC involved in oocyte fertilization (Saunders et al., 2002).

The importance of PLC in Ca^{2+} signalling was suggested about thirty years ago (Michell, 1975; Michell et al., 1977). Later on, it was demonstrated that the molecular basis of this function in Ca^{2+} signalling mainly relies on the formation of the soluble messenger $Ins(1,4,5)P_3$, which derive from PLC-dependent $PtdIns(4,5)P_2$ degradation (Berridge & Irvine, 1984; Streb et al., 1983). $Ins(1,4,5)P_3$ is a soluble messenger and it acts through its receptors, named $InsP_3Rs$, mostly located at the ER, which is the most important intracellular Ca^{2+} store. In the ER, Ca^{2+} concentration is estimated to be 1,1-1 millimolar, which is only a bit lower the extracellular concentration (Bygrave & Benedetti, 1996). The opening of $InsP_3R$ channels allows the leak of calcium from the ER into the cytoplasm. Therefore, $Ins(1,4,5)P_3$ represents a crucial second messenger for the generation of a rapid intracellular calcium increase. Moreover, Ca^{2+}depletion in the ER rapidly induces the opening of CRACs at the plasma membrane triggering the SOCE mechanism (Putney, 2005). Notably, CRACs opening is activated by a decline in ER Ca^{2+} concentration but not by a rise in intracellular Ca^{2+} concentration (Clapham, 2007).

1.6.2 The inositol phosphates metabolism

The metabolism of inositol phosphates is a complex process (Fig.17). As for calcium, $Ins(1,4,5)P_3$ presence within the cell is finely tuned, being $Ins(1,4,5)P_3$ a second messenger as well. Therefore, following the cleavage of $PtdIns(4,5)P_2$ into DAG and $Ins(1,4,5)P_3$, the latter rapidly undergoes different metabolic processes that, in turn,

generate a number of inositol phosphates, some of them also implicated in intracellular signalling. Different kinases and phosphatases are enrolled in these enzymatic reactions, their names usually reflects the position of the inositol ring where they act (Pattni & Banting, 2004).

Ins(1,4,5)P_3 can go through two distinct destinies, the first one leading to inositol (1,4)-bisphosphate (Ins(1,4)P_2), the second one to inositol (1,3,4,5)-tetrakisphosphate (Ins(1,3,4,5)P_4). Ins(1,4)P_2 is formed following the removal of the 5-phosphate from the inositol ring by inositol polyphosphate 5-phosphatases (5-phosphatases). Ins(1,4)P_2 is inactive and is subsequently dephosphorylated to form free inositol, which is then recycled to generate phosphatidylinositol that is then delivered to the plasma membrane (Michell, 1997). On the other hand, Ins(1,3,4,5)P4, is generated after the phosphorylation of the inositol ring by the enzyme Ins(1,4,5)P_3 3-kinase (IP_3-3K) that catalyses ATP-dependent phosphorylation of the hydroxyl on the 3-carbon of inositol (Irvine et al., 1986). Ins(1,3,4,5)P_4 is rapidly metabolised to Ins(1,3,4)P_3, by some of the same inositol polyphosphate 5-phosphatases that dephosphorylate Ins(1,4,5)P_3. The majority of Ins(1,3,4)P_3 is further dephosphorylated to finally give inositol, however Ins(1,3,4)P_3 can be eventually phosphorylated by a IP_3-6K to form Ins(1,3,4,6)P_4 (Shears et al., 1987). Ins(1,3,4,6)P_4 is an intermediate for the production of inositol (1,3,4,5,6)-pentakisphosphate (Ins(1,3,4,5,6)P_5) and the subsequent inositol hexaphosphate ($InsP_6$) (Putney & Bird, 1993) (Fig.17). $InsP_6$ is also the substrate for the generation of pyrophosphate-containing inositol phosphates: $InsP_5PP$ and $InsP_4(PP)_2$, inaccurately referred to as $InsP_7$ and $InsP_8$ respectively (Irvine & Schell, 2001).

An additional complexity to the inositol phosphates metabolism

is given by the fact that a minor product of PLC activity on PtdIns(4,5)P_2 can be the cyclic inositol (2,4,5)-trisphosphate. This compound, which is not a substrate for IP_3-3K, is sequentially dephosphorylated to give free inositol (Wilson et al., 1985) (Fig.17).

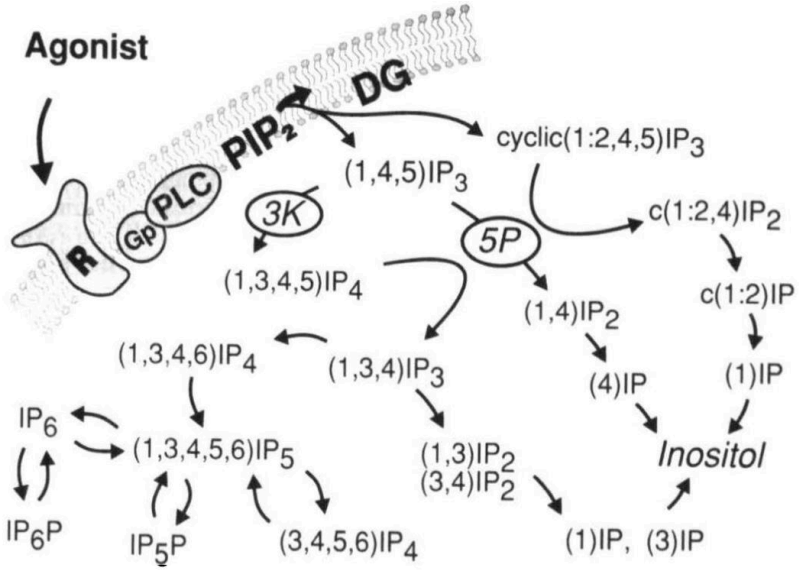

Figure 17 Pathways of inositol phosphate metabolism.
Agonist activation of a surface receptor (R) activates, usually through a G-protein (Gp), a PLC, which catalyses the breakdown of PtdIns(4,5)P_2 (PIP_2 in the figure) into the Ca^{2+}-signalling messenger, Ins(1,4,5)P_3 and DAG (DG in the figure). Ins(1,4,5)P_3 is metabolized by two enzymes, a 5-phosphatase and a 3-kinase, initiating complex degradative and synthetic pathways of inositol phosphate metabolism. A minor product of PLC action on PtdIns(4,5)P_2 is a cyclic derivative of Ins(1,4,5)P_3, cyclic(l:2,4,5)IP_3. Adapted from: Putney & Bird, 1993.

1.6.2.1 Beyond Ins(1,4,5)P_3: IP_3-3 Kinases and Ins(1,3,4,5)P_4 generation

IP_3-3K (also known and hereinafter called ITPK) has a key role in maintaining Ca^{2+} homeostasis within the cells, by regulating the rapid metabolism of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 (Irvine et al. 1986). In mammals, three isoforms of ITPK have been described, ITPKA, ITPKB and ITPKC (Choi et al., 1990; Dewaste et al., 2000). They all share the same structure, consisting of two distinctive domains: a conserved catalytic C-terminal domain and a variable N-terminal regulatory domain. The particular and highly conserved structure of the Ins(1,4,5)P_3 binding site makes ITPKs extremely specific for Ins(1,4,5)P_3.

ITPK isoforms show differential expression within mammalian tissues. Isoform A is mainly expressed in the brain, particularly at the level of the pyramidal neurons of the CA1 region and of the dentate gyrus granule cells of the hippocampus but also at the level of cerebellar Purkinje cells (Mailleux et al., 1991a, 1991b). *Itpk*$A^{-/-}$ mice present an altered hippocampal long-term potentiation (LTP), which is one of the most important mechanisms of synaptic plasticity that underlies functions as learning and memory (Jun et al., 1998). Isoform B is widely expressed, it is highly represented in the brain, the smooth muscle, the lung and also the immune system (Hascakova-Bartova et al., 2004). In the immune system, ITPKB is involved in lymphocyte maturation (Pouillon et al., 2003; Wen et al., 2004), neutrophil activation (Jia et al., 2007) and myelopoiesis (Jia et al., 2008). Finally, isoform C is almost ubiquitously expressed (Dewaste et al., 2000). Recently, a functional

single nucleotide polymorphism (SNP) in ITPKC gene has been associated to the pathogenesis of Kawasaki disease, an autoimmune disease possibly due to the alteration of NFAT transcriptional activity in T cells (Onouchi et al., 2008).

All the three ITPKs are regulated by Ca^{2+}, through the interaction with calmodulin, with ITPKC showing the lower sensibility (Irvine and Schell 2001). Besides, other mechanisms are involved in ITPKs activity regulation. Phosphorylation, by cyclic AMP-dependent protein kinase A (PKA), protein kinase C (PKC) and calmodulin regulated kinase II (CaMKII), differently modulate the activity of each ITPK isoform (Schell, 2010; Woodring & Garrison, 1997). Proteolytic cleavage of ITPKs has been also described. Indeed, ITPKs contain PEST sequences (Choi et al., 1990), namely P (proline), E (glutamine), S (serine) and T (threonine) enriched regions, which make a protein susceptible to proteolysis through Ca^{2+}-regulated proteases (calpains), followed by rapid degradation (Rechsteiner & Rogers, 1996). Finally, the subcellular localization is likely involved in the regulation of ITPKs activity by influencing the proximity to their substrate Ins(1,4,5)P_3 (Schell, 2010).

The question about which is the reason why the cells should consume energy to generate Ins(1,3,4,5)P_4 when they have the possibility to inactivate Ins(1,4,5)P_3 by simple using a 5-phosphatase has struggled many researchers since long time. Some compelling data demonstrated that Ins(1,3,4,5)P_4 functions as second messenger within the cell, rather than being just a by-product of Ins(1,4,5)P_3 metabolism. In addition, Ins(1,3,4,5)P_4 synthesis lays the ground for the generation of higher order inositol phosphates, which have been shown to have

cellular functions as well (Irvine & Schell, 2001).

So far, many functions of Ins(1,3,4,5)P_4 have been pointed out. Ins(1,3,4,5)P_4 appears to have a dual role in the modulation of Ins(1,4,5)P_3 activity. If from one hand the formation of Ins(1,3,4,5)P_4 limits the presence of Ins(1,4,5)P_3, shortening its half-time, from the other hand, it has been shown that the conversion of Ins(1,4,5)P_3 into Ins(1,3,4,5)P_4 seems protective for the first. In particular, Ins(1,3,4,5)P_4 is hydrolysed by the same 5-phosphatases that hydrolyses Ins(1,4,5)P_3 to Ins(1,4)P_2, but this enzyme has higher affinity for Ins(1,3,4,5)P_4 rather than Ins(1,4,5)P_3 (Connolly et al., 1987), which means that upon re-challenge with an Ins(1,4,5)P_3-generating stimulus, the Ins(1,3,4,5)P_4 still present "protects" Ins(1,4,5)P_3 by competitive inhibiting the 5-phoshatase, thus prolonging its half-life (Hermosura et al., 2000). This process is of particular relevance at the level of neuronal dendritic spines where a close in time reapplication of a stimulus is at the base of different neuronal responses (Irvine 2001).

A number of Ins(1,3,4,5)P_4 interacting proteins have been identified, characterized by the presence of the pleckstrin homology (PH) domains that mediate the binding to phosphatidylinositol lipids. Among these, there are Centaurins/p42IP4 (Hammonds-Odie et al., 1996; Stricker et al., 1999) and synaptotagmins (Ibata et al., 1998), which are proteins involved in vesicular trafficking and GAP1 family of Ras GTPases, especially RASA3 (Cozier et al., 2000; Cullen et al., 1995), involved in signal transduction. Ins(1,3,4,5)P_4 seems able to influence the activation state and the subcellular localization of its target protein, mostly by recruiting them to the plasma membrane.

Another class of cellular targets of Ins(1,3,4,5)P_4 are channel

proteins. The activity of Ins(1,3,4,5)P_4 on channels can be both direct and indirect and sometimes displays controversial effects that leave the research in this field still open. Ins(1,3,4,5)P_4 can be dephosphorylated to generate Ins(1,3,5)P_3, which inhibits Ins(3,4,5,6)P_4-1 kinase that is involved in the conversion of Ins(3,4,5,6)P_4 into Ins(1,3,4,5,6)P_5. This leads to an increase of Ins(3,4,5,6)P_4 that has an inhibitory activity on Ca^{2+}-regulated Cl^- channels (Vajanaphanich et al., 1994).

Several data suggest a role for Ins(1,3,4,5)P_4 in the regulation of intracellular Ca^{2+} mobilization. It has been shown that Ins(1,3,4,5)P_4 can modulate Ca^{2+} channels at the plasma membrane. By extending Ins(1,4,5)P_3 half-life, Ins(1,3,4,5)P_4 can facilitate store-operated Ca^{2+} influx in conditions of sequential Ins(1,4,5)P_3 generation (Hermosura et al., 2000). On the other hand, it has been demonstrated that in lymphocytes Ins(1,3,4,5)P_4 formation inhibits store-operated Ca^{2+} channel, thus inhibiting Ca^{2+} entry (Miller et al., 2007). A direct effect of Ins(1,3,4,5)P4 on several Ca^{2+}-permeable channels at the plasma membrane has been described (Lückhoff & Clapham, 1992; Szinyei et al. , 1999; Tsubokawa et al., 1996).

Ins(1,3,4,5)P_4 can also bind InsP_3Rs. It has been shown that Ins(1,3,4,5)P_4 can mobilize intracellular Ca^{2+} by opening InsP_3Rs located at the ER, although with less potency than Ins(1,4,5)P_3 (Sims & Allbritton, 1998). Indeed, the affinity of InsP_3Rs for Ins(1,4,5)P_3 is higher in respect to Ins(1,3,4,5)P_4. It is remarkable that Ins(1,3,4,5)P_4 has higher affinity for InsP_3Rs at the plasma membrane as compared to intracellular ones (Khan et al., 1992b) and this comes out in favour of a role of Ins(1,3,4,5)P_4 in the regulation of Ca^{2+} flux across the plasma membrane. The different affinity of plasma membrane InsP_3Rs for the

two inositol phosphates may rely on the high sialic acid content which is typical of plasma membrane proteins (Khan et al., 1992b).

1.6.3 $InsP_3$ receptors

$InsP_3Rs$ are intracellular ligand-gated Ca^{2+}-permeant channels that play a pivotal role in intracellular Ca^{2+} dynamics, thus in numerous cellular functions. The structure of $InsP_3Rs$ has been elucidated about thirty years ago, when it was demonstrated that $InsP_3Rs$ are very large glycoproteins with tetrameric structure (Supattapone et al., 1988).

So far, three $InsP_3Rs$ isoforms have been described in mammals, $InsP_3R1$ (IP_3R1), $InsP_3R2$ (IP_3R2), $InsP_3R3$ (IP_3R3). Alternative splicing variants have also been identified (Mikoshiba, 2007). Further diversity is given by the formation of both homo- and hetero-tetramers. Most of the cells express all the three isoforms, although in different proportion, suggesting functional redundancy (Wojcikiewicz, 1995). However, each isoform shows different affinity for $Ins(1,4,5)P_3$, approximately summarized as $IP_3R2 > IP_3R1 > IP_3R3$, which implicates a diverse responsiveness to $Ins(1,4,5)P_3$ (Newton et al., 1994) and, likely, to other potential ligands.

$InsP_3Rs$ share 65-85% of homology. The channel region, located at the C-terminal domain, is characterized by six membrane-spanning helices with a short cytoplasmic C-terminal tail that is critical for receptor gating (Uchida et al., 2003). At the N-terminal domain reside both a ligand coupling/suppressor domain, which is responsible for

ligand-affinity features (Iwai et al., 2007) and the Ins(1,4,5)P_3-binding core (Yoshikawa et al., 1996). These two domains are often collectively referred to as Ins(1,4,5)P_3-binding domain. An internal coupling domain confers susceptibility to regulation by several protein kinases, as PKA, PKA, CamKII and also by various intracellular mediators such as calmodulin, ATP and Ca^{2+} itself (Foskett et al., 2007).

The three isoforms of InsP_3R have been shown to mediate different Ca^{2+} mobilization patterns, therefore, Ca^{2+} signalling within the cell depends on the content of InsP_3R isoforms. In particular, IP_3R2 is the most sensitive to Ins(1,4,5)P_3 and mediates robust, long lasting and regular Ca^{2+} oscillations. IP_3R1 activation provokes less regular Ca^{2+} oscillations and can cause Ca^{2+} transients. Finally, IP_3R3 is the least sensitive to Ins(1,4,5)P_3, as well as to Ca^{2+} regulation, and generates only monophasic Ca^{2+} transients (Miyakawa et al., 1999b).

The IP_3Rs are widely distributed in mammal tissues with some preferred localization for each isoform. IP_3R1 is mainly expressed in the central nervous system, with a predominant expression at the level of the cerebellum, especially in the Purkinje cells (Furuichi et al., 1993; Satoh et al., 1990). IP_3R2 is highly expressed in skeletal and cardiac muscles. In particular, cardiac myocytes express almost exclusively this isoform. IP_3R2 is also present in glial cells. IP_3R3 is represented in some neurons but its localization is restricted to the soma, in contrast with IP_3R1 that is widely distributed throughout the cell (Johenning et al. 2002). IP_3R3 is highly represented in DCs (Stolk et al., 2006).

The prevalent view about InsP_3Rs subcellular localization is that they are present at the ER where they regulate Ca^{2+} mobilization from the most effective intracellular Ca^{2+} store (Berridge & Irvine, 1984;

Zhang et al., 2011). However, several studies unveiled the expression of $InsP_3Rs$ in a number of other cellular membranes. $InsP_3Rs$ have been described in the Golgi apparatus (Pinton et al., 1998), in the nucleoplasmic reticulum (Echevarría et al., 2003), which is a network of invagination that is a continuous with the ER and the nuclear envelope (Malhas et al., 2011) and also at the plasma membrane in different cell types. A plasma membrane localization for $InsP_3Rs$ has been described in human T lymphocytes (Khan et al. 1992a), in olfactory neurons of different species (Fadool & Ache, 1992; Restrepo et al., 1992) and in rat liver cells (Dargemont et al., 1988; Guillemette et al., 1988). Although plasma membrane does not embody a Ca^{2+} store in the exact sense of the term, it represents a source of readily available Ca^{2+} coming from the extracellular space. Functional plasma membrane IP_3R3 have been shown to be involved in antigen-induced Ca^{2+} entry in chicken B lymphocytes (Dellis et al., 2006). However, limited data are available about the function of plasma membrane $InsP_3Rs$.

Notably, the subcellular distribution of $InsP_3Rs$ is not static. In many cell types, it has been shown that a redistribution of InsP3Rs can occur, in response to external stimuli, extracellular calcium concentration or during certain processes such as oocytes maturation and cell polarization. The mechanism underlying $InsP_3Rs$ redistribution is still under investigation, likely, it involves interactions of $InsP_3Rs$ with various cytoskeletal and scaffolding proteins (Vermassen et al., 2004).

1.7 Scope of the thesis

Innate immune myeloid cells sense the presence of microbes or microbial products through PRRs. Among these, TLRs are the best-characterized and they are key regulators of anti-bacterial and anti-viral immune responses.

TLR4, together with CD14 and MD-2, forms the multi-receptor complex that recognizes the LPS, the major component of the outer membrane of Gram-negative bacteria. CD14 is a GPI-anchored protein largely expressed on DCs and macrophages and it acts as fundamental regulator of cellular responses to LPS. In particular, CD14 concentrates the LPS signal and mediates the relocation of TLR4 and MD-2 to the endosome for the initiation of the TRAM-TRIF, thus the production of type I IFNs. Moreover, in DCs, LPS-dependent CD14 activation has been shown to elicit a TLR4-independent signalling pathway which leads to the activation of the transcription factor NFAT.

In activated DCs, the CD14-NFAT pathway has been shown to direct distinct biological processes including regulation of IL-2 (Granucci et al., 2001) and PGE2 (Zanoni et al., 2012) production and the induction of apoptosis in terminally differentiated DC (Zanoni et al., 2009).

Granucci and co-workers demonstrated that, after acute exposure to LPS, CD14 leads to the activation of src family kinase (SFK) that, in turn, activates PLCγ2. PLCγ2 fosters a rapid monophasic Ca^{2+} influx, causing an increase in intracellular Ca^{2+} concentration that is the triggering event for NFAT activation and nuclear translocation. CD14 localization in lipid rafts, subdomains of the plasma membrane rich in

cholesterol and glycosphingolipids, is needed for LPS-induced Ca^{2+} mobilization, since soluble CD14 is not able to restore Ca^{2+} mobilization in CD14 deficient DCs. Src family kinase and PLCγ2 are necessary component of this signalling pathway (Zanoni et al., 2009). However, the molecular mechanism responsible for the increase in intracellular Ca^{2+} in DCs downstream LPS-induced CD14-dependent PLCγ2 activation, is still unknown.

With the present work, we intended to evaluate the CD14-dependent signalling pathway evoked by LPS in DCs which leads to Ca^{2+} mobilization and, consequently, NFAT activation and nuclear translocation.

Since it was pointed out that LPS-induced NFAT activation occurs in DCs but not in macrophages (Zanoni et al., 2009), we investigated the reason for the cell-type specificity of the CD14-NFAT pathway.

Finally, we wondered whether the same mechanism of NFAT pathway initiation is active in human DCs after LPS exposure. We concentrated our analysis on $CD1c^{+}CD14^{+}$ DCs, a very recently discovered human DCs subtype present in the blood that was classified as DC3 (Villani et al., 2017).

Chapter 2

In this Chapter, we described our finding on the signalling pathway involved in the CD14-dependent activation of NFAT pathway, both in mouse and in human dendritic cells following LPS exposure. In addition, we proposed an explanation for the lack of CD14-NFAT pathway initiation in macrophages.

Chapter 3

Summary

Conclusions and future perspective

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Chapter. 2 Study of the mechanism of LPS-dependent Ca^{2+} mobilization in dendritic cells

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2.1 Store-operated Ca^{2+} entry is not involved in LPS-induced Ca^{2+} mobilization in dendritic cells

In DCs, a rapid monophasic increase in intracellular Ca^{2+} concentration follows acute LPS exposure. It has been demonstrated that this event completely relies on CD14 engagement while it is fully independent of TLR4 activation and any other TLRs. LPS-induced Ca^{2+} mobilization in DCs needs the activity of SFK and the subsequent activation of PLCγ2 (Zanoni et al., 2009). However, the molecular events coupling PLCγ2 activation to the increase in intracellular Ca^{2+} concentration have not been fully established.

The main mechanism involved in Ca^{2+} entry in non-excitable cells depends on Ca^{2+}-released activated channels (CRACs) opening at the plasma membrane, which occurs in response to Ca^{2+} depletion in the ER due to $InsP_3Rs$ opening, a mechanism known as store-operated Ca^{2+} entry (SOCE) (Prakriya & Lewis, 2015; Putney et al., 2001). However, Ca^{2+} entry through plasma membrane in non-excitable cells can be directed by other two mechanisms that are receptor-operated Ca^{2+} entry (ROCE), when plasma membrane Ca^{2+} channels open in response to agonist-binding and second messenger-operated Ca^{2+} entry (SMOCE), when plasma membrane Ca^{2+} channels open upon binding of intracellular second messengers such as products released in response to PLCγ activation (Putney, 2002).

We, therefore, explored the mechanism underlying Ca^{2+} mobilization in DCs following acute LPS exposure. For these

experiments we used a well-established murine splenic DC line, D1 (Winzler et al., 1997).

To investigate the dynamics of Ca^{2+} mobilization, we compared the profile of Ca^{2+} flux recorded either from LPS-stimulated D1 cells or ATP-stimulated D1 cells, as positive control for SOCE. ATP can signal through both ionotropic and metabotropic receptors (Di Virgilio, 2005). Ionotropic P2X receptors are ATP-gated ion channels, whereas metabotropic P2Y receptors are GPCRs. In DCs, ATP signalling account mostly on P2Y receptors (Hsu et al., 2001), resulting in phosphatidylinositol breakdown, the release of Ca^{2+} from intracellular stores and the opening of CRACs at the plasma membrane (Feske, 2007).

Following LPS challenge, D1 cells display a rapid and monophasic Ca^{2+} transient (Fig.18A). On the contrary, upon ATP stimulation a biphasic profile of Ca^{2+} mobilization occurs, reflecting the rapid Ca^{2+} transient due to Ca^{2+} release from the ER followed by a sustained CRAC-dependent plateau (Fig.18B). The chelation of extracellular Ca^{2+}, by means of EGTA, completely abrogates LPS-induced Ca^{2+} transient, meaning that the source for Ca^{2+} mobilization derives from the extracellular space (Fig.18C). In the case of ATP stimulation, EGTA does not exert any effect on the first phase of Ca^{2+} mobilization, which comes from the intracellular space, while it blocks the second phase, coherently with the position of CRACs at the plasma membrane (Fig.18D). The treatment with a selective CRACs-inhibitor, N-[4-[3, 5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl]-4-methyl-1, 2, 3-thiadiazole-5-carboxamide (YM-58483) (Ishikawa et al., 2003), has the same result of Ca^{2+} chelation on ATP-treated D1 cells (Fig.18F),

whereas does not affect LPS-induced Ca^{2+} transient (Fig.18E), indicating that CRACs are not involved, thus excluding the SOCE mechanism. The pre-treatment of D1 cells with 2-Aminoethyl diphenylborinate (2-APB), a non-selective membrane permeant modulator of several ion channels including $InsP_3R$ (Maruyama et al., 1997), totally prevents LPS-induced Ca^{2+} transient (Fig.18G) as well as ATP-induced Ca^{2+} mobilization (Fig.18H), as expected by blocking the depletion of Ca^{2+} within the ER, which is the triggering event for CRACs opening.

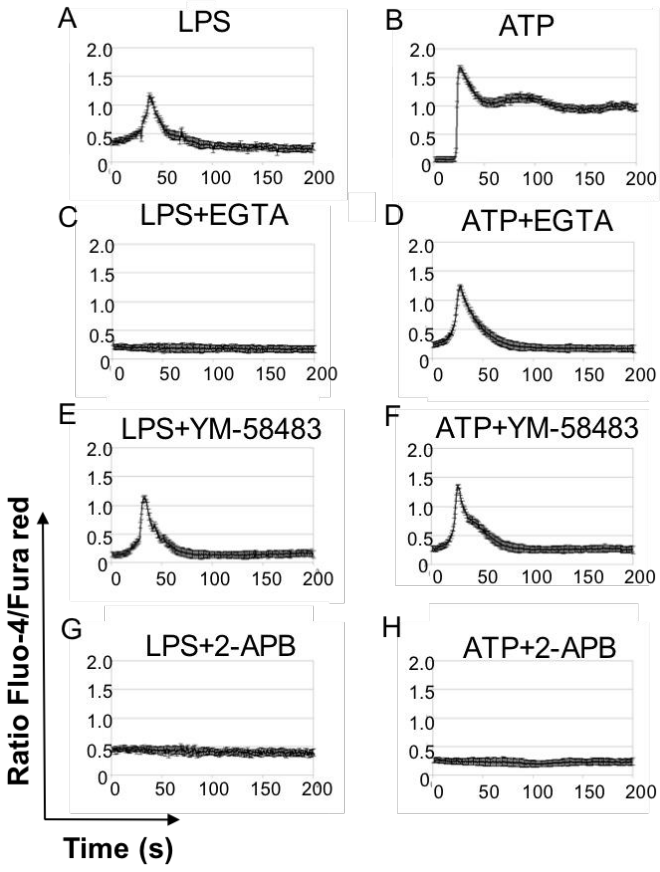

Figure 18 Ca^{2+} mobilization measurement in stimulated D1 cells.

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Figure 18 continued.
D1 cells have been stained with paired ratiometric calcium probes Fluo-4 AM (calcium bound) and Fura Red AM (calcium free). Cells were then treated with LPS (1μg/ml) (A, C, E, G) or with ATP (100μM) (B, D, F, H). EGTA (2mM) (C, D), YM-58483 (E, F) and 2-APB (100μM) (G, H), were added to the culture medium 10 minutes before the stimulus. The cells were analysed by confocal microscopy. The intracellular Ca^{2+} concentration was 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.

The increase in the intracellular Ca^{2+} concentration is the crucial event for NFAT pathway initiation. Therefore, we stimulated D1 cells with LPS and we measured the production of two cytokines: IL-2, which is transcriptionally controlled by NFAT and regulated by the CD14-NFAT pathway in DCs, and TNFα, which, instead, depends on the TLR4/MyD88/TRIF/NF-κB pathway. The pre-treatment with 2-APB selectively inhibits, in a dose-dependent fashion, the production of IL-2, while it does not affect TNFα secretion. No effect due to CRACs inhibition was detected, neither on IL-2 nor on TNFα production (Fig.19).

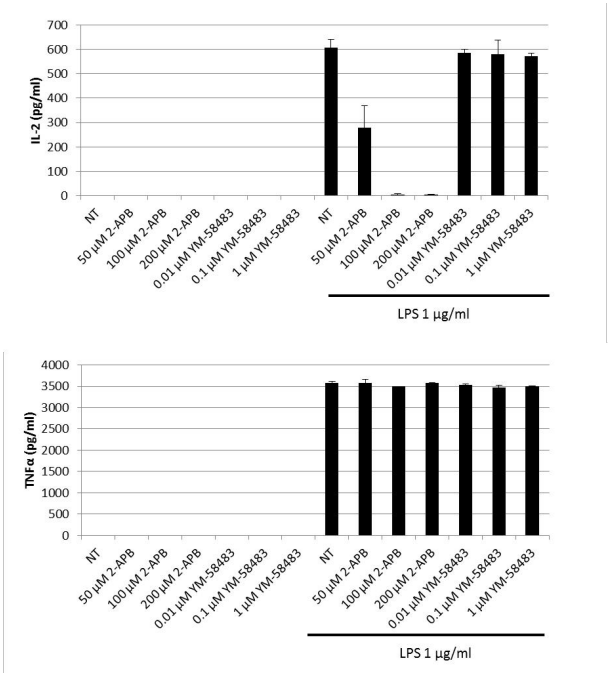


Figure 19 IL-2 and TNFα production by D1 cells in the presence of either 2-APB or YM-58483.

D1 cells were pre-treated with either 2-APB or YM-58483 for 30 minutes at the concentration indicated in the figures and then stimulated with LPS (1µg/ml) for 24 hours. IL-2 and TNFα production was quantified by ELISA assay. Indicated values represent means of at least three independent experiments performed in duplicate (± SEM).

Taken together these results indicate that acute LPS exposure of DCs induces a direct influx of Ca^{2+} across the plasma membrane and rule out a possible involvement of SOCE, differently from NFAT activation in T and B lymphocytes (Feske, 2007).

2.2 Plasma membrane IP_3R3 are required for LPS-induced Ca^{2+} entry in dendritic cells

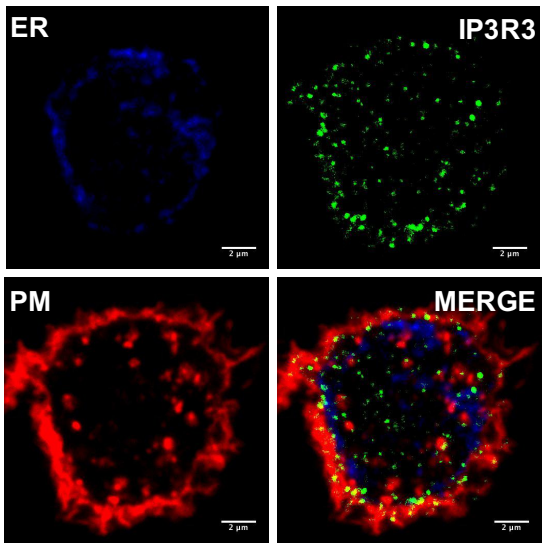
Former results have indicated that LPS-induced Ca^{2+} mobilization in DCs needs PLCγ2 activity, indeed, *Plcg2*$^{-/-}$ DCs fail in Ca^{2+} mobilization after challenge with LPS (Zanoni et al., 2009). PLCγ2 is a lipid metabolizing enzyme that catalyses the hydrolysis of PtdIns(4,5)P_2 in the two second messenger molecules DAG and Ins(1,4,5)P_3 (hereinafter referred to as IP_3 for convenience). PLCγ2 involvement led us consider the hypothesis that IP_3 is the second messenger responsible for LPS-induced CD14-mediated Ca^{2+} entry across the plasma membrane. Once produced, while DAG resides in the plasma membrane, IP_3, which is highly soluble, can diffuse throughout the cell searching for its receptors, $InsP_3Rs$ (hereinafter referred to as IP_3Rs). The prevalent, but not unique, localization of IP_3Rs is the ER (Taylor & Tovey, 2010), here these receptors regulate the emptying of Ca^{2+} stores that is also necessary for SOCE.

Our previous data demonstrated that Ca^{2+} entry in LPS-stimulated D1 cells occurs through the plasma membrane, therefore we wondered whether IP_3Rs could be expressed on the plasma membrane in DCs. It has been shown that IP_3R3 can be localized at the level of the plasma membrane in B cells (Dellis et al., 2006). IP_3R3 is the predominant isoform expressed in DCs (Stolk et al., 2006), moreover, IP_3R3 is reported to generate only monophasic Ca^{2+} transients, which

fits with the Ca^{2+} mobilization profile observed in LPS-treated DCs (Miyakawa et al, 1999).

To assess the presence and subcellular localization of IP_3R subtypes in D1 cells we performed confocal microscopy with simultaneous staining of plasma membrane, ER and IP_3Rs (Fig.20A, B, C). IP_3R3 appears distributed both at the level of the ER and at the plasma membrane (Fig.20A), suggesting that the prediction of a plasma membrane localization of IP_3Rs is indeed valid. Conversely, IP_3R2 is not represented at the plasma membrane, whereas it is present at the ER and in other intracellular structures (Fig.20B). Finally, IP_3R1 appears far less represented and more dispersed (Fig.20C).

A



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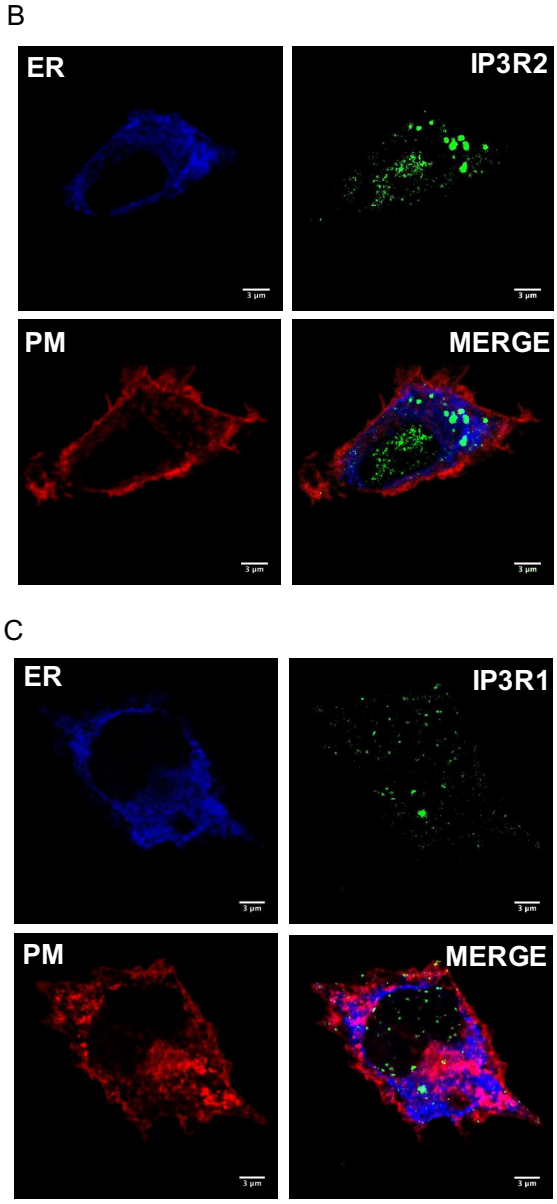
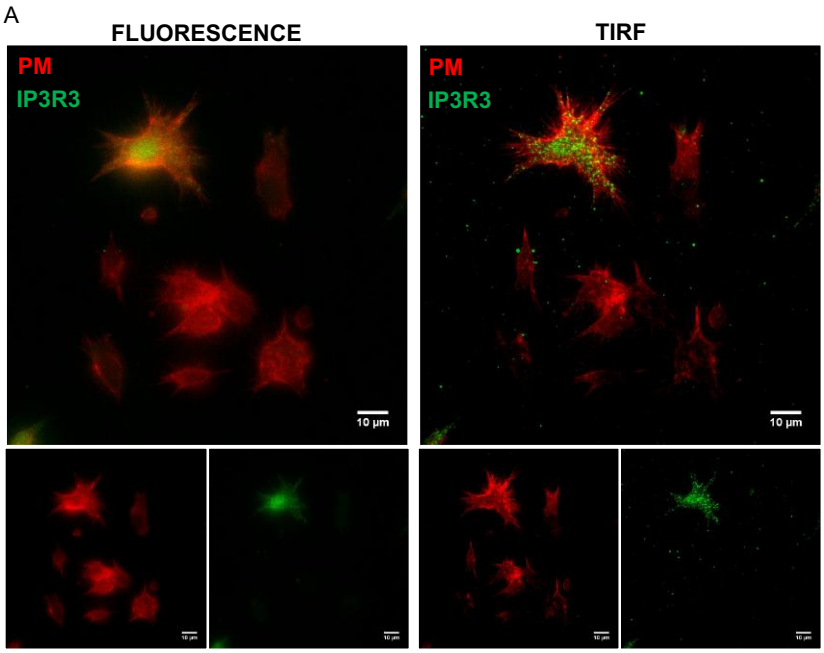


Figure 20 Subcellular localization of IP_3R isoforms in D1 cells revealed by confocal microscopy.

D1 cells were seeded onto glass coverslips. After staining, cells were analysed by confocal microscopy. Images show: the plasma membrane (PM) (red), the ER marker Calnexin (blue), IP_3R3 (A), IP_3R2 (B) and IP_3R1 (C) (green). Images are representative of at least three independent experiments.

To better clear these results, we took advantage of total internal reflection (TIRF) microscopy which is particularly suited for the observation of molecules near the plasma membrane. TIRF microscopy confirmed IP_3R3 localization at the plasma membrane (Fig.21A), while no signal was detected for both IP_3R2 (Fig.21B) and IP_3R1 (Fig.21C) at the cell surface.



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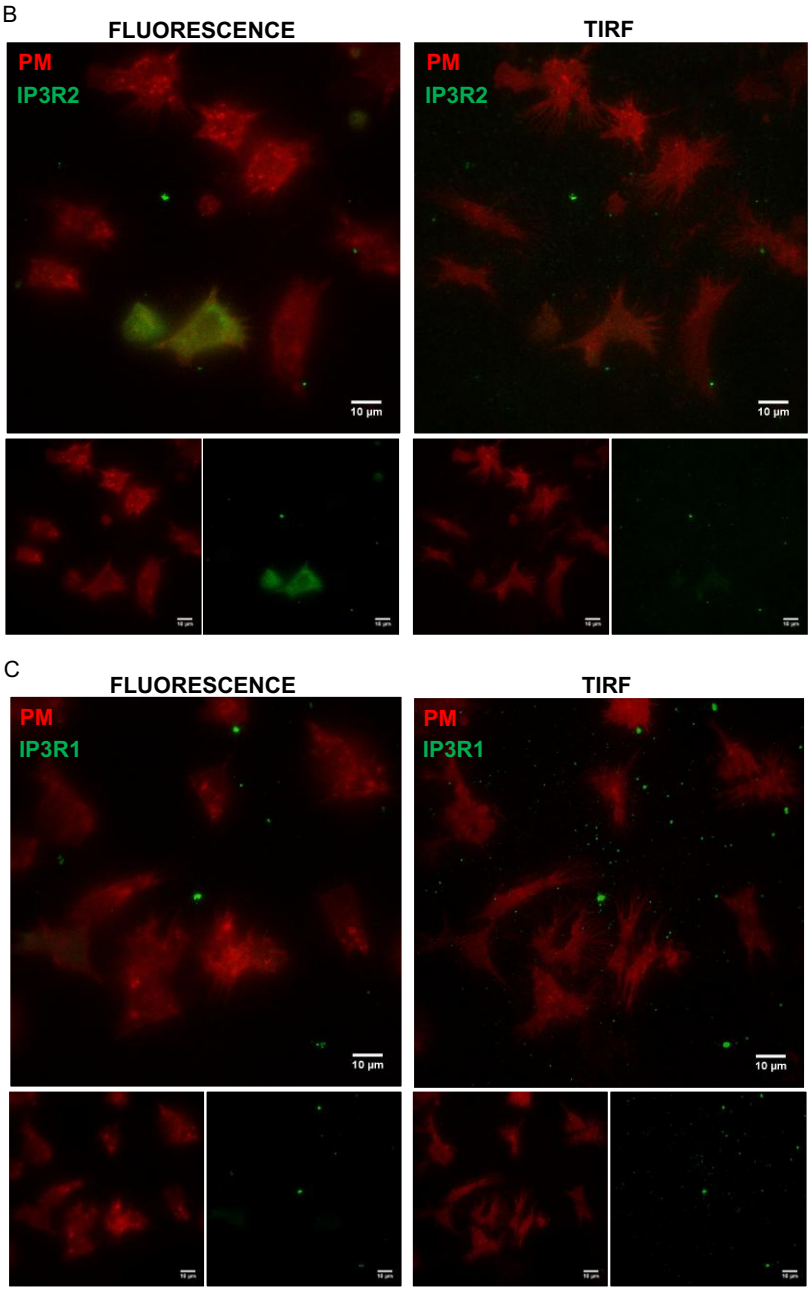

Figure 21 IP3Rs localization at the plasma membrane of D1 cells revealed by TIRF microscopy.

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Figure 21 continued.
D1 cells were seeded onto glass coverslips. After staining, cells were analysed by TIRF microscopy. Images show: the plasma membrane (PM) (red), IP_3R3 (A), IP_3R2 (B) and IP_3R1 (C) (green). The same field was analysed by fluorescence microscopy (on the left) and by TIRF microscopy (on the right). Images are representative of at least three independent experiments.

To address the role of IP_3R3 in LPS-induced CD14-mediated Ca^{2+} mobilization in DCs we specifically silenced IP_3R3 in D1 cells by means of RNA interference (Fig.22). After 48 hours from siRNAs transfection a specific reduction of about 50 % in the expression of IP_3R3 is detectable in D1 cells (Fig.22).

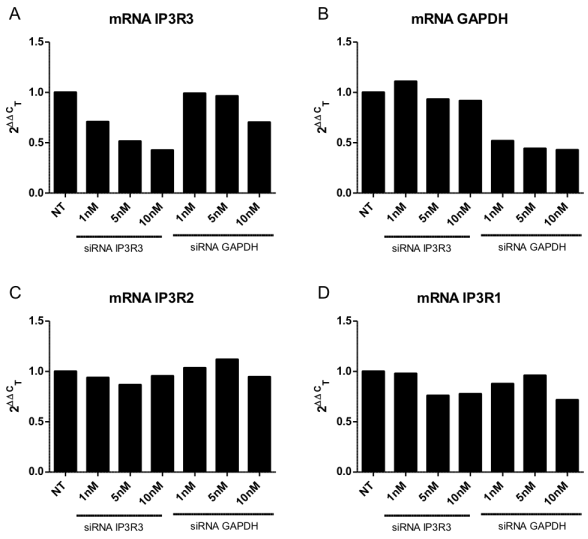


Figure 22 Efficiency and specificity of IP_3R3 silencing in D1 cells.
D1 cells were transfected with increasing concentrations of siRNAs directed either against IP_3R3 or against GAPDH, as control. After 48 hours, quantitative reverse transcription PCR was performed to evaluate the mRNA level of IP_3R3 (A), in order to assess siRNAs efficiency and of GAPDH (B), IP_3R2 (C) and IP_3R1 (D), to assess specificity and off-target of IP_3R3 silencing.

Despite the uncomplete IP_3R3 silencing, D1 cells lacking IP_3R3 totally fail in the mobilization of Ca^{2+} upon LPS exposure, resembling the effect of Ca^{2+} chelation in the culture media (Fig.23A). No effect on LPS-induced Ca^{2+} flux was provoked by GAPDH silencing, as expected (Fig.23B).

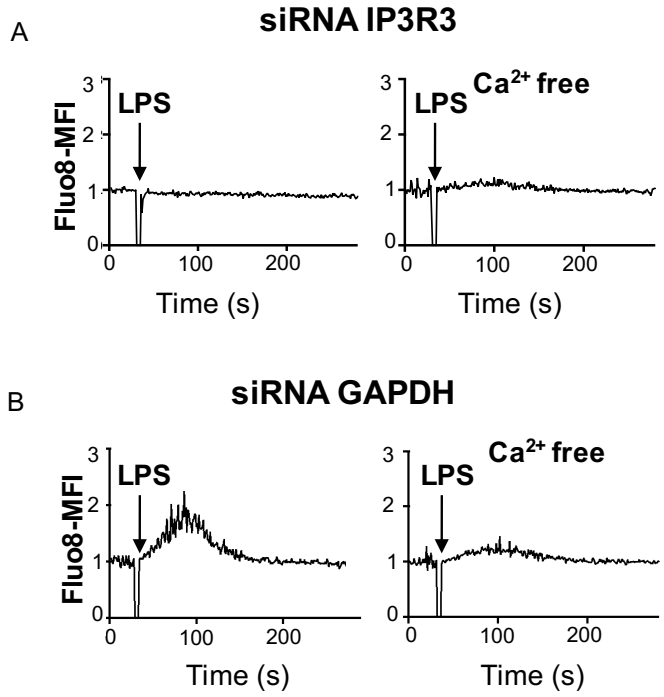


Figure 23 Effect of IP_3R3 silencing on LPS-induced Ca^{2+} mobilization in D1 cells.

D1 cells were transfected with siRNAs either against IP_3R3 (A) or against GAPDH (B) as control. Ca^{2+} mobilization was measured 48 hours later. Cells were stained with Fluo-8-AM, then stimulated with LPS (1µg/ml) in normal culture condition or in Ca^{2+} free conditions (EGTA, 2mM). The cells were analysed using a flow cytometer. Intracellular Ca^{2+} concentration variations were quantified as mean fluorescence intensity (MFI) per second, normalized considering the MFI mean measured 30 seconds before adding the stimuli. Data are representative of at least three independent experiments.

Consistently with the inhibition of Ca^{2+} increase upon LPS exposure, IP_3R3 silencing impairs LPS-induced NFAT-dependent IL-2 mRNA increase in D1 cells, while it does not affect LPS-induced TNFα mRNA increase, which relies on NF-κB activation (Fig.24).

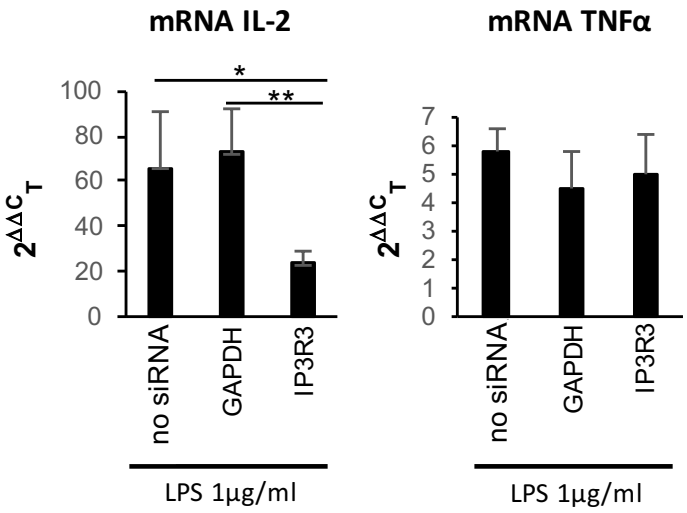
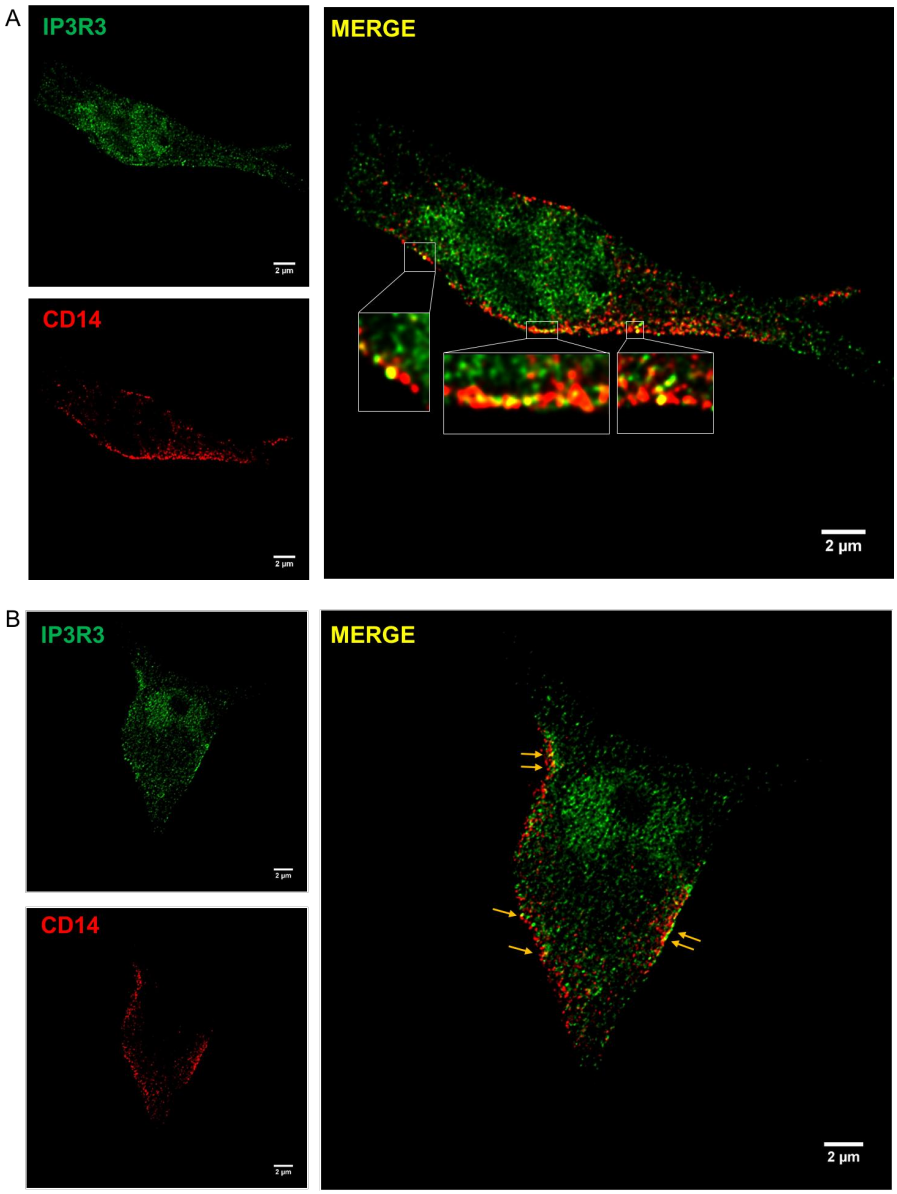


Figure 24 Effect of IP_3R3 silencing on IL-2 mRNA expression.
D1 cells were transfected with siRNAs either against IP_3R3 or against GAPDH, as control. 48 hours later, cells were stimulated with LPS (1 µg/ml) for 4 hours. The RNA was extracted from cell lysates and quantitative reverse transcription PCR of IL-2 or TNFα mRNA was performed. Values represent at least three independent experiments performed in duplicate (± SEM) and are expressed as fold change in comparison with the untreated non-transfected control.

Taken together these data clearly indicate that IP_3R3, besides being expressed at the ER as commonly accepted, it is also present at the plasma membrane of DCs where it directs Ca^{2+} flux across the plasma membrane upon LPS stimulation, demonstrating that LPS-induced Ca^{2+} entry in DCs occurs *via* SMOCE.

2.3 IP_3R3 and CD14 colocalize at the plasma membrane of dendritic cells within lipid rafts

LPS stimulation of DCs induce a monophasic Ca^{2+} flux which takes place entirely through the plasma membrane and relies on IP_3R3, according to the SMOCE process. However, this arises the question about why the second messenger involved in this pathway should open plasma membrane instead of intracellular IP_3R3. To fit this model, we hypothesized that this pathway is strictly compartmentalized at the plasma membrane, likely to optimize the concentration of the second messenger involved. We, therefore, reasoned that IP_3R3 can colocalize with CD14. To verify this hypothesis, we used two approaches: firstly the stimulated emission depletion (STED) microscopy, a technique of super resolution microscopy that, overcoming the diffraction limit of light microscopy, increases the resolution up to 20 nanometres and secondly the proximity ligation assay (PLA), which identifies molecules within 40 nanometres of distance from each other. The results of both techniques demonstrated the colocalization between IP_3R3 and CD14 in D1 cells. By using STED microscopy, we clearly observed colocalization between IP_3R3 and CD14 (Fig.25A, B), as confirmed by signal intensity profiles analysis (Fig.25C).



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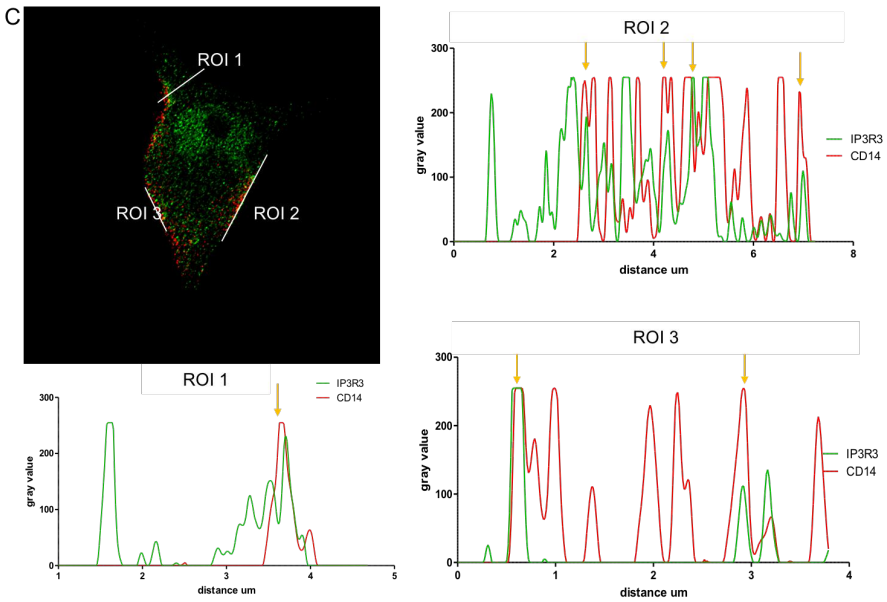


Figure 25 Analysis of IP_3R3-CD14 colocalization in D1 cells using STED microscopy.

D1 cells were seeded onto glass coverslips. After staining, the cells were analysed by STED microscopy. (A, B) Images show: CD14 (red) and IP_3R3 (green). Yellow dots show signal colocalization. (C) Representative plots comparing the pixel intensity profiles analysis of CD14 (red line) and IP_3R3 (green line). Coincident peaks indicate co-localization (yellow arrows). Images are representative of at least three independent experiments.

Consistently, the proximity ligation assay (PLA) revealed several spots of colocalization between IP_3R3 and CD14 in D1 cells (Fig.26A). Since it is known that CD14 colocalizes with TLR4 upon LPS-stimulation, CD14-TLR4 colocalization was used as positive control, whereas CD14-deficient BMDCs were used as negative control. PLA confirmed IP_3R3-CD14 colocalization also in *ex vivo* splenic murine DCs (Fig.26B).

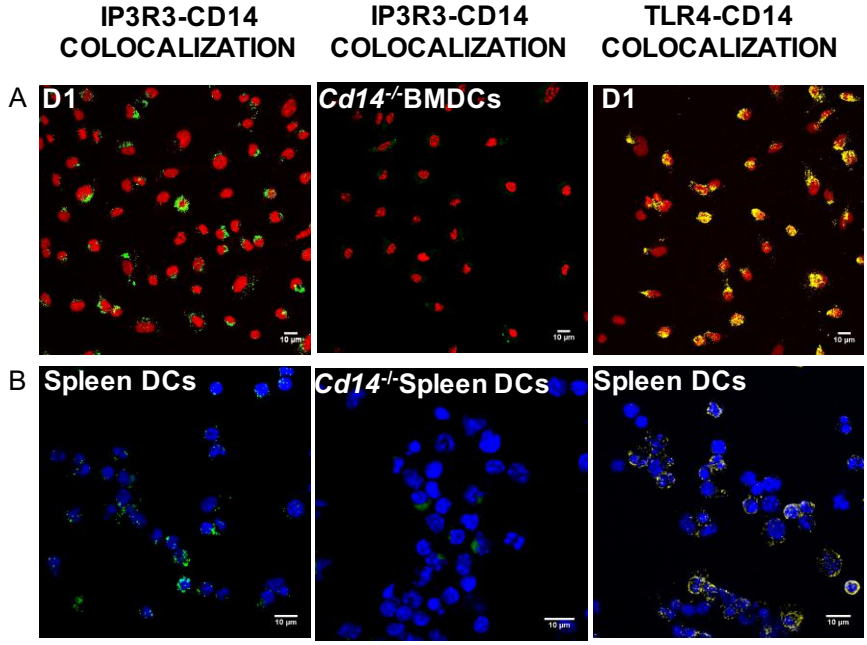


Figure 26 Analysis of IP_3R3-CD14 colocalization in D1 cells and spleen DCs using proximity ligation assay.
Cells were either seeded (A) or spun (B) onto glass coverslips and PLA assay was performed. The cells were analysed by confocal microscopy. TLR4-CD14 colocalization after LPS treatment was used as positive control, whereas *Cd14*$^{-/-}$ BMDCs and spleen DCs were used as negative control. Each spot represents a colocalization signal. The images show: nuclei (blue), IP_3R3-CD14 colocalization (green) and TLR4-CD14 colocalization (yellow). Images are representative of at least three independent experiments.

It is known that CD14 is largely located within lipid rafts, therefore, we wondered whether lipid rafts architecture participates in mediating CD14-IP_3R3 close proximity. To this purpose we looked at the effect of cholesterol depletion, by means of β-cyclodextrin treatment, on the subcellular localization of our proteins of interest in D1 cells (Fig.27). After treatment with β-cyclodextrin, CD14 and IP_3R3

disperse within the cell (Fig.27B), losing their organization at the plasma membrane (Fig.27A).

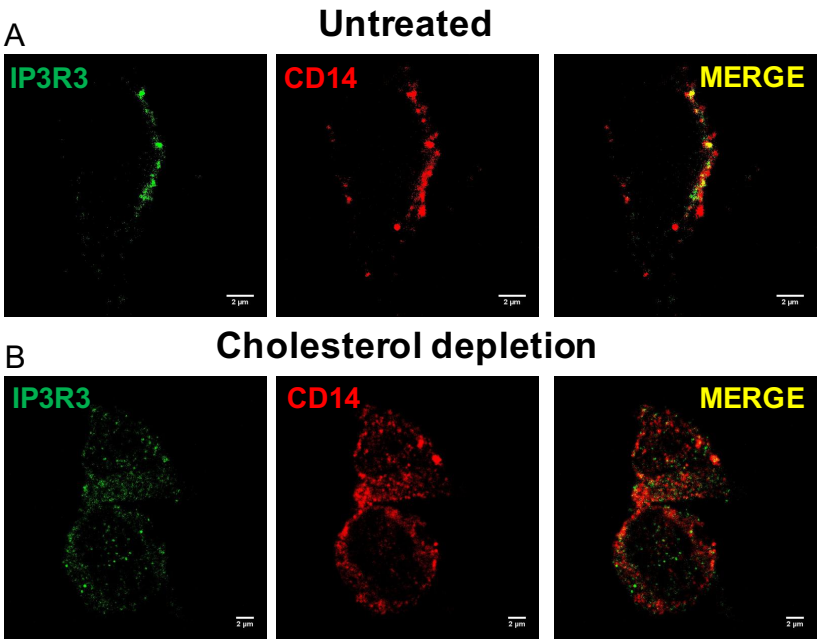


Figure 27 Effect of cholesterol depletion on IP_3R3-CD14 organization in D1 cells.

D1 cells were seeded onto glass coverslips. β-cyclodextrin (5mM) was administered for 30 minutes at 37°C (B). After staining, cells were analysed by confocal microscopy. The images show: IP_3R3 (green) and CD14 (red). Images are representative of at least three independent experiments.

Cholesterol depletion, hence lipid rafts alteration, also affects LPS-induced Ca^{2+} mobilization. Indeed, the treatment with β-cyclodextrin abrogates Ca^{2+} flux in LPS-stimulated D1 cells (Fig.28).

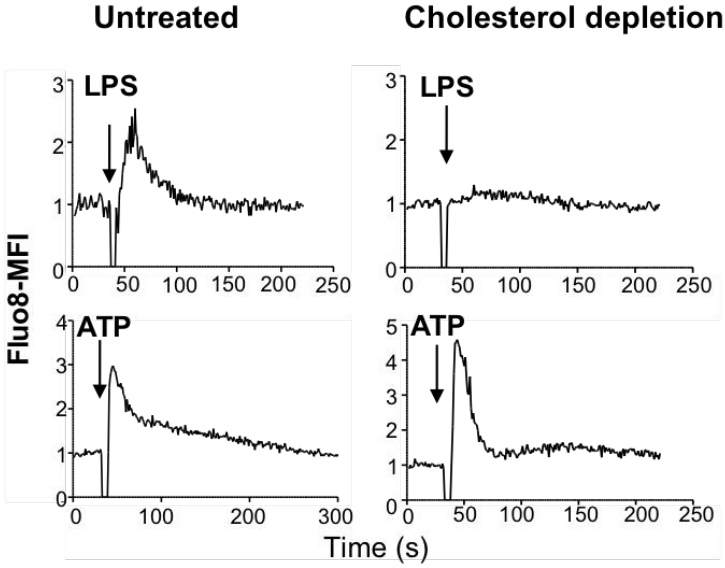


Figure 28 Effect of cholesterol depletion on LPS-induced Ca^{2+} flux in D1 cells.

D1 cells untreated (graphs on the left) or treated with β-cyclodextrin (5mM, 30 minutes at 37°C) (graphs on the right) were stained with Fluo-8 AM. Cells were analysed using a flow cytometer. After 30 seconds of recording, cells were stimulated with either LPS (1μg/ml) or with ATP (100μM). Intracellular Ca^{2+} concentration variations were quantified as mean fluorescence intensity (MFI) per second, normalized considering the MFI mean measured 30 seconds before adding the stimuli. Data are representative of at least three independent experiments.

These observations pointed out that IP_3R3 and CD14 co-localize within lipid rafts in DCs and that this organization is required for LPS-induced Ca^{2+} entry through plasma membrane IP_3R3.

2.4 Ins(1,3,4,5)P_4 is the actual second messenger involved in LPS-induced Ca^{2+} mobilization in dendritic cells

As already mentioned, PLCγ2 involvement in LPS-induced CD14-dependent Ca^{2+} mobilization in DCs (Zanoni et al., 2009), prompted us to think that IP_3 can be the second messenger in this signalling pathway, which would be supported by the evidence that IP_3R3 is required. If this is true, then it remains unclear the reason why IP_3 acts preferentially on plasma membrane IP_3Rs. Indeed, even in the case of a low production of IP_3, the opening of IP_3Rs at the ER would be favoured, since data in literature indicate that IP_3 has about 4-5 times higher affinity for IP_3Rs expressed on the ER rather than those on the plasma membrane (Khan et al., 1992). This suggests that another player is likely taking part in this signalling pathway. We got a cue on this matter looking at IP_3 metabolism. The first step in IP_3 metabolism is the conversion either into Ins(1,4)P_2, which is biological inactive or into Ins(1,3,4,5)P_4 (hereinafter referred to as IP_4), which has been shown to have different cellular functions (Irvine & Schell, 2001). Interestingly, IP_4 has about 10 times higher affinity for IP_3Rs expressed at the plasma membrane as compared with those on the ER (Khan et al., 1992), suggesting a role for IP_4 in the regulation of Ca^{2+} flux across the plasma membrane. This fosters the hypothesis that, in our context, IP_4 could be the actual second messenger downstream CD14-mediated Ca^{2+} mobilization that, once produced at the plasma membrane, opens IP_3R3 located in this compartment. Therefore, we focused our

attention on the involvement of IP_4 in LPS-induced CD14-NFAT pathway activation in D1 cells.

The conversion of IP_3 into IP_4 is catalysed by IP_3 3-kinases (ITPKs). Among ITPKs, the isoform ITPKB is the uniquely expressed in hematopoietic cells (Jia et al., 2008). We evaluated if ITPKB was activated in D1 cell upon LPS exposure. To this purpose, the phosphorylated proteins were extracted and a Western blot was performed to verify the presence of ITPKB among the phospho-proteins. Upon LPS stimulation, an enrichment in phosphorylated ITPKB was found in D1 cells, as compared to untreated cells, meaning that ITPKB is actually activated in response to LPS challenge (Fig 29).

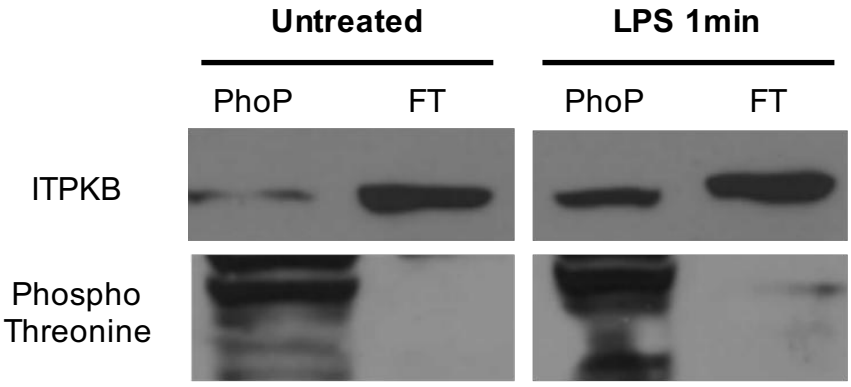


Figure 29 Western blot of phospho-proteins from D1 cells.
Phospho-proteins were extracted from D1 cells either untreated or treated for 1 minute with LPS (1 µg/ml) and then Western blot was performed. The membrane was probed with anti-ITPKB antibody and anti-phospho-threonine antibody, to verify the correct separation of phospho-proteins (PhosP) from unphosphorylated ones (flow through, FT). Data are representative of at least three independent experiments.

To explore the role of ITPKB, hence IP_4 production, in the mobilization of Ca^{2+} in LPS-stimulated DCs, we pharmacologically inhibited ITPKB in D1 cells and we measured Ca^{2+} transients upon LPS exposure (Fig.30). We observed that the inhibition of ITPKB, dramatically impairs LPS-induced Ca^{2+} entry in D1 cells. A residual Ca^{2+} flux can be detected, which occurs independently of the external Ca^{2+} source (Fig.30), suggesting that in the case of an uncomplete conversion of IP_3 into IP_4, the remaining IP_3 acts preferentially on intracellular IP_3Rs, as expected considering its higher affinity for those receptors.

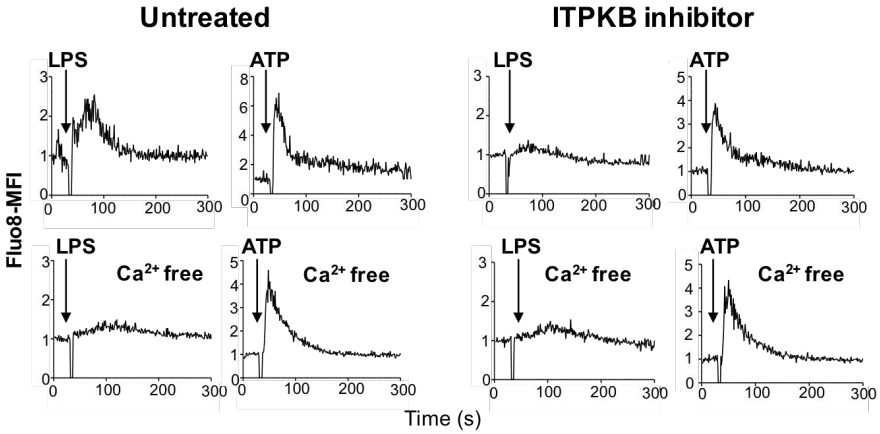


Figure 30 Effect of ITPKB inhibition on LPS-induced Ca^{2+} mobilization in D1 cells.

D1 cells were stained with Fluo-8 AM. The ITPK inhibitor, TNP (20µM) was administered 5 minutes before LPS (1µm/ml) stimulation in normal culture condition or in Ca^{2+} free conditions (EGTA, 2mM). The cells were analysed using a flow cytometer. Intracellular Ca^{2+} concentration variations were quantified as mean fluorescence intensity (MFI) per second, normalized considering the MFI mean measured 30 seconds before adding the stimuli. Data are representative of at least three independent experiments.

Taking advantage of a fluorescent biosensor that allows to trace specifically IP_4 ($Ins(1,3,4,5)P_4$) within the cells (Sakaguchi et al., 2010), we managed to monitor the levels of IP_4 in D1 cells during time. The 15F-IP_4 biosensor uses the pleckstrin homology (PH) domain of the general receptor for phosphoinisitides 1 (GRP1), modified to have high affinity and selectivity for IP_4 ($Ins(1,3,4,5)P_4$). The fluorescence intensity of the 15F-IP_4 biosensor decreases upon binding with IP_4 (Sakaguchi et al., 2010). We found that, following LPS stimulation, IP_4 levels tend to increase in the cells in an ITPKB-dependent manner (Fig.31).

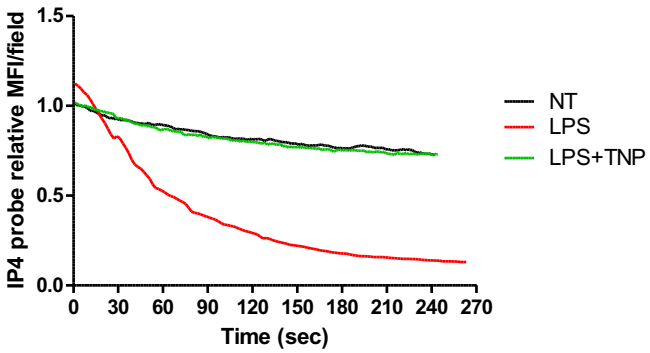


Figure 31 Real-time IP_4 measurement in D1 cells.
D1 cells were transfected with 15F-IP_4 fluorescent probe and then plated on a nunc glass bottom dish and let adhere. Cells were analysed by confocal microscopy. Stimuli were added after 20 seconds of recording: LPS (1 µg/ml), TNP (20 µM).

In accordance with the involvement of ITPKB in Ca^{2+} mobilization in response to LPS, STED microscopy unveiled IP_3R3-ITPKB co-localization of in D1 cells which is observable both in untreated and LPS-treated conditions (Fig.32).

To conclude, these data indicate that ITPKB-dependent IP_4 production is necessary for LPS-induced Ca^{2+} mobilization in DCs.

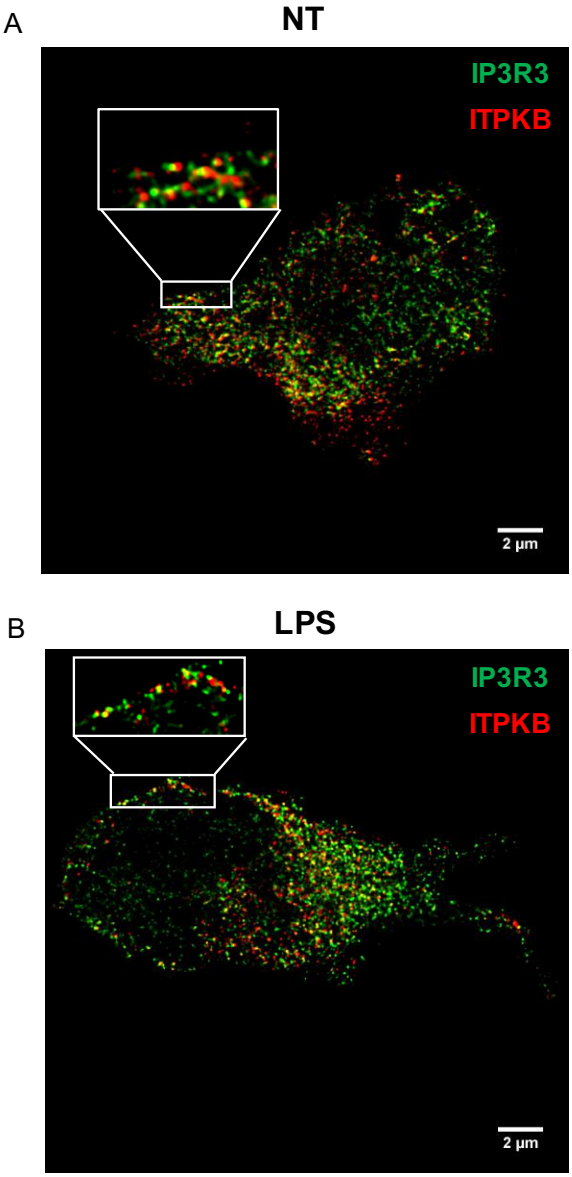
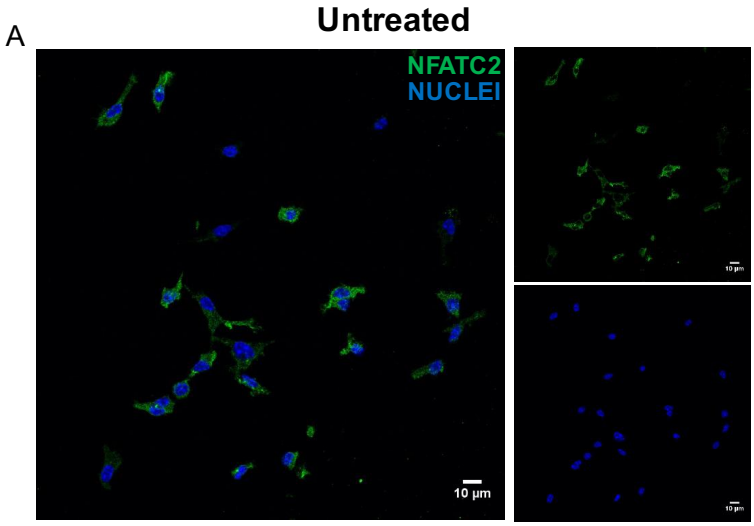


Figure 32 Analysis of IP_3R3-ITPKB colocalization in D1 cells using STED microscopy.

D1 cells were seeded onto grass coverslips. After staining the cells were analysed by STED microscopy. The images show: IP_3R3 (green) and ITPKB (red). Yellow dots show signal colocalization. Images are representative of at least three independent experiments.

2.5 Ins(1,3,4,5)P_4 directs LPS-induced CD14-mediated NFAT pathway activation in dendritic cells.

Since Ca^{2+} mobilization is the crucial event leading to NFAT translocation and we demonstrated that ITPKB is required for Ca^{2+} entry in LPS-stimulated DCs, we further explored the contribute of ITPKB on NFAT pathway activation both *in vitro* and *in vivo*. We focused on NFATc2 which is the most expressed isoform in DCs (Zanoni et al., 2009). Firstly, we evaluated the effect of ITPKB inhibition on LPS-induced CD14-mediated NFATc2 nuclear translocation in D1 cells (Fig.33). We observed that, the ITPKB inhibitor affects nuclear translocation of NFATc2 in D1 cells in response to LPS exposure (Fig.33C).



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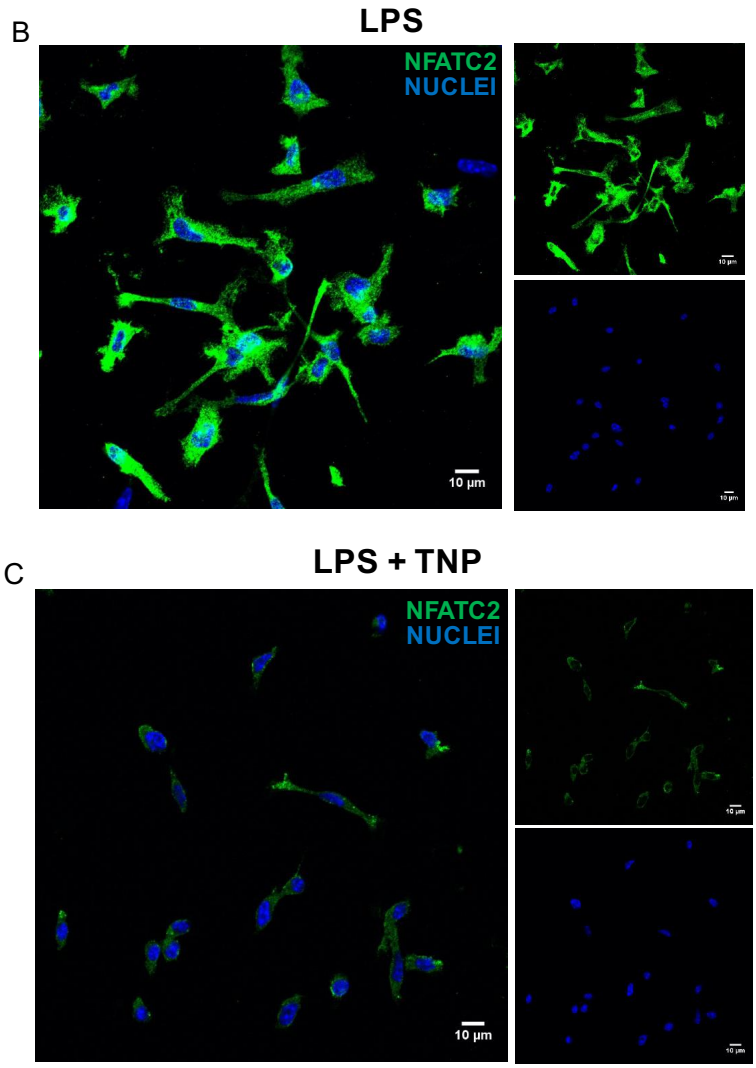
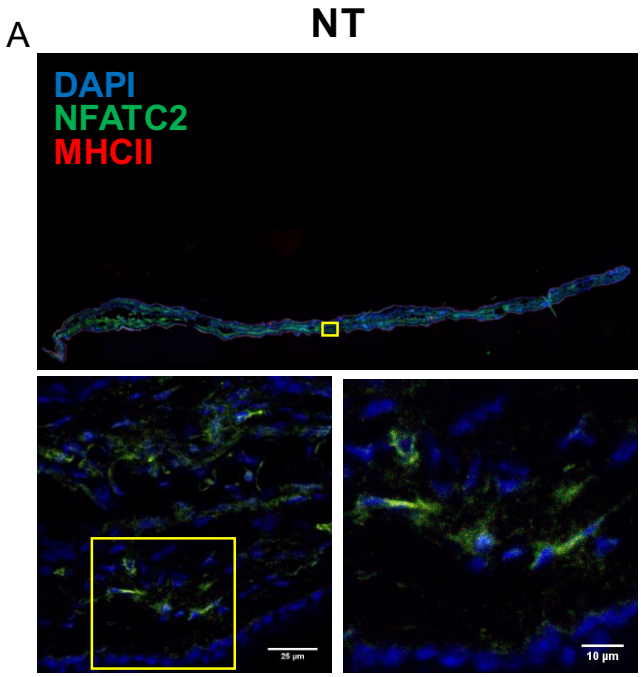


Figure 33 Effect of ITPKB inhibition on LPS-induced nuclear translocation of NFATc2 in D1 cells.

D1 cells were seeded onto glass coverslips and let adhere. (A) Untreated cells. (B) Cells stimulated with LPS (1 µg/ml) for 1 hour and 30 minutes. (C) Cells pre-treated with TNP (20 µM) for 40 minutes and then with LPS (1 µg/ml) for 1 hour and 30 minutes. After staining, cells were analysed by confocal microscopy. Nuclei (blue), NFATc2 (green). Images are representative of at least three independent experiments.

A comparable result was obtained *in vivo*. Indeed, we examined NFATc2 nuclear translocation in MHC II^{+} cells in the ear skin (Fig.34) and we found that, while intradermal LPS injection fosters NFATc2 nuclear localization (Fig.34B), the co-administration of ITPKB inhibitor blocks the translocation of NFATc2 (Fig.34C).



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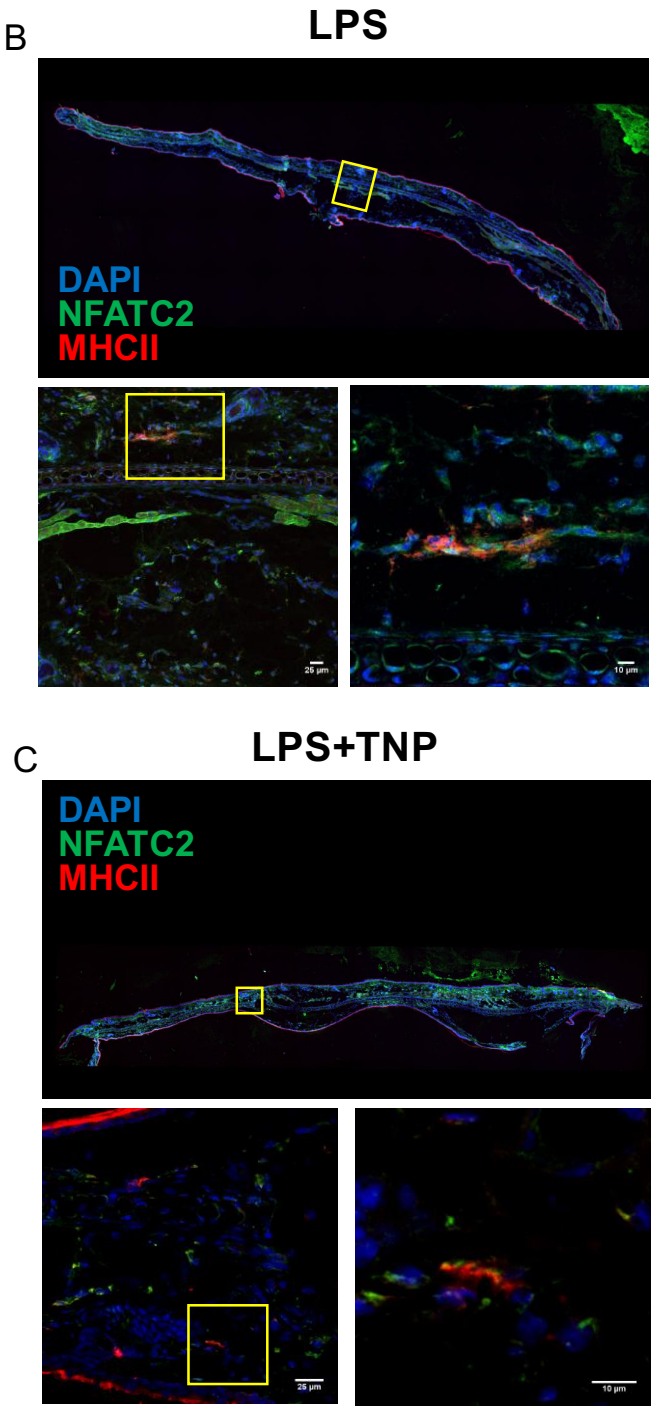

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Figure 34 Effect of ITPKB inhibition on nuclear translocation of NFATc2 in MHC II$^+$ skin cells *in vivo*.

Histological sections of ear skins showing: MHC II (red), NFATc2 (green) and nuclei (blue). Ear skin sections were prepared from mice injected subcutaneously with: PBS (30 μl), as control (A, NT); LPS (10 μg in 30 μl of PBS) (B); LPS + TNP (10 μg LPS + 200 μM TNP in 30 μl of PBS).

To further explore the role of ITPKB in the CD14-NFAT pathway we took advantage of an *in vivo* model of LPS-induced CD14-NFAT-mediated edema formation. In mice, subcutaneous LPS administration has been shown to increase vascular permeability leading to edema formation and that this relies on DC-derived NFAT-dependent PGE_2 production (Zanoni et al., 2012). Our in vitro results suggest that in this model ITPKB should be activated. We therefore evaluated if ITPKB inhibition could interfere with vascular leakage induced by LPS. To this purpose, mice were injected subcutaneously in the ear with LPS and vascular permeability was measured by Evans Blue dye extravasation (Fig.35A, B). Phosphate-buffered saline (PBS) and PGE_2 were used as negative and positive control of extravasation, respectively. We observed that the co-injection of LPS and ITPK inhibitor prevents LPS-induced vascular leakage (Fig.35A, B). Previous findings demonstrated that the transcriptional target of NFAT involved in the regulation of vascular permeability is *Ptges1* (Zanoni et al., 2009), which codes the protein mPGES-1, an enzyme necessary for PGE_2 biosynthesis. Consistently, we found that the ITPK inhibitor prevents LPS-induced upregulation of mPGES-1 mRNA (Fig.35C).

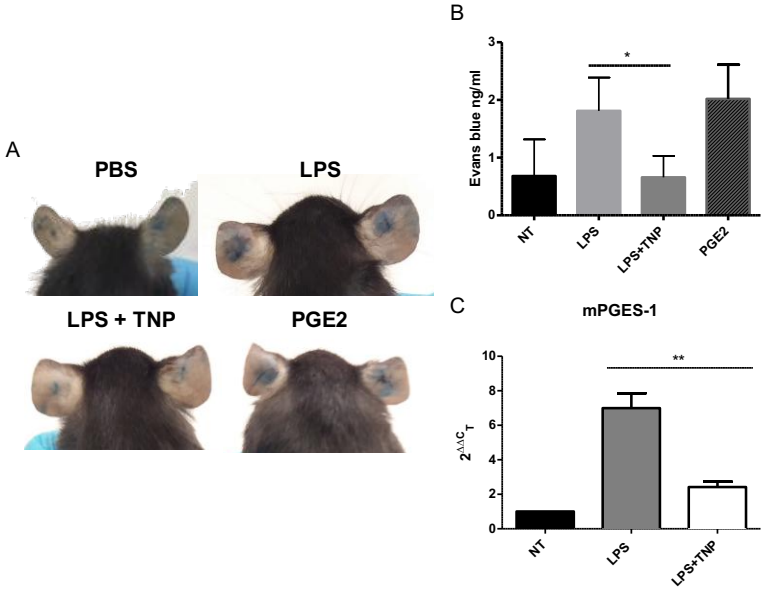

Figure 35 Effect of ITPKB inhibition on LPS-induced vascular permeability *in vivo*.

(A) WT C57BL/6 mice were injected subcutaneously in the ears with PBS (20 µl), LPS (10 µg in 20 µl PBS), LPS+TNP (10 µg LPS + 200 µM TNP in 20µl PBS), PGE2 (50 µg in 20 µl PBS). Immediately after, 100 µl of Evans blue were injected intravenously in the tail. Mice were sacrificed either 30 minutes or 3 hours later. (B) Ears explanted from WT C57BL/6 mice treated as described in (A) were processed, then the absorbance of Evans blue was read using a spectrofluorometer and converted into ng/ml for quantification. (C) quantitative reverse transcription PCR from ear lysates deriving from WT C57BL/6 mice treated as described in (A) and sacrificed 3 hours after injection. Data are expressed as fold change in comparison with the untreated control (NT). Indicated values represent means of three independent experiments (± SD). For each experiment one mouse was injected per condition. Statistics was performed using t-test: * $p < 0,05$; ** $p < 0,01$.

Taken together, these data indicate that the inhibition of ITPKB, thus of IP_4 production, blocks the activation of the NFAT signalling pathway and reduces vascular permeability *in vivo*, which is directed by NFAT activation in DCs.

2.6 The kinetics of CD14 internalization and the extent of IP_3R3-CD14 colocalization could be responsible for the lack of Ca^{2+} mobilization in macrophages

The stimulation with LPS does not elicit Ca^{2+} mobilization in macrophages (Zanoni et al., 2009). Our data demonstrated that IP_3R3 colocalization with CD14 within lipid rafts is necessary for LPS-induced Ca^{2+} entry in DCs, therefore we wondered whether IP_3R3 is expressed at the plasma membrane of macrophages and if it colocalizes with CD14, as well as in DCs. For these experiments we used primary murine bone marrow-derived macrophages (BMDMs). By using TIRF microscopy, we found that IP_3R3 is actually expressed at the plasma membrane of BMDMs (Fig.36).

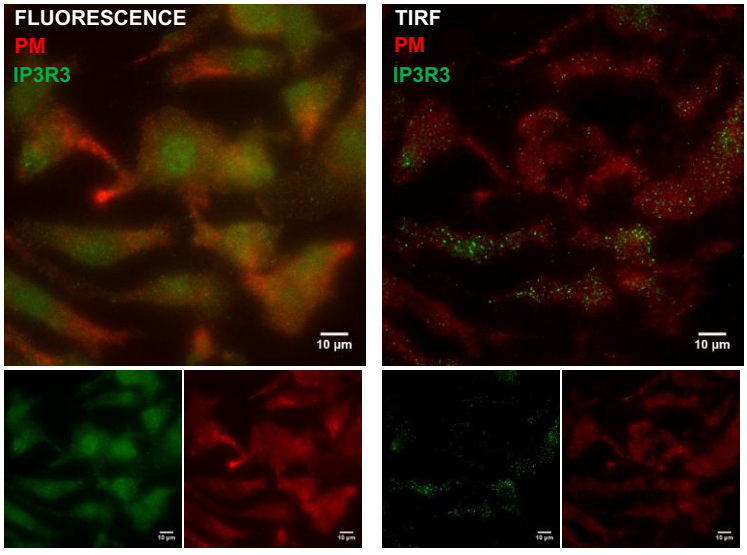

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Figure 36 IP_3R3 localization at the plasma membrane of BMDMs revealed by TIRF microscopy.

BMDMs were seeded onto glass coverslips. After staining, cells were analysed by TIRF microscopy. Images show: the plasma membrane (PM) (red), IP_3R3 (green). The same field was analysed by fluorescence microscopy (on the left) and by TIRF microscopy (on the right).

We compared the expression of IP_3R3 at the plasma membrane between BMDMs and D1 cells by using the PLA assay (Fig.37). TLR4-CD14 colocalization, following LPS stimulation, was measured as positive control, whereas CD14-deficient BMDMs were used as negative control. We found that, compared to D1 cells, BMDMs show fewer spots of colocalization between IP_3R3 and CD14 (Fig.37). Therefore, it is possible that the lower extent of IP_3R3-CD14 colocalization is involved in preventing LPS-induced Ca^{2+} entry in macrophages.

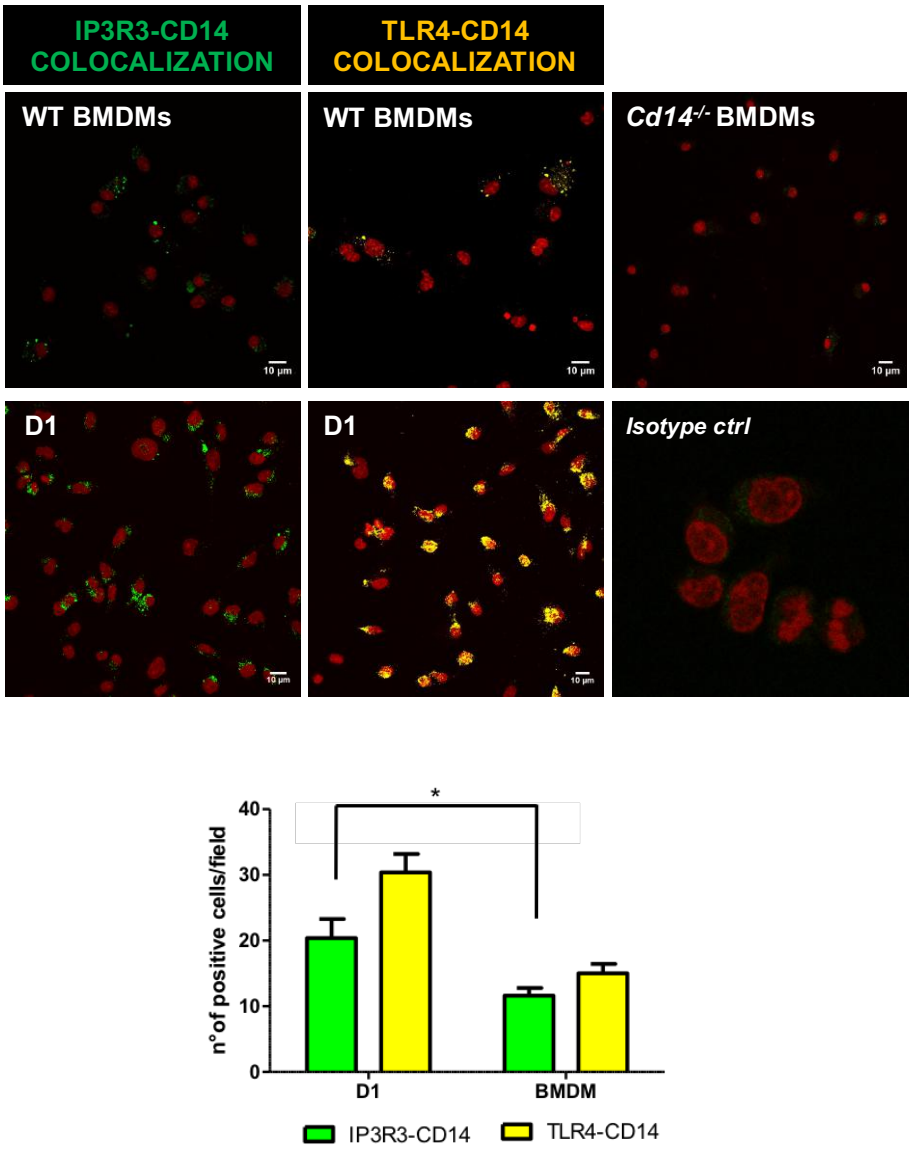

Figure 37 Quantitative analysis of IP_3R3-CD14 colocalization between BMDMs and D1 cells using proximity ligation assay.

Cells were either seeded onto glass coverslips and PLA assay was performed. The cells were analysed by confocal microscopy. TLR4-CD14 colocalization after LPS treatment was used as positive control, whereas *Cd14*$^{-/-}$ BMDMs and isotype control for D1 cells were used as negative control. Each spot represents a colocalization signal.

Figure 37 continued on next page.

Figure 37 continued.
The images show: nuclei (red), IP_3R3-CD14 colocalization (green) and TLR4-CD14 colocalization (yellow). Indicated values represent means of three independent experiments (± SD). Ten fields (containing about 50 cells/field) from three independent experiments were quantified. Statistics was performed using t-test: * $p < 0,05$; ** $p < 0,01$.

Following LPS stimulation, CD14 mediates the relocation of the entire LPS receptor complex to the endosome, which is the site for the initiation of the TRAM-TRIF pathway that regulates type I IFNs production (Zanoni et al., 2011). It has been shown that in immortalized BMDMs, the stimulation with LPS leads to the internalization of 80 % of the CD14 within two minutes (Tan et al., 2015). We investigated whether BMDMs and D1 cells have different kinetics of internalization and, indeed, we observed that CD14 is internalized quicker in BMDMs than in D1 cells (Fig.38).

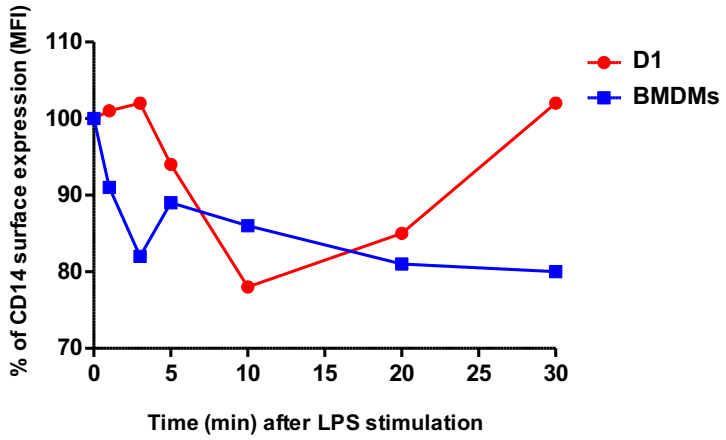

Figure 38 CD14 internalization in BMDMs and D1 cells.
BMDMs (blue line) and D1 cells (red line) were either left untreated or stimulated with LPS (1 µg/ml) for 1, 3, 5, 10, 20, 30 minutes.
Figure 38 continued on next page.

Figure 38 continued.
Immediately after, cells were stained for CD14 and analysed using a flow cytometer. The percentage of CD14 at the cell surface was measured as MFI normalized on the untreated control. Data are representative of at least three independent experiments.

This observation suggests that the increased rapidity of CD14 internalization can participate in preventing LPS-induced Ca^{2+} mobilization in macrophages. To verify this hypothesis, we measured Ca^{2+} mobilization in response to LPS after inhibiting clathrin-dependent endocytosis, both in BMDMs and in D1 cells (Fig.39). Indeed, it has been previously shown that LPS induces CD14 internalization in a clathrin-dependent manner (Zanoni et al., 2011). For these experiments we used dynasore an inhibitor of dynamin activity, which is essential for clathrin-dependent endocytosis. After dynasore exposure, it is possible to measure a Ca^{2+} transient in LPS-treated BMDMs (Fig.39A). The inhibition of endocytosis increased LPS-induced Ca^{2+} entry in D1 cells (Fig.39B), thus confirming that CD14 internalization limits Ca^{2+} mobilization.

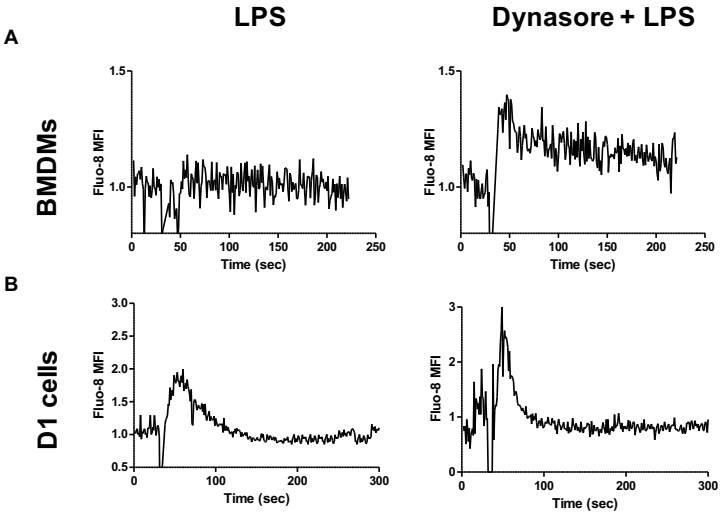

Figure 39 Effect of endocytosis inhibition on Ca^{2+} mobilization in BMDMs and D1 cells after LPS exposure.

BMDMs (A) and D1 cells (B) were either left untreated (graphs on the left) or pre-treated with dynasore (80 μM, 30 minutes) (graphs on the right) and then stained with Fluo-8 AM. Cells were analysed using a flow cytometer. After 30 seconds of recordings cells were stimulated with LPS (1 μg/ml). Intracellular Ca^{2+} concentration variations were quantified as mean fluorescence intensity (MFI) per second, normalized considering the MFI mean measured 30 seconds before adding the stimuli. Data on BMDMs result from the mean of three independent experiments. Data on D1 cells are representative of at least three independent experiments.

Taken together, these results indicate that the lack of Ca^{2+} mobilization in LPS-treated macrophages, could be due to the rapid kinetics of CD14 internalization and, likely, to the reduced level of colocalization between IP_3R3 and CD14.

2.7 In human $CD1c^+CD14^+$ DCs IP_3R3 and CD14 colocalize at the plasma membrane and IP_4 regulates LPS-induced CD14-mediated NFAT pathway activation

Finally, we wondered whether the signalling pathway responsible for LPS-induced NFAT activation in mouse DCs is conserved in human DCs. For these experiments we focused on the $CD1c^+CD14^+$ DC population that has been recently described as a new human DC subtype circulating in the blood, named DC3 (Villani et al., 2017). $CD1c^+$ DCs have been separated from peripheral blood mononuclear cells (PBMCs) of healthy donor by magnetic separation (MACS), these cells represent 0,1 up to 1 % of total PBMCs. A minor proportion, about 20 %, of these cells are $CD14^+$, the DC nature of these cells was verified by co-expression of high levels of CD36 and CD163 as described in literature (Villani et al., 2017). By using TIRF microscopy, we found that in $CD1c^+CD14^+$ DCs IP_3R3 is expressed at the plasma membrane (Fig.40), as observed in D1 cells.

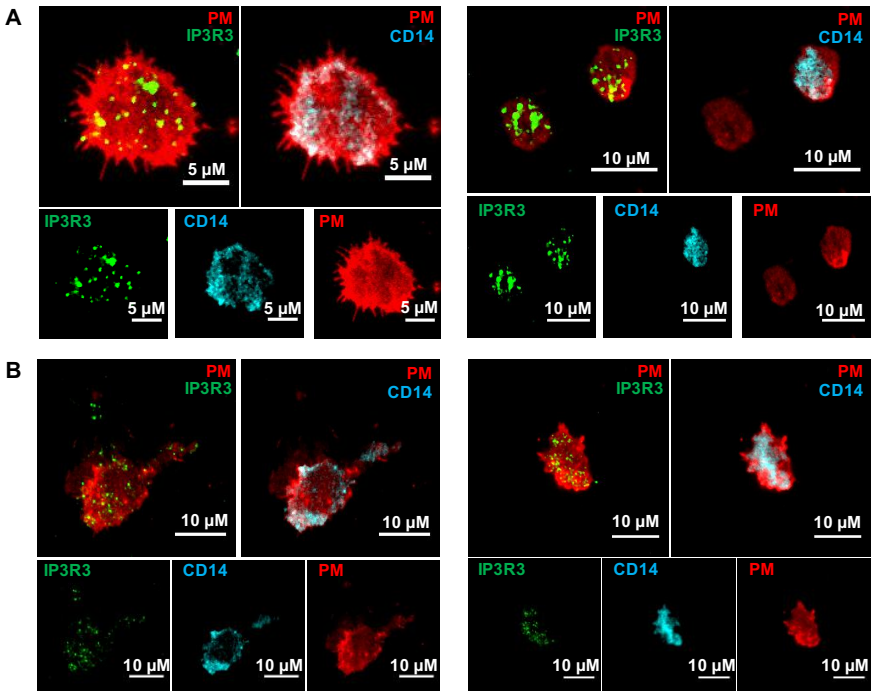

Figure 40 IP_3R3 localization at the plasma membrane of human $CD1c^+CD14^+$ DCs revealed by TIRF microscopy.
$CD1c^+$ DCs were isolated from PBMCs of healthy donors ($CD1c^+$ DCs from donor A and donor B) and seeded on glass coverslips. After staining, cells were analysed by TIRF microscopy. Images show: the plasma membrane (PM) (red), IP_3R3 (green), and CD14 (cyan). Images are representative of at least three independent experiments.

Moreover, consistently with our previous data on D1 cells, IP_3R3 colocalizes with CD14 in $CD1c^+CD14^+$ DCs, as revealed by STED microscopy (Fig.41).

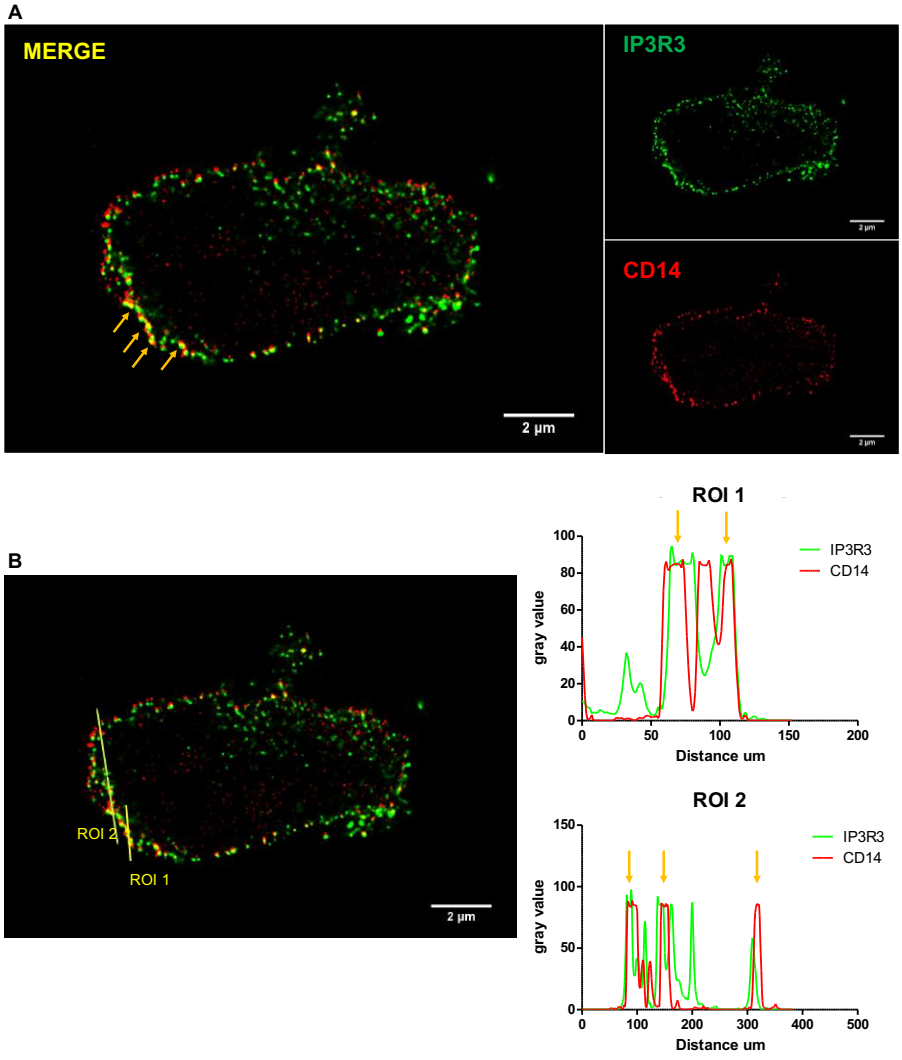

Figure 41 Analysis of IP_3R3-CD14 colocalization in human $CD1c^+CD14^+$ DCs using STED microscopy.

$CD1c^+$ DCs were isolated from PBMCs, seeded onto glass coverslips and let adhere. After staining, cells were analysed by STED microscopy. Images show: IP_3R3 (green) and CD14 (red). Yellow dots show signal colocalization. Representative plots comparing the pixel intensity profiles analysis of CD14 (red line) and IP_3R3 (green line) are also displayed. Coincident peaks indicate co-localization (yellow arrows). Images are representative of at least three independent experiments.

Furthermore, we observed that IP_3R3 colocalizes with ITPKB in $CD1c^+CD14^+$ DCs (Fig.42).

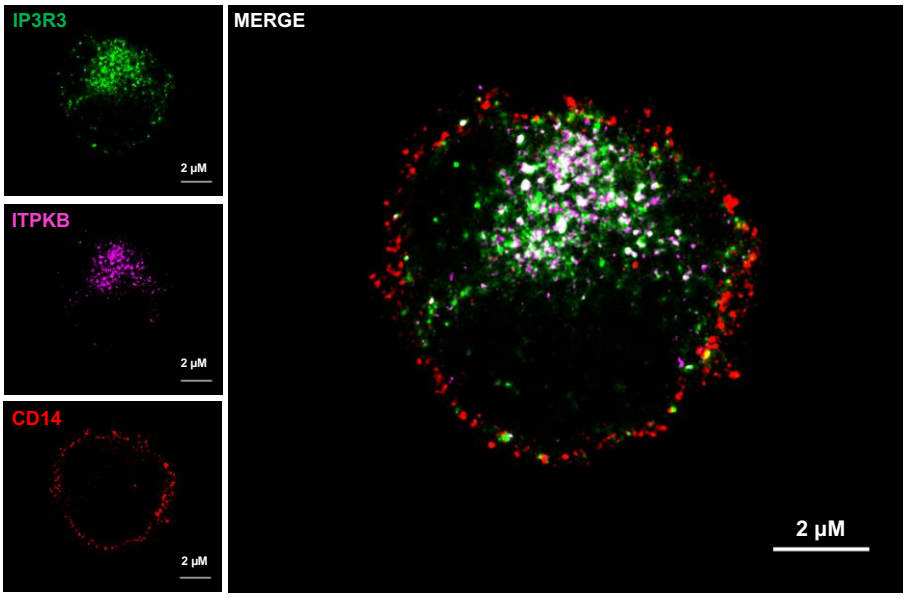

Figure 42 Analysis of IP_3R3-ITPKB colocalization in human $CD1c^+CD14^+$ DCs using STED microscopy.
$CD1c^+$ DCs were isolated from PBMCs, seeded onto glass coverslips and let adhere. After staining, cells were analysed by STED microscopy. Images show: IP_3R3 (green), ITPKB (magenta) and CD14 (red). White dots show IP_3R3-ITPKB colocalization. Images are representative of at least three independent experiments.

Since the low percentage of $CD1c^+CD14^+$ DCs makes Ca^{2+} measurement technically difficult to perform, we analysed NFAT nuclear translocation in response to LPS as direct consequence of Ca^{2+} mobilization. We found that the stimulation of $CD1c^+CD14^+$ DCs with LPS induce NFAT nuclear translocation and that this is prevented by ITPKB inhibition, suggesting that IP_4 production is required for Ca^{2+} mobilization in human DC3 (Fig.43), as well as in D1 cells.

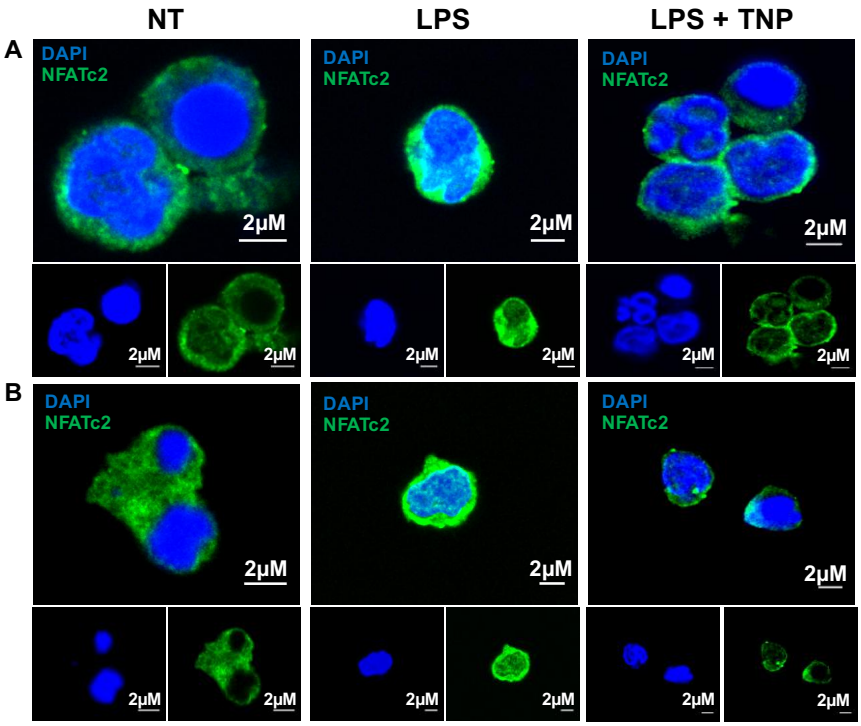

Figure 43 Effect of ITPKB inhibition on LPS-induced nuclear translocation of NFATc2 in human CD1c$^+$CD14$^+$ DCs.
CD1c$^+$ DCs were isolated from PBMCs of healthy donors (CD1c$^+$ DCs from donor A and from donor B), CD14$^+$ cells were sorted and seeded onto glass coverslips and let adhere. Cells were either left untreated or stimulated with LPS (1 µg/ml) for 1 hour and 30 minutes. For ITPKB inhibition, cells were pre-treated with TNP (20 µM) for 40 minutes and then with LPS (1 µg/ml) for 1 hour and 30 minutes. After staining, cells were analysed by confocal microscopy. Nuclei (DAPI, blue), NFATc2 (green). Images are representative of at least three independent experiments.

Accordingly, ITPKB inhibition impairs mPGES-1 mRNA induction in response to LPS exposure, which is known to depend on NFAT regulation (Fig.44).

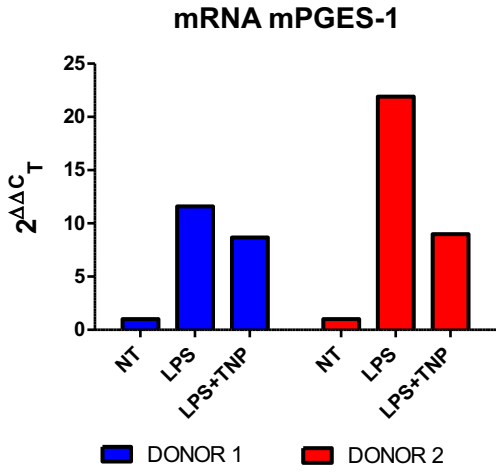

Figure 44 Effect of ITPKB inhibition on LPS-induced NFAT-dependent upregulation of mPGES-1 mRNA.
$CD1c^+$ DCs were isolated from PBMCs of healthy donors ($CD1c^+$ DCs from donor 1 and from donor 2) and plated in multi-well plate. Cells were left untreated or were stimulated with LPS (1 μg/ml) for 4 hours; for ITPKB inhibition, cells were pre-treated with TNP (20 μM) for 40 minutes. The RNA was extracted from cell lysates and quantitative reverse transcription PCR of mPGES-1 mRNA was performed. Data are expressed as fold change in comparison with the untreated control and a housekeeping gene was used for normalization.

These data indicate that, also in human $CD1c^+CD14^+$ DCs, LPS exposure leads to NFAT activation and that this pathway is regulated by IP_4 that likely directs Ca^{2+} mobilization through plasma membrane IP_3R3.

Methods

D1 cell line. The mouse splenic DC line, D1 (Winzler et al., 1997), were cultured as previously described by Granucci et al., 2001.

***Ex vivo* splenic dendritic cells.** Splenic DCs were purified from the spleen of WT C57BL/6 mice. The spleen was enzymatically digested with collagenase for 30 minutes and then mechanically dissociated. The suspension was filtered using 70 μm cell strainer and red blood cells lysis was performed. Splenocytes were resuspended in IMDM containing 10 % heat-inactivated fetal bovine serum (FBS), 2 mM L-Glutamine, 100 IU penicillin, 100 μg/ml streptomycin. $CD11c^+$ cells were isolated by magnetic separation using CD11c MicroBeads by Miltenyi Biotec (Order no. 130-052-001).

Bone marrow-derived dendritic cells (BMDCs). BMDCs were cultured as previously described by Zanoni et al., 2009; Zanoni et al., 2009b.

Bone marrow-derived macrophages (BMDMs). BMDMs were cultured as previously described by Zanoni et al., 2009; Zanoni et al., 2009c.

Human CD1c (BDCA-1)$^+$ dendritic cells. Human $CD1c^+$ DCs were isolated from peripheral blood mononuclear cells (PBMCs) extracted from buffy coats of healthy donors (provided by Niguarda hospital blood bank, Milan) by density gradient centrifugation using

Ficoll (Ficoll-Paque PLUS, GE Helthcare). Briefly, blood was stratified on Ficoll in 3:4 ratio and centrifuged at 400xg for 30 minutes without brake. PBMCs were collected, washed with serial centrifugation with decreasing speed (from 400xg to 200xg) and then BDCA-1 cells were purified using MACS beads according to the manufacturer's instructions (Miltenyi Biotec, Order no. 130-090-506).

Chemicals. TLR4-grade smooth LPS (*Escherichia coli* 055:B5) was purchased from Enzo Life Sciences. 2-APB, YM-58483, EGTA, ATP, dynasore, Evans blue, β-cyclodextrin and TNP were purchased from Sigma-Aldrich. Water soluble probenecid, inhibitor of organic-anion transporters of the plasma membrane, Fluo-4 AM, Fluo-8 AM, Fura red, were purchased from Invitrogen.

Calcium measurement with confocal microscopy. The intracellular Ca^{2+} concentration was determined by a fluorometric ratio technique. Cells were incubated with Fluo-4 AM (3 μM) and Fura red AM (6 μM) at 37°C for 1 hour in complete medium containing probenecid (2,5 mM). Cells were washed twice with complete medium containing probenecid and then incubated at 37°C for 30 minutes. For the experiments in which 2-APB, YM-58483 and EGTA were used, they were added to the medium 10 minutes before starting measuring. The stimulus, LPS (1 μg/ml) or ATP (100 μM), was added after 30 seconds of measurement. Images were acquired with Leica TCS SP2 confocal microscope using 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 second intervals. The change in Ca^{2+} signal was determined by the change in the ratio of Fluo-4 to Fura red fluorescence.

Calcium measurement with flow cytometer. For some experiments, intracellular Ca^{2+} concentration was detected with flow cytometry. Cells were loaded with 3 μM Fluo-4 AM or Fluo-8 AM and incubated for 15 min at 37 °C in completed medium containing probenecid (2,5mM). Cells were then washed with complete medium plus probenecid and immediately analyzed by flow cytometry (FACSCalibur, BD Bioscences). For the experiments in which CD14 endocytosis was inhibited, cells were treated with dynasore (80 μM) at 37°C for 30 minutes and then stained as described. Calcium chelation was performed by adding EGTA to the media 20 minutes before FACS analysis, while inhibition of ITPKB was performed by adding TNP (20 μM) to the cells 5 minutes before starting measuring. Samples were excited with a laser at 488 nm, and the intensity of the fluorescence between 505-550 nm was measured as the Fluo-4 or Fluo-8 signal. The change in Ca^{2+} signal was determined by the change in Fluo-4 or Fluo-8 fluorescence, quantified as mean fluorescence intensity (MFI) per second and normalized considering the MFI mean measured 30 seconds before the addition of the stimuli: LPS (1 μg/ml) or ATP (100 μM). Data were analysed with FlowJo software.

Immunocytochemistry. Cells were seeded onto glass coverslips and let adhere. For plasma membrane staining, cells were incubated with WGA-Alexa Fluor 555 conjugated (3 μl/ml) (Invitrogen) for 10 minutes

directly into the culture media. Cells were fixed with 4 % paraformaldehyde for 10 minutes at room temperature (RT), washed twice in PBS and treated for 1 minute with NH_4Cl (50 mM). After two washes in PBS, blocking was performed with Fc block antibody (CD16/32) (BD Bioscences) diluted 1:100 in 5 % bovine serum albumin (BSA) (w/v) in PBS at RT for 30 minutes. Samples were washed twice, then the staining with primary antibodies, diluted in blocking solution, was performed overnight at 4°C. After two washes in PBS the samples were stained with secondary antibodies, diluted in PBS and applied for 45 minutes at RT. When required, nuclei were stained with DAPI (1 µl/ml). Finally, sample were washed in PBS and then mounted with FluorSave Reagent (Calbiochem). Images were acquired at Nikon $A1^+$ for confocal microscopy, at Leica TCS SP2 for TIRF microscopy, whereas STED microscopy was performed using Leica TCS SP8. Images analysed with ImageJ software.

For confocal microscopy, the following antibodies were used:

Primary Antibody	**Secondary Antibody**
Mouse anti-human/mouse cross-reactive IP_3R3 (BD Bioscences) 1:50	Anti-mouse Alexa Fluor 488 (Thermo Scientific) 1:250
Goat anti-mouse IP_3R2 (Santa Cruz Biotechnology) 1:100	Anti-goat Alexa Fluor 488 (Thermo Scientific) 1:250
Goat anti-mouse IP_3R1 (Santa Cruz Biotechnology) 1:100	Anti-goat Alexa Fluor 488 (Thermo Scientific) 1:250
Rabbit anti-mouse Calnexin (Abcam) 1:250	Anti-rabbit Alexa Fluor 647 (Thermo Scientific) 1:250
Goat anti-mouse CD14 (Santa Cruz Biotechnology) 1:100	Anti-goat Alexa Fluor 647 (Thermo Scientific) 1:250
Rabbit anti-mouse/human cross-reactive NFATc2 (immunoGlobe) 1:200	Anti-rabbit Alexa Fluor 488 (Thermo Scientific) 1:250

For TIRF microscopy, the following antibodies were used:

Primary Antibody	Secondary Antibody
Mouse anti-human/mouse cross-reactive IP_3R3 (BD Biosciences) 1:50 for mouse cells, 1:100 for human cells	Anti-mouse Alexa Fluor 488 or 647 (Thermo Scientific) 1:250
Goat anti-mouse IP_3R2 (Santa Cruz Biotechnology) 1:100	Anti-goat Alexa Fluor 488 (Thermo Scientific) 1:250
Goat anti-mouse IP_3R1 (Santa Cruz Biotechnology) 1:100	Anti-goat Alexa Fluor 488 (Thermo Scientific) 1:250
Mouse anti-human CD14 (eBioscience) 1:500	Anti-mouse Alexa Fluor 488 (Thermo Scientific) 1:250

For STED microscopy, the following antibodies were used:

Primary Antibody	Secondary Antibody
Mouse anti-human/mouse cross-reactive IP_3R3 (BD Biosciences) 1:50 for mouse cells, 1:100 for human cells	Anti-mouse Alexa 594 (Invitrogen) 1:500 or Anti-mouse ATTO 488 (Rockland) 1:500
Goat anti-mouse CD14 (Santa Cruz Biotechnology) 1:100	Anti-goat ATTO647N (Rockland) 1:3000
Mouse anti-human CD14 (eBioscience) 1:500	Anti-mouse Alexa Fluor 488 (1:250)
Rabbit anti-human/mouse cross reactive ITPKB (Thermo Scientific) 1:100	Anti-rabbit ATTO647N (Rockland) 1:500

Proximity ligation assay (PLA). The PLA assay was performed using Duolink kit (Sigma-Aldrich) (Cat. No. DUO92014). Cells were either spun onto glass coverslips (for *ex vivo* splenic DCs) or seeded and cultured overnight onto glass coverslips (D1 cells, BMDCs, BMDMs). Then the cells were fixed with 4 % paraformaldehyde for 10 minutes at RT and washed twice with PBS. Blocking was performed with 2 % BSA

(w/v) in PBS for 30 minutes at RT. Samples were incubated overnight at 4°C with two alternative couples of primary antibodies: goat anti-mouse CD14 (Santa Cruz Biotechnology, clone T-19) (1:100) and mouse anti-human/mouse cross-reactive IP_3R3 (BD, clone 2/IP_3R-3) (1:50) or goat anti-mouse CD14 (Santa Cruz Biotechnology, clone T-19) (1:100) and rabbit anti-mouse TLR4 (Santa Cruz Biotechnology, clone M-300) (1:50), diluted in blocking solution. After two washes in buffer A in gently agitation for 5 minutes, cells were incubated with complementary PLA probe (anti-mouse PLUS and anti-goat MINUS or anti-goat MINUS and anti-rabbit PLUS) diluted in blocking solution; the incubation was performed at 37°C for 1h. Subsequently, sample were washed twice with buffer A in gently agitation for 5 minutes and then the ligase solution was added for 30 minutes at 37°C. Cells were again washed twice with buffer A in gently agitation for 2 minutes. Then, the polymerase solution and the fluorescent dNTPs were added to the sample for 100 minutes at 37°C. Cells were stained with DAPI 1 μg/ml for 5 minutes at RT and finally mounted using FluorSave Reagent (Calbiochem). Images were acquired with Nikon $A1^+$ confocal microscope system and analyzed with ImageJ software.

Immunohistochemistry. WT C57BL/6 mice were injected subcutaneously in ear with the indicated stimuli for 1h and 30 minutes. Explanted skin ears were embedded in optimal cutting temperature freezing media (Bio-Optica). Sections (5 μm) were cut on a cryostat, adhered to a Superfrost Plus slide (Thermo Scientific)

The samples were fixed with acetone for 5 minutes and blocked with 5 % BSA in PBS for 1 hour at RT. Sections were then washed twice in

PBS and stained overnight at 4°C with primary antibodies: anti-mouse MHC II Alexa Fluor 488 conjugated (BioLegend) and rabbit anti-NFATc2 (immunoGlobe), diluted 1:100 in blocking solution. After two washes in PBS sections were stained with anti-rabbit Alexa Fluor 555 (Thermo Fisher Scientific) diluted 1:250 in blocking solution. After washing in PBS, nuclei were stained with DAPI 1 μg/ml and finally washed again and mounted using FluorSave Reagent (Calbiochem). Images were acquired with Nikon A1$^+$ confocal microscope system and analyzed with ImageJ software.

IL-2 and TNFα production measurements. The concentration of IL-2 and TNFα were assessed by ELISA kits purchased from R & D Systems and eBioscience, respectively.

siRNA transfection. 7 x 10^5 D1 cell were plated onto 6-well plate and transfected with siRNAs mix (riboxx) specific either for IP_3R3 or GAPDH; the final concentrations of siRNA transfected were 1 to 10 nM. The transfection, was achieved using INTERFERin (Polyplus) (Cat. No. 409-10) according to manufacturer's protocol.

IP_4 measurement. 5 x 10^5 D1 cells were let in suspension in 1 ml of medium without serum and transfected with 10 μg of 15F-IP_4 fluorescent probe (described in Sakaguchi et al., 2010 and kindly provided by Professor Takashi Morii). Pulsin (Polyplus) (Cat. No. 501-04) was used for IP_4 probe transfection following the manufacturer's protocol. 2,5 x 10^5 cells were then plated on nunc glass based dish (Thermo Scientific) in 2 ml of complete medium and let adhere for 2

hours. Imaging was performed on a Nikon A1+ confocal microscope system 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 IP_4 signal. Images were acquired at 1.2 s intervals.

CD14 internalization. D1 cells were stimulated with LPS (1 μg/ml) for 1, 3, 5, 10, 20 and 30 minutes. Afterwards, untreated D1 cells and LPS-treated cells were collected by centrifugation (200xg for 5 minutes) and then stained for 30 minutes at 4 °C with anti-mouse CD14 PE (eBioscence, clone Sa2-8) diluted 1:200 in PBS. Samples were acquired at Gallios flow cytometer (Beckman Coulter) and analysed with FlowJo software.

Quantitative reverse transcription polymerase chain reaction. Pieces of ears or cells sample were homogenized in TRIzol reagent, then RNA was extracted using quick-RNA MiniPrep or microPrep kits (Zymo research, cat. no. R2050 and R1051, respectively). Single-strand complementary DNA (cDNA) was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, cat. no. 4368814). The NanoDrop (Thermo Scientific) was used to titer mRNA. cDNA amplification was performed using the TaqMan Gene Expression Master Mix (Applied Biosystems, cat. no. 4369016) and TaqMan probes: *Itpr1* Mm_00439907_m1, *Itpr2* Mm_00444937_m1, *Itpr3* Mm_01306070_m1, *Il-2* Mm_00434256_m1, *Tnfα* Mm_004432258_m1, *Ptges1* Mm_00452105_m1, *Gapdh* Mm_99999915_g1, *PTGES1* Hs_0115610_m1, *RPS13* Hs_01011487_g1, *18s*, Mm_03928990_g1. Relative mRNA expression

was calculated using the ΔC_t method, using 18S or RPS13 as a reference gene.

Western blot. Pro-Q Diamond Phosphoprotein Enrichment Kit (Invitrogen, cat. no. P33358) was used to isolate phosphoproteins from cellular extracts. Total proteins were quantified using a bicinchoninic acid assay (Quantum protein assay kit, Euroclone, cat. no. EMP014500). 40 μg of protein were ran on a 10 % polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Thermo Scientific) for 90 minutes at 260 mA at 4°C. Afterwards, the nitrocellulose membrane was incubated for 1 hour with blocking solution (Tris-buffered saline, TBS + 0,1 % Tween + 5 % skim milk powder) and then incubated overnight at 4°C with rabbit anti-ITPKB (Thermo Scientific) diluted 1:750 in TBS + 5 % BSA. The nitrocellulose membrane was incubated for 1 hour with horseradish peroxidase (HRP)-conjugated secondary antibody and then developed using an enhanced chemiluminescence substrate reagent (Thermo Scientific). Subsequently, the nitrocellulose membrane was stripped for 20 minutes, incubated with blocking solution and then with anti-phospho-threonine (Cell Signaling Technology), diluted 1:750 in TBS + 5 % BSA. Finally, the nitrocellulose membrane was incubated with HRP-conjugated secondary antibody and developed as described above.

Vascular permeability assay. WT C57BL/6 mice were injected subcutaneously in the ear with different stimuli as indicates in the figure. Immediately after, 100 μl of Evans blue were injected intravenously in

the tail. 30 minutes later mice were sacrificed, ears were explanted and incubated O/N in formalin (Sigma Aldrich) at 55°C. Evans blue in formalin solution was quantified by measure the absorbance at 650 nm. Known concentration of Evan blue were used to create a calibration curve in order to convert absorbance values into ng/ml values.

Animals. WT and *Cd14*$^{-/-}$ C57BL/6 mice were housed under pathogen-free conditions. All experiments were carried out in accordance with the relevant laws and institutional guidelines.

Statistical analysis. Means were compared by t-tests. Statistical significance was defined as $P < 0.05$. Sample size for each experimental condition is provided in the figures and the respective legends.

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Chapter. 3 Final considerations

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3.1 Summary

Innate myeloid cells are equipped with a repertoire of PRRs which allow them to sense the presence of microbes or microbial products. Among PRRs, TLRs are the best characterized and play a critical role in the regulation of the immune response (Akira et al., 2006). TLR4, together with CD14 and MD2, forms the multi-receptor complex that is responsible for the recognition of LPS (Triantafilou & Triantafilou, 2002), the major component of the outer membrane of Gram-negative bacteria.

CD14 is a GPI-anchored protein widely expressed on DCs and macrophages. CD14 is extremely important in the modulation of cellular responses to LPS. Indeed, it concentrates the LPS signal, mediates the relocation of the LPS-receptor complex to the endosome (Zanoni et al., 2011) and, specifically in DCs, it is able to initiate a TLR4-independent signalling pathway that culminates on the activation of the transcription factor NFAT (Zanoni & Granucci, 2013).

In DCs, CD14 engagement by LPS leads to the activation of src family kinase (SFK) that activate PLCγ2. Somehow, a rapid monophasic Ca^{2+} influx follows PLCγ2 activation. The increase in the intracellular Ca^{2+} concentration induces the activation of calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, which dephosphorylates NFAT allowing the exposition of the nuclear localization signal, thus its translocation from the cytoplasm into the nucleus (Zanoni et al., 2009). Once in the nucleus, NFAT cooperates with transcriptional partners to regulate gene expression. In DCs the activation of NFAT regulates the production of IL-2 (Granucci et al.,

2001) and PGE_2 (Zanoni et al., 2012) and the induction of apoptosis (Zanoni et al., 2009).

In the present work, we focused on the mechanism of LPS-induced CD14-dependent Ca^{2+} mobilization that leads to NFAT activation in DCs. Moreover, we proposed an explanation for the lack of CD14-NFAT pathway activation in macrophages in response to LPS.

The results of this study revealed that, in mouse DCs, IP_3R3 is expressed not only in the intracellular compartments, such as the ER, but also at the plasma membrane and interestingly, it colocalizes with CD14 in lipid rafts. We found that, Ca^{2+} mobilization in LPS-stimulated DCs is due to a direct Ca^{2+} influx from the extracellular space, that relies on IP_3R3 and requires IP_4, rather than IP_3, as second messenger. Indeed, the silencing of IP_3R3 or the inhibition of ITPKB, which is the kinase implicated in the IP_3 to IP_4 conversion in DCs, abolishes Ca^{2+} entry and NFAT activation. Conforming to our *in vitro* results, the inhibition of ITPKB *in vivo*, prevents the activation of NFAT, thus reducing vascular permeability, which depends on NFAT-driven PGE_2 production by DCs.

The key results obtained in mouse DCs were confirmed in a newly discovered $CD1c^+CD14^+$ DC subtype present in the blood (Villani et al., 2017). In these cells, coherently with the previous data, IP_3R3 can be also found at the plasma membrane where it colocalizes with CD14. ITPKB has been shown to colocalize with IP_3R3 and to be required for LPS-induces NFAT pathway activation in $CD1c^+CD14^+$ DCs. Even if further elucidation would be required, our data indicates a pivotal role

for IP_4 in LPS-induced CD14-dependent NFAT activation also in $CD1c^+CD14^+$ DCs.

Differently from DCs, although IP_3R3 is also expressed at the plasma membrane of macrophages, it shows a low level of colocalization with CD14. Besides, upon LPS stimulation, CD14 internalization occurs more rapidly in macrophages compared to DCs. These data can explain, at least in part, the absence of CD14-NFAT pathway activation in macrophages in response to LPS.

3.2 Conclusions and future perspectives

DCs are innate immune cells that act as antigen-sampling "sentinels" of the immune system and stand out as the APC par excellence. By means of PRRs, DCs are able to detect antigens of different origin, which are then processed and presented to naïve T cells to initiate and orchestrate complex immune responses (Banchereau & Steinman, 1998). Among PRRs, TLRs are widely expressed on DCs and are key player in the immune responses. TLR4, together with CD14 and MD2, recognizes the LPS of Gram-negative bacteria. In DCs, CD14 acts not only as co-receptor for LPS recognition but is also able to signal independently of TLR4, regulating the Ca^{2+}-dependent activation of the transcription factor NFAT (Zanoni et al., 2009).

In the present work, we described that, LPS stimulation of mouse DCs induces Ca^{2+} mobilization that relies on CD14 engagement

and is directed by the ITPKB-dependent generation of IP_4. In particular, IP_4 opens IP_3R3 placed at the plasma membrane, which are colocalized with CD14 at the level of lipid rafts. The opening of IP_3R3 allows an inward flux of Ca^{2+} from the extracellular space and leads to NFAT activation.

Although further elucidation would be required, we confirmed that IP_4-mediated NFAT activation in response to LPS also occurs in human $CD1c^+CD14^+$ DCs, a newly described blood DC subtype (Villani et al., 2017).

These results shed light on a previously unknown mechanism of NFAT activation, directed by a transient monophasic increase in intracellular Ca^{2+} concentration. In most other leukocytes NFAT activation is regulated by SOCE mechanism, characterized by a first increase in intracellular Ca^{2+} concentration, due to the opening of IP_3Rs at the ER, followed by a sustained Ca^{2+} entry through CRACs at the plasma membrane. The observation that SOCE mechanism is dispensable for NFAT activation in LPS-stimulated DCs is in accordance with data showing that Stim1 and 2, key proteins of SOCE, are not necessary for several DCs functions (Vaeth et al., 2015).

Although IP_3Rs are generally considered receptors located at the ER, there is evidence indicating that they can be present in several membrane structures (Taylor & Tovey, 2010). The expression of IP_3Rs at the plasma membrane has been already pointed out in different cell types such as human T cells (Khan et al., 1992a), olfactory neurons of different species (Fadool & Ache, 1992; Restrepo et al., 1992) and rat liver cells (Dargemont et al., 1988). More recently, it has been shown that IP_3R3 can be expressed at the plasma membrane of chicken B cells

and can participate in Ca^{2+} mobilization (Dellis et al., 2006), however the meaning of this mechanism of Ca^{2+} entry remained vague.

A crucial point in the LPS-induced CD14-NFAT pathway in DCs is that IP_3R3 must colocalize with CD14 for the Ca^{2+} mobilization to happen. In this context, lipid rafts are active players, since the alteration of their architecture affects IP_3R3-CD14 colocalization and completely abolishes Ca^{2+} entry following LPS. This role of lipid rafts is in accordance with data in literature describing lipid rafts as optimal sites for dynamic protein-protein interactions that favour signal transduction (Simons & Toomre, 2000). The co-clustering of IP_3R3 with CD14 is, likely, a way for DCs to facilitate the activation of IP_3R3 even at low concentration of the second messenger. This co-clustering has been shown for IP_3Rs with TCR (deSouza et al., 2007) and with PLC-coupled receptors in neurons, such as B2 bradykinin receptors, where the co-clustering forms "signalling micro-domains" to ensure efficient IP_3R activation by creating locally elevated IP_3 concentration (Delmas et al., 2002).

In DCs, IP_3 conversion into IP_4 further optimizes the availability of the second messengers. IP_4 has been shown to have greater affinity for sialylated IP_3Rs of the plasma membrane in comparison to the intracellular IP_3Rs (Khan et al., 1992b). Indeed, when IP_4 production is blocked, by inhibiting ITPKB, intracellular channels are preferentially opened, consistently with the higher affinity of IP_3 for intracellular IP_3Rs. However, the increase in the intracellular Ca^{2+} concentration that arises from the opening of IP_3Rs at the ER is not sufficient to elicit SOCE and NFAT activation, likely due to the low IP_3 concentration.

The relevance of the colocalization between IP_3R3 and CD14, which also depends on CD14 permanence at the cell surface, is confirmed by our data on macrophages. In LPS-stimulated macrophages no Ca^{2+} mobilization can be measured (Zanoni et al., 2009). We described that macrophages display a lower extent of IP_3R3-CD14 colocalization and a higher kinetics of CD14 internalization upon LPS stimulation, as compared to DCs. These data may explain the reason for the cell type-specificity of the CD14-NFAT pathway.

The CD14-NFAT pathway has been shown to direct important processes in LPS-stimulated DCs, such as IL-2 (Granucci et al., 2001) and PGE_2 (Zanoni et al., 2012) production and the control of DCs apoptosis (Zanoni et al., 2009). IL-2 and PGE_2 are important inflammatory mediators. IL-2 is required for fully activation of NK cells (Granucci et al., 2004), T cell priming (Granucci et al., 2001) and T_{reg} homeostasis (Kulhankova et al., 2012; Zanoni & Granucci, 2011). PGE_2 is a vasoactive eicosanoid involved in inducing vasodilation and increased vascular permeability that sustain edema formation during inflammation (Legler et al., 2010).

The identification of the signalling cascade that regulates NFAT activation in DCs allows to target NFAT in a cell type-specific manner. Our data suggest that ITPKB inhibitors can be taken in consideration as a new target for anti-inflammatory therapies aimed at inhibiting specific DC functions.

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