The University of Milano Bicocca

THE PhD PROGRAM DIMET

The PhD Program in Translational and Molecular Medicine (DIMET) is an inter-departmental project between the School of Medicine and the Faculty of Science, organized by the University of Milano-Bicocca.

DIMET - Dr. Renato Ostuni - A.A. 2009-10

PhD

PROGRAM IN TRANSLATIONAL AND MOLECULAR MEDICINE

DIMET

UNIVERSITY OF MILANO-BICOCCA
SCHOOL OF MEDICINE AND FACULTY OF SCIENCE

The role of CD14-NFAT pathway in the regulation of innate immune functions

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XXIII CYCLE
ACADEMIC YEAR
2009-2010

Alla mia famiglia, a cui devo tutto il mio percorso. So che l'unico modo che ho per ringraziarvi è rendervi orgogliosi… Io ci provo…

A Vania, il mio angelo custode.

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Chapter 1

Introduction - Section 1.

Immunobiology of Dendritic Cells and Macrophages

The decision to activate an immune response against invading microorganisms is made by cells of the innate immune system, such as macrophages and dendritic cells (DCs), which are quiescent until they encounter inflammatory stimuli. They use a repertoire of non-clonal receptors to transmit the information of what is present in the environment (quality and quantity) and the duration of this signal. This complex activity is regulated by transcriptional responses involving the differential expression of thousands of genes and the integration of a number of signaling pathways. The active transcriptional response leads to the acquisition of diverse DC and macrophages functional phenotypes, orchestrating the appropriate immune responses. Following activation with inflammatory stimuli DCs lose their antigen uptake capacity, but become particularly efficient in antigen processing and acquire the ability to migrate to the T cell areas of secondary lymphoid organs where they present antigens to naïve T cells to initiate primary adaptive responses. Finally, after the achievement of their effector functions, DCs undergo terminal differentiation and die by apoptosis[1]. On the other hand, macrophages contribute to the initiation of the inflammatory process in the tissue and, diversely from DCs, do not die following activation. Tissue-resident macrophage survival is, indeed, crucial for

the resolution of inflammation. Late-activated macrophages produce anti-inflammatory mediators, such as some lipid mediators and TGFβ, that extinguish the inflammatory process and start tissue repair.

Conventional DCs in homeostatic conditions

DCs can be subdivided in conventional DCs (cDCs), cells having phenotypic and functional characteristics of DCs, and pre-DCs, which require a further step of development to acquire phenotypic and functional DC features[2,3]. Conventional DCs are of hematopoietic origin and are found in many different organs and tissues, including the heart, liver, thyroid, pancreas, bladder, kidney, ureter, gut, lungs, skin and mucosa. Fully developed DCs have also been observed in the circulatory networks of the body, including blood and afferent lymphatic vessels, and in lymphoid organs. DCs display a high degree of plasticity within organs and lymphoid tissues and their effector functions are often regulated by the tissue microenvironment[4]. However, two broad groups of DCs can be defined on the basis of their homeostatic location: those located in non lymphoid tissues, such as skin, mucosa and internal organs (tissue-resident DCs), and those found in lymphoid organs, blood and afferent lymphatic vessels (lymphoid tissue-resident DCs).

Mucosal and skin DCs perform a sentinel function, continually scanning the environment for the presence of incoming pathogens. Different subtypes of conventional DCs have been identified in both tissues. In the mouse the skin contains two populations of langerin$^+$

DCs: the epidermal Langerhans cells (LCs) and the dermal DCs (DDCs). The dermis also contains migratory LCs and langerin$^-$ DCs. LCs and langerin$^+$ DDCs can be distinguished on the basis of CD103 expression (negative for LCs and positive for DDCs). Six different DC populations, all expressing CD11c, have been identified in skin draining lymph nodes: CD8$^+$DEC205$^+$ resident DCs, CD8$^-$DEC205$^-$ (both CD4$^-$ and CD4$^+$) resident DCs, CD8^{low}CD205^{int} DCs (migratory dermal DCs) and CD8^{low}DEC205^{high} DCs (migratory Langerhans cells, LCs)[5,6]. LCs are almost immobile in the skin, displaying only occasional repetitive dendrite movements known as dendrite surveillance extension and retraction cycling habitude (dSEARCH)[7]. By contrast, DDCs are highly motile, frequently finding their way into the dermal interstitial space[8], facilitating encounters with pathogens and favoring an immediate response to danger signals[8]. Under homeostatic conditions, DDCs and LCs undergo continuous, slow migration to the draining lymph nodes[9]. A few motile immature LCs loaded with tissue-specific antigens (*e.g.* components of apoptotic keratinocytes and melanocytes, including melanin[10]) continually make their way to the draining lymph node. These cells are presumably required to maintain the tolerance of T cells to self-antigen[7,11,12]. Indeed, recent data have suggested that skin DCs may be involved in tolerogenic responses. For instance, stronger contact sensitivity responses have been shown to occur in mice in the absence of langerin$^+$ cells[13]. Moreover, in a transgenic mouse model in which a membrane-bound form of ovalbumin is selectively expressed by keratinocytes, DDCs and LCs have been shown to cross-present ovalbumin to ovalbumin-

specific $CD8^+$ T-cells and to induce the deletion of these cells in steady-state conditions[14].

A large number of DCs, the lamina propria (LP) DCs, are present immediately beneath the epithelium in the mucosa of the small intestine in rodents and humans. Resident DCs are also present in the Peyer's Patches and isolated lymphoid follicles (ILFs). It has been suggested that LPDCs are responsible for maintaining tolerance to commensal bacteria, food and self-antigens in homeostatic conditions[15]. Indeed, the continuous migration of LPDCs transporting apoptotic bodies to mesenteric lymph nodes has been described in rats[16]. Moreover, the adoptive transfer of LPDCs recovered from OVA-fed mice to naive mice reduces delayed-type hypersensitivity reactions[17]. A possible explanation for the immunoregulatory function of these cells in the gut relates to their ability to constitutively express interleukin-10 (IL-10) and type I interferons (IFNs), but not IL-12[17]. In the large intestine, very few DCs are present in the LP, these cells being mostly concentrated in the subepithelial regions and ILFs. In steady-state conditions, human DCs in the LP of the colon produce large amounts of IL-10, presumably exerting immunoregulatory functions in the gut through the control of T-cell responses[18]. Thus, a deregulation of DC function in the colon may contribute to the development of inflammatory diseases, such as inflammatory bowel disease (IBD)[18]. Mesenteric lymph nodes (MLNs) contain different populations of DCs originating from three different sources: the PP, the LP and blood precursors. $CCR7^+$ LPDCs, which migrate to MLNs via afferent lymphatic vessels, play a fundamental

role in inducing tolerance to orally applied soluble antigens[19]. An important new function has recently been attributed to conventional $CD11c^{high}$ DCs residing in lymphoid organs in mice: the control of homeostatic hematopoiesis. In the absence of $CD11c^{high}$ DCs, an increase in secondary lymphoid organ cell content is observed, due to a robust expansion of $CD11b^{+}$ myeloid cell population comprising $Gr1^{int}$ monocytes and $Gr1^{high}$ neutrophils[20]. In addition to the described conventional DC subtypes, a DC population producing large amounts of type I IFN following microbial infections, named IFN-producing plasmacytoid DCs (pDCs), has been described in mouse blood and lymph nodes[21]. In steady state conditions, these cells can be classified as preDC[2]. Upon activation they acquire not only the capacity to produce large amount of type I IFNs but also some DC antigen processing and presentation properties[22]. Compared to mouse DCs, human DC phenotypes have been in general less well typed. DCs expressing CD11b, CD11c and CD4 have been described in the spleen and tonsils[23]. Human IFN-producing pDCs have been described as $CD45RA^{+}CD123^{+}$ and $CD11c^{-}$[24].

Conventional DCs in inflammatory conditions

Upon activation in response to an inflammatory stimulus (exogenous or endogenous), migratory and lymphoid tissue-resident DCs display a decrease in phagocytic activity, accompanied by an increase in processing capacity and an upregulation of MHC and costimulatory molecules at the cell surface[6]. Migratory DCs also acquire the

capacity to migrate to lymph nodes, and spleen marginal zone DCs begin to mobilize to the T-cell zone. Cells reaching the T-cell zone in the lymph nodes and spleen have the most suitable phenotype for T-cell activation[2]. This migration to the T-cell areas of the lymph nodes and spleen is regulated by CCR7 expression[25], which renders DCs responsive to CCL19 and CCL21. In the absence of CCR7, DCs within the spleen are mostly located in the marginal zone and outside the white pulp[26,27]. Another molecule has recently been associated with the regulation of DC migration in mice: the MHC class II-associated invariant chain or CD74[28]. LPS-activated, CCR7-expressing DCs move faster in the absence of CD74, suggesting that antigen processing and migration are jointly regulated. This coregulation may ensure the efficient detection and uptake of the antigen by DCs in a defined space before migration to the lymphoid organs[28]. Once activated by inflammatory stimuli, DCs acquire the ability to prime first NK cells and then T cells[29].

Early events, priming of NK cells

NK cells were originally described as cells with spontaneous cytotoxic activity, but it has been consistently shown that a fraction of the NK cells purified from human peripheral blood or mouse spleen has no cytotoxic function[30]. Thus, NK cells are in a resting state in homeostatic conditions and their effector functions may be induced by accessory cells. Cytokines released by DCs, such as type I IFNs, IL-15 and IL-18, affect NK cell functions, such as IFNβ production[31], migration[32], cytotoxic function[33], and proliferation[34]. In particular, IL-

18 seems to play an important role in enabling NK cells to migrate to secondary lymphoid organs, where they interact with DCs[32]. Human NK cells exposed to IL-18 display rapid CCR7 induction and an increase in responsiveness to CCL21, with no increase in lytic activity. Once in the lymph nodes, these NK cells produce large amounts of IFNβ in response to IL-12, TNFα and IL-2. It has been shown in a mouse model in which DC ablation can be induced that DC-derived IL-15 and its presentation in trans are required to prime resting NK cells recruited to the draining lymph nodes after peripheral Toll-like receptor stimulation[35]. The first reports of IL-2 production by activated DCs in the hours immediately following stimulation[1] led to suggestions of a possible physiological role of this early production of IL-2 in NK cell activation. Following activation with Escherichia coli, DC-derived IL-2 is required for the activation of NK cells, in terms of IFNβ production, both in vitro and in vivo. By contrast, type I IFNs and other unknown factors are required for optimal NK cell cytotoxicity[36]. Monocyte/DC-derived IL-2 has also been shown to play a role in NK cell activation in humans. Newman *et al.*[37] demonstrated that the capacity of human NK cells to produce IFNγ in response to stimulation with Plasmodium falciparum-infected red blood cells was strictly dependent upon cell contact and IL-2/type I IFN-mediated signals derived from monocytes and myeloid DCs.

Late events, priming of T cells

The requirement of DCs for T-cell activation in lymph nodes was initially demonstrated in vivo for $CD8^+$ T lymphocytes. Mice

temporarily deprived of $CD11c^+$ DCs cannot mount efficient specific $CD8^+$ T-cell responses to infections with the intracellular bacterium Listeria monocytogenes, the parasite Plasmodium yoelii, LCMV or antigen immunization[38,39]. It was subsequently shown that $CD11c^{high}$ cDCs are required for the priming of both $CD8^+$ and $CD4^+$ T-cells in the spleen, whereas these cells are dispensable for $CD4^+$ T-cell priming in lymph nodes. Indeed, within lymph nodes, pDCs can replace cDCs for this function[40]. In the absence of cDCs, pDC-mediated $CD4^+$ T-cell priming occurs, but is not associated with $CD8^+$ T-cell activation, indicating that pDCs prime $CD4^+$ T-cell-dominated immune responses only[40].

DCs can enter the lymph node via the blood or lymph[41]. Studies in mice have shown that DCs arriving in the blood are initially found clustered together, close to high endothelial venules (HEV). However, their distribution subsequently changes, such that, one day after reaching the lymph node, DCs are distributed throughout the T-cell area[42]. The process by which DCs reach the lymph node via the lymph and undergo redistribution has been well characterized in the mouse skin compartment. Following skin inflammation, DDCs are the first cells to migrate to the draining cutaneous lymph node, where they are first detected after 24 hours, their levels peaking after two days. LCs migrate to the lymph nodes slightly later, their numbers peaking in the draining lymph nodes four days after the stimulus[43]. LCs and DDCs are localized in different areas of the lymph node. Within the T-cell area, DDCs are localized in the outer paracortex, just beneath the B-cell area, whereas LCs are localized in the inner paracortex,

suggesting that these two DC populations may have different functions and encounter different T-cell subsets[43]. In cutaneous viral infections, such as herpes virus infections, the antigen is transported to the draining lymph node principally by DDCs, which transfer the antigen to resident $CD8^+$ DCs in the cortex, leading to the activation of $CD8^+$ T-cell responses through cross-presentation[44]. It has recently been shown, in the mouse, that antigen processing and presentation by both lymphoid resident and migratory DCs is required for $CD4^+$ T-cell priming after skin antigen administration. Lymphoid tissue-resident DCs initiate antigen-specific T-cell activation within the draining lymph nodes, whereas migratory DC antigen presentation is required to induce T-cell proliferation[45].

The different DC subtypes present in the lymph nodes, whether lymphoid tissue-resident or migratory, display considerable diversity in terms of specialization for antigen presentation. For instance, $CD8^-$ $33D1^+$ DCs preferentially present antigens in association with MHC class II molecules and these cells increase the efficiency of the class II processing machinery when activated. Thus, these cells are specialized in $CD4^+$ T-cell activation[46]. By contrast, $CD8^+DEC205^+$ cells efficiently present antigens to both $CD4^+$ and $CD8^+$ T cells[46]. These cells are the only cells able to prime $CD8^+$ T-cell responses in the lymph node[38]. There is at least one other important difference between the two DC subtypes. $CD8^+DEC205^+$ DCs primarily trigger Th1 responses, whereas $CD8^-33D1^+$ DCs induce the production of either IL-4 alone or both IFNγ and IL-4 by T-cells [47,48]. IL-12 has been shown to be an essential cofactor for skewing toward Th1 responses

in various experimental systems[49]. $CD8^+DEC205^+$ DCs produce much larger amounts of IL-12 than $CD8^-33D1^+$ cells[50]. However, these cells are thought to induce Th1 skewing via the membrane-bound cofactor CD70, a member of the tumor necrosis factor family[51], in the presence of TLR4 and TLR3 agonists, rather than through IL-12.

Apoptosis of DCs

Following activation and the achievement of terminal differentiation, DCs progress toward apoptotic death. Once DCs have completed their antigen presentation function, they are eliminated by apoptosis, to dampen immune responses and to free the spaces they occupy after migration[52]. Significant DC accumulation has been observed in patients with autoimmune lymphoproliferative syndrome type II, who have a defect in apoptosis[53], and in *lpr* mice[54]. These findings suggest that defects in DC apoptosis may be responsible for the development of autoimmunity. The mechanisms of DC apoptosis may be FAS-dependent or FAS-independent[52]. Two different mouse models have been generated for investigating the importance of DC apoptosis in immune system homeostasis. In the first, DC apoptosis is inhibited by constitutive expression of the baculoviral caspase inhibitor, p35. This model consists of transgenic animals expressing p35 exclusively in DCs, under the control of the CD11c promoter. No marked DC or lymphocyte population expansion is observed in young transgenic mice, whereas significant expansion of the DC population

is observed in aged mice, indicating that the inhibition of DC apoptosis in homeostatic conditions leads to the accumulation of these cells. A remarkable decrease in the rate of DC apoptosis is particularly evident in transgenic animals following the in vivo administration of strong inflammatory stimuli, such as complete Freund's adjuvant, resulting in a higher efficiency of T-cell priming. Consistent with this observation, DC-p35 mice spontaneously developed autoimmune manifestations, such as anti-nuclear antibody production, in old age[55]. Similar results were obtained by inducing conditional DC-specific FAS ablation[56]. In this case, several manifestation of autoimmunity, including anti-nuclear antibodies, hyperimmunoglobulinemia, splenomegaly and histological modifications to the spleen and liver, were apparent at 12 months of age. Thus, FAS ablation exclusively in DCs is sufficient to cause autoimmunity[56].

Macrophages in homeostatic conditions

The term "macrophage" was first used by Ellie Metchnikoff when he inserted a rose thorn into a starfish larva and observed ameboid phagocytosis around the foreign matter. Macrophages were discovered more than 100 years ago and we now know a considerable amount about them, although many of their features remain to be clarified. The ancient unicellular eukaryotic entameba, a protozoan parasite displaying active phagocytosis, is a prototype

macrophage[57]. This simple cell undergone considerable modification during evolution and the macrophages of multicellular eukaryotic organisms are complex cellular systems with diverse tissue distributions and cell phenotypes. They have complex and, in some cases, opposite functions in homeostasis, innate and adaptive immunity. Macrophages are derived from myeloid precursors in the bone marrow, spleen and fetal liver. Macrophages precursors (monocytes) enter the bloodstream and are transported into the peripheral tissues, where they encounter a large number of different agents (including cytokines, chemokines, hormones and other molecules), which determine the functional and phenotypic characteristics of the fully differentiated cell, the tissue-resident macrophage[58]. During an inflammatory response, some of the features of tissue-resident macrophages change and these cells, together with newly recruited monocytes destined to become inflammatory macrophages, contribute to the innate and subsequent adaptive immune response.

Monocyte heterogeneity

Monocytes originate in the bone marrow from a common myeloid precursor that also gives rise to neutrophils. They enter the bloodstream, in which they circulate for several days before entering peripheral tissues to replenish the pool of tissue-resident macrophages[59]. Murine monocytes can be identified as $F4/80^+$ $CD11b^+$ cells and can be subdivided into two subpopulations as a function of their expression profiles for CCR2, CD62L, Ly6C, and

CX3CR1. One subset is $CCR2^{+}$, $CD62L^{+}$, $Ly6C^{+}$, and $CX3CR1^{low}$ (corresponding to "classic" human monocytes, which are $CD14^{high}$ $CD16^{-}$), whereas the second subset expresses CX3CR1 at high levels but no Ly6C, CCR2 or CD62L (corresponding to $CD14^{+}$ $CD16^{+}$ human monocytes)[60]. CCL2 (also known as MCP1) is released in inflamed tissues and induces the recruitment of $CCR2^{+}CX3CR1^{low}$ monocytes. This subset of monocytes is therefore considered to be the "inflammatory" subset[60,61]. $CCR2^{+}CX3CR1^{low}$ monocytes do not survive long after adoptive transfer and are difficult to detect in peripheral tissues. However, as expected, they are strongly recruited to tissues in which inflammation has been experimentally induced [60]. The $CCR2^{-}CX3CR1^{high}$ subset persists for longer periods after transfer and can replenish, at least partially, the tissue-resident populations of macrophages and some of the DCs. Following the experimental depletion of monocytes[62], the first population to reappear is the $CCR2^{+}$ (inflammatory) subset, suggesting that these monocytes are generated before those of the $CCR2^{-}$ subset. Thus, it is currently thought that $CCR2^{+}CX3CR1^{low}$ monocytes are released into the bloodstream, where their phenotype is altered such that the cells are in an intermediate $CCR2^{+}CCR7^{+}CCR8^{+}Ly6C^{mid}$ state. Both these populations can respond to inflammatory stimuli and are recruited to inflamed tissue, where they can differentiate into inflammatory macrophages or DCs. In the absence of inflammation, monocytes enter tissues and become tissue-resident monocytes ($CCR2^{-}$ $CX3CR1^{high}Ly6C^{-}$) able to replenish populations of tissue

macrophages. They can also give origin to a subpopulation of DCs, the monocyte-derived DCs[63].

Tissue-resident macrophages

Macrophages play a key role in controlling tissue homeostasis, by controlling the metabolic activity of adipocytes, regulating the turnover of epithelial cells, tissue repair and the engulfment of apoptotic debris[63,64]. Cells dying purposefully by programmed death processes are taken up by mechanisms that do not induce an inflammatory response. Apoptotic cells provide “eat me signals” ensuring that they are efficiently and rapidly engulfed and preventing the release of noxious agents. Macrophages may recognize different apoptotic cell structures, including, in particular, the exposed anionic phospholipid phosphatidylserine, which is normally located in the inner leaflet of the plasma membrane but is exposed during apoptosis[65]. The release of pro-inflammatory cytokines is inhibited in macrophages that have ingested apoptotic cells[66]. Apoptotic cells are thus eliminated in a silent manner during normal tissue homeostasis. Macrophages constitute a highly heterogeneous population of cells. This heterogeneity reflects the specialized functions of these cells in different peripheral tissues and anatomical locations within a given tissue, such as in the spleen or central nervous system. The macrophages present in adults are thought to be derived from circulating monocytes, which continually replenish the population of tissue-resident cells. Local proliferation has also been shown to play a major role in maintaining macrophage homeostasis in non-

inflammatory conditions, whereas the dependence on monocyte precursor recruitment to support tissue macrophage replenishment becomes more marked during inflammatory processes.

Osteoclasts. Osteoclasts are probably the best example of a differentiated lineage of tissue-resident macrophages. Bones undergo a continual process of bone resorption and synthesis known as remodeling. Bone remodeling is the principal metabolic process regulating bone structure and osteoclasts play a key role in this process. An osteoclast is a tissue-specific macrophage polykaryon created by the differentiation of monocyte/macrophage precursor cells at or near the surface of the bone. Two hematopoietic factors are both necessary and sufficient for osteoclastogenesis: the TNF-related cytokine RANKL and the polypeptide growth factor M-CSF[67]. The end-stage cell is clearly differentiated and displays cell body polarity. However, macrophages can be rapidly induced to express osteoclast genes and to reabsorb bone. Osteopetrotic (*op/op*) mice have a genetic defect resulting in a total absence of functional M-CSF[68,69]. The phenotype of *op/op* mice is very marked, with an absence of teeth, low body weight, slow growth and many skeletal defects. These mice are osteopetrotic because they lack osteoclasts.

Kupffer cells. Kupffer cells are specialized macrophages present in the liver. Their principal function is to clean the blood in the portal circulation, by phagocytosis. Kupffer cells have both C3 complement receptors and Fc receptors and can take up a wide variety of opsonized and non-opsonized particles by phagocytosis. Unlike

hepatocytes, these cells can be induced to express high levels of heme oxygenase-1, an enzyme required to break heme down into biliverdin and, subsequently, into bilirubin[70]. The functional phenotype of Kupffer cells depends on their proximity to the portal vein and differences may be observed in the receptors, enzymes and subcellular structures present in different subpopulations of cells. For example, Kupffer cells in periportal regions are more phagocytic and larger than those in centrilobular regions[58].

Alveolar macrophages. Alveolar macrophages reside within the alveolus and are often seen protruding from the alveolar epithelial walls into the lung lumen. Their position within the body is unique, because they are in close contact with both air- and blood-derived substances. Alveolar macrophage populations are probably maintained by the proliferation of local precursors, and it seems likely that precursors (probably monocytes) are recruited from blood and proliferate in situ only in cases of major cell loss (*e.g.* after irradiation in bone-marrow transplantation)[71]. Alveolar macrophages play a key role in the regulation of many physiological processes, as highlighted by studies of GM-CSF-deficient mice. GM-CSF is another key hematopoietic growth factor required for the correct proliferation and differentiation of neutrophils, DCs and monocytes/macrophages. The key characteristic of these mice is their development of abnormalities in the lungs resembling those observed in the human disease pulmonary alveolar proteinosis (PAP). This disease is characterized by an accumulation of lung surfactant

that, at least in some forms of PAP, is considered to be due to the impaired phagocytosis of surfactant by alveolar macrophages. The administration of rGM-CSF to patients with this disease restores alveolar macrophage function, demonstrating the major role of this growth factor as a stimulator of terminal differentiation and highlighting macrophage key function in the control of homeostasis by resident macrophages in the lung[70].

Intestinal macrophages. The intestine is the most abundant reservoir of macrophages in the body. The location of the intestinal macrophages, in close contact with the resident intestinal bacteria, provides an interesting example of the microenvironmental control of macrophage differentiation. Intestinal macrophages are derived from monocyte precursors continually recruited to the lamina propria by endogenous chemoattractants. These precursors are then induced, under the influence of TGFβ in particular, to differentiate into non-inflammatory macrophages. These cells have normal levels of MHC class II molecules, but lack many innate receptors, including those for Fc, complement and LPS. Their pro-inflammatory functions are thus strongly downregulated. Despite these features, intestinal macrophages maintain a high capacity for phagocytosis. Thus, these cells retain their host defense functions, but simultaneously promote the absence of inflammation typical of the intestine, despite the presence of the intestinal flora[72].

Microglia. The central nervous system contains a wide variety of macrophages with different functions. Some are located close to

neurons (microglia), whereas others are found near the blood vessels (perivascular macrophages) or in the meninges (meningeal macrophages). Although derived from monocytes, the microglial cells of the CNS have few of the features of fully differentiated macrophages. For example, microglia cells express only low levels of the membrane receptors essential for macrophage function. In normal brain, ramified microglial cells with down-regulated macrophage function may be useful for cleaning up the extracellular environment. In conditions in which the brain is faced with some kind of threat (presence of foreign or endogenous signals, such as microbes or T-cell activation), microglial cells take on the characteristics of active macrophages, displaying changes in morphology and proliferation and the upregulation of surface molecules (*i.e.* increase in the expression of CR-3, MHC class I and II). Activated microglia are involved in inflammation, the removal of cell debris and wound healing, and may also play a role in neural regeneration and revascularization. There is also evidence to suggest that activated ramified microglial cells are involved in T-cell apoptosis and are therefore important for control of the extent of damage during the inflammatory response[73-75].

Spleen macrophages. The rodent spleen is a clear example of macrophage's heterogeneity. The spleen is rich in different macrophages subpopulations differing in function, location and receptor expression profile. In the red pulp (the region of the spleen responsible for hematopoietic functions and for the filtration and

degradation of red blood cells) a population of F4/80^{+} macrophages is actively involved in the clearance of dying erythrocytes. At the interface between the spleen circulation and lymphoid tissue (the marginal zone) there are two other subpopulations: the metallophilic macrophages (with a function currently unknown but thought to be important during systemic infections) and the phagocytic marginal zone macrophages. These macrophages express many different pattern recognition receptors and interact with soluble or particulate antigens. Another subpopulation present in the white pulp is the "tingible body" macrophage population that plays an important role in clearing the apoptotic B-cell debris produced in the germinal center[76].

Tissue-resident macrophages in inflammatory conditions

In response to endogenous or exogenous inflammatory signals, macrophages initiate specialized and polarized genetic reprogramming, leading to the development of specific functional properties that are similar in most cases in human and mice.

Early events

During the initial phases of inflammation, macrophages acquire specific properties as a function of the stimulus they encountered and become polarized. It has long been recognized that CCR2^{+}Ly6C^{+} inflammatory monocytes are strongly recruited to infection sites.

Once they have reached the tissue, inflammatory monocytes stop expressing CCR2, begin to express CCR1 and CCR5 and differentiate into macrophages, to sustain the activity of tissue-resident cells. Polarized macrophages have been broadly classified into two groups, mirroring the Th1/Th2 nomenclature used for T cells: M1 and M2 macrophages. M1 macrophages are induced by the combination of IFNγ and a microbial stimulus (such as LPS) or a cytokine (GM-CSF and TNFα), in an activation process described as "classical". M2 macrophages constitute a heterogeneous group of macrophages activated via "alternative" (IL-4 and IL-13) pathways or by encounters with immune complexes or TLR ligands (also called type II macrophages, induced during "innate" activation processes)[63,77]. Furthermore, deactivated macrophages that have previously come into contact with IL-10 are also often classified as M2 macrophages and play an important role in tissue remodeling and repair, which will be described below [78].

M1 macrophages. Classical macrophages can produce large amounts of various cytokines (IL-12, IL-23, TNFα, and IL-1β, the production of this last cytokine requiring efficient activation of the caspase system), reactive oxygen species (ROS), and, through efficient activation of the inducible form of NO synthase (iNOS), they also release large amounts of nitric oxide. They also have the capacity to present antigens and to upregulate costimulatory molecules, such as CD80 and CD86. Thus, M1 macrophages are actively involved in Th1 immune responses, killing microorganisms and tumor cells and

producing copious amounts of proinflammatory cytokines. They also produce many different chemokines of major importance during inflammation. IFNγ and LPS strongly induce CXCL9 (also known as MIG), CXCL10 (also known as IP-10) and CCL5. These chemokines recruit cytotoxic $CD8^+$ T-cells and NK cells, strongly enhancing immunity against intracellular bacteria and tumors. Studies in IFN receptor-deficient mice and STAT1-deficient mice have shown that IP-10 induction in response to LPS is secondary to IFNβ induction and subsequent STAT activation[79-81]. Macrophages activated in the presence of IFNγ and TNFα produce large amounts of CXCL1 and CXCL16, transmembrane chemokines acting on Th1 T-cells and NKT cells[82,83] and favoring a positive feedback loop leading to efficient Th1 immune responses. M1 macrophages exert key functions during bacterial infections, as highlighted by experiments with Listeria monocytogenes. L. monocytogenes is a gram-positive bacterium that causes disease in pregnant women and immunocompromised hosts. It is normally efficiently controlled by M1 responses, which prevent phagosomal escape and stimulate the killing of the bacteria[84]. L. monocytogenes can replicate in non-polarized macrophages, in which it escapes from the phagosome. Activated M1 macrophages kill the bacterium by blocking phagosome escape through the generation of ROS and NO soon after bacterial uptake. Mice lacking the M1-polarizing TNFα and IFNγ cytokines or receptors succumb to L. monocytogenes infection. The IL-12 pathway and, more recently, the IL-23 pathway[85,86] of M1 macrophages have been shown to be

essential for immune responses directed against Mycobacterium tuberculosis and Salmonella.

M2 macrophages. M2 cells generally express high levels of mannose, galactose and scavenger receptors. The "alternative" activated macrophages do not produce IL-12, IL-23 and TNFα, but produce large amounts of IL-1 receptor antagonist (IL-1ra), a decoy receptor for IL-1 and IL-10. Alternative activated M2 macrophages upregulate MHC class II molecules, but inefficiently present peptides to T-cells. Indeed, in many cases they have been shown to inhibit T-cell proliferation. These macrophages also display inhibitory activity with mitogen-activated T-cells, which proliferate to a lesser extent and have a weaker secretory response in the presence of alternatively activated macrophages than in the presence of classical macrophages[87]. These cells may provide negative regulatory signals in the lung, protecting the host against excessive inflammatory responses to environmental stimuli[88]. The ability of M2 alternative macrophages to kill intracellular microbes is compromised by their lack of NO production, due to their expression of arginase. When macrophages are exposed to IL-4 and IL-13, arginase is induced and transforms arginine, normally used by iNOS to produce NO, into urea and ornithine. It has been clearly demonstrated that arginase levels are correlated with diseases due to the presence of schistosome eggs, whereas iNOS levels are inversely correlated with these diseases[89]. Thus, alternatively activated macrophages may have more regulatory and recovery functions than classically activated

macrophages, which tend to be more involved in effector killing functions. Type II macrophages, induced by immune complexes and TLR or IL-1R ligands, by contrast, retain some of the features of M1 macrophages. Indeed, these cells produce large quantities of IL-10 and small amounts of IL-12 but maintain the ability to secrete TNFα, IL-1 and IL-6. They promote humoral and Th2 immunity and protect against LPS toxicity. Their name, "type II-activated macrophages", is derived from their preferential induction of Th2 adaptive immune responses. Whereas IL-12 secretion by classically activated macrophages induces IFNγ production by T cells, IL-10 secretion by type II macrophages results in IL-4 production by T-cells[90]. The influence of type II macrophages on antibody responses has been investigated by injecting type II or classical macrophages loaded with ovalbumin in mice and assessing the antibody response. Mice treated with classical macrophages have only low IgG titers[91]. By contrast, mice treated with type II macrophages produce much larger amounts of IgG, particularly of isotype 1, which is classically associated with Th2 immune responses. This observation suggested that the presence of type II macrophages was sufficient to induce Th2 IgG1 class switching. Binding to the Fc receptor on activated macrophages induces the production of IL-4 by T-cells, which, in turn, induces the production of IgG1 by B cells in response to the antigen. Protection against LPS endotoxemia was demonstrated by injecting mice with type II macrophages generated in vitro and treating these animals with a lethal dose of LPS. The mice that received type II macrophages remained completely healthy, whereas control mice succumbed to

lethal endotoxemia[92]. The secretion of large amounts of IL-10 by M2 cells of this type suggests that this protective effect might be due to the production of this cytokine. This hypothesis was tested in parallel experiments with type II-activated macrophages from IL-$10^{-/-}$ mice. These macrophages failed to prevent the death of these mice. Thus, the IL-10 secreted by type II macrophages has a potent anti-inflammatory effect that can be used to prevent acute diseases, such as LPS toxicity.

Late events

The resolution of inflammation is an active process in which endogenous mediators induce the phagocytosis of apoptotic cells and anti-inflammatory cytokines and simultaneously abolish cell recruitment and pro-inflammatory cytokine production. Inflammation is a beneficial host response to tissue injury[93] but, if prolonged, it may result in pathological processes, such as asthma, rheumatoid arthritis and myocardial infarction. Thus, after entering tissues, the cells that promote inflammation induce the switch from proinflammatory prostaglandins and leukotrienes to lipoxins, cyclopentenone prostaglandins[94] and resolvins and protectins derived from omega-3 polyunsaturated fatty acids[95,96], which initiate the “termination” process. These events coincide with a decrease in neutrophil recruitment and an increase in neutrophil apoptosis[95,96]. Macrophages have long been recognized to play a central role in the resolution of inflammation and tissue repair. Macrophage activation has been shown to be plastic and reversible, highlighting the dynamic

nature of macrophage populations, allowing a given cell to contribute to the induction of inflammation and then to its resolution. M2 macrophages often have anti-inflammatory properties and have been detected during the resolution of inflammatory responses and in wound healing. It has also been shown that M1 cells can revert to M2 cells, switching from pro-inflammatory to anti-inflammatory functions[97]. Macrophages play a key role in the termination process. Indeed, these cells are responsible for the phagocytosis of apoptotic neutrophils and the release of anti-inflammatory and reparative cytokines, including TGFβ. Endogenous anti-inflammatory mediators seem to play a key role in inducing the switch in macrophage function. On the one hand, lipoxins and cyclopentenone prostaglandins affect cell recruitment, decreasing neutrophil recruitment and increasing the recruitment of monocytes that could potentially become anti-inflammatory macrophages[98-100]. On the other hand, lipoxins strongly promote the phagocytic clearance of apoptotic cells by macrophages[101], whereas cyclopentenone prostaglandins efficiently inhibit the production of pro-inflammatory cytokines by activated macrophages[102,103], simultaneously promoting cell apoptosis[104,105]. Neutrophil apoptosis is normally rapid but may be slowed in inflammatory settings[106]. However, the arrival of pro-inflammatory macrophages at the site of infection may counteract this anti-apoptotic effect, by triggering the release of "death mediators" or Fas ligand[107]. The recognition of apoptotic cells[108] by macrophages induces a shift in the transcription program, resulting in the production of TGFβ and abolition of the production of pro-

inflammatory cytokines[109-111]. This process has been demonstrated, through the experimental transfer of apoptotic cells into LPS-treated lungs. This treatment results in a TGFβ-dependent decrease in proinflammatory cytokine production and leukocyte recruitment[110]. Furthermore, the impaired clearance and uptake of apoptotic bodies has been correlated with various diseases, including systemic lupus erythematosus and cystic fibrosis[112,113]. The recognition of dying cells by phagocytes involves a complex molecular mechanism. First, bridging molecules, such as thrombospondin 1, may facilitate the binding of apoptotic cells. Phagocyte receptors (CD36, integrins and phosphatidylserine receptors) may then be responsible for initiating phagocytosis[114]. These molecules also play an important role in inducing the production of TGFβ and vascular endothelial growth factors essential for epithelial and endothelial injury repair.

Introduction - Section 2.

Sensing and Signaling Strategies of Toll-like Receptors

Although the seminal discovery of phagocytosis by Elie Metchnikoff dates back to 1883, the field of innate immunity has gained the appropriate attention only in recent years, thanks to Janeway's theory of innate immune recognition[1]. Until the early 90s, the investigation of adaptive immunity and antigen-specific receptors was the major focus of almost all immunologists, the "clonal selection" and the "two signals" theories being the milestones of self-nonself discrimination. However, the adjuvant and immunogenic properties of LPS and other microbial stimuli, together with the typically innate mechanisms of phagocytosis, degranulation, and ROS production were well appreciated at the time. What was lacking was a unifying view that could synthesize these two otherwise absolutely distinct branches of immunology. In 1989 Janeway proposed that the costimulatory signal for lymphocyte activation was provided by antigen presenting cells (APCs) that had been previously primed by conserved pathogen-associated molecular patterns (PAMPs). PAMPs were postulated to be selectively recognized by pattern-recognition receptors (PRRs) different from the antigen receptors[1]. Nevertheless, the formal demonstration for this brilliant hypothesis came only about a decade later, when Medzhitov and Janeway cloned the first Toll-like receptor, known as TLR4, and linked its ectopic expression to activation of NF-κB, expression of the costimulatory molecules B7.1 and B7.2 as well as several proinflammatory cytokines[2]. For this

discovery Medzhitov took advantage of a previously published work by Hoffmann and colleagues showing that the Drosophila melanogaster Toll receptor, known for its role in dorso-ventral development, was indeed essential for antifungal defense in flies[3]. The identification of a human homolog of Drosophila Toll that was involved in immune defense represented a major breakthrough in immunology, as it suggested that TLRs could couple innate immune recognition with activation of adaptive immunity. However, the ligand for TLR4 was not known at that time and human Toll was proposed to function, similar to the Toll-Spatzle axis in flies, downstream of a proteolytic cascade triggered by microbial recognition. Finally, in 1998, Beutler and colleagues positionally cloned the Tlr4 genetic locus showing a correspondence with the Lps locus in the C3H/HeJ and C57BL/10ScCr mouse strains[4], previously shown to be hyporesponsive to LPS-induced septic shock. These findings, implemented by the generation of TLR4ko mice[5], definitively established TLR4 as the long sought-after receptor for LPS and paved the way for a whole new line of research in immunology, i.e. the identification of other members of the TLR family and the characterization of their signaling pathways. So far, more than a dozen of different TLRs have been identified, of which TLRs 1-9 are conserved among humans and mice, TLR10 is selectively expressed in humans and TLR11 is functional in mice[6]. Although much research has provided a considerable wealth of information about the biological functions and modes of action of TLRs 1-9 and 11, we still lack a basic knowledge on the physiology of the newly discovered

family members TLRs 10, 12 and 13, which will be therefore not discussed further in this thesis. In the first part of this work I review and update our current knowledge on the structural biology and ligand recognition strategies of TLRs. In the following sections, by using it as model system for the whole TLR family, I attempt to provide a comprehensive description of the signal transduction pathways triggered by TLR4, with a particular emphasis on the molecular and cell biological aspects regulating its function.

TOLL-LIKE RECEPTORS

TLR structure

TLRs are type I transmembrane glycoproteins composed of an extracellular, a transmembrane and an intracellular signaling domain[7]. The extracellular domains of TLRs are responsible for ligand recognition and contain 16-28 leucine-rich repeat (LRRs) modules, which provide the TLR ectodomains with a characteristic horse-shoe-shaped folding[8]. TLRs are "typical" LRR proteins, displaying 24 aa-long LRR modules with the conserved hydrophobic motif "xLxxLxxLxLxxNxLxxLPxxxFx"[9]. The N- and C-terminal LRRs (LRRNT and LRRCT, respectively) do not have real LRR motives but frequently contain clustered cysteines forming disulfide bonds. These modules stabilize the protein by protecting its hydrophobic core from exposure to a polar solvent[9]. The ''LxxLxLxxN'' motives are located in the inner concave surfaces of the horseshoe-like structure formed

from parallel b strands. The variable parts of the modules form the convex surface generated by parallel 310 helices, b turns, and/or loops[7]. The resultant structure is exemplified by TLR3, and probably TLR5, TLR7, TLR8 and TLR9, with a flat and uniform horse-shoe-like shape[10-12]. Notably, the ectodomains of TLR1, TLR2 and TLR4, and perhaps TLR6 and TLR10, show two sharp structural transitions in the b sheets that define three distinct subdomains, the central markedly deviating from standard values in terms of radius, twist and tilt angles[13-15]. This atypical conformation seems to be generated by unusual, non-uniform, LRR modules distribution in the central domain and may provide some degree of ligand or co-receptor specificity to these TLRs.

Ligand binding by TLR ectodomains readily triggers homo-/hetero-dimerization between TLRs, and resulting in the recruitment of different adaptor proteins to intracellular TIR (Toll/Interleukin-1 Receptor) domains. As suggested by the denomination, TIR domains share homology sequence with the intracellular domain of IL-1R and typically consist of a five-stranded b-sheet surrounded by five a-helices, folding into a globular structure. Of particular importance for receptor dimerization and adaptor recruitment is the BB loop that connects the second b-sheet and the second a-helix. Indeed, a single point mutation (Pro681His) in the BB loop of TLR2 TIR domain has been shown to impair the recruitment of the adaptor MyD88[16], resulting in a defective signaling in response to yeast and Gram-positive bacteria[17]. Notably, the Pro681His mutation in TLR2 TIR corresponds to the Pro712His mutation in TLR4 TIR domain originally

identified in the LPS hyporesponsive C3H/HeJ mouse strain[18]. Even though some TIR domains have been successfully crystallized, namely TLR1, TLR2 and TLR10[16,19], what is still lacking is a deeper knowledge on how interactions between TIR domains take place. In this regard, it has been predicted that the major binding force may be the electrostatic complementarity between interacting portions of TIR domains[20-21]. This hypothesis is intriguing, as it could provide a structural explanation to why some TLRs directly recruit the adaptor proteins MyD88 and TRIF upon ligand-induced dimerization whereas others require the intermediate adaptors TIRAP and TRAM (see below).

TLR ligand specificity

TLR1, TLR2 and TLR6. Contaminating lipoproteins in the LPS preparations used in early experiments generated the misleading hypothesis that TLR2 could be the receptor for LPS[22]. This statement was later shown to be incorrect, with bacterial lipoproteins being the actual TLR2 ligands[23]. Since then TLR2 has been demonstrated to have a crucial role in the recognition of a wide variety of bacterial, fungal as well as host ligands[24]. The broad specificity of TLR2 ligand recognition relies on its ability to function as a heterodimer with TLR1 or TLR6[25-27], TLR1/2 heterodimer recognizes lipoproteins and lipopeptides from a wide panel of bacteria including mycobacteria and meningococci[26-27], OspA lipoprotein from Borrelia burgdoferi[28],

PorB porin from Neisseria meningitidis[29] as well as the synthetic lipoprotein structure Pam3CSK4[27]. TLR2/6 heterodimer recognizes micoplasma lipoproteins (e.g. MALP-2), zymosan[25], lipoteichoic acid[30] and possibly peptidoglycan[27]. The major feature conferring these ligand specificities has been identified in the differential recognition of diacylated versus triacylated lipoproteins by TLR1/2 and TLR2/6, respectively. Accordingly, most bacterial lipoproteins and Pam3CSK4 are triacylated whereas MALP-2, lipoteichoic acid and peptidoglycan are usually diacylated. In addition to what reported above, a role for TLR2 independently of TLR1 or TLR6 has been demonstrated in the recognition of many other microbial ligands including: cell wall preparations and lipotheichoic acid from Gram-positive bacteria[31-32], mycobacterial lipoarabinomannan[33], a phenol-soluble staphylococcal modulin[34], glycoinositolphospholipids from Trypanosoma cruzi[35], glycolipids from Treponema maltophilum[36], atypical LPS from Leptospira interrogans[37], Porphyromonas gingivalis[38], Legionella pneumophila and Bordetella pertussis[39]. TLR2 also plays an important role in the recognition of some endogenous danger signals as HSP60/70[40-41], hyaluronan[42] and, in coordination with TLR6 and the co-receptor CD14, the proteoglycan versican[43]. Importantly, hyaluronan and versican are highly expressed in lung cancer and it has been shown that versican recognition by TLR2 generates an inflammatory environment that in turn promotes tumor metastasis[43]. As described above, the breadth of TLR2 ligand repertoire is impressive and can be only partially explained with the ability of TLR2 to form heterodimers. Maximum flexibility and specificity in ligand

recognition is achieved through the usage of the co-receptors CD36 and CD14. CD36 is a double-spanning plasma membrane protein belonging to the class B scavenger receptor family that has been shown to function as a crucial sensor of microbial dyacilglicerides that signal through the TLR2/6 heterodimer, namely MALP-2 and lipoteichoic acid[30]. The structural and functional features of CD14 will be described below in this section. The observation that TLR2 functions independently of TLR1 or TLR6 in response to a considerable number of ligands strongly suggests that TLR2 may form homodimers or, alternatively, that it may interact with additional TLRs (candidates include TLR11, TLR12 and TLR13) or other non-TLR proteins to recognize these molecular species. Although in vitro studies would exclude the first hypothesis[25] a formal demonstration of its inconsistency is still lacking.

TLR3. The potent immunostimulatory activity exerted by virus infections has long been known to depend on the recognition of peculiar nucleic acids. One such molecule is double-stranded RNA (dsRNA), which can be considered a bona fide marker of viral infection. Indeed, dsRNA molecules are never generated during the physiological life cycle of uninfected cells but can be synthesized as a consequence of the infection by both RNA and DNA viruses. The source of dsRNA could be: (i) the genome itself, as well as mRNA secondary structures, for dsRNA viruses; (ii) replicative intermediates for ssRNA viruses and (iii) the overlapping convergent transcription for DNA viruses[44]. Viral infections can be experimentally mimicked by

using a synthetic analog of viral dsRNA, polyribocytidylic acid [poly(I:C)], which has been shown to be the main ligand for TLR3[45]. Few other TLR3 ligands have later been identified in addition to viral dsRNA, namely endogenous cellular mRNA[46], and sequence-independent small interfering RNAs[47]. Despite the ability of TLR3 to recognize a universal marker of viral infection suggests it may play a major role in recognizing and limiting infections by virtually all viral species, various studies heavily questioned its real biological role[48], which is now restricted only to a limited selection of viruses. This lack of requirement for TLR3 in response to many viral infections can be easily explained by the overlapping activities of other nucleic acid-sensing TLRs (TLR7, TLR8 and TLR9, see below) as well as a growing number of cytosolic receptors for DNA and RNA[49]. It is interesting to note that also TLR3 makes use of CD14 as a co-receptor, even though the mechanistic details of this interaction are quite different from TLR2 and TLR4. Indeed, it has been demonstrated that CD14 binds to poly(I:C) on the cell surface and then actively promotes its uptake and delivery into the endosomal compartment where TLR3 is located[50].

TLR4. TLR4 is the most thoroughly studied TLR because of its outstanding role in antibacterial defense and its peculiar modes of signal transduction. It is well established that LPS from Gram-negative bacteria is the main ligand for TLR4[4], even though TLR4 alone is not sufficient for mounting an effective immune response to LPS. LPS recognition is indeed a complex process that is orchestrated

by numerous players other than TLR4 itself, the most important being the LPS-binding protein LBP and the co-receptors CD14 and MD-2. However, due to the massive body of information about LPS recognition and considered the physiological importance of these interactions, the features of this particular sensing system will not be described here, but a section of this introduction will be specifically dedicated to this aspect (see below). Apart from LPS, TLR4 has been implicated in the recognition of several other ligands, both of exogenous or endogenous origin. The plant product taxol from Taxus brevifolia is commonly used in cancer chemotherapy due to its potent anti-mitotic activity. Interestingly, taxol shows a marked LPS-mimetic immunostimulatory activity in mice that is dependent on TLR4 and MD-2, with no contribution from CD14[51-53]. TLR4 has also been shown to contribute to the antiviral response, through the recognition of the fusion protein of respiratory syncytial virus[54], and the envelope proteins of mouse mammary tumor virus and Moloney murine leukemia virus[55]. TLR4 seems to play a role in the recognition of several endogenous danger signals. Heat shock proteins primarily function as chaperone for nascent misfolded proteins and are highly expressed upon a variety of stress conditions, including heat shock, UV radiation and microbial infections. Upon cellular damage, HSPs are released and can activate innate immune cells[56]. HSP60/70 of endogenous origin[57-58] as well as from Chlamydia pneumoniae[59], Gp96[60] and the small HSP family members αA crystalline and HSPB8[61] have all been shown to be recognized through TLR4, although some of these ligands may require a contribution from TLR2. Extracellular

matrix components are produced and released upon tissue injury and may constitute potent inflammatory signals that act through TLR4 stimulation. Accordingly, TLR4 is involved in the recognition and response to type III repeat extra domain A of fibronectin[62], oligosaccharides of hyaluronic acid[63], polysaccharide fragments of heparan sulfate[64], fibrinogen[65] and, together with TLR2, hyaluronan fragments and biglycan[66-67]. TLR4 has also been shown to participate in the recognition of HMGB1 released by necrotic cells in a model of hepatic ischaemia reperfusion injury[68], murine β-defensin 2[69], minimally modified LDL[70], lung surfactant protein-A[71] and the phagocyte-specific S100 proteins Mrp8 and Mrp14[72]. TLR4 has recently been reported to mediate the recognition of oxidized LDL and β-amyloid, two endogenous proinflammatory signals underlying atherosclerosis and Alzheimer's disease[73]. Notably, these interactions are dependent on CD36, which acts as a common receptor for these disparate ligands, and triggers inflammation through a TLR4:TLR6 heterodimer[73]. The ability of TLR4 to recognize this number of ligands highlights its importance in the induction of an inflammatory state, even in a "sterile" pathogenic setting. However these data must be interpreted cautiously because, due to technical difficulties in isolating highly purified quantities of endogenous ligands, it remains possible that they are contaminated with traces of true TLR ligands such as LPS, flagellin or peptidoglycan. Indeed, it should be noted that all of these endogenous ligands activate TLR4 only at high doses, sharply contrasting with the low concentrations required for LPS.

TLR5. TLR5 was identified as the receptor for flagellin, a bacterial protein that constitutes the main bacterial motility apparatus, the flagellum[74]. Despite proteins do not generally make good PAMPs due to the difficulty in discriminating their origin between self and non self, flagellin fulfills Janeway's criteria by virtue of a highly conserved central core structure that is essential for protofilament assembly and that constitutes the actual TLR5 ligand[75]. The TLR5 recognition site of flagellin is normally masked in the flagellum, which suggests that flagellin can only be recognized as a monomer. Different from other TLRs, TLR5 does not seem to require any co-receptorial contribution but rather directly binds its ligand at residues 386-407 of the ectodomains[76].

TLR7 and TLR8. Mouse TLR7 was originally described for its ability to detect imiquimod and R-848[77], two synthetic compounds with potent antiviral and antitumor activities belonging to the class of imidazoquinolines. Both human TLR7 and TLR8, but not mouse TLR8, have been shown to recognize R-848[78-79]. Additional variants of R-848 have been generated that specifically activate TLR7 (3M-001), TLR8 (3M-002) or both (3M-003)[80]. Loxoribine and bropirimine, other immunomodulator compounds with a strong type I IFN-inducing activity, have also been shown to stimulate immune cells through TLR7[81]. Notably, all of these small compounds are nucleic acid base analogs, showing high structural similarity with adenosine, guanosine and pyrimidine. This observation is consistent with the features of the natural ligand for TLR7 and TLR8, which has been reported to be

single-stranded RNA[82-84]. TLR7 and TLR8 are therefore involved in the recognition of different viral species, including human immunodeficiency virus (HIV), influenza virus, Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), Coxsackie virus and human parechovirus 1[82-86]. However, also siRNA has been shown to activate immune cells through TLR7 and TLR8, raising the possibility that both receptors may also recognize dsRNA[87]. Even though a complete understanding of the relationship between the sequence of the stimulatory RNA and the recognition by TLR7 and TLR8 is still lacking, the presence of uridines seems to be crucial for these interactions. Additionally, enrichment in guanosines or adenosines appears to differentially target the ssRNA to TLR7 or TLR8, respectively[83,88]. It should also be noted that the recognition of ssRNA by TLR7 and TLR8 is strongly inhibited by the incorporation of modified nucleosides, such as 5-methylcytosine, N6-methyladenosine, 5-methyluridine, 2-thiolated uridine or pseudouridine, which are commonly found in endogenous but not in bacterial or viral RNA[89]. This observation clearly suggests a system to discriminate between self and non-self ligands and therefore preventing autoimmune reactions.

TLR9. Bacterial DNA has been demonstrated to have a potent immunostimulatory activity, which is dependent on the presence of unmethylated CpG dinucleotides in a particular base context named CpG motif[90]. Differently from bacteria, where CpG motifs are found at the expected rates and are normally non-methylated, the

vertebrate genome is poor of CpG motifs (a strategy called "CpG suppression")[91]. Additionally, in vertebrates cytosines in CpG motifs are highly methylated, suggesting that the differential expression and methylation of CpG motifs could account for a rather specific self-non self discrimination between host and microbial or viral DNA[90]. CpG-containing oligonucleotides (CpG-ODN) are recognized by means of TLR9[92], which is accordingly known to play a role in the recognition of viruses, including murine cytomegalovirus (MCMV and herpes-simplex virus 1 and 2 (HSV-1 and HSV-2)[93-96], and bacteria[97-98]. However, recent studies questioned the paradigm stating that DNA recognition by TLR9 is strictly CpG-dependent. By using CpG-ODN containing natural phosphodiester linkages (PD-CpG-ODN) instead of the commonly used, DNase-resistant, phosphothioate-stabilized ODN (PS-CpG-ODN), it was shown that activation of TLR9 by DNA is actually determined by recognition of the DNA sugar backbone[99]. Base-free PD 2'deoxyribose backbone is a basal TLR9 agonist, and its activity is enhanced by sequence-aspecific base addition. On the contrary, base-free PS 2'deoxyribose backbone acts as an antagonist for TLR9, and its activity is transformed into stimulatory only by addition of CpG motifs[99]. In light of these findings, TLR9 should not be considered as different from the other nucleic acid-sensing TLRs, which all recognize general chemical and structural, rather than sequence-specific, features of DNA or RNA.

TLR11. TLR11 plays a crucial role in the response to uropathogenic E.coli infections in mice, although the precise ligand is unknown[100].

However, it is believed that TLR11 recognizes a protein ligand since the immunostimulatory activity of these bacteria is sensitive to proteinase K treatment[100]. Other TLR11 ligands include a class of profilin-like molecules from protozoan[101]. Notably, human TLR11 is nonfunctional, due to the presence of a stop codon in its genetic sequence.

TLR localization

The TLR family members described above can be classified into two groups according to their subcellular localization. Namely, TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are expressed on the plasma membrane, whereas TLR3, TLR7, TLR8 and TLR9 are found in the endolysosomal compartment. Although this distinction is based on a mere phenotypical analysis, recent research showed that it is highly informative of the functional role played by the two categories. Plasma membrane TLRs bind lipid or protein structures that are expressed on the surface of pathogens and are therefore readily available for recognition in the extracellular space. Instead, the distinctive trait of intracellular TLRs is the shared ability to bind nucleic acids, which are normally confined inside invading pathogen but can be encountered by immune cells upon phagocytosis or endocytosis and subsequent fusion with TLR-containing vesicles with phagosomes, endosomes or macropinosomes. It should be noted that this nucleic acid sensing strategy, although unavoidable since

viruses lack conserved non-nucleic patterns, is not costless[102]. The inappropriate TLR activation by host DNA or RNA in pathologic circumstances may indeed contribute to the emergence of autoimmune disorders, including SLE[103]. However, it has been shown that the intracellular localization of TLR9 is crucial for discriminating between self and non-self DNA, a hypothesis that may well be extended to the other nucleic acid sensing TLRs[104]. An additional strategy that limits the possibility of host DNA/RNA recognition has been identified in the proteolytic processing of TLR7 and TLR9 ectodomains that specifically occurs in endolysosomes and that is required for the generation of a functional receptor[105-106]. Accordingly, a pool of full-length TLR7 or TLR9 can still be found on the cell membrane but these receptors are not able to recruit the downstream adaptor MyD88 upon ligand binding[105]. A thorough cell biological approach has recently been used to study the features of TLR4 signaling, despite its apparently static localization on the plasma membrane. As it turned out, also the functions of TLR4 strictly rely on basic cellular mechanisms that regulate the ability of TLR4 to dimerize and recruit the adaptors MyD88 and TRIF[102]. A detailed description of that will be provided in the following sections.

TOLL-LIKE RECEPTOR 4: A CASE STUDY

Since its discovery, TLR4 has been the focus of much attention because of its peculiar features in terms of ligand recognition and signal transduction, two key aspects that this section will specifically deal with. TLR4 shows a highly orchestrated usage of co-receptors to discriminate between ligands and this multifaceted receptor system additionally plays a role in triggering several signal transduction pathways through the sequential recruitment of at least four adaptor proteins. Since the resultant biological outcomes recapitulate the whole spectrum of TLR responses, I will use TLR4 as a model to describe the molecular events linking receptor stimulation with downstream activation of transcription factors.

LPS Structure

Lipopolysaccharide (LPS) is a highly expressed component of the cell wall of all Gram-negative bacteria, and it plays a crucial role in maintaining the structural and functional integrity of the outer membrane[107]. Due to its vital biological importance, the general structure of LPS is conserved among all Gram-negative bacteria, making it a prototypical PAMP to be recognized by the innate arm of the immune system. LPS recognition by innate immune cells triggers the rapid release of proinflammatory cytokines (e.g. TNFα, IL-6), which promote a local inflammation that is beneficial for bacterial clearing. However, if the response to LPS is spatially uncontrolled, a

systemic inflammatory reaction can occur, leading to multiorgan failure, endotoxic shock and potentially death[108]. LPS from most Gram-negative bacteria conforms to a general architecture composed of three separate regions, namely the lipid A, the core region and the O-chain. The lipid A moiety is highly hydrophobic and it is largely responsible for the endotoxic activity of the whole LPS molecule, which can be effectively mimicked by a synthetic lipid A. Lipid A is inserted into the external face of the outer membrane of the bacterial cell wall and it is typically composed of a β-D-GlcN-(1,6)-α-D-GlcN disaccharide bearing two phosphates at positions 1' and 4'. This structure is further modified by the attachment to the disaccharide module of up to four primary acyl chains, which can in turn be substituted by additional fatty acids. The number and lengths of acyl chains or the phosphorylation state of the lipid A represent the major factors contributing to its endotoxicity. Accordingly, lipid A structures either lacking a phosphate group or showing a wrong number of acyl chains are much less active than classic E.coli lipid A[109]. It appears that the lipid A structure associated with the highest endotoxic activity is the E.coli-like diphosphorylated β(1,6)-linked D-GlcN dimer with six acyl chains, as evidenced by the observation that deviations from this pattern invariably reduce the activity of the molecule[109]. Consistent with this hypothesis are at least three clinically relevant observations. First, the compound Eritoran (E5564), a synthetic derivative of the lipid A from the non pathogenic LPS of R.sphaeroides bearing only four lipid chains, acts as a strong antagonist of TLR4:MD-2[110] and is currently in a phase III trial for the

treatment of sepsis. Second, lipid IVa (compound 406), an intermediate in E.coli LPS biosynthesis, is an antagonist of TLR4:MD-2 in humans but a mild agonist in mice and horses[111-113]. Third, monophosphoryl lipid A (MPLA) from S.enterica is a variant form of lipid A that awaits approval for use as a vaccine adjuvant, since it generates clinically useful immune responses associated with a very low toxicity compared to LPS[114]. The core region is a rather conserved polysaccharidic structure that can be formally subdivided into inner and outer core. The inner core is proximal to the lipid A and it contains unusual sugars, such as Kdo and heptose, which are absolutely required for bacterial viability and therefore are well conserved among all LPS species. The variability of the whole region is concentrated in the outer core, which typically consists of common hexose sugars. In particular, depending on the degree of completion of the core oligosaccharide, five unique structures have been determined and classified from Ra to Re in E.coli. The hydrophilic O-chain is the outer region of the LPS molecule and it provides bacteria with an effective protection against hydrophilic antibiotics or complement proteins. The O-chain is a highly variable region composed by repeating saccharidic units formed by up to eight glycosyl residues that differ between bacterial strains in terms of sugars, sequences, linkages and substitutions used. Additionally, these forming units can be repeated up to 50 times and a single organism will produce a wide range of these lengths due to incomplete synthesis of the chain. Since the O-chain is also generally targeted by antibodies it is also referred to as O-antigen, and the

serology of O-antigens has become an effective tool in typing strains of organisms and LPSs. Around 170 serotypes of E.coli LPS have been identified, demonstrating the high degree of variability of the O-antigen. However, the O-chain is not ubiquitous, as some Gram-negative strains seem to express a truncated version of LPS. Notably, bacteria carrying mutations in the genes involved in the synthesis and attachment of the O-chain do not express it at all. These mutants are called “rough” (R-) because of the morphology of the colonies they form in a plate that is different from what observed for wild-type, “smooth” (S-)bacteria.

LPS sensing machinery

Well before the definitive discovery of TLR4 as the transducing receptor for LPS, some of the key players involved in its recognition had already been discovered and characterized. After the isolation and cloning of LBP (LPS binding protein)[115], it was shown that its primary function was to extract monomers of LPS from aggregates or bacterial membranes[116] and facilitate LPS recognition by the receptor CD14[117]. However, since CD14 is a GPI-anchored protein that lacks an intracellular domain for canonical signal transduction, it appeared evident that it could not function as the sole receptor for LPS[118]. The seminal discoveries of TLR4[2,4] and its associated co-receptor MD-2 (myeloid differentiation protein 2)[119], contributed to establish a model in which CD14 acts as an LPS sensing receptor that accepts LPS

monomers from LBP and then transfers them to the TLR4:MD-2 complex, thereby promoting its ligand-induced dimerization.

LBP. LBP is a 58-60 kDa glycoprotein that is secreted in the serum mainly by hepatocytes as an acute phase protein. It belongs to the family of lipid transfer/LPS binding protein (LT/LBP) and, among all the members, it shows the highest sequence similarity with BPI (bactericidal permeability increasing protein)[116]. The crystal structure of murine BPI has been determined, which allows the construction of a reliable tertiary structural model of LBP[120]. BPI has a boomerang-shaped structure composed by two barrel domains, connected by a proline-rich linker, each of which can accommodate a phosphatidylcholine molecule[120]. The LPS binding site of LBP has been identified by mutagenesis analysis and it consists of a cluster of cationic residues that bind the phosphorylated head of the lipid A moiety[121]. Based on the crystal structure of BPI, the LPS binding site of LBP is fully exposed at the N-terminal tip of the protein[122], whereas the C-terminal domain is required for the interaction of LBP with the cell membrane or with CD14[116,123-124]. At low concentrations, LBP has been shown to facilitate the recognition of LPS by extracting it as a monomer from aggregates or bacterial membranes[125-126]. LBP then catalyzes the transfer of LPS to both membrane-bound and soluble CD14[117,127] by means of a first order enzymatic reaction[128-129]. Notably, acute-phase high serum concentrations of LBP strongly inhibit LPS recognition, protecting the host from LPS- or Gram-negative bacteria-induced septic shock[130]. LBP is believed to exert

this inhibitory role at least partly by transferring LPS to serum lipoproteins such as HDL[131] or by forming inactive aggregates with LPS[132]. It is interesting to note that, although the inhibitory effect on LPS signaling by high LBP concentrations is equally observed for all LPS chemotypes, the features of LPS clearance seem to be rather specific. Indeed, it has been shown that S-form LPS is predominantly cleared by the LBP-dependent transfer to serum lipoproteins, whereas R-form LPS is cleared by means of both mCD14-dependent and –independent mechanisms[133].

CD14. CD14 is a 55 kDa glycoprotein expressed on the surface of myelomonocytic cells as a GPI-anchored receptor or secreted in a soluble form[134]. Sequence and crystallographic analyses revealed that CD14 contains 11 LRR modules folding into a truncated horse shoe-shaped tridimensional structure. Notably, CD14 has been crystallized as a dimer in which two monomers are bound by means of their C-terminal regions and the total number of LRRs in the CD14 dimer is similar to that in TLR4, suggesting that the overall shape of the two proteins may coincide[135]. The concave surface is largely composed of β-sheets, whereas the convex face contains an irregular pattern of helices and loops. This structure implies the presence of a number of grooves and pockets that are crucial for ligand binding. The LPS binding site of CD14, identified by mutagenesis and epitope mapping experiments[136-138], is located at the N-terminal region of the protein in a large hydrophobic pocket with a cluster of positively charged residues at the rim that accommodates the phosphorylated lipid A

moiety[135]. Additionally, since also the carbohydrate portion of LPS contributes to the binding to CD14[118], it has been proposed that the LPS binding site extends beyond the N-terminal pocket to include additional hydrophilic grooves in the neighboring area[135]. Such an irregular and flexible structure provides CD14 with the ability to bind different forms of LPS with a comparable high affinity[139], and probably explains its ligand promiscuity. CD14 has indeed been involved in the recognition of a number of other ligands, acting as a co-receptor for TLR1, TLR2, TLR6, TLR4 and TLR3 (see sections above). CD14 functions as an LPS sensing receptor whose role is to enhance the sensitivity of innate immune cells to LPS by binding to picomolar concentrations of LPS and facilitating its recognition by TLR4:MD-2[140]. This was demonstrated by studies showing that CD14-deficient mice are highly resistant to LPS- or Gram-negative bacteria-induced septic shock[141] and that CD14-deficient macrophages display heavily impaired sensitivity to low concentrations of LPS[142]. Nevertheless, the detailed mechanism of how LPS is transferred to TLR4:MD-2 has not been completely unraveled. The simplest scenario would be that CD14 directly contacts TLR4:MD-2 and operates the LPS presentation. Alternatively, since CD14 has been consistently shown to catalyze the rapid insertion of LPS into the cell membrane[143-144] it is plausible to envisage a model in which CD14 leaves the LPS molecule in the cell membrane to be picked up by TLR4:MD-2. In support of both hypotheses is the identification of several residues of CD14 that are not required for LPS binding but for its signaling[145-146] and that could mediate the interaction with TLR4:MD-2. Furthermore, it has been

shown that LPS is brought by CD14 in close proximity of TLR4:MD-2[147] and it is released before CD14 internalization[148]. Although its crucial role in the recognition of low LPS doses is established, CD14 has been shown to be largely dispensable for the response to high concentrations of LPS, which occur almost normally in CD14-deficient macrophages[142]. This notable observation suggests that an excess of LPS can be also sensed by means of a CD14-independent pathway, which possibly implies either direct LPS recognition by TLR4:MD-2[149] or the participation of different LPS binding proteins[150] (discussed below). Nevertheless, the biological relevance of these CD14-independent pathways in response to high doses of LPS must be cautiously analyzed, since the LPS concentrations measured during bacterial infections in vivo are several orders of magnitude lower than the ones used in the in vitro experiments described above. In addition to its concentration, the chemotype of LPS determines the extent of CD14 requirement for ligand recognition. It has been demonstrated that the O-glycosylated, S-form LPS typically triggers a response in terms of TNFα production that relies on CD14 activation, whereas CD14 is at least partially dispensable for the response to R-form LPS, which lacks a typical O-antigen[151]. This observation has been confirmed by a phenotypic screening of N-ethyl-N-nitrosurea-mutated mice, which showed that mice carrying a truncated version of CD14 (*Heedless*) lose the ability to activate the MyD88-dependent pathway and produce TNFα after stimulation with S-LPS but not with R-LPS or lipid A[152]. These data suggest that CD14 is essential for the presentation of S-LPS to TLR4:MD-2, whereas R-LPS or lipid A can be

directly sensed by the receptor complex, consistent with other reports[149,153]. Strikingly, Heedless mice are also unable to produce type I IFNs, a TRIF-dependent class of cytokines, when challenged with both S- or R-LPS and lipid A[152]. Therefore, it appears that CD14 is strictly required for the induction of the TRIF-dependent signaling pathway irrespective of the LPS chemotype. Even though different selection of each pathway by different LPS chemotypes has been observed[154], it should be stressed that the discrimination between LPS chemotypes seems to rely largely on TLR4:MD-2 rather than CD14 itself, since specific responses are observed even in the absence of CD14. The latter retains the ability to effectively bind both S- and R-LPS and its physiological expression nullifies their distinction in terms of signaling[152]. It has been proposed that CD14 specifically promotes TRIF-dependent signaling by acting as a chaperone or by inducing a conformational change in TLR4:MD-2 that results in the engagement of TRIF[152]. Regardless of the molecular mechanism, the observation that CD14 selectively regulates the production of type I IFNs can be interpreted as a major objection to the long-standing dogma that CD14, as a GPI-anchored receptor, can not have a direct signaling capability. As a matter of fact this theory needs to be revisited, also in light of recent reports showing that various GPI-anchored proteins, exemplified by CD59, transiently recruit and activate downstream kinases after antibody-mediated cross-linking[155-156]. Notably, GPI-lated CD14 itself has been demonstrated to trigger a phospholipase C (PLC)–dependent calcium mobilization following treatment with cross-linking antibodies[157]. Even though the

biological significance of these findings could be argued due to the artificial clustering of the GPI-anchored proteins, we recently established a direct signaling role for CD14 in triggering a DC-specific pathway after recognition of its natural ligand S-LPS[158]. The features of this CD14-dependent and TLR4-independent signaling pathways will be described in detail in Chapter 2 of this thesis.

MD-2. MD-2 is a 25-30 kDa glycoprotein that can be classified into the ML superfamily of lipid-binding proteins. It has been identified as a TLR4-interacting molecule that is essential for conferring LPS responsiveness to cells expressing TLR4[119]. Among all the TLR4 accessory molecules MD-2 is the only one that is absolutely required for the response to LPS, as evidenced by the observation that MD-2- and TLR4-deficient mice display the same LPS unresponsive phenotype[5,159]. Even though it has been demonstrated that MD-2 is the LPS binding component[160], it is not clear whether TLR4 may also directly bind LPS, as suggested by genetic evidence and recent structural studies[15,149]. Nevertheless, the TLR4:MD-2 complex binds LPS with higher affinity than MD-2 alone or CD14[161] and the binding affinity of MD-2 or MD-2:LPS complex for TLR4 is the same[160], suggesting that either CD14 or soluble MD-2 may shuttle LPS to TLR4:MD-2. MD-2 has also been identified in the form of covalently linked oligomers[162]. However, the biological function of MD-2 oligomers has remained elusive, since it has been shown that only the monomeric form of MD-2 is competent for binding LPS and forming a stable LPS:MD-2 complex able to transduce the signal

through TLR4[140,163]. The crystal structure of MD-2 has recently been solved , both alone and complexed with TLR4[14-15,164]. MD-2 is a β cup-folded protein with two antiparallel β-sheets that are separated on one side, with the internal hydrophobic core forming a large internal pocket, lined with cationic residues, which can accommodate LPS. The interaction between MD-2 and TLR4 relies on hydrogen and electrostatic bonds between two complementary charged patches located on each molecule[14] that have been identified as crucial for the formation of the TLR4:MD-2 complex[165-167]. The crucial role of MD-2 in LPS recognition has been identified in its being instrumental for ligand-induced receptor dimerization[168]. In initial structural analyses, MD-2 has been complexed with the antiendotoxic, tetra-acylated phospholipids Eritoran and lipid IVa[14,164]. Both studies showed that the four acyl chains of the molecules completely fill the hydrophobic pocket of MD-2 and that additional electrostatic interactions are operated between the phosphate groups and the cluster of positively charged residues at the edge of the pocket. Notably, Eritoran was shown not to make direct contact with TLR4 and not to promote TLR4 dimerization[14]. It was proposed that the binding of a bigger, agonistic LPS molecule carrying six acyl chains could have resulted in a major conformational change of MD-2, thus exposing otherwise hidden interaction sites (Phe126, Gly129, His155) for binding to the C-terminal domain of a second TLR4 molecule[14,168]. The authors alternatively hypothesized that four of the six acyl chains of LPS could insert into the hydrophobic pocket of MD-2, whereas the two remaining lipid could protrude from the molecule and

directly interact with a second TLR4 molecule. As a compendium of both models, the recently reported structure of a crystallized TLR4:MD-2:LPS complex clearly revealed that, upon LPS binding, a symmetric “m”-shaped multimer composed of two TLR4:MD-2 heterodimers is formed[15]. The hydrophobic pocket of MD-2 accommodates five acyl chains of LPS, with the remaining chain exposed to interact with a secondary TLR4 by means of hydrophobic interactions. The phosphate groups also contribute to the dimerization by interacting with cationic residues of primary MD-2 and TLR4, as well as secondary TLR4[15,169]. Additionally, LPS binding induces a localized structural change in MD-2, triggering the protrusion of its Phe126 loop that makes hydrophilic contact with a secondary TLR4[15]. In light of these recent findings, it seems that many local forces and interactions determine the extent of receptor dimerization and the resultant signaling. This experimental framework will be essential to systematically investigate how the TLR4:MD-2 system discriminates between different LPS molecules and how specific recognition is achieved.

Accessory Molecules and Structures. Despite the crucial importance of the LBP:CD14:TLR4:MD-2 system described above, an LPS sensing machinery composed of such a small number of proteins may be an oversimplification that hardly explains the whole complexity of the LPS response. How a system will react to LPS may indeed vary significantly according to a number of parameters, including the host species, the cell type or the cell differentiation/activation state and

the nature, the concentration or the duration of the stimulus. Additionally, the toxicity of an uncontrolled LPS response inevitably requires a panel of negative regulators. A growing list of accessory molecules, either positively or negatively involved in LPS recognition, has been identified that may compose a combinatorial cluster whereby the differential usage of co-receptors results in a specific LPS response[150,170].

RP105 (radioprotective 105 kDa protein) is an LRR-containing type I transmembrane protein that shows significant homology with TLR4. However, in contrast to TLRs, RP105 lacks a TIR domain but its cytoplasmic portion is composed of a short sequence consisting of 6-11 aminoacids[171]. Similarly to TLR4, whose proper function requires MD-2, RP105 both surface expression and activity are strictly dependent on the MD-2 homologue, MD-1[172]. RP105 was originally identified and characterized in B cells as a protein governing anti-RP105-mediated proliferation[173]. Since RP105-deficient B lymphocytes display a blunted LPS-driven proliferative response[174-175], RP105 is believed to positively regulate TLR4-mediated LPS recognition in this cell type. Nevertheless, the role of RP105 in cells of the myeloid lineage appears to be quite different, since it has been reported as a negative regulator of TLR4 in cell lines or in primary DCs and macrophages[176]. No ligand for RP105 has been reported nor any direct interaction between RP105 and LPS has been demonstrated[176-177], suggesting that RP105 may not function as a sensor or as a molecular sink for LPS. On the other hand, RP105:MD-1 and

TLR4:MD-2 co-immunoprecipitate bidirectionally and these interactions seem to be directly mediated by MD-1 and MD-2[176]. These observations led to the hypothesis that in myeloid cells, where TLR4 is highly expressed, RP105 may inhibit the LPS binding ability of TLR4:MD-2 by forming heterodimeric complexes. In B cells, where TLR4 expression is limiting, the formation of these heterodimers may facilitate TLR4 multimerization instead[178]. However, formal proof for this theory is lacking and additional experiments will be required for explaining the opposite effects of RP105 in LPS signaling between different cell types.

TIR8, also known as SIGIRR (single Ig IL-1-related receptor) is a IL-1R-like glycoprotein with a single Ig domain, an atypical TIR domain lacking two cysteines that are normally conserved and play essential roles in IL-1R signaling, and an additional cytosolic tail whose function is unknown[179-180]. It is widely expressed in a number of tissues, as well as B lymphocytes and innate immune cells[181-182] but its transcription is downregulated upon LPS stimulation[183]. Even though no specific ligand has been reported[179], TIR8 is a well known negative regulator of the signaling triggered from some IL-1R and TLR family members, including TLR4, TLR7 and TLR9[184]. The inhibitory mechanism differs between the two receptor families, as both the Ig and the TIR domain are required for inhibiting IL-1R signaling but only the TIR domain is necessary to block LPS signaling[185]. Indeed, whereas the mutated TIR domain of TIR8 affects both IL-1R and TLR4 signaling by interfering with the recruitment of downstream

components, namely IRAK1 and TRAF6, the Ig domain is selectively responsible for inhibiting the heterodimerization of IL-1R[185].

ST2 (suppressor of tumorigenicity 2) is a transmembrane negative regulator of TLR signaling that sequester downstream adaptor proteins, namely TIRAP and MyD88, through its TIR domain[186]. The extracellular domain of ST2 contains three Ig-like domains that do not bind LPS, but have been reported to recognize the IL-1 family member IL-33[187]. ST2 also exists in a soluble form, sST2, that inhibits TLR signaling through binding to a putative ST2 receptor and suppressing the transcriptional expression of TLR4[188].

The complement receptors *CR3* (also called Mac-1 or CD11b/CD18) and *CR4* (CD11c/CD18) are transmembrane glycoproteins that belong to the β2-integrin family[189]. At variable extents and combinations, they are expressed by neutrophils, monocytes, macrophages and NK cells, and they play a major role in a number of cellular functions, including cell adhesion, cell migration and phagocytosis[189]. Additionally, CR3 and CR4 have been reported to bind LPS[190-191] and to trigger LPS-induced, serum-independent, NF-κB activation after heterologous expression in the TLR4-expressing CHO cell line[192-193]. Direct interaction between CR3 and CD14 has also been shown in neutrophils stimulated with LPS in the presence of serum or LBP, implying a receptor crosstalk[194]. This observation led to a model of CR3 function in which LPS-induced CD14 clustering increases the local concentration of LPS and CR3, thereby potentiating the binding of LPS to CR3. This in turn results in a marked increase of CR3 adhesive

properties, bridging bacteria to the surface of phagocytes[125], and in the subsequent detachment from CD14[194], which then transfers LPS to TLR4:MD-2. Notably, CR3-mediated adhesion and phagocytosis that are up-regulated by LPS require its cytoplasmic domain, whereas a tail-less CR3 mutant can still contribute to NF-κB activation[193]. This suggests that CR3 may function, similarly but less efficiently than CD14, by presenting LPS to TLR4:MD-2. However, the significance of CR3 in mediating the cellular response to LPS has been questioned by a study reporting that CD18-deficient human monocytes and macrophages respond normally to LPS treatment in terms of TNFα and IL-1β production[117]. These data are in agreement with other reports showing that monoclonal antibodies against CD11b or CD18 do not impair TNFα release by human monocytes[195-196]. On the other hand, CR3 has been shown to be important for the production of TNFα by membrane-bound, particulate LPS[196] as well as for the expression of a panel of LPS-induced genes, including COX-2, IL-12p35, IL-12p40[197] and IL-6[198]. A recent report also points to a crucial role for CD11b in regulating LPS signaling in macrophages, whose implications will be described below[198].

Lipid rafts are nanoscaled, spatially defined, assemblies of sphingolipid and cholesterol that fluctuate in lipid bilayers[199]. The lipid raft concept is opposed to the classical model of the cell membranes as a uniform and passive solvent for proteins. Instead, it proposes the existence of discrete microdomains that are laterally segregated from the cell membrane in which they navigate, due to

the preferential interaction between certain lipids. The raft hypothesis originated from the observation that the lipid composition between the apical and the basolateral membrane of epithelial cells are distinct, but only recently the technological advances in the field of fluorescence and electron microscopy demonstrated their existence in living cells. The lateral organization of lipid rafts, which renders them resistant to Triton X-100 solubilization at 4°C, stems from the close association of sphingolipids bearing saturated hydrocarbon chains in which cholesterol molecules intercalate. Accordingly, cholesterol-depleting agents have been used as a tool for disrupting lipid raft in order to study their biological function. The key feature of lipid rafts is that they incorporate proteins: GPI-anchored, doubly acylated, cholesterol-linked and palmitoylated but also some transmembrane proteins have been reported to show a significant raft affinity[200]. Selective protein residency in lipid rafts mainly defines their secretion route as well as their mode of endocytosis[201]. However, for the purposes of this thesis, the most interesting aspect of lipid rafts is that they act as signaling platform by increasing the local concentration of receptors and downstream effectors. A crucial role for raft-mediated protein clustering has been clearly demonstrated for the induction of IgE and TCR signaling, but recent works also included the LPS sensing molecules in the list of receptors whose activity is regulated by their localization into microdomains. As a GPI-anchored protein, CD14 normally resides in lipid rafts together with hsp70 and hsp90[118,202-203]. Upon LPS stimulation, TLR4:MD-2, CXCR4,

and GDF5 as well as MAPKs are transiently recruited and segregated to microdomains, as determined by biochemical and FRAP measurement[202-205]. Consistent with these observations, pharmacological disruption of raft integrity sensibly impairs the cellular response to LPS in terms of cytokine production[203,206]. A significantly reduced TLR4 mobilization into lipid rafts has also been observed in LPS-pretreated cells, suggesting that the regulation of this mechanism may contribute to endotoxin tolerance[207]. Notably, we have recently shown that the sub-localization of CD14 in lipid microdomains is crucial for its own, TLR4-independent, signaling induced by LPS[158].

As described above, a conspicuous number of regulators of TLR4 signaling has been identified that exert their function by acting at each step of receptor activation. Ligand binding affinity, receptor sublocalization, clustering and dimerization as well as downstream effector recruitment and activation are all subjected to both positive and negative forces that altogether shape the host response to LPS. In light of the complexity of this recognition system, further experiments are required to fully appreciate the relative contribution of each participant and to characterize how the interplay between them relates to the different, highly specific response triggered by bacterial infections.

TLR4 SIGNALING PATHWAYS

Upon ligand binding, TLRs trigger several signaling pathways that culminate in the activation of specific transcription factors. Independently of the nature of the pathogenic stimulus, every TLR stimulates the production of proinflammatory cytokines (e.g. TNFα, IL-6, proIL-1β) whose transcriptional regulation depends on NF-κB (nuclear factor-κB) and AP-1 (activator protein-1). All TLR family members, except from TLR3, signal NF-κB and AP-1 activation by means of the adaptor MyD88 (myeloid differentiation primary response gene 88). In the case of TLR3, the same biological output is obtained through the usage of a different adaptor, TRIF (TIR-domain containing adaptor inducing IFN-beta). Whereas the activation of NF-κB and AP-1 is a common feature of the signaling induced by all TLRs, only a subset of them is additionally able to trigger the production of type I interferons (IFNα and IFNβ), a class of cytokines with potent antiviral and antibacterial activities. Indeed, only the intracellular, nucleic acid-sensing TLRs (TLR3, TLR7, TLR8 and TLR9) can activate the transcription factors IRF (interferon regulatory factor)3 and IRF7, which largely regulate the expression of type I IFNs. This implies that the induction of type I IFNs is TRIF-dependent for TLR3, whereas it is MyD88-dependent for TLR7, TLR8 and TLR9.

A notable exception to the rules outlined above, i.e. (i) a single TLR can signal through either MyD88 or TRIF and (ii) only intracellular TLRs trigger a type I IFN response, is represented by TLR4. Indeed, TLR4 recruits both MyD88 and TRIF to induce the activation of NF-κB

and AP-1 and, similarly to TLR3, it uses TRIF to stimulate the production of type I IFNs, though in response to non-nucleic acid ligands. Furthermore, TLR4 requires the two additional upstream adaptors TIRAP (TIR-domain containing adaptor protein) and TRAM (TRIF-related adaptor molecule), which mediate the recruitment to TLR4 of MyD88 and TRIF, respectively. TLR1,2 and 6, (but not TLR5, 7,8 and 9) share the usage of TIRAP as a MyD88 bridging adaptor, whereas TRAM is specifically recruited to TLR4 (and not TLR3). These features of TLR4 effectively synthesize the complexity of TLR signaling, making it a prototype for the whole family. The next section will deal with the signaling pathways emanating from TLR4 in response to LPS, describing the modes of adaptor recruitment as well as the downstream cascades that lead to the biological outcome.

TLR4 Adaptor Recruitment

Originally showed to mediate IL-1R-dependent activation of NF-κB[208-209], MyD88 is now known to play a crucial role for the signaling pathways induced by almost every TLR[195,210-211]. MyD88 has a modular structure[212], which consists of a C-terminal TIR domain that is responsible for the interaction with the TIR domain of recruiting TLRs, an intermediate domain, and an N-terminal death domain (DD) that performs homotypic interactions with downstream proteins of the IRAK family[208-209]. Whereas MyD88 can be directly recruited to some TLRs (TLR5, 7, 8 and 9), it is not able to efficiently interact with

TLR4 (and TLR1, 2 and 6), probably due to a lack of electrostatic complementarity between the respective TIR domains[20]. Therefore, TLR4 (and TLR1, 2 and 6) requires the intermediate adaptor TIRAP, also known as MAL (MyD88 adapter-like), to bind MyD88[213-214]. Limited to TLR4 and TLR2 stimulation, the phenotypes of MyD88- and TIRAP-deficient mice are largely overlapping, with a completely abolished proinflammatory cytokine production that demonstrates a crucial role for TIRAP upstream of MyD88 in signaling by TLR4 and TLR2 but not by TLR9, IL-1R or IL-18R[215-216]. However, despite their total inability to produce proinflammatory cytokines, a reduced and delayed activation of NF-κB and AP-1 is still detectable in MyD88- or TIRAP-deficient mice after TLR4 but not TLR2 stimulation[211,215-216]. This late wave of NF-κB activation, as well as the expression of type I IFN genes, is a hallmark of the MyD88-independent signaling pathway that is specifically triggered by TLR4 by means of the adaptor TRIF, also known as TICAM-1 (toll-like receptor adaptor molecule 1)[216-217]. Accordingly, TRIF-deficient mice do not produce measurable amounts of proinflammatory cytokines in response to TLR4 or TLR3 stimulation, but the early activation of NF-κB is still observed after LPS treatment. Any response to LPS is instead totally eliminated in mice doubly deficient for MyD88 and TRIF, showing that TRIF accounts for the MyD88-independent aspect of TLR4 signaling. Furthermore, no detectable expression of type I IFNs induced by TLR4 or TLR3 is observed in TRIF-deficient mice[218-219]. Additionally, a fourth adaptor, TRAM (or TICAM-2), is specifically required for TLR4 to recruit TRIF and thereby promote TRIF-

dependent NF-κB and IRF3/7 activation[217,220], two biological processes that are inhibited by the TIR-containing protein SARM (sterile α- and armadillo-motif)[221]. SARM is thought to negatively regulate TRIF, but not MyD88, by directly binding to it and to prevent the recruitment of key downstream effectors.

Since TIRAP and TRAM are TIR-containing proteins that do not possess any obvious effector domain (i.e. DD) they are believed to function as bridging adaptors for MyD88 and TRIF, respectively. Recently, this hypothesis has been experimentally validated by several studies that also provided a detailed molecular description of the early events triggered by LPS recognition[198,222]. Opposite to the common view of TIR-containing adaptors as cytosolic proteins that are recruited to membranes by means of TIR-TIR interactions with dimerized TLRs, TIRAP is normally found at actin-rich membrane ruffles as well as in intracellular vesicles and its localization is TIR-independent[198]. Instead, both its localization and its function rely on an N-terminal phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$)-binding domain that is essential for targeting TIRAP to the membrane microdomains where TLR4 is located[198]. Since MyD88 is cytosolic, the biological function of TIRAP is to recruit MyD88 by means of a TIR-TIR interaction and to sort it to specific $PI(4,5)P_2$-rich membrane microdomains where signaling can initiate[198]. Consistent with the role of TIRAP as a sorting adaptor, MyD88-deficient macrophages, in which the localization of TIRAP is normal, do not produce cytokines after LPS stimulation. Instead, forced localization of MyD88 to $PI(4,5)P_2$-rich membrane microdomains restores the response to LPS

in TIRAP-deficient cells, demonstrating that MyD88 is the signaling adaptor[198]. PI(4,5)P_2 is a phospholipid that plays many critical functions in the cytoplasmic leaflet of the plasma membrane, of which it is a minor constituent (about 1%)[223]. Apart from being the source of the second messengers inositol 1,4,5-trisphosphate (I(1,4,5)P_3), diacylglycerol (DAG) and PI(3,4,5)P_3, it regulates several processes at the cell membrane, including endocytosis, exocytosis, actin polymerization and cytoskeletal rearrangements and enzyme activation[223]. Many of these functions are operated by virtue of its ability to recruit proteins that bear a suitable PI(4,5)P_2-binding domain, such as the PH (pleckstrin homology) domain. It is believed that PI(4,5)P_2 is concentrated in lipid raft, although this claim is somehow controversial and it can not be taken as a general assumption. Nevertheless, several reports have consistently shown that PI(4,5)P_2 is concentrated in nascent phagosomes and membrane ruffles[224-225]. The observations that TLR4 relocates to lipid rafts after activation[203] and that the PI(4,5)P_2-binding protein TIRAP colocalizes with TLR4[198] strongly suggest that PI(4,5)P_2 may indeed be found in TLR4-containing microdomains. Further evidence for this is provided by our discovery of a CD14-dependent pathway induced by LPS that promotes the activation of PLCγ2 , whose substrate is PI(4,5)P_2[158]. One of the mechanisms that can explain the existence of pools of PI(4,5)P_2 at the plasma membrane is the localized synthesis of this lipid through the phosphorylation of PI(4)P by a phosphatidylinositol 4-phosphate 5-kinase (PI(4)P5K)[226]. Interestingly, it has been reported that ARF6 (ADP-ribosylation factor 6), a known positive

regulator of PI(4)P5K[225] is essential for the localization of TIRAP and for the resultant TLR4 signaling[198]. ARF6 activation is in turn inhibited by AIP1, a newly described ARF6-GAP (GTPase-activating protein)[227]. Since β2 integrins can promote $PI(4,5)P_2$ synthesis at least partly by activating ARF6[189], the observation that CD11b-deficient macrophages show a defective TIRAP localization and an impaired IL-6 production in response to LPS suggests a model in which LPS recognition by CD11b, probably mediated by CD14 (see above), stimulates an ARF6-dependent acute synthesis of $PI(4,5)P_2$. This allows TIRAP to locally concentrate and sort a pool of cytoplasmic MyD88 to the membrane microdomains where activated TLR4 is recruited[198]. It should be noted that this mode of integrin function, although convincingly demonstrated, is not consistent with previous reports showing that a tail-less version of CD11b is unable to promote phagocytosis but still activates NF-κB after LPS stimulation[193]. However, these discrepancies may result from an unphysiological behavior of CD11b-transfected cells as well as from a differential membrane organization between the cell types (CHO and primary macrophages) used. Based on the observation that CD11b is the only β2 integrin expressed in macrophages but DCs also express CD11c[228], one might speculate that in the latter cell type the two integrins may act comparably, since both have been shown to bind LPS[190-191]. Specific experiments will be required to test this hypothesis. Additionally, it is plausible that a similar MyD88 recruitment strategy might be shared by TLR2, which also requires TIRAP to signal MyD88-dependent activation of NF-κB[229]. Consistent

with this hypothesis, also TLR2 relocates to lipid raft after stimulation[204], it uses CD14 as a co-receptor and it is assisted by β2 integrins in the recognition of Gram-positive bacteria[230].

The functional distinction of TIRAP and MyD88 acting as a sorting and signaling adaptor has recently been shown to hold true also for the TRAM-TRIF axis, even though the modes by which the two adaptor pairs are recruited to TLR4 vary significantly[222]. The resultant model that describes the early phases of LPS-induced signal transduction is in sharp contradiction with the long-standing dogma that TLR4 simultaneously induces the TIRAP-MyD88 and the TRAM-TRIF pathways from the cell membrane. Indeed, unlike TIRAP, TRAM is found both at the plasma membrane[231] and in the $Rab5^{+}$ early endosomal compartment in resting cells[222]. The peculiar localization of TRAM is totally TIR-independent but it is due to an N-terminal bipartite domain that contains a myristoylation motif[222,231] (aminoacids 1-7) and a polybasic region (8-20)[222]. A careful deletion analysis has revealed that both halves of the bipartite motif are required for targeting TRAM to the plasma membrane but only the myristoylation site is necessary for endosomal localization[222]. A myristoylation-deficient TRAM (either by deletion of the first 7 aminoacids or by substitution of a crucial glycine residue) is indeed uniformly distributed in the cytosol[222,231] whereas a TRAM version lacking the polybasic region selectively resides in early endosomes[222]. Strikingly, unlike the cytosolic or a strict plasma membrane version, the endosome-targeted TRAM is able to fully restore the production of TRIF-dependent cytokines, such as IL-6 and RANTES, in TRAM-

deficient macrophages treated with LPS[222]. This notable observation suggests that the LPS-induced TRAM-TRIF signaling occurs from endosomes, rather than the cell membrane. In resting cells, TLR4 is found predominantly at the plasma membrane but it also recycles to $Rab5^{+}$ early endosomes by means of a dynamin-dependent mechanism that is different from the one used by TRAM, whose localization is insensitive to dynamin inhibition[222]. Within 15-30 minutes of LPS stimulation, TLR4, together with CD14 and LPS, is actively endocytosed through a dynamin-dependent mechanism[222,232-233]. Although it has been reported that the main function of LPS-induced TLR4 internalization is to promote its degradation[232], the inhibition of TLR4 endocytosis results in a selective impairment of TRAM-TRIF signaling, as measured by IRF3 activation and IFNβ production[222]. This suggests that, after LPS stimulation, plasma membrane TLR4 moves into endosomes to interact with TRAM, which in turn recruits TRIF to trigger MyD88-independent NF-κB and IRF3 activation. Not only these findings demonstrate that the TIRAP-MyD88 and TRAM-TRIF signal transduction pathways occur from spatially separated cellular locations, but that they also originate sequentially in time. Therefore, a new model for the initiation of TLR4 signaling can be envisaged whereby initial LPS recognition by CD14 at the plasma membrane triggers the recruitment of TLR4 into $PI(4,5)P_2$-rich lipid rafts, as well as the acute synthesis of $PI(4,5)P_2$ through CD11b- and ARF6-mediated activation of PI(4)P5K. Here, TIR-TIR interactions drive the recruitment to TLR4 of the $PI(4,5)P_2$-binding protein TIRAP, which in

turn sorts MyD88 to TLR4. This leads to the MyD88-dependent early wave of NF-κB and AP-1 activation. Soon after LPS stimulation, dynamin-dependent receptor endocytosis is initiated. At this early phase of membrane rearrangement, a synthesis of $PI(4,5)P_2$ is observed, probably because it provides docking sites for cytoskeletal components that regulate actin capping and nucleation[224,234]. However, a subsequent drop in the local concentration of this phospholipid is required for the severing and the cytosolic release of a vesicle[224,234] containing LPS, as well as TLR4 and CD14. Since this compartment is then devoid of $PI(4,5)P_2$[235], TIRAP detaches from the endosomal membrane, leaving the TIR domain of TLR4 available for interaction with TRAM. TLR4-bound TRAM acts as a sorting adaptor for TRIF, which is recruited to TLR4 at the endosome to signal late NF-κB and AP-1 activation as well as IRF3 nuclear translocation. As the early endosome matures to $Rab7a^+$, a splicing variant of TRAM, TAG (TRAM adaptor with GOLD domain), inhibits TRAM-TRIF signaling by competing with TRIF for binding TRAM[236]. Instead of TRAM bipartite motif, TAG has an N-terminal GOLD domain that targets it to the endoplasmic reticulum as well as early endosomes in resting cells. Upon LPS stimulation TAG moves to late endosomes, where it functions as a signal switch-off by displacing TRIF from TRAM and by promoting the endolysosomal degradation of TLR4[232, 236]. Triad3A is an E3 ubiquitin ligase that also promotes TLR degradation, even though the mechanisms regulating its participation to TLR trafficking is unknown[237]. How LPS-induced TLR4 endocytosis is initiated and which receptor proteins govern it, what is the role of $PI(4,5)P_2$ in this

mechanism and how $PI(4,5)P_2$ metabolism is regulated remain key questions that will need to be addressed by specific experimental approaches. Additionally, the functional reason for TRAM to localize at plasma membrane instead of residing exclusively in early endosomes remains unclear. A likely explanation comes from the analysis of the N-terminal bipartite domain of TRAM. This unstructured motif is also found on several proteins that are known to shuttle between the plasma membrane and the endosomes[222]. Among them the most notable is MARCKS (myristoylated alanine-rich protein kinase C substrate), a protein that shows a particular affinity for acidic phospholipids as $PI(4,5)P_2$ or $PI(3,4,5)P_3$[238]. Since the myristoyl group alone is not sufficient for anchoring proteins to the plasma membrane, a second signal is required for effective protein localization. Basic residues in the polybasic region of the N-terminal unstructured domain of MARCKS have been reported to interact with the negatively charged heads of three molecules of $PI(4,5)P_2$[239] and MARCKS has been proposed to contribute to the lateral sequestration of $PI(4,5)P_2$ in the cell membrane. According to this model, there are two ways to displace MARCKS and release $PI(4,5)P_2$ at the cell membrane, both of which result in the disruption of the electrostatic interactions between the two molecules: (i) a Ca^{2+}-activated calmodulin binds the polybasic region of MARCKS, and (ii) phosphorylation of three serines in the effector domain by a PKC[238]. Similar to MARCKS, membrane-bound TRAM preferentially colocalizes with TLR4 in $PI(4,5)P_2$-rich microdomains. Interestingly, a serine residue in the basic domain of TRAM is phosphorylated by

PKCε after LPS stimulation and this event has been shown to play an important role in TRAM signaling[240]. Since the initial colocalization with TLR4 allows TRAM to be co-endocytosed with the receptor and that phosphorylation by PKCε may well be important for their internalization , it is likely that this is a system for maximizing TLR4-TRAM interactions in endosomes. It is also tempting to speculate that TRAM initially acts as a "pipmodulin" that concentrates $PI(4,5)P_2$ at the cell membrane and that, upon PKCε phosphorylation, it releases $PI(4,5)P_2$ favoring its lateral dispersion, a condition for effective endocytosis.

Finally, a recent study has reported the striking ability of TLR2 to signal type I IFN production in response to viral ligands, although only in $Ly6C^{hi}$ inflammatory monocytes[241]. Receptor internalization has been shown to be crucial for TLR2-induced type I IFN production[241], suggesting that also TLR2 induces distinct signal transduction pathways from both plasma membrane and intracellular locations. However, since TLR2 does not use TRAM or TRIF as adaptors, it is plausible that a $PI(4,5)P_2$-consuming endocytosis as the one proposed for TLR4 may not function for TLR2, as this would result in TIRAP-MyD88 detachment. Still, this observation reinforces the idea that TLR signaling must be studied from a cell biological point of view in order to integrate innate immune responses with the cellular infrastructure in which they operate[102] (**Fig. 1**).

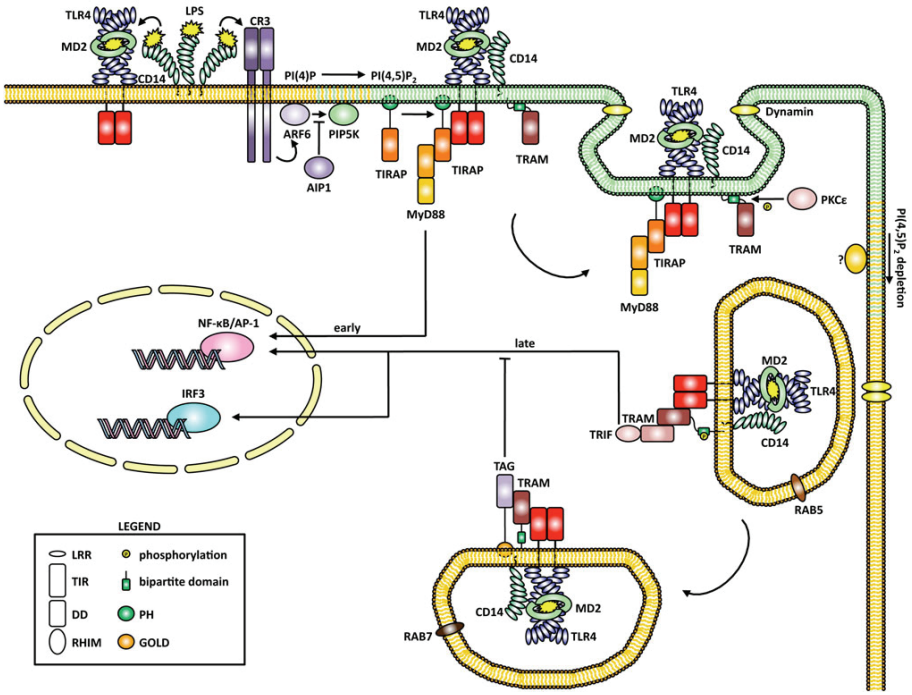

Fig. 1. Cell biology of TLR4 adaptor recruitment. Upon LPS recognition, CD14 mediates endotoxin transfer to TLR4 and promotes its homodimerization. CD14 also triggers CR3 activation by LPS, resulting in a localized ARF6-dependent synthesis of PI(4,5)P2 through phosphorylation of PI(4)P by PIP5K. Activation of the latter by ARF6 is negatively regulated by AIP1. Newly generated $PI(4,5)P_2$ allows TIRAP recruitment and consequent sorting of MyD88 to the activated TLR4:CD14 complex, which signals early NF-κB/AP-1 activation from the plasma membrane. LPS recognition by TLR4:CD14 also promotes receptor endocytosis through a dynamin-dependent process that is initially promoted by $PI(4,5)P_2$ synthesis but requires $PI(4,5)P_2$ consumption for its termination. As a consequence of the drop in $PI(4,5)P_2$ concentration, whose regulation is currently unknown, TIRAP:MyD88 detach from $PI(4,5)P_2$–depleted $Rab5^+$ early endosomes, thereby freeing TLR4 for subsequent interaction with TRAM:TRIF. Due to its

bipartite localization domain, TRAM resides both in early endosomes that fuse with TLR4:CD14-containing vesicles upon recycling and is coendocytosed with the receptor complex. This allows TRAM to sort TRIF to TLR4:CD14, resulting in the TRIF-dependent late wave of signaling to NF-κB/AP-1 and IRF3. As $Rab5^+$ early endosomes mature, TAG, which resides in $Rab7^+$ late endosomes, displaces TRIF from TRAM and thereby inhibits TRIF-dependent signaling.

MyD88-dependent Signaling Pathway

General features of NF-κB and MAPK activation. NF-κB and AP-1 are crucial transcription factors that regulate a plethora of biological functions in both physiological and pathological conditions, including cell proliferation, cell development and inflammation. Since much research has focused on these key regulators of cell physiology in recent years, we refer the readers to other review articles for an extensive and comprehensive description of the subject[242-246]. Briefly, the NF-κB family of transcription factors is composed of five members, p50 (NF-κB1) and its precursor p105, p52 (NF-κB2) and its precursor p100, RelA (p65), RelB and c-Rel all of which share an N-terminal Rel-homology domain (RHD) that mediates homo- and heterodimerization as well as sequence-specific DNA binding. Only RelA, RelB and c-Rel contain a transactivation domain (TAD) that regulates gene transcription, whereas p50 and p52 act as dimerization or DNA-binding partners. NF-κB transcriptional activity is largely regulated by subcellular localization, as in resting cells NF-κB dimers are typically sequestered in the cytosol by the “classical”

IκB (inhibitor of NF-κB) proteins, namely IκBα, IκBβ and IκBε. IκBα is the most studied family member and it is involved in the regulation of the NF-κB dimer RelA:p50 that is activated in response to inflammatory stimuli. IκBs bind NF-κB dimers through multiple ankyrin repeats and, albeit incompletely, mask their nuclear localization sequence (NLS). The strong nuclear export sequence (NES) of IκBs results in an apparent steady-state cytoplasmic localization of I-κB:NF-κB complexes that derives from their constant shuttling between the nucleus and the cytosol. Receptor stimulation triggers a rapid K48-linked ubiquitination of IκB proteins and their subsequent degradation by the proteasome, resulting in the release of NF-κB proteins that translocate into the nucleus to promote gene expression. Notably, one of the earliest genes induced by NF-κB upon LPS stimulation is IκBα, which negatively regulates NF-κB activation by means of a negative feedback loop. IκB degradative ubiquitination is dependent on a previous site-specific phosphorylation event that is operated by an activated IKK (IκB kinase) complex, composed of two kinase subunits (IKKα and IKKβ) and a regulatory component (IKKγ/NEMO). IKKβ accounts for the largest part of kinase activity in canonical NF-κB signaling, with IKKα playing a minor role that is still sufficient to compensate for IKKβ deficiency. NEMO (NF-κB essential modulator) is absolutely required for classical NF-κB activation, since it interacts with both IKKs and it induces the formation of a hexameric (NEMO)2:(IKKα:IKKβ)2 complex upon stimulus-induced dimerization. The biochemical details of how IKK catalytic activity is achieved are still unclear, but it probably results from IKK trans-

autophosporylation in the activation loop as a consequence of NEMO-mediated oligomerization of IKK complexes. Alternatively, this initial phosphorylation step may be operated by an upstream IKK kinase, namely TAK1 (TGFβ-activated kinase 1), whose activation is linked to receptor stimulation. Regardless of the molecular mechanism of IKK activation, post-translational modifications other than phosphorylation (K63-linked polyubiquitination, see below) are necessary for recruiting NEMO to the activated receptor and thus linking extracellular signals to intracellular responses.

AP-1 is a dimeric transcription factor that is composed by members of the Jun, Fos, Maf and ATF subfamilies of basic leucine zipper (bZIP) proteins. AP-1 activation is regulated by various mechanisms affecting transcriptional expression, protein turnover, and dimer formation of AP-1 subunits. In this process, a crucial role is additionally played by post-translational modifications, namely phosphorylation by MAPKs (mitogen activated protein kinases) in the nucleus. AP-1 activation by inflammatory stimuli is mostly mediated by the JNK (Jun N-terminal kinase), p38 and ERK (extracellular signal-related kinase) groups of MAPKs, which are in turn phosphorylated by the MAPK-kinases (MAPKK) MKK4/7, MKK3/6 and MKK1/2, respectively. A MAPKK-kinase (MAPKKK), namely TAK1, which is directly linked to the signaling pathway emanating from the stimulated receptor, mediates upstream activation of MAPKK. It has to be stressed that, whereas stimulus-induced MAPKKK activation may occur upon recruitment to the receptor complex at the plasma

membrane, MAPKKs and MAPKs are localized in the cytosol, the cellular compartment where they can be most efficiently activated and where they can encounter substrates. Therefore, the cytosolic translocation of receptor signaling complex is a prerequisite for MAPK cascade activation. As we will describe below, this represents a key signaling event whose tight regulation ensures a full biological response to TLR stimulation (**Fig. 2**).

IRAKs. The first effectors downstream of MyD88 are the IRAK (IL-1 receptor-associated kinase) family proteins. Originally identified as signaling partners of IL-1R[247], IRAKs have later been shown to play crucial roles also in TLR-mediated responses (except from TLR3). However, much of our current knowledge about how IRAKs work is still based on early observations of IL-1R signaling pathways. Four different IRAKs have been identified in humans and mice, all of them sharing a basic domain organization[248]. All IRAKs have an N-terminal DD, which is typically responsible for recruitment to MyD88 and a central kinase domain (KD) that differentially confers them the ability to function as serine/threonine kinases. Between this two domains is located a region with an unknown function, named undetermined (UD). Functionally, IRAKs transduce signals from the activated receptor to their downstream effector TRAF6, although each member plays a specific role in this process. IRAK-4 is the first protein to interact with MyD88, and it is the only IRAK that is absolutely essential for TLR/IL-1R signal transduction[249-250]. Another feature that renders IRAK-4 unique among the family is that its biological activity

strictly relies upon a functional kinase activity[249]. Indeed, IRAK-4-deficient mice show profoundly impaired responses to IL-1 as well as TLR ligands[250] and overexpression of a kinase-deficient IRAK-4 in cells inhibits NF-κB activation[249]. IRAK-4 functions as a typical Ser/Thr kinase that, upon stimulation, phosphorylates the activation loop of IRAK-1[249], thereby triggering a massive autophosphorylation of IRAK-1[247,251]. This phosphorylation event appears to be negatively targeted by the phosphatase SHP1 (SH2-containing protein tyrosine phosphatase 1), which therefore inhibits NF-κB and MAPK activation[252]. The recruitment of IRAK-1 to MyD88 occurs with a slower kinetics than IRAK-4, due to the initial interaction of unphosphorylated IRAK-1 with the adaptor Tollip (Toll interacting protein)[253]. In resting cells, Tollip binds IRAK-1 in the cytosol and suppresses its kinase activity, consistent with an inhibitory effect of Tollip overexpression[253-254]. Upon activation, Tollip interacts with both TLR and IL-1R complexes, bridging IRAK-1 to MyD88 and favoring IRAK-1 signal transduction[255]. IRAK-1 and IRAK-4 do not form heterodimers[249] but they are brought into close proximity by means of MyD88, which acts as a signaling platform[256]. Accordingly, in the presence of MyD88s, a shorter splice variant of MyD88 that is induced by LPS, IRAK-4 is not recruited to MyD88 and no IRAK-1 phosphorylation is observed[256]. Upon hyperphosphorylation, IRAK-1 weakens its binding affinity for MyD88, while increasing its ability to bind TRAF6 (TNF-R-associated factor 6)[208, 257]. This results in a rapid and transient recruitment of TRAF6 to a conserved C-terminal motif of IRAK-1, followed by their dissociation from the receptor[258]. IRAK-1

and TRAF6 initially remain at the plasma membrane, where TRAF6 interacts with a pre-assembled complex composed of TAK1, an important downstream effector kinase (see below), and the TAK1 binding proteins (TAB)1, TAB2 and TAB3[258-259]. Upon binding to the IRAK-1:TRAF6 complex, TAK1 and TAB2 are phosphorylated by an unknown kinase, even though the meaning of this modification is not clear since it is not able to induce TAK1 kinase activity[258]. Catalytically active TAK1 is only observed in the cytosol, where TAK1, together with TAB1, TAB2, TAB3 and TRAF6, is released shortly after interacting with IRAK-1[258]. The process regulating the cytosolic translocation of the putative TRAF6:TAK1:TAB1:TAB2:TAB3 multimeric complex is poorly defined, although it has been shown to depend on IRAK-1[258,260-261]. Interestingly, IRAK-1 itself does not move into the cytosol but it remains at the cell membrane where it is degraded[251] through a mechanisms that conflicting reports have reported to be either proteasome-dependent[251] or -independent[262]. In this regard it should be noted that IRAK-1 contains two PEST sequence in its UD region, which might be involved in IRAK-1 degradation. It is plausible that IRAK-1 degradation functions as a system to promote the cytosolic release of the signaling complex containing TRAF6 and TAK1 and also as a negative feedback control that regulates excessive activation by TLR/IL-1R.

Despite the model depicted above may imply an essential role of IRAK-1 in inflammatory signal transduction, it is known that it is not sufficient in this process. IRAK-1-deficient mice are more resistant than wild-type to the toxic effects of LPS but their phenotype is much

less evident than IRAK-4- or MyD88-deficient mice, also in terms of cytokine production[263-264]. This suggests that other IRAK family members may take over IRAK-1 functions and compensate for its absence. Additionally, although IRAK-1 is undoubtedly functional as a kinase, it has been reported that IL-1-induced activation of NF-κB in cells lacking IRAK-1 can be fully restored by transfection of a kinase-deficient version of IRAK-1[265]. Therefore, rather than by phosphorylating substrates, IRAK-1 (and also IRAK-2 and IRAK-M, see below) appears to exert its role through protein-protein interactions, basically behaving as a signaling adaptor. Since its discovery, IRAK-2 has always been though to share functional, other than structural, properties with IRAK-1. Similarly to IRAK-1, IRAK-2 interacts with both MyD88 and TRAF6 and its overexpression in cells activates NF-κB[209]. The two proteins seem to act redundantly, as IRAK-2 can largely restore IL-1-induced NF-κB activation in IRAK-1-deficient cells[265], and they can also form heterodimers[266]. However, the physiological role of IRAK-2 has only recently been unveiled, thanks to the generation of gene-targeted mice[267]. The mechanical features of IRAK-2 function are comparable to those of IRAK-1, as also IRAK-2 is recruited to MyD88, where the initial phosphorylation by IRAK-4 triggers IRAK-2 own kinase activity and subsequent interaction with TRAF6[267]. IRAK-2 differs from IRAK-1 in that its action is delayed and it is important for sustaining NF-κB activation, rather than inducing it. Accordingly, cells from IRAK-2-deficient mice show an impaired cytokine production upon TLR/IL-1R stimulation as a result of a defective late-phase transcriptional expression[267]. This sustained activity of IRAK-2 is

consistent with a prolonged half-life of the protein, which is not degraded upon activation. Interestingly, the UD region of IRAK-2 lacks the PEST sequences that are observable in IRAK-1 and that may regulate its destruction. Although IRAK-1 and IRAK-2 act in a kinetically distinct fashion, they can largely compensate for each other's functions. This is clearly demonstrated by the observation that genetic ablation of either IRAK-1 or IRAK-2 in mice generates only a partially impaired phenotype compared to mice doubly deficient in IRAK-1 and IRAK-2, which are almost totally unresponsive to TLR/IL-1R stimulation[267]. An additional feature of IRAK-2 is that its biological function seems to depend on an intact kinase activity[267]. This observation is notable because the kinase domain of IRAK-2 is predicted to be non-functional, due to a mutation in a critical catalytic residue[266]. However, further experiment will be required to assess the real significance of IRAK-2 kinase activity, since it has been hypothesized that experimental artifacts may contaminate these data[268]. The fourth member of the IRAK family is IRAK-M, a kinase-inactive protein that is selectively expressed in monocyte and macrophage populations[266,269]. Although early studies have shown that its overexpression leads to NF-κB activation[265-266], IRAK-M has been reported to negatively regulate signaling through TLR/IL-1R by inhibiting the dissociation of IRAK-4 and IRAK-1 from the receptor[270]. IRAK-M expression is strongly increased upon LPS stimulation and it seems to play a crucial role in endotoxin tolerance, as shown by the observation that pre-stimulated IRAK-M deficient macrophages are not hyporesponsive to a second LPS administration[270].

In addition to IRAKs, several other tyrosine kinases regulate TLR4 signaling[271]. The Src-family kinase members Src, Hck and Lyn have been shown to phosphorylate TLR4 upon LPS stimulation, although the functional significance of this modification is unclear[272]. Btk (Burton's tyrosine kinase) is also involved in TLR4 signaling through phosphorylation of TIRAP, an event that is important for NF-κB activation[273] as well as for the SOCS1 (suppressor of cytokine signaling 1)-mediated TIRAP degradation resulting in inhibition of TLR4 signaling[274].

TRAF6. The TRAF family comprises seven family members that play important roles in the signal transduction to NF-κB triggered by a number of receptors, including TNF-R, IL-1R, TLRs, BCR and TCR[275]. The distinctive feature of all TRAF proteins is a C-terminal domain, termed TRAF[276], which is in turn composed by an N-terminal coiled-coil region (TRAF-N) and a C-terminal β-sandwich (TRAF-C)[277]. The TRAF domain mediates protein-protein interactions, with TRAF-N regulating self-oligomerization and TRAF-C conferring binding to upstream molecules[277]. Apart from TRAF1, all TRAFs contain a RING finger domain at their N-termini, followed by a variable number of zinc fingers[276]. The effector function of the RING domain has been revealed by biochemical studies on TRAF6, the crucial TRAF protein for MyD88-depedent NF-κB activation[278]. TRAF6 acts as an E3 ubiquitin ligase that, in coordination with the E2 ubiquitin-conjugating complex Uev1A:Ubc13, promotes the attachment of Lysine-63(K63)-linked polyubiquitin chains to several substrate

molecules, including TRAF6 itself[279]. Notably, K63-linked polyubiquitination differs from the classical K48-linked polyubiquitination in that it does not constitute a signal for proteasomal degradation[280], but it functions as a signaling moiety in many NF-κB regulatory pathways. This regulatory role relies upon the ability of a number of signaling molecules to recognize and interact with K63-linked polyubiquitin chains through specific ubiquitin-binding domains[281]. Therefore, K63-polyubiquitinated proteins act as scaffolds that recruit downstream effector molecules by providing them with suitable docking sites. Consistent with a crucial role of this post-translational modification in NF-κB signaling, many proteins involved in this pathway have been shown to function either as acceptor or interacting partners for K63-linked polyubiquitin chains. Key targets for K63-linked polyubiquitination in the TLR/IL-1R-induced MyD88-dependent pathway include TRAF6 itself[279], IRAK-1[262] and TAK1[282], whereas TAB2 and TAB3[283] and NEMO[284-285] have been shown to bind K63-linked polyubiquitins through specific ubiquitin-binding domains. These data have led to a model of MyD88-dependent signal transduction whereby, upon TLR/IL-1R stimulation, TRAF6 is recruited by IRAK-1 at the cell membrane. Here, due to the clustering of receptors and adaptors, several TRAF6 molecules oligomerize via their TRAF domains in a process that is assisted by the TRAF6-interacting protein TIFA (TRAF-interacting protein with forkhead-associated domain)[286-287]. Multimerized TRAF6 triggers its own E3 ubiquitin ligase activity[288] and, after interacting with Ubc13 and Uev1A[289], promotes its auto-K63-linked

polyubiquitination. K63-linked polyubiquitinated TRAF6 directly binds to TAB2 and TAB3[283,290] that, together with TAB1, interact with TAK1 at the cell membrane. Upon IRAK-1 degradation, this TRAF6-nucleated complex is released into the cytosol, where the interaction between TRAF6 and TAB2/TAB3 is though to result in TAK1 oligomerization mediated by TAB2/TAB3, followed by TAK1 transactivation[283].

Oligomerization of proteins is a driving force for many signaling pathways because it allows the formation of platforms where auto- or cross-interactions can take place. The TLR/IL-1R pathway is no exception to this rule, as MyD88 and IRAK-4 have been shown to form large complexes with 7:4 or 8:4 stoichiometry, the "Myddosome"[291], and artificial clustering of MyD88 is sufficient to mediate NF-κB and AP-1 activation without the need for receptor stimulation[292]. Therefore, it should be kept in mind that this signaling pathway generally does not act linearly (protein A activates protein B), but it rather relies on multiple, cooperative interactions that altogether bring molecules in close proximity to each other. In this context, K63-linked polyubiquitin chains represent a means for modified proteins to perform additional, long-range interactions. Despite the model of TLR/IL-1R-induced TAK1 activation is strongly based upon the role of K63-linked polyubiquitination of target proteins, a direct experimental evidence for this assumption has been provided only recently. Various studies have reported conflicting data about the importance of Ubc13 for the activation of NF-κB, at least in some signaling pathways, suggesting the existence

of additional E2 enzymes[293-295]. The formal demonstration for an essential function of K63-linked polyubiquitination in NF-κB activation has also been hampered by technical difficulties in mutating the multiple ubiquitin genes in vivo. However, the recent generation of a tetracycline-inducible ubiquitin replacement system in human cells has ultimately shown that Ubc13-mediated K63-linked polyubiquitination is essential for NF-κB activation by IL-1β (and presumably also by TLRs)[296]. Also the in vivo importance of the E3 ubiquitin ligase activity of TRAF6 has remained controversial[297]. However, another recent report has shown that, in a cell-free system, the essential role of TRAF6 for TAK1 activation depends on its ability to generate free, unanchored K63-linked polyubiquitin chains that act as second messengers to promote TAB2/TAB3-mediated TAK1 oligomerization without the need for a direct interaction with TRAF6[298]. This seminal study identifies a novel regulatory apparatus for activation of protein kinases by ubiquitin and it suggests a mechanism for NF-κB activation in vivo. Regardless of the molecular details of how K63-linked polyubiquitin polymers participate in cell signaling, the biological relevance of this post-translational modification is underlined by the existence of K63-specific deubiquitinating enzymes[299]. A20 is a crucial regulator of the inflammatory response, a role that it exerts by deconjugating K63-linked polyubiquitin chains from TRAF6 and RIP1[300]. Downstream of TLRs, A20 inhibits the E3 ligase activity of TRAF6 by promoting degradative K48-linked polyubiquitination of the E2 enzymes Ubc13 and UbcH5c[301]. It is possible that a similar biological function is

played by CYLD (cylindromatosis), which is likely to act redundantly with A20[302].

TRAF3 is another MyD88-interacting TRAF protein[292,303] whose function in TRIF-dependent TLR4 signaling is well established (see below). However, how it works in MyD88-dependent TLR4 signaling has remained elusive until recently. Differently from TRAF6, which promotes both NF-κB and MAPK activation, TRAF3 has been shown to play no role in NF-κB signaling but to act as a negative regulator of MAPK activation by CD40[304] and TLR4[305]. MAPK signaling occurs in the cytosol and it depends on prior intracellular translocation and consequent activation of TAK1, which acts as a MAPKKK in TLR signaling. Upon recruitment to the activated receptor complex at the plasma membrane, TRAF3 exerts its negative function by preventing the cytosolic release of the TRAF6-nucleated signaling complex containing TAK1[305]. Either directly or indirectly, MyD88 also recruits the E3 ubiquitin ligases cIAP(cellular inhibitor of apoptosis)1 and cIAP2, which specifically catalyze the transfer of K48-linked polyubiquitin chains to TRAF[304-305]. K48-linked polyubiquitination of TRAF3 by cIAP1/2 serves as a signal for its proteasomal degradation, an essential event for the detachment of the TAK1-containing signaling complex from the cell membrane and the subsequent TAK1 activation. How TRAF3 is recruited to MyD88 is unclear, but it is plausible that, similarly to TRAF6, it interacts with IRAK1 at the cell membrane. Since IRAK-1 has been proposed to undergo both non-degradative K63- (see below) and degradative K48-linked polyubiquitination through a process of ubiquitin editing[306], this

hypothesis raises the possibility that cIAP1/2 may also promote IRAK-1 degradation through K48-linked polyubiquitination. This would provide a mechanistic explanation for the cIAP1/2-dependent dissociation of the TRAF6-nucleated, TAK1-containing signaling complex. It is notable that cIAP1/2 catalytic activation depends on their previous K63-linked polyubiquitination mediated by TRAF6[305], which therefore links TLR4 stimulation with the relieving of TRAF3-mediated inhibition of MAPK signaling. Although neither E3 ubiquitin ligase activity nor K63-linked polyubiquitination of TRAF3 are observed in MyD88 signaling, TRAF3 works differently in TRIF-dependent signaling, where its alternative, non-K48-linked polyubiquitination provides it with an important signaling function.

TAK1. TAK1 is a crucial signaling intermediate in TLR/IL-1R and TNFα signaling to NF-κB and AP-1[307-308], a function that derives from its ability to induce IKK as well as MAPK activation[288]. Therefore, TAK1 constitutes the point in which MyD88-dependent pathway diverges into the NF-κB and AP-1 signaling branches. These biological processes regulated by TAK1 strictly rely on co-expression of TAB1, TAB2 and TAB3, since overexpression of TAK1 alone does not result in significant NF-κB activation[259,288,290]. TAB1 aids in TAK1 autophosphorylation acting as an activating subunit in the TAK1 complex[309], whereas TAB2 and TAB3 play redundant roles in facilitating TAK1 recruitment to TRAF6 through specific binding to K63-linked polyubiquitin chains. The physiological importance of TAB2 and TAB3 in TLR signaling is underlined by the observation that

the function of these two proteins is negatively regulated by the RING finger-containing TRIM30α[310]. TRIM30α promotes TAB2 and TAB3 degradation through the endolysosomal compartment, rather than by catalyzing K48-linked polyubiquitination[310]. Although TAK1 is undoubtedly required for NF-κB activation by proinflammatory stimuli, the mechanism by which TAK1 activates IKK is still unclear. TAK1 has been shown to function in vitro as an IKK-K by phosphorylating IKKβ in its activation loop[288]. In light of this observation, a model of TAK1-mediated IKK activation has been proposed in which TRAF6 K63-linked polyubiquitin chains recruit both TAK1 and NEMO, as NEMO contains an ubiquitin binding domain named NUB that confers specificity for K63-linked polyubiquitin chains and is required for NF-κB activation[284, 311]. Through this interaction, NEMO brings the catalytic subunits IKKα and IKKβ into close proximity with TAK1 at the receptor complex, thereby facilitating IKK activation through direct phosphorylation by TAK1. Notably, TAK1 itself has been recently reported to undergo TRAF6-mediated K63-linked polyubiquitination, a modification that is important for IKK recruitment[282]. As anticipated above, also IRAK-1 is a substrate for K63-linked polyubiquitination and modified IRAK-1 interacts with NEMO to promote NF-κB activation[262,312]. The E3 ubiquitin ligase responsible for K63-linked polyubiquitination of IRAK-1 has been identified as TRAF6[312] or Pellino isoforms[313] in conflicting studies. Importantly, Pellinos appear to be activated through phosphorylation by IRAK-1 and IRAK-4, whose kinase activity might be at least initially required to promote IRAK-1 K63-linked

polyubiquitination. All these observations suggest that TAK1-dependent IKK activation occurs at the plasma membrane through the concomitant recruitment of TAK1 and IKK at the receptor complex. However, formal demonstration for the existence and the biological significance of TAK1 phosphorylation of IKKβ in vivo is still lacking. Instead, several evidences point to a role of TAK1 in activating IKK that is independent of its kinase activity. Catalytically active TAK1 is only observed in the cytosol[258] after cIAP1/2-dependent detachment of TAK1-containing complex from the plasma membrane[304-305], whereas IKK activation is though to occur at the cell surface. Accordingly, inhibition of the cytosolic translocation of this complex by interfering with cIAP1/2 functionality completely blocks TAK1 catalytic activation, as measured by MAPK phosphorylation, but has no consequence on IKK activation, which proceeds normally[305]. These results strongly suggest that, whereas MAPK signaling is strictly dependent on TAK1 kinase activity, activation of NF-κB relies on TAK1 functions other than phosphorylation. As the relative importance of K63-linked polyubiquitination on specific proteins involved in IKK activation is difficult to ascertain, due to a certain degree of redundancy between potential scaffolding adaptors, all these observation are consistent with a model whereby K63-linked polyubiquitin chains act as second messenger to generate a complex molecular net that facilitates protein-protein interactions. Further complicating the picture is the discovery that additional E2 enzymes with different ubiquitin linkage specificity may participate in NF-κB activation. The E2 UbcH5c can interact with TRAF6 to synthesize

ubiquitin polymers containing both K48 and K63 linkages that trigger IKK activation by NEMO recruitment[298]. Interestingly, linear head-to-tail polyubiquitin chains can activate NF-κB by binding to another ubiquitin-binding region of NEMO[314] or by modifying NEMO itself[315], even though this type of ubiquitin polymer has been reported to inefficiently activate IKK in vitro[298].

Differently from its role as an IKK-K, TAK1 function as a MAPKKK is solidly documented. Upon cytosolic activation, TAK1 phosphorylates crucial substrates that in turn promote activation of MAPK signaling. TAK1 has been reported to phosphorylate MKK3/6[288] and MKK4[316], thereby promoting subsequent activation of p38 and JNK, respectively. ERK activation, which is regulated by MKK1/2 is believed to occur independently of TAK1 but to rely on the Tpl2 (tumor progression locus 2) MAPKKK. Interestingly, Tpl2 activation is promoted by IKK-dependent phosphorylation and subsequent degradative ubiquitination of p105, a negative regulator of Tpl2[317]. Additional MAPKKK whose activation is induced by TLRs and that may contribute to classic MAPK signaling include MLK (mixed lineage kinase)2 and MLK3, at least for p38 and JNK[318]. Finally, MEKK3 is an important MAPKKK that is believed to function similarly to TAK1 in TNFR-induced NF-κB and MAPK signaling[319]. An important role for MEKK3 has also emerged in TLR4 and IL-1R signaling, as its deficiency abrogates NF-κB as well as p38 and JNK MAPK activation[320]. MEKK3 is recruited to TRAF6 upon receptor stimulation, and it appears to function as the same hierarchical level of TAK1, albeit with a delayed activation kinetics[321]. This partial redundancy may explain why the

requirements for TAK1 in promoting NF-κB and MAPK activation vary significantly from cell type to cell type[308]. However, the molecular mechanisms regulating MEKK3 activation remain to be determined, particularly with respect to the role of K63-linked polyubiquitination of MEKK3.

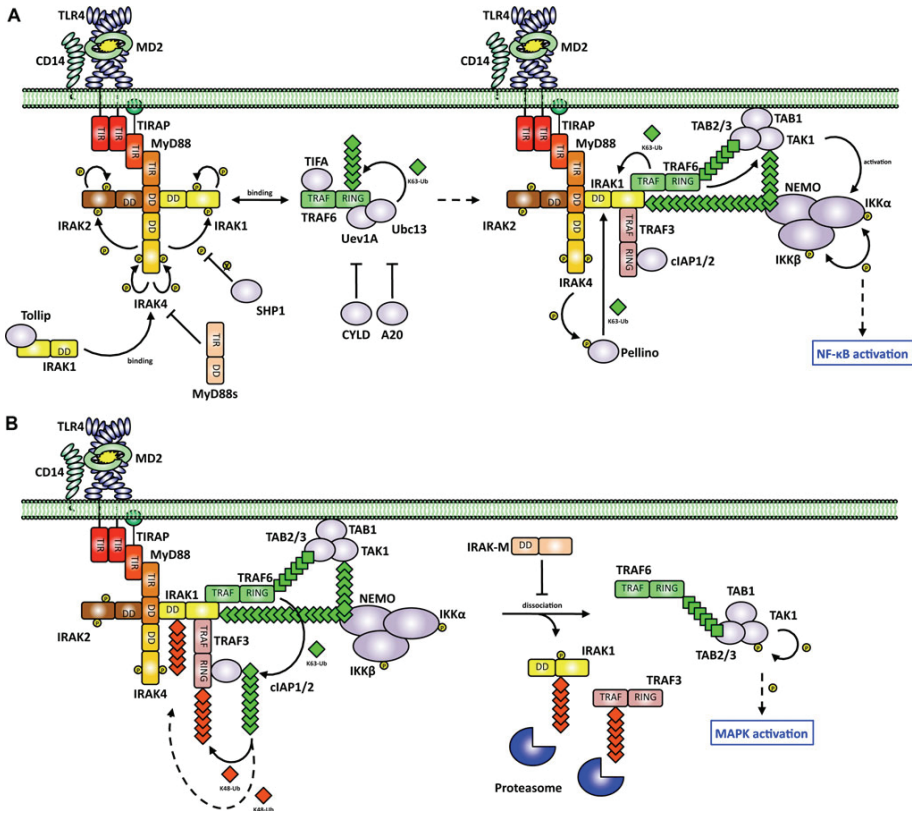

Fig. 2. MyD88-dependent NF-κB and AP-1 activation. **a**) MyD88 mediates recruitment to TLR4 of IRAK4, IRAK1 (through Tollip) and IRAK2 by means of a homotypic death domain (DD) interaction. This results in the activation of IRAK4 (by autophosphorylation), IRAK1 and IRAK2, which are initially phosphorylated by IRAK4 and then undergo additional autophosphorylation. Recruitment of IRAK4 and IRAK1 phosphorylation by

IRAK4 are negatively regulated by MyD88s and SHP1, respectively. Phosphorylated IRAK1 shows an increased binding affinity for TRAF6, which is in turn recruited to the receptor. TIFA-dependent oligomerization of TRAF6 stimulates its E3 ubiquitin ligase activity and, in coordination with the E2 complex Uev1A:Ubc13, TRAF6 catalyzes the attachment of K63-linked polyubiquitin chains on a number of substrates, including itself, in a process that is inhibited by CYLD and A20. Ubiquitinated TRAF6 interacts with TAK1 via TAB2/3. TRAF6 also promotes K63-linked polyubiquitination of IRAK1 and TAK1, which directly recruit NEMO to the receptor complex. Ubiquitination of IRAK1 may also be operated by Pellino, which is in turn activated by IRAK4 and IRAK1 itself (not shown). Upon corecruitment at the receptor complex, TAK1 promotes IKKα/β activation through a process that is independent on TAK1 kinase activity and that occurs at the plasma membrane. This ultimately results in MyD88-dependent activation of NF-κB. Even if not shown, it has to be noted that IRAK2 is likely to behave similarly to IRAK1 in promoting sustained NF-κB activation after IRAK1 degradation (see below). **b**) In addition to TRAF6, IRAK1 probably mediates the recruitment of TRAF3 and cIAP1/2 to the receptor complex where TRAF6 catalyzes K63-linked polyubiquitination of cIAP1/2. K63-linked polyubiquitinated cIAP1/2 is enzymatically active as an E3 ligase that promotes degradative K48-linked polyubiquitination of TRAF3 and possibly IRAK1. Upon subsequent proteasomal degradation of TRAF3 and IRAK1, the TRAF6-nucleated complex containing TAK1 dissociates from the receptor and is released into the cytosol in a process that is inhibited by IRAK-M. Once in the cytoplasm (the cellular compartment where TAK1 substrates are located), TAK1 triggers effective MAPK activation by initiating a cascade of phosphorylating events. The kinase activity of TAK1 is therefore absolutely required for cytosolic MAPK activation.

TRIF-dependent Signaling Pathways

RIP1-dependent NF-κB activation. The adaptor TRIF is structurally different from MyD88 in that it does not contain a DD but it has an N-terminal domain that has been reported to directly bind TRAF6 to promote NF-κB activation[322]. However, several studies in TRAF6-deficient cells have contradicted this observation, ruling out any contribution for TRAF6 in this biological process[278,292]. Instead, the key player for TRIF-dependent NF-κB activation induced by TLR3 and TLR4 is the kinase RIP1 (receptor-interacting protein 1), which interacts with TRIF via a RHIM (RIP homotypic interaction motif) domain that is located at the C-terminal region of TRIF[323]. This process is inhibited by RIP3, a related, inactive protein that competes with RIP1 for TRIF binding[323]. Proinflammatory cytokine, but not type I IFN production is impaired in RIP1-deficient cells after TLR3 or TLR4 stimulation, showing a selective involvement of RIP1 in NF-κB activation[323-324]. RIP1 is known to play a crucial role in TNFR-induced NF-κB activation, where it is recruited to the receptor complex through a homotypic DD interaction with the adaptor TRADD (TNFR1-associated death domain protein)[325-327]. Via its intermediate domain, RIP1 then binds the E3 ubiquitin ligase TRAF2/5 that, analogously to TRAF6, catalyzes the K63-linked polyubiquitination of RIP1 and the subsequent recruitment of NEMO[284,328]. Polyubiquitinated RIP1 also interacts with TAK1 via TAB2[283], and with MEKK3, which in turn activate IKKs. Recent studies have reported that the way in which RIP1 drives TRIF-dependent signaling to NF-κB after TLR stimulation is

reminiscent of what has been described for TNFR. After TLR stimulation, TRIF directly recruits RIP1, which in turn interacts with the adaptor TRADD via a homotypic DD interaction[326-327]. RIP1 then undergoes K63-linked polyubiquitination[324] independently of TRAF6, a process that facilitates IKK activation through TAB2:TAK1 recruitment. TRADD is functionally involved in TRIF-dependent NF-κB activation, as TRADD-deficient cells are impaired in their ability to produce proinflammatory cytokines upon LPS or poly(I:C) stimulation[326-327,329]. How TRADD acts at a molecular level in TLR signaling is still unclear, as TRAF2, which is a typical TRADD binding partner, plays no role in this pathway. It is tempting to speculate that TRADD may act as a scaffolding adaptor for an E3 ubiquitin ligase different from TRAF6 that mediates K63-linked polyubiquitination of RIP1. In this regard, a recent report has identified Pellino1 as a non-redundant E3 enzyme that, upon interaction with RIP1, catalyzes its post-translational modification and regulates NF-κB activation[330].

In addition to the pathway described above RIP1 can also promote NF-κB activation through a different mechanism involving FADD (Fas-associated death domain protein) and caspase 8. The FADD-caspase 8 axis is a well established regulatory system for the initiation of death receptor-induced extrinsic apoptosis. According to this paradigm, FADD and the inactive pro-caspase 8 are part of a death-induced signaling complex (DISC) that drives pro-caspase 8 oligomerization and its "proximity-induced self activation"[331]. Activated, self-cleaved caspase 8 functions as an initiator caspase that triggers the catalytic activity of downstream executioners caspases, which in turn cleave a

large panel of substrates, leading to apoptotic cell death[332]. Interestingly, FADD and caspase 8 have been shown to mediate the pro-apoptotic effect of TRIF overexpression, a cellular process that relies on RIP1 recruitment by TRIF[333]. The TRIF-mediated, caspase 8-dependent, pathway has also been reported to contribute to the early apoptosis of bacterially infected innate immune cells[334-335] through a mechanism that is distinct, but not mutually exclusive, from the DC-specific CD14-NFAT pathway described below[158]. However, several studies have shown that, in addition to its pro-apoptotic effect, caspase 8 can contribute to NF-κB activation upon overexpression[336] or stimulation of TRIF-dependent TLRs. Activation of NF-κB by TLR3 and TLR4, but not TLR9, is significantly impaired in caspase 8-deficient B cells, due to a delayed nuclear translocation of the transcription factor[337-338]. Additionally, both caspase 8 and FADD have been involved in TLR3- and TLR4-induced proliferative B cell response, suggesting that they act together upon TLR stimulation[338-339]. How caspase 8 participates to NF-κB signaling is unclear, but it is believed that caspase 8 acts as a scaffold to recruit the IKK complex independently of its protease activity[336, 338]. Nevertheless, these observations can be synthesized in a model of TRIF-dependent NF-κB activation that relies on RIP1 binding to TRIF. The DD of RIP1 then interacts with the DD of FADD, which in turn recruits caspase 8 via a death-effector domain (DED). Caspase 8 brings the IKK to TAK1, whose recruitment to the signaling complex is dependent on RIP1 K63-linked polyubiquitination (see above). It has to be noted that TRADD is also recruited to RIP1 and it can potentially bind FADD via a

DD-DD interaction. Therefore it remains to be determined whether TRADD participates to FADD-caspase 8 recruitment downstream of RIP1. Although further experimental support will be required to substantiate this model of caspase 8-dependent NF-κB activation, its biological importance is likely to be relevant, as this pathway seems to be activated also by other, non-TLR, virus-sensing receptors (RIG-I and MDA5)[49] (**Fig. 3**).

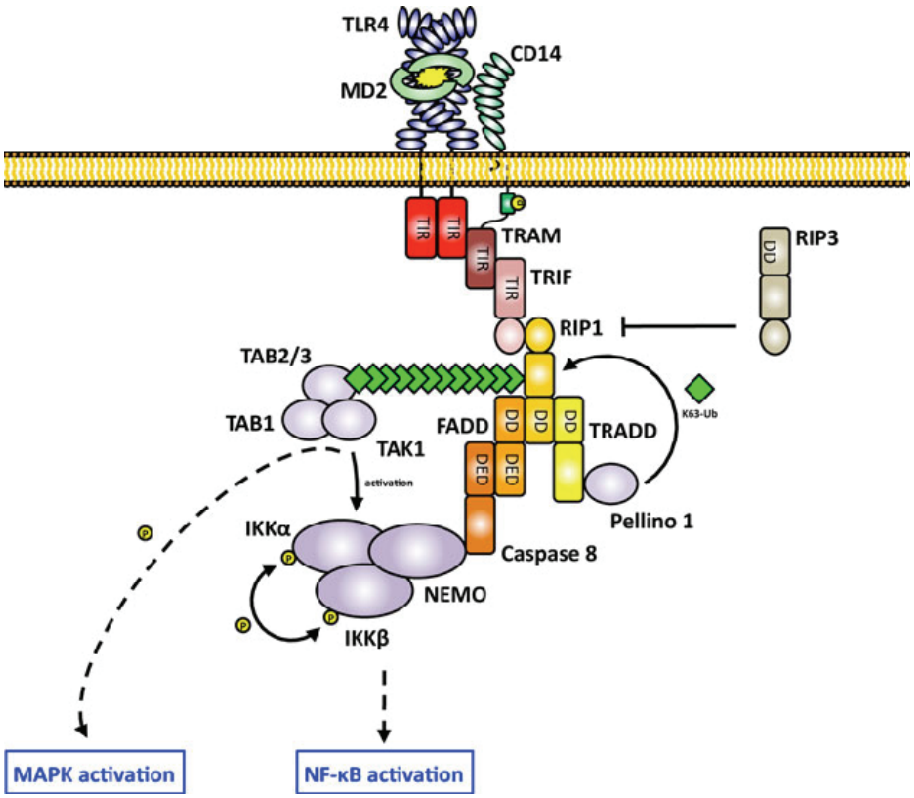

Fig. 3. TRIF-dependent NF-κB/AP-1 activation. TRAM and TRIF are recruited to endosomal TLR4 after TIRAP:MyD88 dissociation from the internalized receptor. Through its RHIM, TRIF mediates direct recruitment of RIP1, which acts as a scaffold for the DD-containing proteins FADD and TRADD.

Inactive RIP3 negatively regulates this process by competing with RIP1 for binding TRIF. TRADD binds Pellino1, an E3 ligase that catalyzes K63-linked polyubiquitination of RIP1. NF-κB/AP-1 activation is operated through the concomitant recruitment to the receptor complex of TAK1, which interacts with modified RIP1 via TAB2/3, and IKKs. IKK recruitment is thought to occur by means of uncleaved caspase 8, which bridges FADD and NEMO. TAK1 in turn mediates IKK activation independently of its kinase activity, which is instead required for downstream MAPK activation.

TRAF3-dependent IRF3/7 activation. Type I IFNs are pleiotropic cytokines that regulate critical cellular functions, most notably innate immune antiviral and antibacterial defense. IFN-α and IFN-β are the most representative and biologically relevant family members, as they are released upon viral or bacterial insults to signal auto/paracrine cell activation[340]. Type I IFN production is controlled at the transcriptional level by the IRF family of transcription factors, with IRF3 and IRF7 acting as key regulators of type I IFN gene expression. In resting cells, a conformationally inactive monomer of IRF3 (and IRF7) localizes in the cytosol. Upon receptor stimulation, IRF3 undergoes phosphorylation by a stimulus-activated kinase, followed by homo- or heterodimerization with IRF7. IRF3:IRF3 or IRF3:IRF7 dimers then translocate into the nucleus, where they promote specific gene expression programs[341]. IRF3 and IRF7 are differentially involved in TRIF-dependent signaling, as they are both required for TLR3-mediated type I IFN production but IRF3 only is

activated downstream of TLR4. However, under certain conditions (i.e. IFN-β pre-treatment) IRF7 can participate to TLR4 signaling[342-344]. IRF3 phosphorylation induced by TLR3 and TLR4 is operated by two IKK-related protein kinases, TBK1 (TANK-binding kinase) and IKKε[345-347], with TBK1 being the major contributor to IRF3-dependent type I IFN production[348-349]. TBK1 is also the target of negative regulation by the phosphatase SHP2[350]. Although TBK1 and IKKε share significant sequence and structure homology with IKKα and IKKβ, their role in NF-κB activation is unclear, and probably limited to a direct, IKKβ-independent, phosphorylation of the p65 subunit leading to an increase in NF-κB transcriptional activity[351]. IRF3 activation by TBK1 and IKKε has been proposed to rely on the upstream adaptors TANK (TRAF-associated NF-κB activator)[352], NAP1 (NAK-associated protein 1)[351] and SINTBAD (similar to NAP1 TBK1 adaptor)[353]. TANK is a binding partner for several TRAF family members[352, 354] as well as for TBK1 and IKKε[355-356], and it has been reported to regulate virus- and TLR-induced type I IFN production by bridging TBK1 and IKKε with TRAF3 in vitro[357-358]. TANK has also been shown to interact with NEMO[359] and to promote the formation of a functional IKK complex that is required for IRF3 activation by RIG-I[360]. Also NAP1 and SINTBAD, which share several structural features with TANK, have consistently been shown to be required for IRF3 phosphorylation[353,361-362] and they are likely to play nonredundant roles in bridging TBK1 and IKKε to upstream activators. IRF3 activation by TLR3 or TLR4 is strictly dependent on the recruitment to TRIF of the adaptor TRAF3[292,303], a protein whose additional role in

limiting MyD88-dependent MAPK activation has already been described in this chapter. The function of TRAF3 in TLR-induced IRF3 activation is to recruit TBK1 and IKKε into the receptor complex as a consequence of the interaction of TRAF3 with TANK (or a TANK-related adaptor). Once at the receptor, the resultant oligomerization of TBK1 and IKKε mediated by TRAF3 probably triggers their own kinase activity through a previous trans-autophosphorylating event. However, the molecular mechanism by which TRAF3 exerts its task is the subject of much current research, since TRAF3 also retains an E3 ubiquitin ligase activity that is essential for proper IRF3 activation. Upon receptor-induced clustering, TRAF3 promotes its own K63-linked polyubiquitination through a process that only partially involves Ubc13 as the E2 enzyme[305]. An additional contribution to TRAF3 polyubiquitination is likely to come from Ubc5, which a recent study identified as the major E2 in RIG-I/MDA5 antiviral signaling[363]. The discovery of DUBA (deubiquitinating enzyme A) as a negative regulator of K63-linked polyubiquitination of TRAF3 further witnesses the importance of this post-translational modification in TRIF signaling to IRF3[364]. Similarly to TRAF6 in MyD88-dependent signaling, K63-linked polyubiquitination of TRAF3 is believed to promote the recruitment of ubiquitin-binding proteins. One such protein is NEMO, which has been shown to interact in vitro with TBK1 and IKKε through TANK[359] and to be essential for RIG-I-induced IRF3 activation[360] through its ubiquitin binding ability[363]. Notably, K63-linked polyubiquitination of TANK has also been reported[358], suggesting that TRAF3 and/or TANK K63-linked polyubiquitination

triggers TBK1 and IKKε recruitment through binding NEMO, which in turn interacts with TANK. Nevertheless, this mechanical model can not be considered consistent at the moment, since several points are still obscure or contradicting. First of all, no contribution of NEMO for IRF3 activation by TLR3 or TLR4 has been reported yet but all data have been generated using RIG-I as the activating receptor. Additionally, the generation of TANK-deficient mice has revealed that TANK is not involved in type I IFN response but it acts as a negative regulator of TLR-induced proinflammatory cytokine production, probably through suppression of K63-linked polyubiquitination of TRAF6[365]. The observation that TANK is dispensable for IRF3 activation in vivo[365] raises the question of whether NAP1 or SINTBAD may operate this function by binding NEMO. Another possibility is that TBK1 itself recruits NEMO, as TBK1 has recently been shown to be the target of K63-linked polyubiquitination by the E3 ligase Nrdp1[366]. Further clarification of these points will result in a reliable knowledge of how IRF3 is mechanically activated by K63-linked polyubiquitination of TRAF3 following TRIF stimulation (**Fig. 4**).

All type I IFN-inducing TLRs trigger IRF activation from an endosomal location, a generalization that includes the TRIF-dependent TLR4 signaling pathway. Functional specialization of receptors is therefore underlined by spatial separation, even though the molecular determinants that mediate site-specific signal transduction are still not perfectly clear. TRAF3 is thought to discriminate between plasma membrane and endosomal signaling, since it is shared by all intracellular (including the TRIF branch of TLR4), but not cell

membrane TLRs (including the MyD88 branch of TLR4). TRAF3 has been reported to localize in pleiomorphic cytosolic structures, a subcellular distribution that precludes its interaction with MyD88 at the cell surface[222]. Artificial targeting of TRAF3 to the plasma membrane allows TLR2 to induce type I IFN expression, suggesting that TRAF3 localization per se regulates the ability of endosomal TLRs to activate IRFs[222]. However, it has to be noted that TRAF3 localization might not be the only discriminating element, since TRAF3 can be recruited at the plasma membrane by some receptors[222], including TLR4 itself[305]. As described above, TRAF3 efficiently interacts with MyD88 upon LPS stimulation where it prevents cytosolic MAPK activation until it gets degraded as a consequence of K48-linked polyubiquitination by the E3 enzymes cIAP1/2[305]. After TLR4 endocytosis, TRAF3 is recruited to TRIF, where it is K63-linked polyubiqutinated by Ubc13 (and possibly Ubc5) to trigger NF-κB activation[305]. Therefore, it appears that the localized differential functionalization of TRAF3 through K48- or K63-linked polyubiquitination, rather than its simple subcellular distribution, is responsible for inducing IRF activation from endosomal locations (**Fig. 4**). A careful investigation will be required to validate this model also for other TLRs and to unveil the mechanisms regulating the selective localization (or activation) of specific ubiquitinating enzymes.

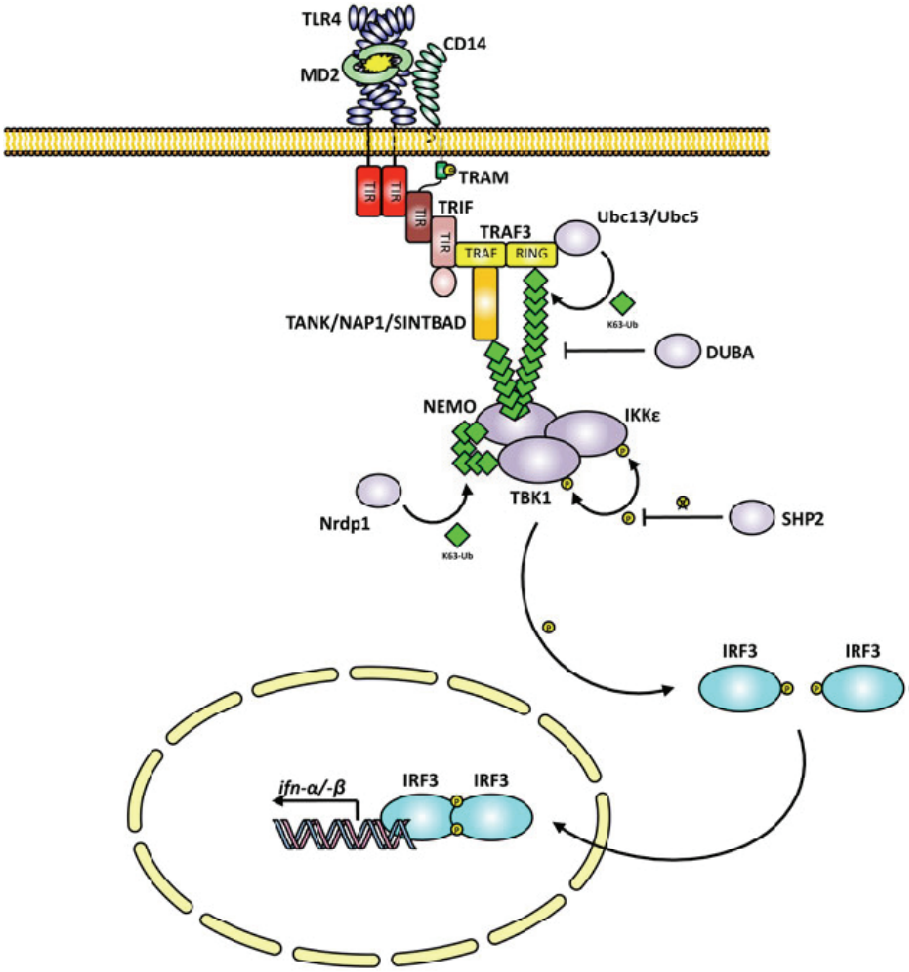

Fig. 4. TRIF-dependent IRF3 activation. TRIF mediates direct recruitment of TRAF3 to endosomal TLR4 and promotes its consequent oligomerization, resulting in Ubc13/Ubc5-dependent K63-linked polyubiquitination of TRAF3 and possibly of the downstream adaptors TANK/NAP1/SINTBAD. K63-linked polyubiquitination of TRAF3 is negatively regulated by the deubiquitinase DUBA. TRAF3 and/or one of the TRAF3-interacting adaptors recruit TBK1 and IKKε via the ubiquitin binding domain of NEMO, thereby linking upstream signaling with IRF3 activation. The interaction of TBK1 with NEMO is also favored by Nrdp1-dependent K63-linked polyubiquitination of TBK1.

Upon transautophosphorylation, TBK1/IKKε get activated and phosphorylate IRF3 monomers, which in turn dimerize and translocate into the nucleus to promote type I IFN gene expression.

Scope of the thesis

This doctoral thesis has been aimed at the identification of signal transduction pathways underlying DC-specific biological functions in response to LPS. The identification of one such pathway will be described in **Chapter 2**, together with its molecular and functional characterization. Cell-specific differences in the ability to activate this signaling pathway will also be reported, with a particular attention to dissimilarities between DCs and macrophages. The content of **Chapter 3** will uncover an additional important biological function that DCs carry on by means of this newly-described pathway and it will also suggest important therapeutic application, which will be discussed in **Chapter 4**.

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Chapter 2

CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation

Published in: **Nature**, 2009 Jul 9;460(7252):264-8

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Toll-like receptors (TLRs) are the best characterized pattern recognition receptors[1]. Individual TLRs recruit diverse combinations of adaptor proteins, triggering signal transduction pathways and leading to the activation of various transcription factors, including nuclear factor-κB (NF-κB), activating protein 1 (AP-1) and interferon regulatory factors (IRFs)[2]. Interleukin-2 (IL-2) is one of the molecules produced by mouse dendritic cells after stimulation by different pattern recognition receptor agonists[3–6]. By analogy with the events after T-cell receptor engagement leading to IL-2 production, it is therefore plausible that the stimulation of TLRs on dendritic cells

may lead to activation of the Ca^{2+}/calcineurin and nuclear factor of activated T cells (NFAT) pathway. Here we show that mouse dendritic cell stimulation with lipopolysaccharide (LPS) induces Src-family kinase (SFK) and phospholipase Cγ2 (PLCγ2) activation, influx of extracellular Ca^{2+} and calcineurin-dependent nuclear NFAT translocation. The initiation of this pathway is independent of TLR4 engagement, and dependent exclusively on CD14. We also show that LPS-induced NFAT activation via CD14 is necessary to cause the apoptotic death of terminally differentiated dendritic cells, an event that is essential for maintaining self-tolerance and preventing autoimmunity[7,8]. Consequently, blocking this pathway *in vivo* causes prolonged dendritic cell survival and an increase in T-cell priming capability. Our findings reveal novel aspects of molecular signaling triggered by LPS in dendritic cells, and identify a new role for CD14: the regulation of the dendritic cell life cycle through NFAT activation. Given the involvement of CD14 in disease, including sepsis and chronic heart failure[9,10], the discovery of signal transduction pathways activated exclusively via CD14 is an important step towards the development of potential treatments involving interference with CD14 functions.

Wild-type LPS, termed smooth LPS (the form of LPS used here), comprises three covalently linked moieties: lipid A, rough core oligosaccharide and an O-antigenic side chain determining serotype specificity. The TLR4:MD-2 complex and the CD14 co-receptor are necessary and sufficient for a full response to smooth LPS[11]. Both

$CD8\alpha^+$ and $CD8\alpha^-$ mouse dendritic cells express CD14, although at lower levels than expressed by macrophages (Supplementary Fig. 1). We tested whether LPS-induced IL-2 production by dendritic cells was dependent on NFAT. Ca^{2+} mobilization is the first event in NFAT activation. We observed that LPS was able to induce Ca^{2+} flux in bone marrow-derived dendritic cells (BMDCs) and in a homogeneous dendritic cell line, D1 cells[12] (**Fig. 1**, Supplementary Fig. 2 and Supplementary Movies).

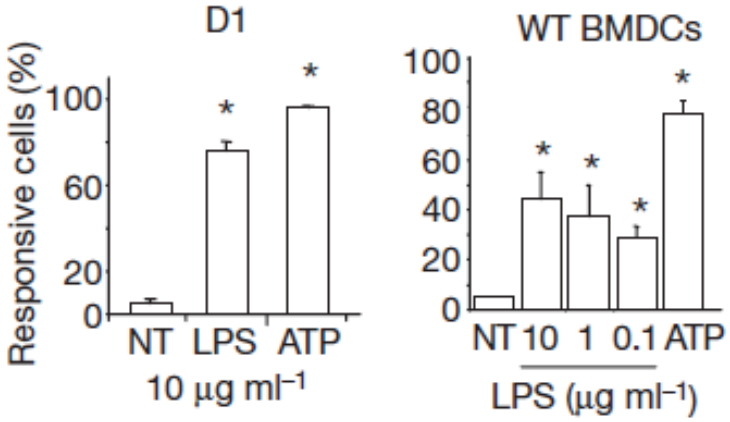


Fig. 1. Percentage of D1 cells and BMDCs showing Ca^{2+} mobilization in the absence (NT) and presence of LPS or ATP. *, $P < 0.05$ versus NT.

Next, we investigated the features of Ca^{2+} mobilization by LPS in BMDCs in detail, by recording Ca^{2+} transients in individual cells. The intracellular calcium concentration ($[Ca^{2+}]_i$) was increased by LPS and ATP ($P < 0.05$ versus untreated cells, **Fig. 2a**). Experiments in Ca^{2+}-free medium demonstrated that the increase in $[Ca^{2+}]_i$ induced by LPS was due to the influx of extracellular Ca^{2+} (**Fig. 2b**, responding cells in Ca^{2+}-free medium less than 1%). To determine the molecular

component of the LPS receptor responsible for Ca^{2+} flux directly, we measured Ca^{2+} influx in TLR4- and CD14-deficient BMDCs. Ca^{2+} transients were fully preserved in TLR4-deficient BMDCs (43 $\pm$ 6% of cells responding, **Fig. 2c**), whereas they were completely abolished in CD14-deficient BMDCs (< 1% of cells responding, **Fig. 2c**).

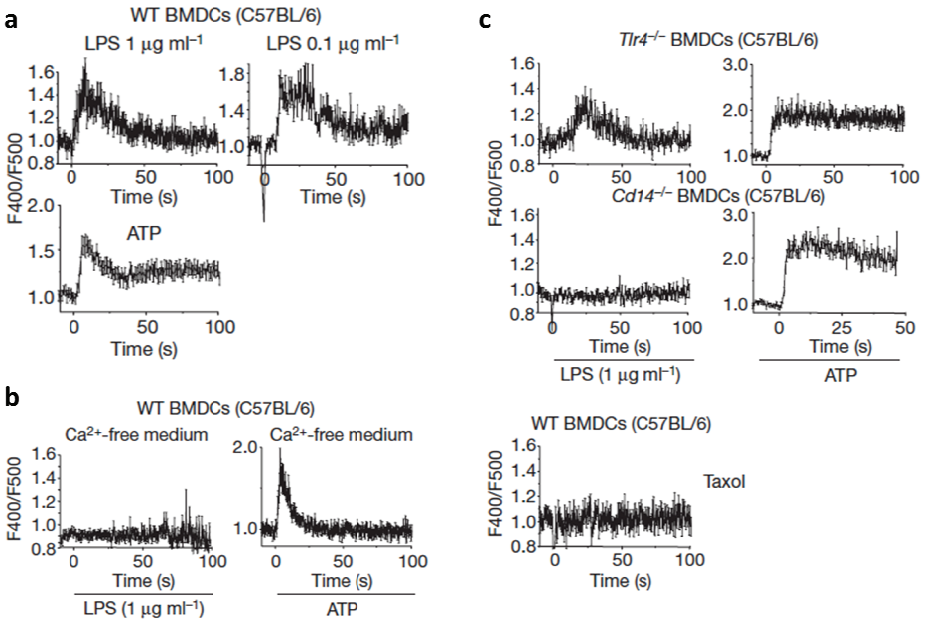


Fig. 2. (**a, b, c**) Ca^{2+} transients in wild-type and mutant. Point 0 indicates the time of stimulus administration. Means and s.d. for a minimum of 30 cells are shown. Experiments were repeated at least three times. The ratio of fluorescence emissions at 400 nm/40 nm band-pass to those at 500 nm/20 nm band-pass was recorded (F400/F500) and used as an index of $[Ca^{2+}]_i$.

To confirm that TLR4 is not involved in this process, Ca^{2+} mobilization was tested in each of the following mutant cell types: MyD88-, MD-2- and TRIF-deficient, and MyD88/TRIF- and TLR4/TLR2-double-deficient

BMDCs. In all of these mutant cells Ca^{2+} mobilization was fully preserved (Supplementary Fig. 3), confirming that CD14 was the receptor responsible for Ca^{2+} influx after LPS activation. Also, there was no Ca^{2+} mobilization in BMDCs in response to taxol (responding cells < 1%), a selective TLR4 agonist[13] (**Fig. 2c**); this is further confirmation of the involvement of CD14. We next conducted various tests to ensure that the results obtained concerning Ca^{2+} transients could not have been due to the presence of contaminants in our LPS preparations (see Supplementary Fig. 4).

CD14 is a glycosylphosphatidylinositol-anchored receptor (GPI-AR). Cross-linking of GPI-ARs, such as CD59[14], triggers the activation of the intracellular inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$)/Ca^{2+} pathway through the association with lipid rafts and SFK activation[15]. Therefore, we investigated whether CD14 membrane localization, presence of lipid rafts and SFK activation were conditions required to obtain Ca^{2+} mobilization. As shown in **Fig. 3**, soluble CD14 did not restore extracellular Ca^{2+} influx in CD14-deficient dendritic cells, and disruption of lipid rafts by cholesterol depletion totally impaired Ca^{2+} mobilization. Thus, consistent with previous results[16], CD14 localization in lipid rafts[17] seems to be required for LPS-induced Ca^{2+} mobilization. Moreover, SFK inhibition completely abolished Ca^{2+} mobilization (**Fig. 3**), indicating direct involvement of SFK in this process. The induction of Ca^{2+} transients by LPS was also fully dependent on PLCγ2 (responding PLCγ2-deficient BMDCs <1%; **Fig. 3**). Consistent with these results, PLCγ2 activation was observed in

wild-type but not in CD14-deficient BMDCs on LPS exposure (Supplementary Fig. 5).

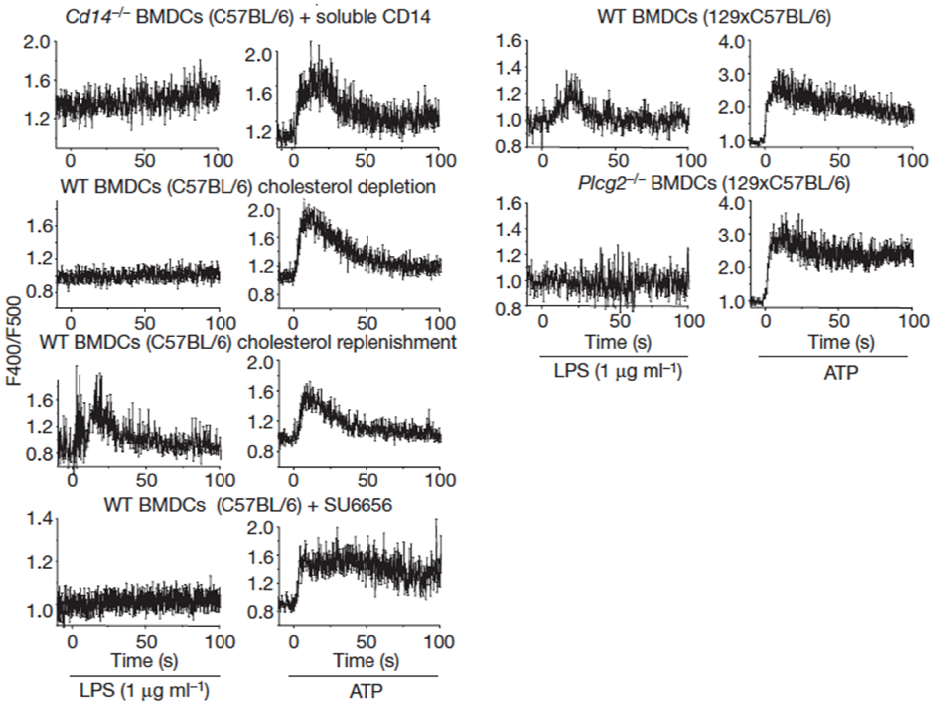


Fig. 3. Ca^{2+} transients in wild-type and mutant BMDCs and wild-type BMDCs in the presence of the SFK inhibitor (SU6656, 260 nM) or soluble CD14 (2 µg ml^{-1}) and upon cholesterol depletion/replenishment.

We then analyzed NFAT activation after LPS treatment in wild-type and CD14-deficient BMDCs. As shown in **Fig. 4**, NFATc2 nuclear translocation occurred in wild-type but not in CD14-deficient BMDCs. In agreement with the Ca^{2+} mobilization studies, NFAT was translocated to the nucleus in wild-type, TLR4-, MD-2- and MyD88/TRIF-deficient BMDCs but not in CD14- and PLCγ2-deficient BMDCs or in wild-type BMDCs stimulated with LPS in the presence of

the SFK inhibitor (Supplementary Fig. 6). These observations demonstrate that CD14 is directly responsible for activating, in dendritic cells, a TLR4-independent, SFK- and PLCγ2-dependent signal transduction pathway leading to NFAT activation.

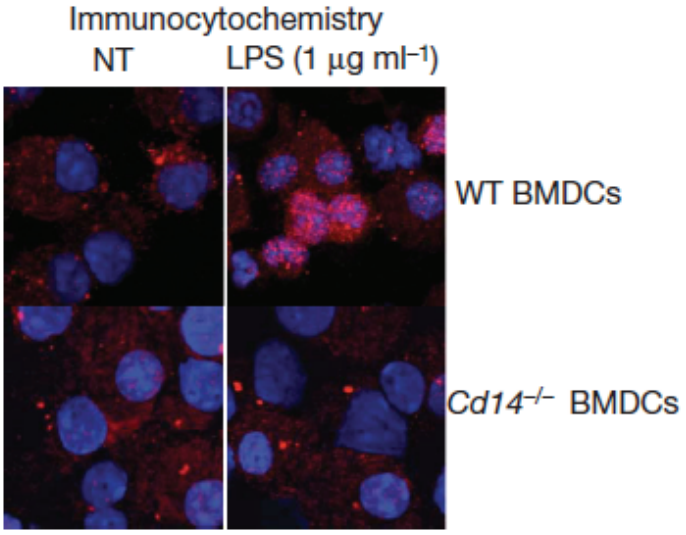


Fig. 4. NFATc2 translocation before (NT) and after LPS challenge for 2 h in BMDCs. Blue, DAPI; red, anti-NFATc2; original magnification, X630.

This is in addition to CD14 enhancing cellular responses to low doses of LPS[18,19] and being required for the LPS-induced recruitment of TRAM and TRIF[20]. We observed that, diversely from macrophages[21] (**Fig. 5**), the production of TNFα and IL-6 by dendritic cells after LPS stimulation was TRIF-independent and, consequently, it was also CD14-independent[11,20], at least at high LPS doses (**Fig. 5**). It is therefore possible that the selective engagement of TLR4 without CD14 activation allows the production of NF-κB-dependent and

NFAT-independent cytokines, such as TNFα[22] and IL-6[23], whereas it selectively prevents the production of cytokines, such as IL-2, that are transcriptionally dependent on NFAT, in addition to NF-κB and AP-1.

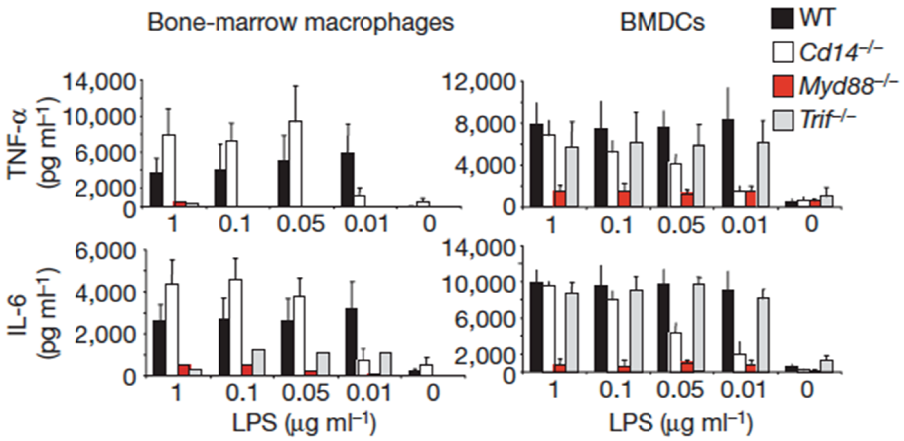


Fig. 5. TNFα and IL-6 production by bone-marrow macrophages and BMDCs stimulated with LPS for 24 h. Results are representative of at least three experiments. Error bars represent standard errors.

We analyzed the behavior of CD14-deficient BMDCs after LPS stimulation and that of wild-type BMDCs after activation with the TLR4-selective stimulus taxol to confirm the existence of a CD14-specific pathway. IL-2 production was impaired after the stimulation of CD14-deficient BMDCs with various concentrations of LPS, including very high concentrations at which the production of TNFα and IL-6 was largely preserved (**Fig. 6a**). Similarly, stimulation of wild-type BMDCs with taxol induced the production of TNFα and IL-6, but not IL-2 (**Fig. 6a**). Nevertheless, coupling TLR4 stimulation with Ca^{2+} mobilization, using thapsigargin, restored IL-2 production (**Fig. 6a** and

Supplementary Fig. 8). Similar results were obtained in vivo (Supplementary Fig. 8).

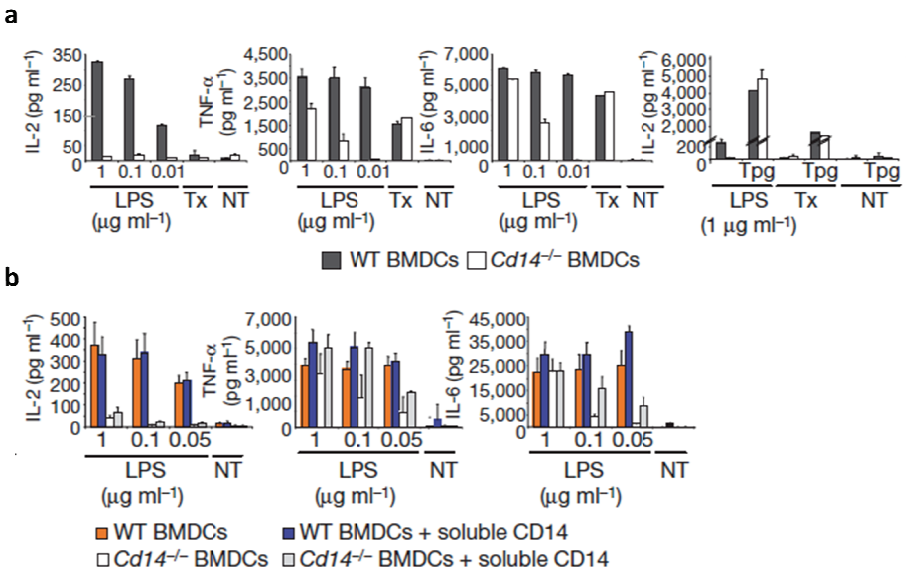


Fig. 6. (**a**) Cytokine production by BMDCs stimulated with LPS or taxol (Tx, 100 μM) and LPS- or taxol-induced IL-2 production by BMDCs with and without thapsigargin (Tpg, 50 nM). (**b**) LPS-induced cytokine production by BMDCs with and without soluble CD14 (2 μg ml^{-1}). Results are representative of at least three experiments. Error bars represent standard errors.

We used various inhibitors blocking the NFAT pathway to confirm CD14-dependent activation of the Ca^{2+}/calcineurin pathway induced by LPS. In particular, inhibition of the influx of external Ca^{2+} by the Ca^{2+} chelator EGTA selectively impaired IL-2 production but did not downregulate TNFα or IL-6 synthesis (Supplementary Fig. 9a). This was also observed in the presence of thapsigargin (Supplementary

Fig. 9b). Inhibition of SFK, PLCγ2 or calcineurin downregulated IL-2 production; however, the production of TNFα and IL-6 was unaffected by the treatment (Supplementary Fig. 9a, c). We also used the VIVIT peptide[24] to inhibit NFAT proteins and obtained similar results (Supplementary Fig. 10). In agreement with the Ca^{2+} mobilization data, addition of soluble CD14 to CD14-deficient BMDCs restored TNFα production, as already observed in macrophages[20], but not IL-2 production at either low or high LPS concentrations (**Fig. 6b**). This observation supports the notion that a membrane localization of CD14 is required to activate the Ca^{2+} pathway. As NFAT proteins are major regulators of many biological processes, we investigated the physiological role of CD14-mediated NFAT activation in dendritic cells, in addition to IL-2 production. We performed a kinetic microarray analysis to identify genes modulated specifically by NFAT in LPS-treated dendritic cells (see Supplementary Fig. 11 for details). From this analysis we hypothesized that LPS-activated dendritic cells in conditions allowing NFAT activation were exhausted cells with little or falling gene transcription and protein translation; in contrast, dendritic cells activated with LPS in conditions inhibiting NFAT activation were transcriptionally and translationally active, presumably not exhausted and not undergoing apoptosis (see Supplementary Table 1 for details). To test whether CD14-mediated activation of the NFAT pathway was required to induce apoptotic death of terminally differentiated cells, wild-type BMDCs and CD14-, NFATc2- and PLCγ2-deficient BMDCs were activated with LPS and the percentage of living cells measured at various times thereafter. The

c2 isoform of NFAT was chosen because it has a pro-apoptotic function in T- and B-cells[25]. Less than 30% of wild-type BMDCs survived after activation, whereas 70–100% of CD14-, NFATc2- and PLCγ2-deficient BMDCs were still alive five days after exposure to LPS (**Fig. 7**). CD14- and PLCγ2-deficient BMDC death after LPS activation was restored by thapsigargin (**Fig. 7**); thapsigargin alone had no effect (data not shown). Similar results were obtained using different LPS doses and LPS from different sources (Supplementary Fig. 12).

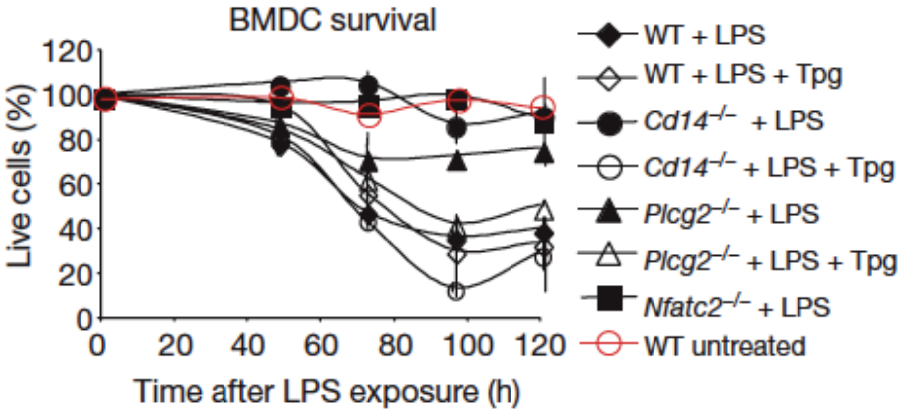


Fig. 7. BMDC survival after incubation with LPS (1 μg ml^{-1}, $P < 0.00001$, wild-type versus mutant BMDCs) or LPS plus thapsigargin (50 nM). Survival of unstimulated cells kept in culture with GM-CSF is also shown. Results are representative of at least three experiments. Error bars represent standard errors.

A new microarray experiment performed at later time points (48 and 60 h) allowed us to identify four putative pro-apoptotic genes specifically modulated by NFAT: *nur77*, *Gadd45g*, *Ddit3* and *Tia1* (Supplementary Table 2). In vivo, splenic dendritic cell numbers

declined quickly in wild-type C57BL/6 mice upon LPS administration, whereas absolute dendritic cell numbers remained almost constant in CD14-deficient and NFATc2-deficient mice after the same treatment (**Fig. 8**). Although we cannot exclude an indirect effect in vivo, these findings provide further evidence that NFAT pathway activation via CD14 is required for dendritic cell apoptosis. The partial dendritic cell decline observed in NFATc2-deficient mice may have been the consequence of the effect of other NFAT isoforms with overlapping functions.

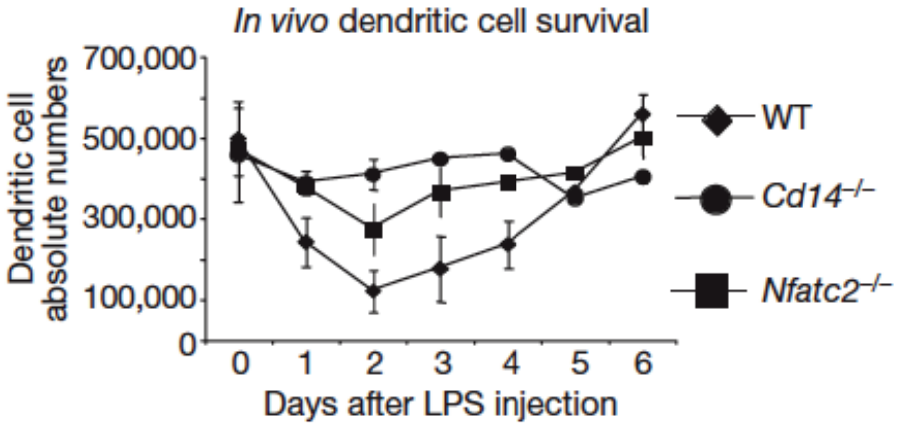


Fig. 8. Absolute numbers of CD11c^{+} cells in spleens after intravenous LPS (1 mg g^{-1}) injection ($P < 0.0006$, wild-type versus mutant mice). Data are representative of two independent experiments (four mice per group).

Apoptotic death is the terminal step of fully matured dendritic cells. This process is essential for regulating peripheral tolerance because dendritic cell accumulation resulting from apoptosis deficiency can induce over-activation of responder lymphocytes, resulting in

systemic autoimmunity[7,8]. We tested whether blockade of the CD14/NFAT pathway leading to dendritic cell persistence after activation could be responsible for an increase in T-cell priming in vivo.

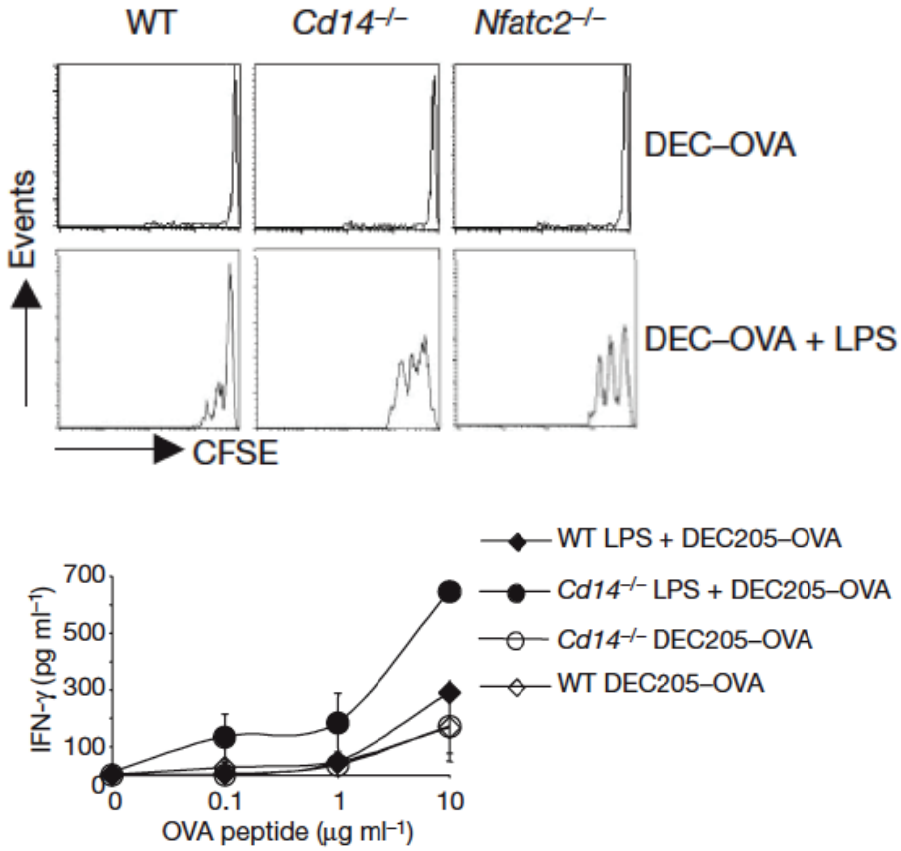


Fig. 9. Top panel: antigen-specific T-cell proliferation in vivo. Lower panel: IFNγ production after in vitro re-stimulation. The experiment was repeated twice with similar results. Error bars represent standard errors.

Wild-type, CD14- and NFATc2-deficient mice were injected with LPS and anti-DEC205–OVA conjugate[26] and 8 h later received

carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled $CD4^+$ T-cells from OVA-specific OT-II mice. As shown in **Fig. 9**, T-cell proliferation in vivo was stronger in mutant than wild-type mice and, consistently, the capacity to produce IFNγ after re-stimulation in vitro was stronger when T-cells were recovered from mutant than wild-type mice. Thus, mutant mice had an enhanced capacity to induce T-cell-specific immune responses.

Diversely from dendritic cells, macrophages do not die after activation with LPS but simply become refractory to a further rechallenge, a process known es endotoxin tolerance[27]. Additionally, tissue-resident macrophage survival after activation is crucial for the resolution of inflammation and the tissue repair response[28]. Therefore, we asked whether macrophage survival after LPS encounter was due to their inability to activate the Ca^{2+} pathway. Bone marrow-derived macrophages were unable to mobilize Ca^{2+} (**Fig. 10a**). Moreover, Supplementary Fig. 13 shows that after LPS encounter NFAT activation and NFAT-dependent gene transcription were not measurable in macrophages. To investigate whether the lack of Ca^{2+}-NFAT pathway activation in macrophages was responsible for their survival upon LPS encounter, we induced Ca^{2+} mobilization with thapsigargin concomitantly with LPS stimulation. In these conditions, bone marrow macrophage death could be induced in an NFATc2-dependent manner (**Fig. 10b**). In addition, splenic macrophages (which were identified as $CD11b^+CD11c^{dim}$ cells) did not

decline in number after a single intravenous injection of LPS (**Fig. 10b**).

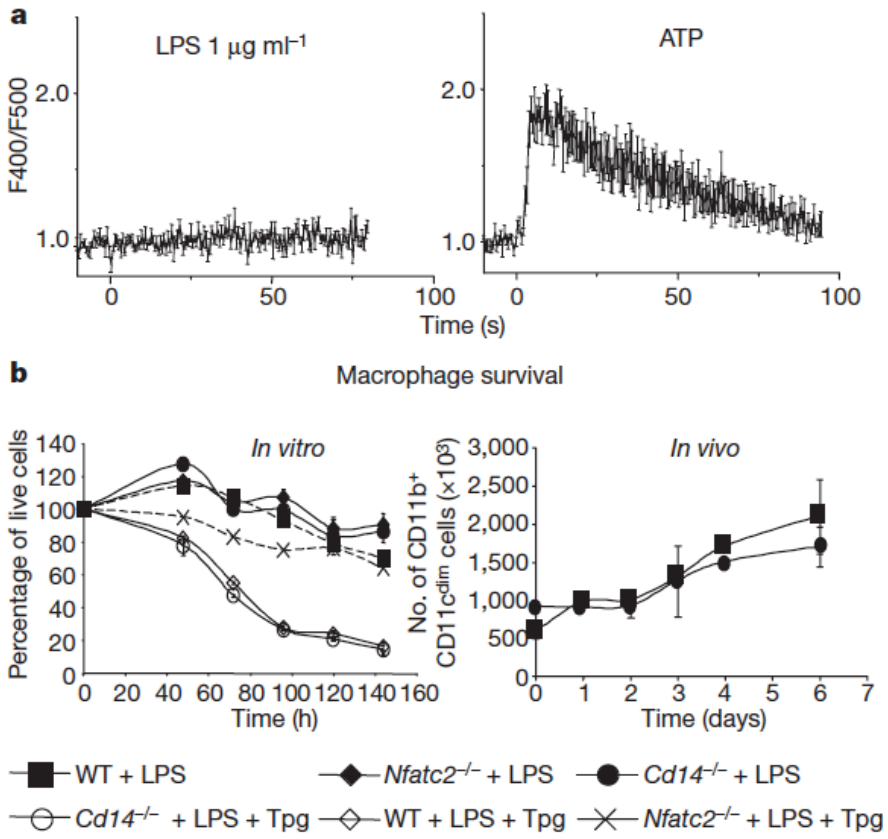


Fig. 10. (**a**) Ca^{2+} transients in bone marrow macrophages. Point 0 indicates time of stimulus administration. Means and s.d. of 30 cells minimum are shown. Experiments were repeated at least three times. (**b**) Left panel: survival of wild-type and mutant bone marrow macrophages after incubation with LPS (1 μg ml^{-1}) or LPS plus thapsigargin (50 nM). Right panel: absolute numbers of $CD11b^{+}CD11c^{dim}$ cells in spleens of wild-type and CD14-deficient mice after intravenous LPS injection (30 mg/mouse). Data represent two independent experiments (four mice per group).

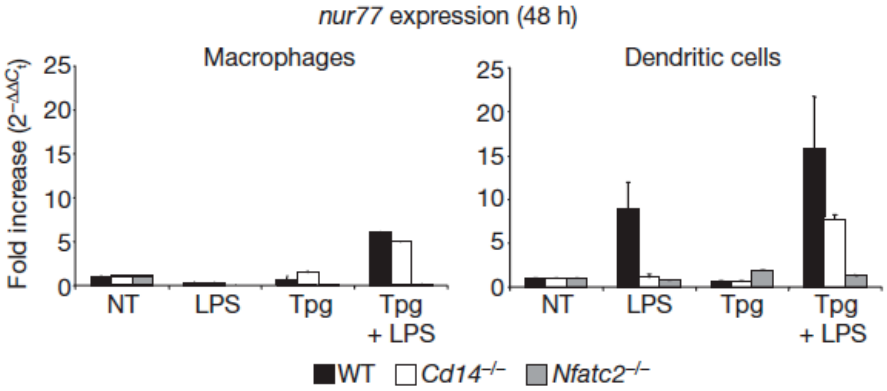


Fig. 11. Fold increase of *nur77* expression in stimulated versus unstimulated wild-type and mutant cells.

As shown in **Fig. 11**, *nur77* was not modulated in macrophages after LPS stimulation. Nevertheless, *nur77* messenger RNA upregulation could be induced in an NFAT-dependent manner after macrophage co-stimulation with LPS and thapsigargin. Moreover, in this validation experiment, we also confirmed that the upregulation of *nur77* mRNA in dendritic cells after LPS stimulation was dependent on NFATc2 (**Fig. 11**). These observations further corroborate our findings concerning the functional consequences of CD14-mediated Ca^{2+} influx and NFAT activation in dendritic cells. Nevertheless, they highlight significant differences in the signal transduction pathways induced by LPS in dendritic cells and macrophages. By revealing novel aspects of molecular signaling triggered by LPS in dendritic cells, this work provides insights into the complexity of cellular responses to bacterial infections.

Materials and Methods

Calcium Measurement. $[Ca^{2+}]_i$ was determined by a fluorometric ratio technique. Cells were loaded with 2 µM indo-1 (Molecular Probes) by incubation at 37 °C for 20 min. Cells were then washed three times with PBS to allow for intracellular de-esterification of indo-1. A direct optical microscope (Olympus, BX51) with a two-photon Ti-Sapphire laser source (720-nm wavelength; Mai Tai, SpectraPhysics) was used for indo-1 excitation. The fluorescence signals emitted by indo-1-loaded cells were digitized at 200 Hz and recorded every 0.5–0.8 s. The ratio of fluorescence emissions at 400 nm/40 nm band-pass to those at 500 nm/20nm band-pass was recorded (R400/500) and used as an index of $[[Ca^{2+}]_i$. Data were normalized to baseline. This approach overcame possible problems of uncertainty related to the calibration of fluorescent Ca^{2+} indicators. In some cases, cells were analyzed in calcium-free PBS or calcium-free PBS supplemented with thapsigargin (50 nM). Images of Ca^{2+} responses were measured by a laser-scanning confocal system using the membrane-permeable dye Fluo4-AM.

Dendritic cells and macrophages. D1 cells and fresh bone marrow cells from C57BL/6 or mutant mice were cultured as previously described[29].

Mice and cells. C57BL/6 mice were purchased from Harlan. *Cd14*$^{-/-}$ mice were purchased from CNRS. OT-II mice were purchased from Charles River. *Tlr4*$^{-/-}$ mice were provided by S. Akira. *Plcg2*$^{-/-}$

129XC57BL/6 and wild-type 129XC57BL/6 mice were provided by M. Colonna. TLR2/TLR4-doubledeficient mice were provided by C. Kirschning. NFATc2-deficient mice were provided by E. Serfling, and MD2-deficient mice were provided by K. Miyake. All animals were housed under pathogen-free conditions. All experiments were carried out in accordance with the relevant laws and institutional guidelines.

Antibodies and chemicals. All the antibodies used for FACS analysis were purchased from BD Biosciences. TLR4-grade LPSs (Escherichia coli, 055:B5 and Salmonella Typhimurium S-form) were purchased from Alexis Corporation; non-TLR4-grade LPS (E. coli O55:B5) was purchased from Sigma. Recombinant soluble CD14 was purchased from CellSciences and SFK inhibitor SU6656 from Sigma. Tacrolimus/FK506 (Fujisawa Pharmaceutical) was used at a concentration of 10 ng ml^{-1}. Partial depletion and replenishment of cholesterol in the plasma membrane was performed according to ref. 14. Indo1-AM and Fluo4-AM (Molecular Probes) were dissolved in DMSO. Stock solutions were diluted in Tyrode solution (154mM NaCl, 4mM KCl, 2mM CaCl2, 1mM MgCl2, 5mM HEPES-NaOH, 5.5mM D-glucose, adjusted to pH 7.35). The concentration of DMSO in the medium did not exceed 0.1%.

Western blot analysis. Wild-type and *Cd14*$^{-/-}$ BMDCs were stimulated for the indicated times with LPS (1 μg ml^{-1}) and lysed in the presence of Protein Inhibitor Cocktail (Sigma) and Phosphatase Inhibitor Cocktails (Sigma). Proteins from cell lysates were separated by

standard SDS–PAGE and analyzed by immunoblotting with antibodies specific for phosphorylated PLCγ2 (Cell Signaling) and for β-actin (Sigma).

Electrophoretic mobility shift assay (EMSA). 32P-labelled DNA oligonucleotide probes were incubated for binding with 5 mg of nuclear extracts[30] for 20 min at room temperature in a buffer containing 5% glycerol, 50mM NaCl, 20mM Tris pH7.9, 0.5mM EDTA, 5mM MgCl, 1mM dithiothreitol, 100 ng ml21 poly(dI-dC), and 50 ng ml^{-1} bovine serum albumin (BSA) in a final volume of 15 ml. The protein–DNA complexes were then separated on a 5% polyacrylamide gel (29:1 acrylamide/bisacrylamide ratio) and were visualized by autoradiography. The sequences of the oligonucleotide probes (sense strand) used in the gel shift assay were as follows: NFATc consensus site, 5'-GCCCAAAGAGGAAAATTTGTTTCATACAG-3'; NFATc mutant site, 5'-GCCCAAAGcttAAAATTTGTTTCATACAG-3'; Oct consensus site, 5'-TCGTCAAACTCTGCTAATTAGCAATGCTGAGAAA-3'.

Immunocytochemistry. Cells were fixed in formaldehyde and spun onto glass slides ($2X10^5$ cells per slide in 200 µl) at 300 r.p.m. for 5 min. Cells were then permeabilized in chilled methanol and washed extensively with PBS. Fc block antibody (CD16/CD32, BD Pharmingen), rabbit anti-mouse NFATc2 (1:250, ImmunoGlobe), rabbit anti-mouse NFATc3 (1:250, Santacruz) and donkey anti-rabbit Alexa Fluor 555 antibodies (1:500, Molecular Probes) diluted in PBS plus 0.1% BSA were added and incubated at room temperature. DAPI

(1:1,000, Sigma Aldrich) was added and the samples incubated for 5 min at room temperature.

IL-2, TNF-a and IL-6 measurements. ELISAs were performed with the DuoSet kits (R & D).

Plasmids and retroviral infection of dendritic cells. VIVIT–GFP DNA was inserted into the PINCO retroviral vector[31], using the HindIII and NotI restriction sites. Standard molecular biology methods were used for cDNA cloning. All the reagents used were from Invitrogen. The production of high-titre vectors and the D1 infection protocol have been described elsewhere[31]. Transduction efficiency was evaluated by FACS analysis and was generally around 30%. GFP1 cells were sorted and cultured as previously described[31].

In vivo production of IL-2 and TNF-α by spleen cells. Aliquots of 10 μg of LPS or 10 μg of LPS and 5mM Tpg (in a final volume of 50 μl) were injected into mouse spleens. After 3 h, spleens were removed and single-cell suspensions prepared. The cell suspensions were incubated with brefeldin A (10 μg ml^{-1}, Sigma-Aldrich) for 5 h. The cells were fixed using BD Phosflow Lyse/Fix buffer (BD Biosciences), permeabilized with BD Phosflow Perm Buffer III (BD Biosciences) and stained with PE-labelled anti-CD11c, APC-labelled anti-IL-2 and anti-TNFα monoclonal antibodies or APC-labelled isotype control antibody. Cells were then analyzed on a FACScalibur (Becton Dickinson).

Microarray experiment. Total RNA was extracted by the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol Invitrogen) followed by a Qiagen RNeasy clean-up procedure. Total RNA integrity was assessed with a Agilent Bioanalyser and the RNA Integrity Number (RIN) was calculated. Only high-quality RNA preparations, with RIN greater than 8.5, were used for microarray analysis. Three micrograms of total RNA were used for cRNA target preparation according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix) using the one cycle target labelling kit and according to the manufacturer's instructions. Ten micrograms of biotinylated cRNA was hybridized to the Affymetrix GeneChip Mouse Genome 430A 2.0 arrays. Bioconductor32 was used for most data handling. The Guanine Cytosine Robust Multi-array Analysis GCRMA33 method was used to calculate probe set intensity. The normalization method applied was the quantile.

In vitro survival assay. Cells were seeded in 96-well plates at a concentration of $0.5X10^6$ cells ml^{-1} in 100 µl of complete IMDM plus GM-CSF (for BMDCs) or M-CSF (for bone-marrow macrophages) in the presence or absence of LPS (1 µg ml^{-1}) and/or thapsigargin (50 nM). After 24 h, 100 µl of complete IMDM was added to the culture. Survival of cells was measured using the CellTiter-Blue Cell Viability Assay (Promega).

In vivo survival assay. Four-to-six-week-old mice, showing an average of 90 million total spleen cells, were injected intravenously

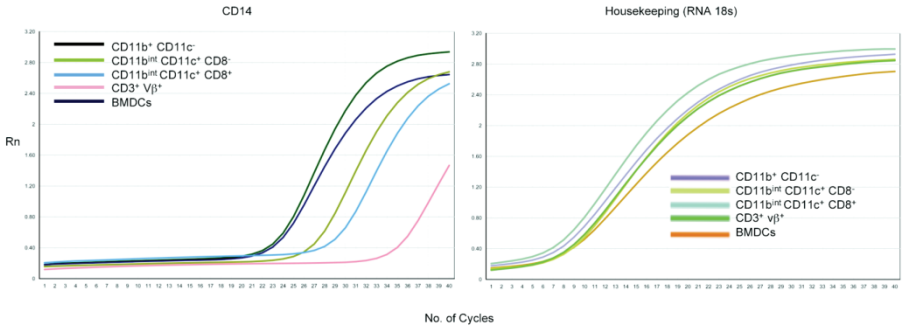
with 1 mg g^{-1} of LPS. At various times thereafter, spleens were collected, single-cell suspensions produced and total spleen cells counted. Absolute numbers of dendritic cells were determined by FACS analysis using CD11c–APC-conjugated, CD8α–PE-conjugated, and CD11b–FITC-conjugated antibodies.

T-cell activation. Mice were injected intravenously with LPS (1 mg g^{-1}) and anti-DEC205–OVA complex (10 μg) and 8 h later received CFSE-labelled $CD4^+$ T cells ($4X10^6$) from OT-II mice. T-cell proliferation was measured in the spleen after 48 h. One week after immunization total $CD4^+$ T-cells were recovered and re-stimulated in vitro with OVA peptide in the presence of splenic APCs. IFNγ production was measured by ELISA after 48 h of culture.

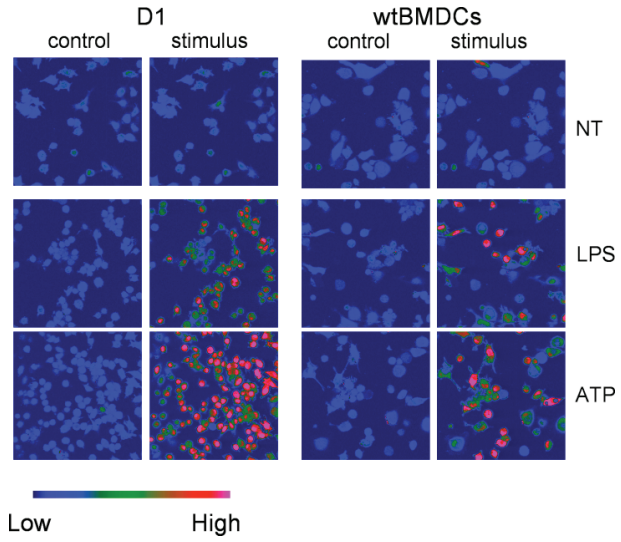
Real-time quantitative PCR. Total RNA was extracted from 10^6 cells using the TRIZOL reagent according to the recommended procedure (Gibco-BRL). Single-strand cDNA was synthesized using High-capacity cDNA Reverse Transcription Kits (Perkin-Elmer, Applied Biosystems Division). The NanoDrop (ThermoScientific) was used to titre mRNA and amplification was performed using the Power Sybr Green PCR Master Mix (Perkin Elmer).

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

Supplementary information

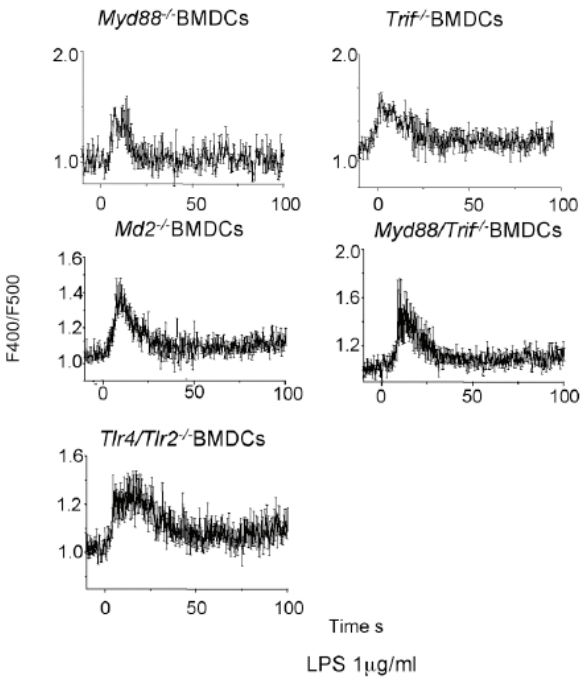


Supplementary Fig. 1. CD14 is expressed by CD8α^{+} and CD8α^{-} DCs. RT-qPCR was performed on the indicated ex vivo sorted populations and on BMDCs. *Ex vivo* T cells were used as negative control. RNA 18s was used as loading control. The experiment was repeated twice with similar results.



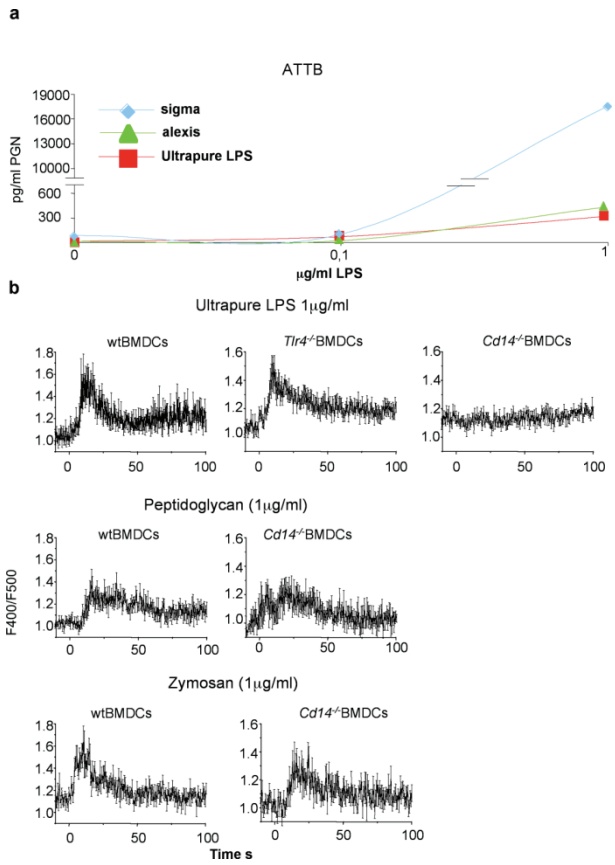
Supplementary Fig. 2. Ca^{2+} mobilization in DCs. Representative images of D1 cell and BMDC fields in control conditions and after steady-state superfusion with LPS and ATP; the Ca^{2+} concentration is indicated by

colored bars. We used confocal images acquired before and during stimulus superfusion to determine whether LPS induced Ca^{2+} flux in BMDCs and in D1 cells. Basal Ca^{2+} activity was recorded, and the cells were then superfused with LPS for up to 2 min. ATP at a saturating dose of 200 µM was used as a positive control[1]. In both cell types, a small number of elements displayed spontaneous sub-baseline Ca^{2+} activity, which was significantly increased by LPS ($P < 0.05$). The response to LPS was stronger in D1 cells than in BMDCs ($P < 0.05$), due to the greater homogeneity of D1 cells.



Supplementary Fig. 3. Ca^{2+} mobilization in wild-type and mutant DCs following LPS treatment. Means and standard deviations for a minimum of

30 cells are shown. Experiments were repeated at least three times for each set of conditions, with similar results.

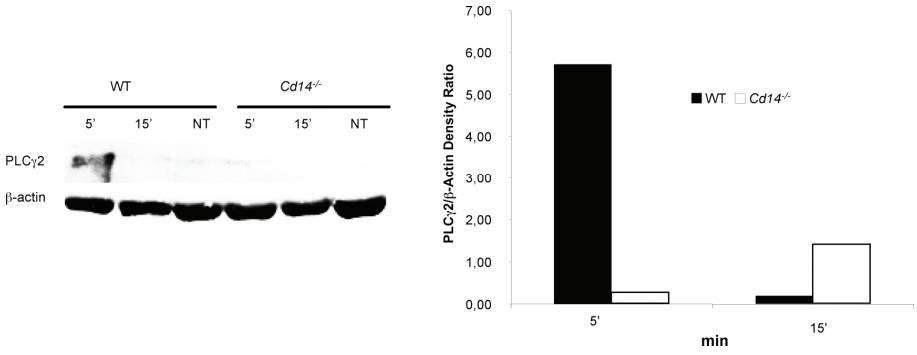


Supplementary Fig. 4. *Introductory explanation*. It is possible that LPS preparations are contaminated with different bacterial components, such as lipoproteins and peptidoglycan. We could exclude the presence of TLR2 ligands (lipopetides) by using TLR2/TLR4-double deficient cells (see Supplementary Fig. 3). To exclude the eventuality that the observed TLR4-independent and CD14-dependent Ca^{2+} mobilization was due to peptidoglycan contaminants we used the following approach. It has been

shown that insect cells can be activated by peptidoglycan while they are unresponsive to LPS[2]. Therefore, contaminant peptidoglycan in LPS preparations can induce insect cell activation measured in terms of anti-microbial peptide transcript upregulation. We, thus, exploited insect cells to analyze the presence of peptidoglycan in different LPS samples. We treated insect cells with LPS from Sigma (non-TLR4 grade, positive control) and LPS from Alexis (TLR4 grade) and we measured the upregulation of the anti-microbial peptide attacin B transcript (ATTB) by quantitative real time (qRT) PCR. The results are shown in (**a**). While a clear peptidoglycan contamination was observable in the LPS preparation from Sigma, the Alexis LPS showed a much-reduced level of contaminant peptidoglycan, near the detection limit. The Sigma LPS was then re-purified according to Kaneko et al.[2] and the level of contamination analyzed again. As shown in (**a**) the re-purified LPS (ultrapure) showed a sensibly reduced level of peptidoglycan. Notably, Alexis LPS and re-purified LPS had a similar level of contamination. A dose of peptidoglycan corresponding to the amount of contaminant present in 1 μg/ml of LPS (Alexis) was never capable of stimulating Ca2+ fluxes (data not shown). Consistent with the data obtained using LPS from Alexis, ultrapure LPS was able to induce Ca^{2+} mobilization in a TLR4-independent, CD14-dependent manner (**b**, upper panels). To further prove that the observed phenomenon was not due to peptidoglycan contamination, we analyzed the ability of peptidoglycan to induce Ca^{2+} mobilization in BMDCs. As shown in (**b**), peptidoglycan was able to induce Ca^{2+} mobilization but in a CD14-independent manner, thus with a modality completely different from LPS. As IL-2 production by BMDCs can be strongly induced by Zymosan[3, 4], we analyzed the ability of this microbial product to induce Ca^{2+} fluxes. As shown in (**b**), Zymosan is able to induce Ca^{2+}

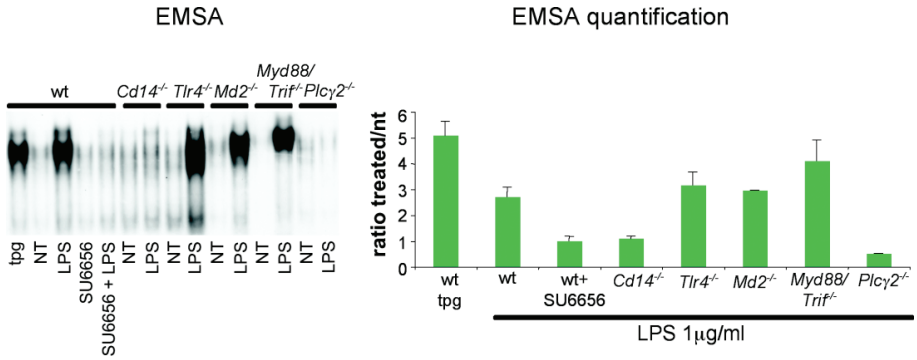
mobilization but in a CD14-independent manner. This highlights the existence of a specific pathway for LPS.

Figure legend. (**a**) Amounts of peptidoglycan (PGN) contamination in the indicated LPS preparations. S2 insect cells have been incubated with graded amounts of PGN for 3h after 24h of differentiation with the insect hormone 20-hydroxyecdysone. A dose-response curve has been produced by correlating the amount of PGN to the antimicrobial peptide attacin B (ATTB) transcript levels as measured by qRT-PCR. The amount of contaminant PGN in LPS preparations has been determined by comparing ATTB mRNA level induced in LPS treated S2 cells with the PGN dose-response curve. (**b**) Ca^{2+} mobilization in wt and mutant DCs following LPS, peptidoglycan and Zymosan treatment. Time point 0 indicates the time at which the stimulus was administered. Means and standard deviations for a minimum of 30 cells are shown. Experiments were repeated at least three times for each set of conditions, with similar results obtained in each case.

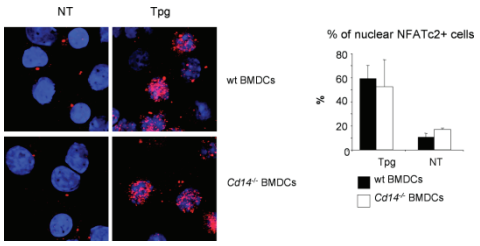


Supplementary Fig. 5. PLCγ2 is activated in wt but not in CD14-deficient BMDCs. WT and CD14-deficient BMDCs were treated with LPS at the indicated time points and equal amounts of lysates were immunoblotted

with anti-phospho-PLCγ2 antibody. The experiment was repeated twice with similar results obtained in each case. Left panel, western blot analysis; right panel, western blot analysis quantification. NT, untreated cells.

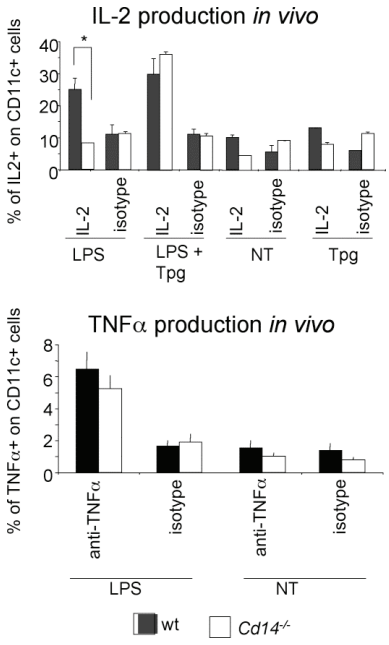


Supplementary Fig. 6. NFAT nuclear translocation in BMDCs. NFAT nuclear translocation was determined by EMSA in wt, the indicated mutant BMDCs and BMDCs in presence of the SFK inhibitor (SU6656, 260nM) before (NT) and after LPS treatment. Thapsigargin (tpg) was used as a positive control. Quantification analysis indicating the fold-difference in nuclear NFAT is also shown. Data were normalized to the nuclear abundance of the unrelated transcription factor, Oct1, that remains constant during activation.



Supplementary Fig. 7. NFATc2 nuclear translocation induced by thapsigargin. Left panel, NFATc2 nuclear translocation determined by

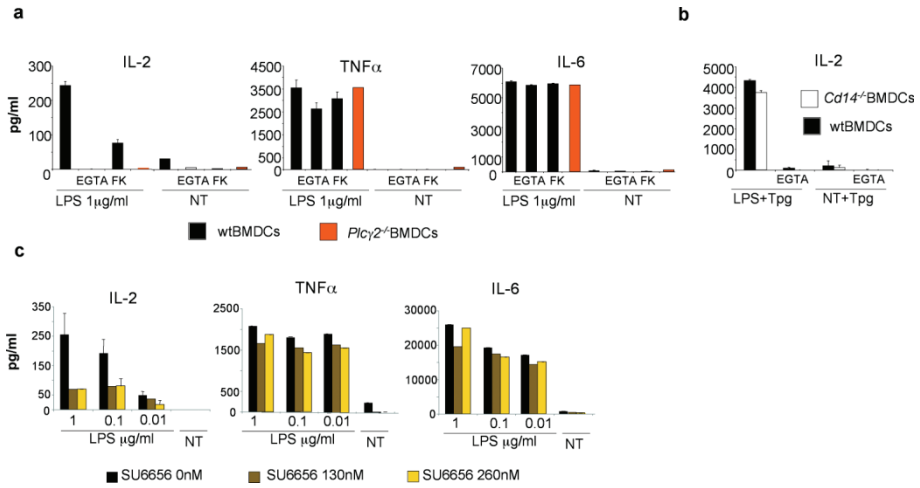
immunofluorescence analysis in wt and CD14-deficient BMDCs before (NT) and after challenge with thapsigargin (Tpg) for 2 h. Stained cells were analyzed by confocal microscopy. Blue, DAPI; red, anti-NFATc2; original magnification, X 630. Right panel, percentage of wt and CD14- deficient BMDCs showing NFATc2 inside the nucleus before and after thapsigargin treatment. Data represent means and standard errors of ten fields (containing around 50 cells each) analyzed in two independent experiments.



Supplementary Fig. 8. *Introductory explanation*. We used IL-2 and TNF-α as reporter genes to evaluate whether CD14 was required to activate the NFAT pathway in DCs *in vivo*. Consistent with *in vitro* observations, *in vivo* treatment of CD14-deficient mice with LPS did not induce IL-2 production by $CD11c^{+}$ splenic DCs. Nevertheless, the concomitant administration of

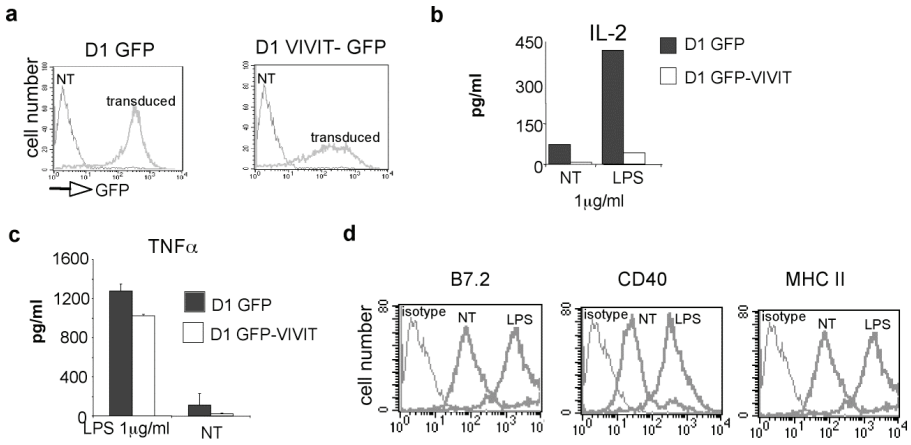
thaspigargin with LPS restored the ability of $CD11c^+$ splenic DCs to produce IL-2. Similarly, *in vivo* TNFα production by DCs following LPS administration in both wt and CD14-deficient mice was observed. These findings confirmed that CD14 was required to transduce Ca^{2+}-mediated signals in DCs following LPS stimulation *in vivo*.

Figure legend. IL-2 production *in vivo* following LPS administration depends on CD14. Upper panel, percentage of $CD11c^+$ cells producing IL-2 identified by FACS analysis in the spleen of wt and CD14-deficient mice after the intra-splenic administration of LPS or LPS plus thapsigargin. * = P < 0.05. NT, untreated mice; Tpg, mice treated with thapsigargin; Isotype, splenocytes stained with the isotype control antibody; IL-2, splenocytes stained with the anti-IL-2 antibody. Data are representative of three independent experiments conducted with four mice per group.



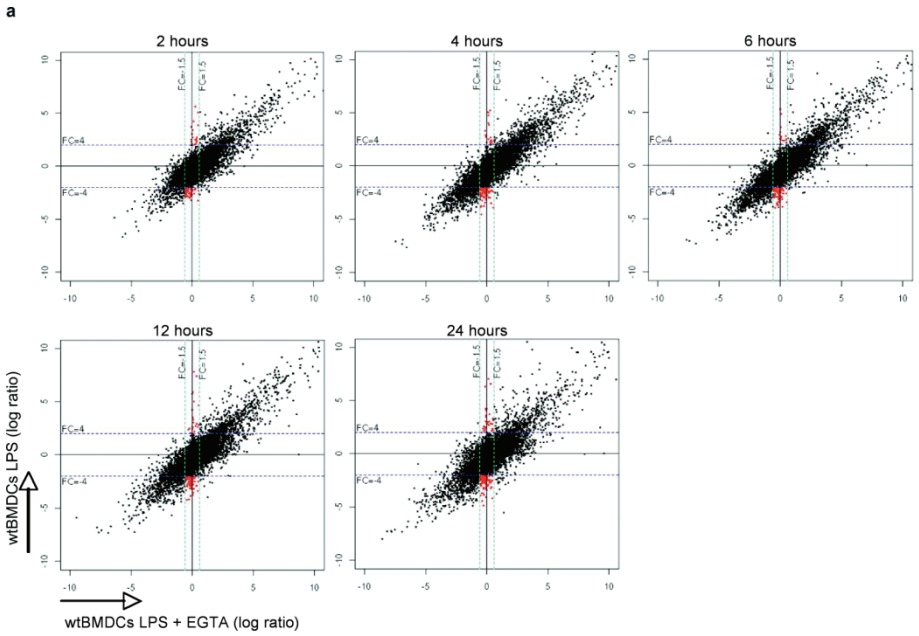
Supplementary Fig. 9. Blocking the Ca^{2+} pathway at different levels inhibits IL-2 production. (**a**) IL-2, TNF-α and IL-6 production by wt BMDCs stimulated by incubation for 18 h with LPS in the presence or absence of the calcium

chelator EGTA (2 mM) or the calcineurin inhibitor tacrolimus (FK, 50 nM). (**b**) IL-2 production by wt and CD14-deficient BMDCs stimulated by incubation for 18 h with LPS and thapsigargin (Tpg) in the presence or absence of EGTA. (c) IL-2, TNF-α and IL-6 production by PLCγ2-deficient BMDCs and by wt BMDCs in presence of the indicated concentrations of the SFK inhibitor (SU6656). Experiments were repeated at least three times for each set of conditions, with similar results obtained in each case.



Supplementary Fig. 10. *Introductory explanation*. The VIVIT peptide selectively interferes with the calcineurin-NFAT interaction, without affecting calcineurin phosphatase activity[5], by binding the calcineurin-docking motif of NFAT. Thus, the VIVIT peptide specifically inhibits the production of cytokines dependent on NFAT activation, without inhibiting the production of cytokines that require calcineurin but not NFAT[5]. D1 cells transduced with the VIVIT peptide fused to the green fluorescent protein (GFP) produced very little IL-2 following activation with LPS. In contrast, TNF-α production and the upregulation of activation markers were not affected.

Figure legend. IL-2 production by VIVIT-GFP-transduced D1 cells. (**a**) GFP expression by D1 cells transduced with the empty vector and with the VIVIT-GFP vector. (**b**) IL-2 production by D1 cells transduced with the VIVIT-GFP chimeric protein (D1 GFP-VIVIT) or with the GFP protein as a control (D1 GFP), after 6 h of stimulation with LPS. (**c**) TNF-α production by D1 cells transduced with the VIVIT-GFP chimeric protein after stimulation with LPS for 18 h. (**d**) Upregulation of activation markers at the surface of VIVIT-GFP-transduced D1 cells 24 hours after stimulation with LPS, as measured by FACS analysis. Isotype, cells stained with isotype control antibodies; NT, unstimulated cells. The experiment was repeated twice, with similar results.



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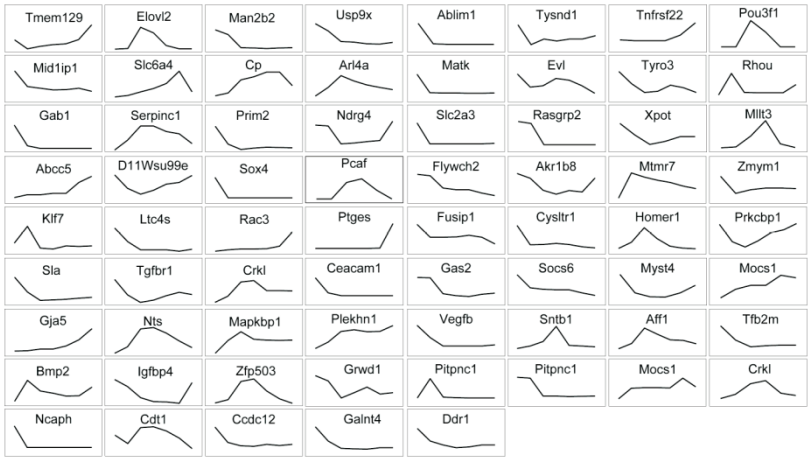
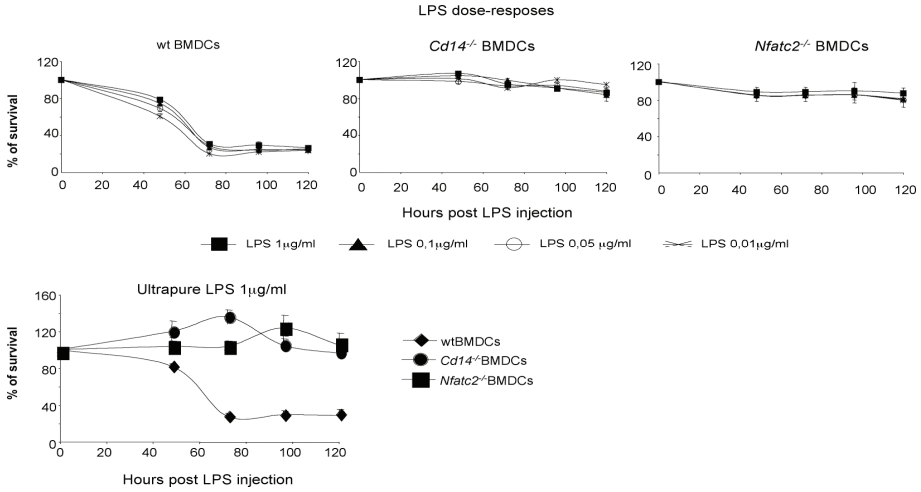


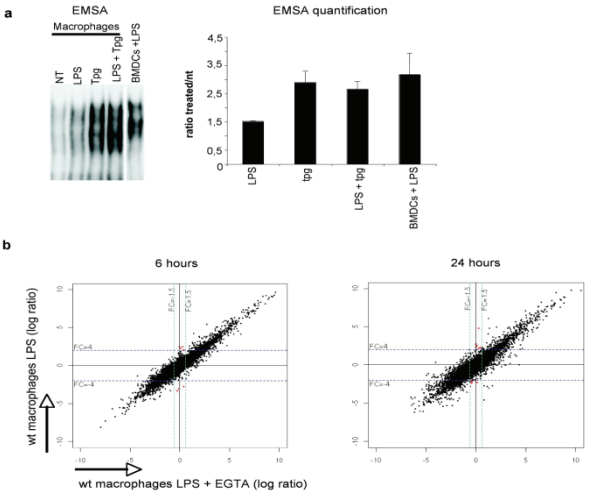
Supplementary Fig. 11. *Introductory explanation*. To identify the genes selectively modulated by NFAT, comparative kinetic microarray analyses were performed in the following conditions: 1) CD14-deficient BMDCs stimulated with LPS; 2) wtBMDCs stimulated with LPS in presence of EGTA; 3) wtBMDCs stimulated with LPS. This experimental setting allowed us to

select for effects due to Ca^{2+} fluxes and exclude the effects due to other causes, particularly the block of TRIF recruitment in CD14-deficient cells and the EGTA effects unrelated to Ca^{2+} chelation. Thus, we selected those genes showing a fold change higher than 4 with respect to the basal level in any kinetic time points in wt BMDCs and not showing any change (fold change lower than 1.5) in EGTA-treated BMDCs (**a**). These genes were further selected for not showing any change in CD14-deficient cells. The selected genes are shown in the panels **b**.

Figure legend. Comparative global gene expression analyses showing the NFAT regulated genes. (**a**) Signal Log Ratio plots of genes expressed by wt BMDCs at different time points after LPS vs LPS-EGTA treatments. Red dots represent genes showing a fold change in the expression levels higher than 4 with respect to the basal level in LPS treated cells and a fold change lower than 1.5 in EGTA plus LPS treated cells. (**b**) Expression regulation of genes showing a fold change higher than 4 with respect to the basal level in any kinetic time points in wt BMDCs and not showing any change in EGTA-treated wt BMDCs and in CD14-deficient cells.



Supplementary Fig. 12. Survival curves of wt and mutant BMDCs following incubation with the indicated doses of Alexis LPS or 1 μg/ml of re-purified (ultrapure) Sigma LPS (see Fig. S-4).



Supplementary Fig. 13. *Introductory explanation*. We performed an EMSA analysis in BM-macrophages to investigate NFAT activation. This analysis showed only a minimal NFAT presence in the nucleus of BM macrophages

compared to DCs following LPS stimulation. To further investigate whether the Ca^{2+}-NFAT pathway played any role in LPS-stimulated macrophages, we performed a comparative kinetic microarray analysis in the same conditions used for DCs (see Fig. S-11): 1) CD14-deficient macrophages stimulated with LPS; 2) wt macrophages stimulated with LPS in presence of EGTA; 3) wt macrophages stimulated with LPS. Based on the previous analysis, we reduced the kinetic to 0, 6 and 24 h following LPS stimulation. Microarray analysis was performed with the same procedure used for DCs (see Supplementary Fig. 11). As shown in this supplementary figure, very few genes were modulated in LPS-treated compared to EGTA and LPS-treated wt macrophages at 6 and 24 h, indicating that the EGTA treatment had a minimal or no effect. When we further selected the obtained genes for not showing any change in CD14-deficient cells, no genes remained. Moreover, none of the genes regulated by NFAT in DCs were modulated in macrophages following LPS stimulation (data not shown). All together these results indicate that, diversely from DCs, the Ca^{2+}-NFAT pathway does not play any role in macrophages following LPS stimulation.

Figure legend. (**a**) NFAT nuclear translocation determined by EMSA in BM-macrophages 2 h after the indicated treatments. LPS-treated BMDCs were loaded onto the same gel for positive control. Quantification analysis indicating the fold difference in nuclear NFAT is also shown. Data were normalized to the nuclear abundance of an unrelated transcription factor that remains constant during activation. (**b**) Signal Log Ratio plots of genes expressed by BM macrophages at 6 and 24 h after LPS vs LPS-EGTA treatments. The analysis has been done as described in Supplementary Fig. 11.

Supplementary Table 1

Selected NFAT-modulated genes in DCs following LPS stimulation

Gene	Protein Function	Regulation	Ref.
Tfb2m	TFB2M is a mithocondrial auxiliary factor necessary for promoter recognition by mithocondrial RNA polymerase (POLRMT)	Down	[6, 7]
Kl7	KL7 is involved in growth arrest and cell differentiation	Up	[8]
Myst4	MYSTs are a family of histone acetyltransferases involved in activation of gene transcription	Down	[9, 10]
Pcaf	PCAF is a hystone-modifying co-factor involved in regulation of differentiation	Up	[11, 12]
Xpot	Exportin T is the tRNA transport from nucleus to cytoplasm	Down	[13, 14]

Supplementary Table 2

Apoptosis genes regulated by NFAT in DCs following LPS stimulation

Gene	Protein Function	Regulation	Ref
Nur77	The encoded protein acts as a nuclear transcription factor and mediates apoptosis.	Up	[15]
Ddit3	When the functions of the ER are severely impaired, transcription is upregulated for the induction of cell apoptosis	Up	[16]
Gadd45g	Stress responsive gene, involved in the elimination of activated T_H1 cells by promoting apoptosis and inhibiting proliferation.	Up	[17]
Tia1	Regulator of alternative pre-mRNA splicing of FAS, upregulated in presence of Cyclosporin A	Down	[18, 19]

Experimental setting used. To identify apoptosis genes selectively modulated by NFAT, a comparative kinetic (time points 0, 48 and 60 h) microarray analysis was performed in the following conditions: 1) wt BMDCs stimulated with LPS; 2) CD14-deficient BMDCs stimulated with LPS; 3) wt BMDCs stimulated with LPS in presence of thapsigargin; 4) CD14-deficient BMDCs stimulated with LPS in presence of thapsigargin. Thus, we selected apoptosis genes showing a fold change higher than 4 with respect to the basal level in any kinetic time points in wt BMDCs stimulated with LPS. Among these genes we further selected those showing the same modulation additionally in wt and CD14-deficient BMDCs costimulated with

LPS and thapsigargin. From this subgroup we then selected those genes not showing any change (fold change lower than 1.5) in CD14-deficient BMDCs

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Chapter 3

CD14-NFAT signaling in dendritic cells regulates PGE_2 synthesis and edema formation in response to LPS

Manuscript submitted

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Prostaglandin E_2 (PGE_2) is a proinflammatory mediator whose biosynthesis is targeted by non-steroidal anti-inflammatory drugs (NSAID). Here we show that dendritic cells (DCs) are the main source of PGE_2 in a model of lipopolysaccharide (LPS)-induced inflammation and that CD14-NFAT pathway regulates this function. Therefore, CD14 signaling in DCs is a valuable target for the development of a novel class of anti-inflammatory compounds, alternative to COX-2 inhibitors, selectively blocking PGE_2 production.

PGE_2 is a potent eicosanoid lipid mediator involved in numerous homeostatic and inflammatory processes, most notably the induction

of inflammatory swelling through its vasodilating activity[1]. Its production, together with that of pro-inflammatory cytokines, is triggered by Toll-like receptor (TLR)-driven pathogen recognition[2-4]. Upon LPS recognition, TLR4-expressing innate immune cells mediate the initial release of PGE_2 through a multistep biosynthetic process requiring the coordinated up-regulation of cyclooxygenase-2 (COX-2)[5] and microsomal PGE synthase-1 (mPGES-1)[6,7]. In a scrutiny of data sets identifying genes regulated by the DC-specific CD14-NFAT signaling pathway triggered by LPS[8], we identified *Ptges1* as a potential transcriptional target (Supplementary Fig.1). We validated this observation by qRT-PCR in mouse bone marrow-derived DCs (BMDCs) and observed a strong induction of mPGES-1 mRNA in wild-type (wt) BMDCs after LPS stimulation, a response that was greatly impaired in $CD14^{-/-}$ BMDCs (**Fig. 1**).

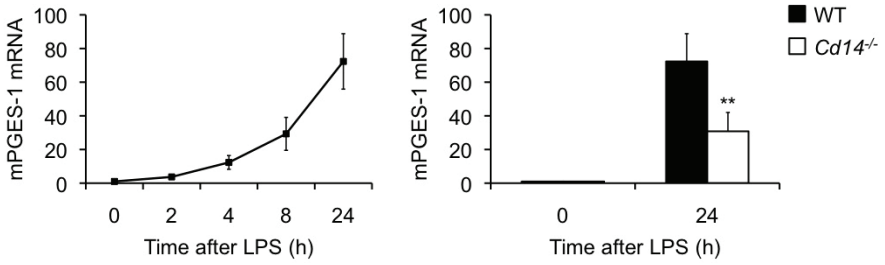


Fig. 1. Left panel: qRT-PCR analysis of mPGES-1 mRNA induction kinetics by wt BMDCs stimulated with LPS (1 µg/ml). Right panel: Up-regulation of mPGES-1 mRNA after LPS administration by wt and $CD14^{-/-}$ BMDCs Values represent at least three independent experiments performed in duplicate + s.e.m. ** $P < 0.01$, N.S. not significant.

Blocking NFAT activation in wt BMDCs by pre-incubating cells with the Ca^{2+} chelator EGTA or the calcineurin inhibitor FK506 also resulted in a blunted mPGES-1 expression (**Fig. 2**), suggesting that CD14-dependent NFAT activation regulates this process. We excluded that a reduced activation of NF-κB accounted for the defective mPGES-1 up-regulation in $CD14^{-/-}$ BMDCs[9] by using doses of LPS (1 μg/ml) that allowed direct agonist detection by TLR4 without an absolute requirement for CD14, as evidenced by the ability of $CD14^{-/-}$ BMDCs to normally secrete TNF-α (**Fig. 3**). Similarly, an impairment of IRF3 activation[10] could not explain our observations on mPGES-1 transcription, since co-administration of IFN-β did not restore mPGES-1 induction in LPS-treated $CD14^{-/-}$ BMDCs (**Fig. 2**).

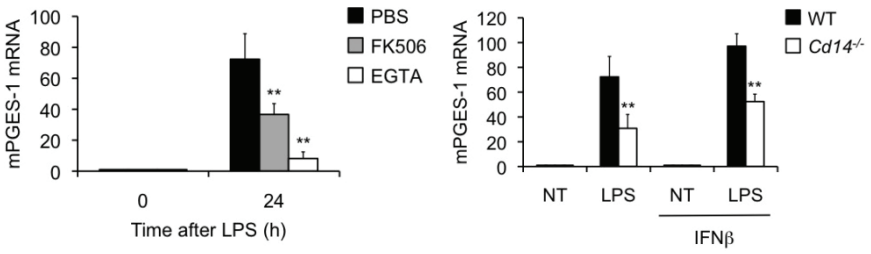


Fig. 2. Up-regulation of mPGES-1 mRNA after LPS administration by wt BMDCs pre-treated with PBS, FK506 (0,5 mM) or EGTA (2 mM) (left) and by wt and $CD14^{-/-}$ BMDCs treated or not with IFN-β (50 U/ml) 2 hours after LPS (right). Values represent at least three independent experiments performed in duplicate + s.e.m. ** $P < 0.01$, N.S. not significant.

Supporting the hypothesis of NFAT being the key factor, mPGES-1 induction by LPS correlated with production of IL-2, a bona fide marker for NFAT activation in DCs[8,11], but not of TNF-α (**Fig. 3**).

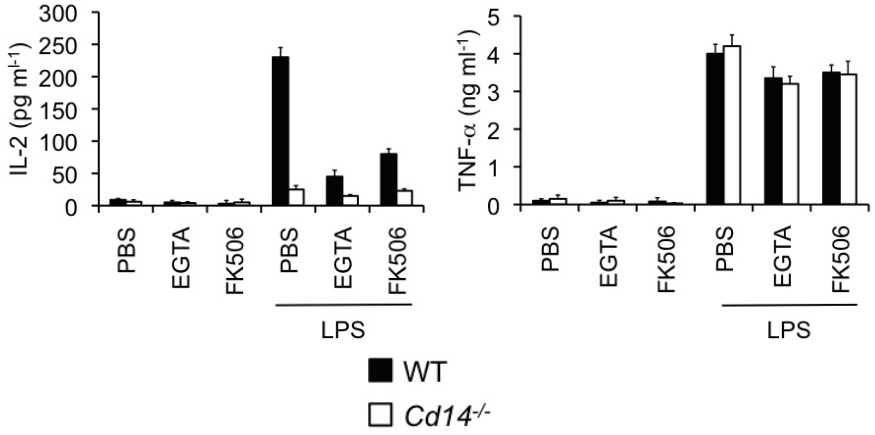


Fig. 3. Production of IL-2 (left) and TNF-α (right) by wt and $CD14^{-/-}$ BMDCs treated with LPS or LPS plus thapsigargin (50 nM) in the presence/absence of EGTA or FK506. Values represent at least three independent experiments performed in duplicate + s.e.m. ** $P < 0.01$, N.S. not significant.

PGE_2 release *in vitro* was also strongly impaired in $CD14^{-/-}$ BMDCs, as well as in EGTA- or FK506-pre-treated wt BMDCs relative to controls (**Fig. 4**). However, we were able to restore PGE_2 production in $CD14^{-/-}$ BMDCs by coupling LPS stimulation with the artificial NFAT activation obtained by administration of thapsigargin and this effect was NFAT-dependent since it could be blocked by EGTA or FK506 (**Fig. 4**). Thapsigargin co-treatment additionally restored IL-2 production in $CD14^{-/-}$ BMDCs (**Fig. 4**). Another key enzyme for PGE_2 production is COX-2, whose transcriptional expression has been reported to be regulated by NFAT in other experimental settings[12]. However, COX-2 induction by LPS in BMDCs was not affected by CD14 deficiency (Supplementary Fig. 2). These data show that, by regulating LPS-

mediated mPGES-1 (but not COX-2) induction, CD14-NFAT signaling drives the *in vitro* production of PGE_2 by BMDCs.

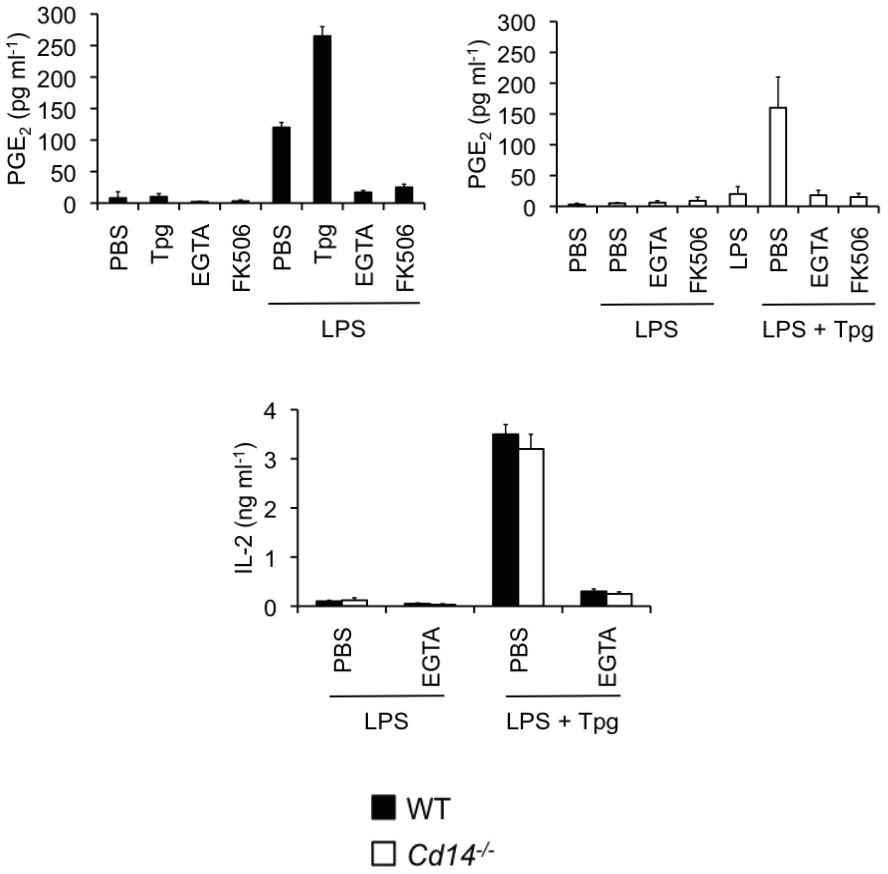


Fig. 4. Upper panel: PGE_2 production by wt and $CD14^{-/-}$ BMDCs treated with LPS or LPS plus thapsigargin (50 nM) in the presence/absence of EGTA or FK506. Lower panel: production of IL-2 by wt and $CD14^{-/-}$ BMDCs in the indicated conditions. Values represent at least three independent experiments performed in duplicate + s.e.m. ** $P < 0.01$, N.S. not significant.

Before migrating to draining lymph nodes, activated DCs are likely to participate to the inflammatory process in vivo, as described for monocyte-derived DCs[13,14]. Given their ability to produce PGE_2, which regulate edema formation through local vasodilation[1], we investigated whether DCs participate to LPS-induced tissue swelling. Using the CD11c.DOG transgenic mouse model, in which expression of the CD11c promoter (active in DCs) controls the expression of diphtheria toxin receptor (DTR)[15], we were able to conditionally ablate DCs in lymphoid and non-lymphoid organs and tissues (including the skin) by consecutive DT injections (**Fig. 5** and Supplementary Fig. 3). Importantly, such treatment did not cause any significant alteration in either macrophage or granulocyte populations, as measured by qRT-PCR of cell-specific mRNAs[16] in the whole footpad (**Fig. 5**).

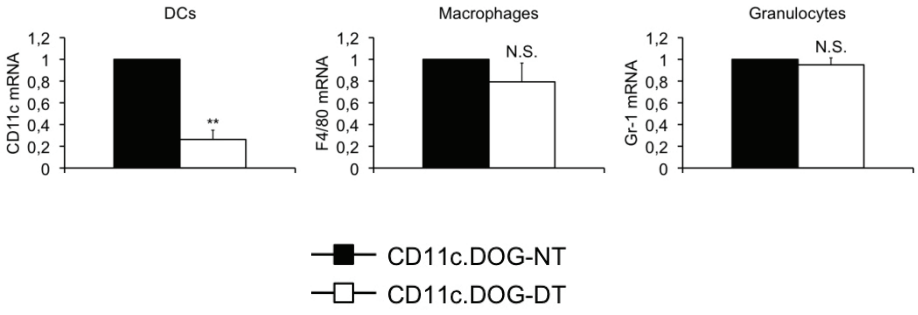


Fig. 5. qRT-PCR analysis of CD11c (DCs), F4/80 (macrophages) and Gr-1 (granulocytes) mRNA in the footpad of CD11c.DOG mice before (CD11c.DOG-NT) or after 3 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). Values represent at least two independent experiments (n=5) + s.e.m. ** $P < 0.01$, N.S. not significant.

We compared paw edema formation after a single injection of LPS into the footpad of CD11c.DOG mice that were previously administered DT (CD11c.DOG-DT) or PBS (CD11c.DOG-NT). Notably, DC depletion strongly impacted on tissue edema formation (**Fig. 6**), indicating that DCs play a major role in this process.

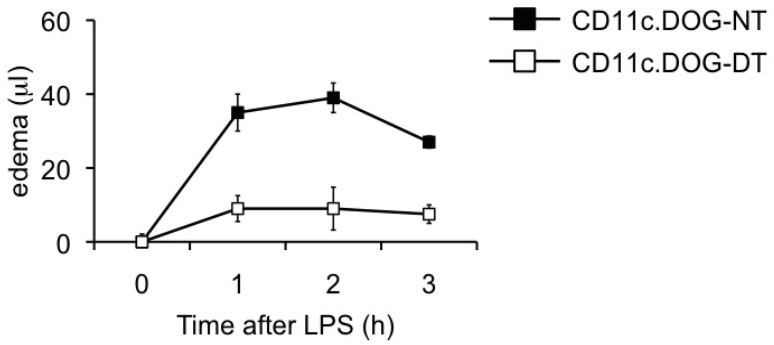


Fig. 6. Inflammatory swelling in the footpad of CD11c.DOG-NT and CD11c.DOG-DT mice measured at the indicated time points after s.c. injection of LPS (25 µg/mouse). Values represent at least three independent experiments performed in duplicate + s.e.m. ** $P < 0.01$, N.S. not significant.

Inflammatory swelling was mainly induced by tissue-resident DCs, since no local recruitment of DCs, macrophages or granulocytes was observed early after LPS administration (Supplementary Fig. 4). These results are consistent with the faster kinetics of tissue edema formation (30-60 minutes) as compared to immune cell recruitment, and they are supported by the observation that *in vitro* LPS-stimulated BMDCs secrete much higher levels of PGE_2, as well as TNF-α, than bone marrow-derived macrophages (Supplementary Fig. 5).

According to this model, alterations in the PGE_2 biosynthetic pathway of DCs should recapitulate the LPS-unresponsive phenotype in terms of tissue swelling of DC-depleted mice. To this purpose, we compared LPS-induced paw edema in wt and $CD14^{-/-}$ mice and observed that $CD14^{-/-}$ mice did not develop significant swelling as compared to wt controls (**Fig. 7**). The phenotype could be restored by co-treating $CD14^{-/-}$ mice with LPS and thapsigargin, indicating a role for NFAT activation also in this in vivo model of PGE_2-dependent inflammation (**Fig. 7**). Thapsigargin alone did not trigger a detectable inflammatory response in the paw (Supplementary Fig. 6).

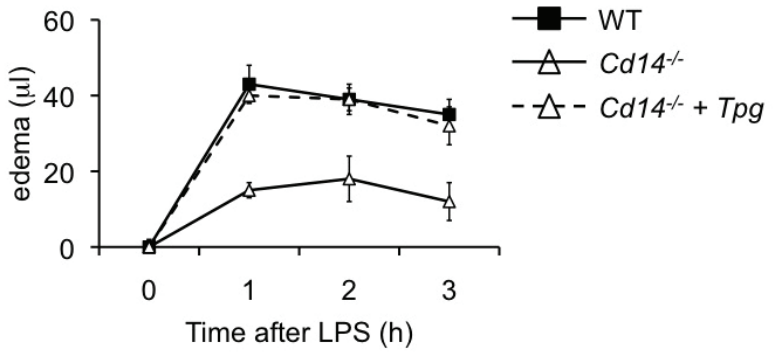


Fig. 7. Inflammatory swelling in the footpad of wt and $CD14^{-/-}$ mice treated with LPS (25 µg/mouse) in the presence or absence of thapsigargin (5 µM) measured at the indicated time points. Values represent at least three independent experiments performed in duplicate + s.e.m. ** $P < 0.01$, N.S. not significant.

Mechanistically, the reduction in paw edema observed in mice in which DCs were either depleted or impeded in their CD14-NFAT signaling pathway was due to a defective mPGES-1 up-regulation,

and consequently flawed PGE_2 production, in response to LPS. The *in vivo* analysis of mPGES-1 mRNA in the footpad of wt mice revealed a global 3-fold transcriptional induction of this gene upon LPS treatment that was lost in $CD14^{-/-}$ mice (**Fig. 8**). TNF-α mRNA in the whole tissue was similarly up-regulated in the two mouse strains (**Fig. 8**), showing that reduced footpad swelling in $CD14^{-/-}$ mice was indeed due to a defective PGE_2 production, rather than to a general defect in LPS sensing.

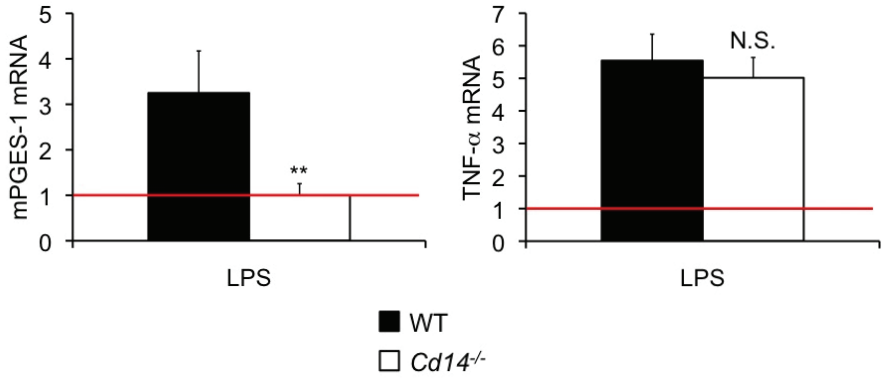


Fig. 8. qTR-PCR analysis of mPGES-1 (left) and TNF-α (right) mRNA induction in the footpad of wt and $CD14^{-/-}$ mice 1 hour after LPS treatment (25 µg/mouse) Values represent at least two independent experiments (n=5) + s.e.m. ** $P < 0.01$, N.S. not significant.

Depletion of DCs in CD11c.DOG-DT mice also affected *in vivo* mPGES-1 up-regulation in the paw after LPS treatment (**Fig. 9**). However, local induction of TNF-α mRNA was considerably reduced in this condition (**Fig. 9**), strongly reinforcing the idea that DCs are crucial

innate immune players that directly regulate the onset of inflammation.

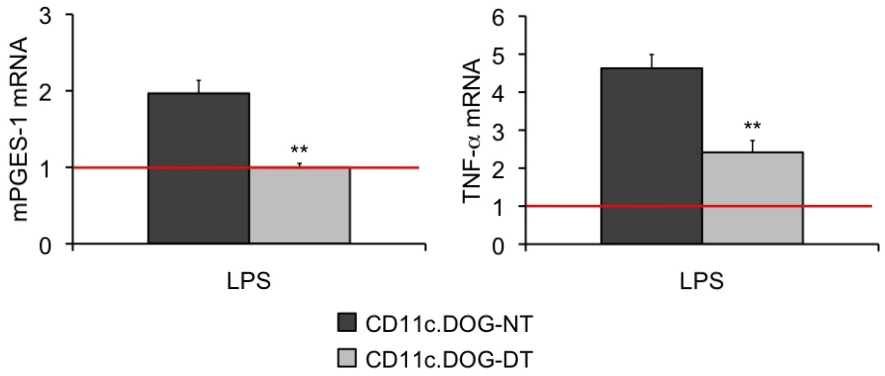


Fig. 9. qRT-PCR analysis of mPGES-1 (left) and TNF-α (right) mRNA induction in the footpad of CD11c.DOG mice pre-treated (-DT) or not (-NT) with DT and stimulated with LPS. Values represent at least two independent experiments (n=5) + s.e.m. ** $P < 0.01$, N.S. not significant.

Prostanoid synthesis is a key target of NSAIDs, which typically act as COX-2 inhibitors. However, the usage of such drugs has been limited by the existence of severe toxic secondary effects related to their systemic route of administration[17,18]. This suggests that inhibitors of the DC-specific CD14-NFAT pathway might act to relieve the symptoms of an ongoing inflammatory pathology. As a proof-of-principle experiment to investigate the potential anti-inflammatory effects of pharmacological inhibition of CD14-NFAT pathway, we administered daily FK506 to wt mice systemically (intraperitoneally) or locally (subcutaneous injection in the footpad) for two days. After LPS treatment in the footpad, we measured the local up-regulation of mPGES1 and TNF-α mRNAs. As shown in **Fig. 10**, the induction of

mPGES1 mRNA in the footpad triggered by LPS was almost completely abolished by both FK506 treatments. Importantly, no significant impairment of TNF-α mRNA induction was observed as a consequence of calcineurin inhibition (**Fig. 10**).

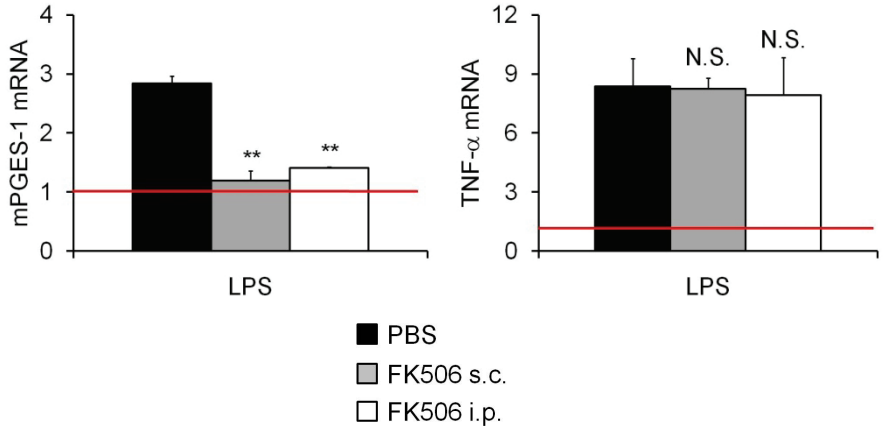


Fig. 10. qRT-PCR analysis of mPGES-1 (left) and TNF-α (right) mRNA induction in the footpad of wt mice pre-treated with 3 mg/Kg of FK506 in the footpad (s.c.) or systemically (i.p.) and stimulated with LPS. Values represent at least two independent experiments (n=5) + s.e.m. ** $P < 0.01$, N.S. not significant.

DCs are antigen-presenting cells that link innate and adaptive immune responses. However, their ubiquitous localization in peripheral tissues, as well as the existence of DC-specific innate immune pathways, makes them suitable for local proinflammatory purposes. Our data show that DCs are the major regulators in a model of LPS-induced inflammation in vivo and they exert this role through their peculiar ability to respond to LPS through a CD14-NFAT pathway that transcriptionally regulates mPGES-1 expression and

PGE_2 synthesis. Since the usage of COX-2 inhibitors as anti-inflammatory drugs[5] has been hampered by the existence of severe toxic secondary effects[17,18], our findings suggest that targeting CD14-NFAT signaling in DCs may constitute a strategy to overcome such problems by selectively blocking the biosynthesis of PGE_2 in specific inflammatory settings.

Materials and Methods

Mice and cells. C57BL/6 mice were purchased from Harlan. $Cd14^{-/-}$ mice were purchased from CNRS d'Orléans (Orléans Cedex 2, France). N. Garbi kindly provided CD11c.DOG mice. All animals were housed under pathogen-free conditions and all experiments were carried out in accordance with the relevant laws and institutional guidelines. Dendritic cells and macrophages were derived from bone marrow progenitors of wt or mutant mice as previously described[8].

Antibodies and chemicals. Antibodies against mouse CD11b and CD11c used for FACS analysis were purchased from BD Biosciences. TLR4-grade smooth LPS (Escherichia coli, 055:B5) were purchased from Alexis Corporation. EGTA, FK506 and thapsigargin were purchased from Sigma Aldrich. Recombinant murine IFN-β and diphtheria toxin were purchased from R&D Systems. Antibody against murine COX-2 was purchased from Cayman Chemical.

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

***In vitro* mRNA expression level measurement**. Cells (2*106) were lysed with the TRIZOL reagent (Applied Biosystems) and total mRNA was extracted with an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. NanoDrop spectrophotometer (ThermoScientific) was used to quantify mRNA and to assess its

purity. 600 ng of mRNA were retro-transcribed to cDNA using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). 10 ng of cDNA were amplified using the Power Sybr Green PCR Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems) and data were analyzed using the built-in software. Primer pairs used were: 5'-ACGACATGGAGACAATCTATCCT–3' and 5'-TGAGGACAACGAGGAAATGT-3' (mPGES-1), 5'-CGAAAGCATTTGCCAAGAAT-3' and 5'-AGTCGGCATCGTTTATGGTC-3' (18s). 18s mRNA was used as internal reference for relative quantification studies.

DC depletion. Diphtheria toxin (16 ng/g) was administered daily to CD11c.DOG mice through an i.p. injection for 3 consecutive days. Control mice were given PBS. Effective DC depletion was assessed by FACS and qRT-PCR analysis.

Tissue edema. Following anesthesia with pentobarbital (60 mg/kg), sex- and age-matched mice were subcutaneously injected with LPS (25 μg/25 μl), LPS plus thapsigargin (5 μM) in the footpad or PBS as a control. The paw volume of the LPS-, as well as the PBS-treated contralateral paw was then measured by a plethysmometer (Ugo Basile) at the indicated time points. At the 2 h time point, most of the animals had recovered from the anesthesia, and at the 4 h time point all animals had recovered. The volume of the contralateral paw was subtracted from the volume of the injected paw, to obtain the edema volume.

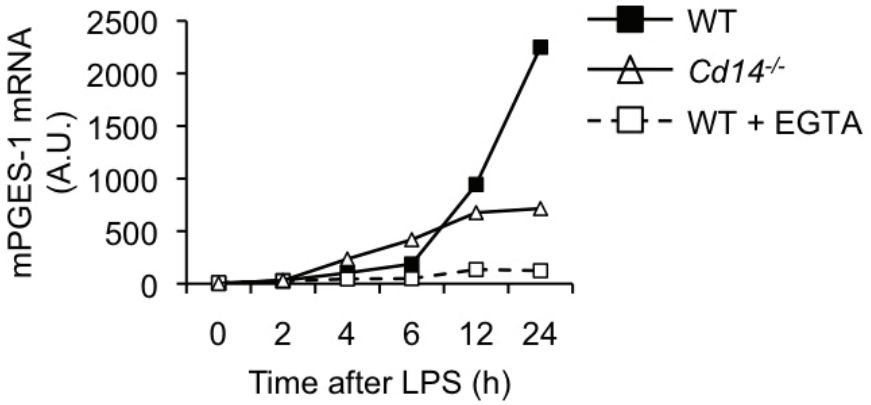
***In vivo* mRNA expression level measurement**. Whole footpad tissue from treated or control mice was cut, briefly washed in cold PBS and immersed in RNAlater® solution (Ambion) at 4°C for 24 hours. Paws were then lysed in TRIZOL® and mechanically disrupted using a TissueLyser (Qiagen) (30 shakes/sec for 3 minutes). Subsequent mRNA processing was performed as described above. Primer pairs used were: 5'-TTTGTTTCTTGTCTTGGCTTCAA-3' and 5'-TTAGTGGCTTTTATTTCCTTTGGT-3' (CD11c), 5'-CACCTTCATTTGCATCAACA-3' and 5'-TCTGAAAAGTTGGCAAAGAGAA-3' (F4/80), 5'-TGCTCTGGAGATAGAAGTTATTGTG-3' and 5'-TTACCAGTGATCTCAGTATTGTCCA-3' (Gr-1). Primer pairs for mPGES-1 and 18s are indicated above. Pre-validated Quantitect® primer pairs for TNF-α and HPRT1 (reference gene) were purchased from Qiagen.

***In vivo* calcineurin inhibition**. Mice were administered 3 mg/Kg FK506 (dissolved in Castor oil, Sigma) once a day for two consecutive days. FK506 was given through i.p. or s.c. (footpad) injections and LPS stimulation was performed as described above one day after the end of FK506 treatment.

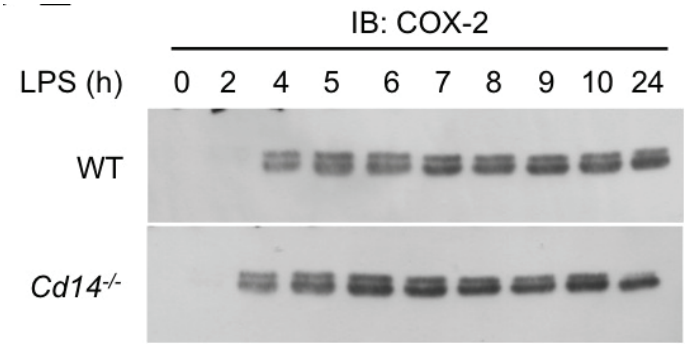
Western blot. Cells were lysed with a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1% NP-40 supplemented with protease and phosphatase inhibitor coktails (Roche). Cell debris were removed by centrifugation at 16,000 x g for 15 minutes (4°C) and proteins were quantified using a BCA assay (Thermo Scientific). 10 µg of cell lysate were run on a 10% polyacrilamide gel and SDS-PAGE was operated following standard procedures. After protein

transfer, nitrocellulose membranes (Thermo Scientific) were incubated with the indicated antibodies and developed using an ECL substrate reagent (Thermo Scientific).

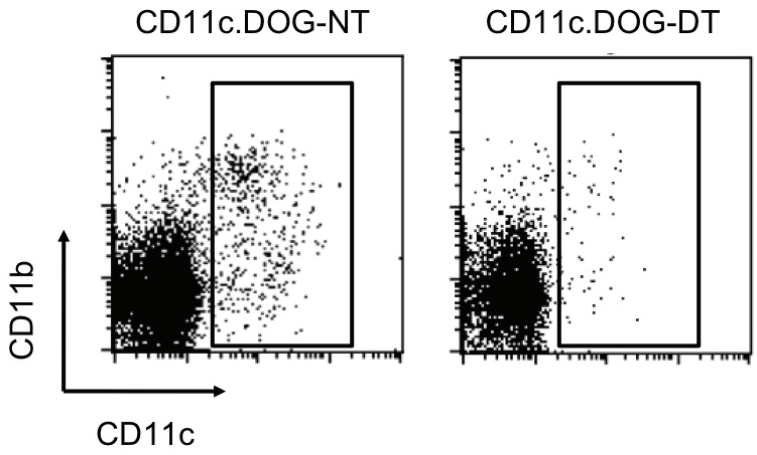
Supplementary Information



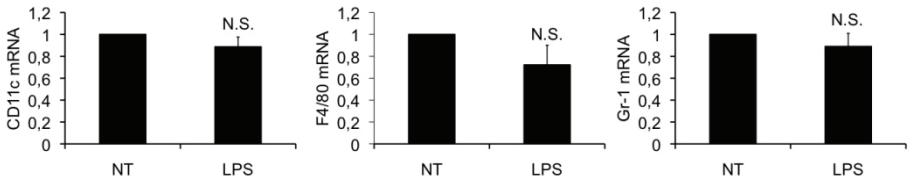
Supplementary Fig. 1. mPGES-1 is a potential target of CD14/NFAT signaling in DCs. GeneChip analysis of mPGES-1 mRNA expression by wt BMDCs +/- EGTA (2mM) or $CD14^{-/-}$ BMDCs stimulated with LPS (1 μg/ml) at the indicated time points.



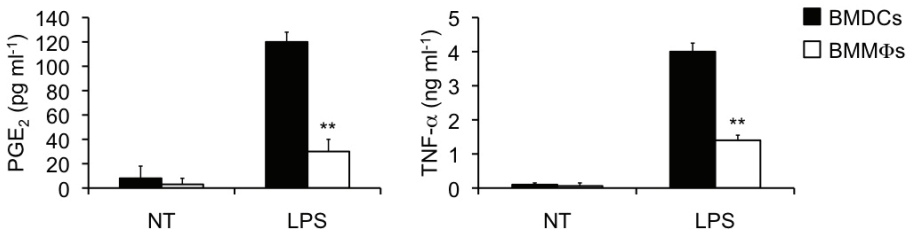
Supplementary Fig. 2. COX-2 up-regulation in response to LPS is CD14-independent in DCs. Western blot analysis of COX-2 induction by wt and $CD14^{-/-}$ BMDCs treated with LPS (1 μg/ml) at the indicated time points.



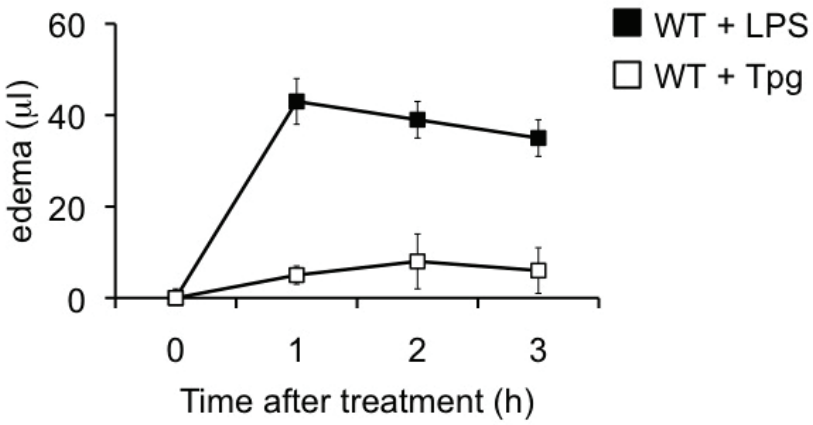
Supplementary Fig. 3. DCs are effectively depleted from the spleen of CD11c.DOG mice after DT treatment. Representative flow cytograms of splenocytes from CD11c.DOG mice before (CD11c.DOG-NT) or after 3 rounds of DT (16 ng/g) treatment (CD11c.DOG-DT). $CD11c^{+}$ $CD11b^{+/-}$ DC populations are shown.



Supplementary Fig. 4. LPS stimulation does not induce significant recruitment of inflammatory cells after 1 hour. Real-Time PCR analysis of CD11c, F4/80 and Gr-1 mRNA in the footpad of CD11c.DOG mice before and after 1 hour of s.c. LPS injection (25 μg/g).



Supplementary Fig. 5. Differential responsiveness of DCs and macrophages to LPS stimulation in vitro. PGE_2 and TNF-α production in vitro by wt BMDCs and bone marrow macrophages (BMMFs) after LPS stimulation.



Supplementary Fig. 6. Thapsigargin alone does not induce tissue edema. Inflammatory swelling in the footpad of wt mice measured at the indicated time points after s.c. injection of LPS (25 µg/mouse) or thapsigargin (5 µM).

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Chapter 4

Summary, Conclusions and Future Perspectives.

CD14: a co-receptor with crucial signaling functions

In the introduction to this thesis I have described the known functions of CD14 as a sensing receptor for TLR4, as well as other TLRs. However, we have described a novel signal transduction pathway induced by LPS that exclusively relies on CD14 for activation of the transcription factor NFAT (nuclear factor of activated T cell) in DCs[1]. These findings provide the definitive demonstration that CD14 can act independent of TLRs as a transducing receptor (**Fig. 1**).

DCs retain the ability to release IL-2 upon stimulation with several pattern recognition receptors, including TLR4[2,3], a key event for natural killer (NK) cell activation[4]. Since in T lymphocytes IL-2 expression is regulated at the transcriptional level by NFAT[5], we have undertaken a study to assess whether LPS stimulation in DCs is also able to induce activation of this transcription factor. As it turned out, CD14 is responsible for the induction of a rapid and transient influx of Ca^{2+} ions in LPS-stimulated DCs. The consequent increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) triggers the activation of calcineurin, a phosphatase that in turn removes phosphate groups from cytosolic, inactive NFAT proteins. Constitutive phosphorylation by GSK3β (glycogen synthase kinase 3β) in resting cells is a strategy for sequestering NFAT in the cytosol through masking its NLS. LPS-induced activation of calcineurin relieves inhibition of NFAT, thereby

promoting its nuclear translocation. Strikingly, experiments in DCs doubly deficient in TLR4 and TLR2 as well as MyD88 and TRIF clearly reveal that activation of NFAT by LPS in DCs occurs with no contribution at all from TLR4 or any other TLR. Instead, CD14 is necessary for inducing Ca^{2+} influx and NFAT activation after LPS stimulation in DCs. The downstream effectors of this CD14-dependent pathway have also been identified, with Src family kinases (SFKs) and PLCγ2 playing essential roles in triggering Ca^{2+} influx and NFAT activation. These and other data, in addition to previous knowledge, allow the definition of a reliable model for CD14-dependent signal transduction in DCs whereby LPS stimulation triggers the clustering of CD14 molecules in lipid rafts. Through an ill-defined mechanism, CD14 clustering results in SFK activation (possibly Lyn), which in turn activates PLCγ2 by phosphorylation. PLCγ2 acts by hydrolyzing its substrate $PI(4,5)P_2$ into the second messengers $I(3,4,5)P_3$ and diacylglycerol (DAG). Whereas DAG probably signals NF-κB activation through PKCs (protein kinase C), $I(3,4,5)P_3$ triggers the opening of $I(3,4,5)P_3$-regulated ion channel receptors on the cell surface, resulting in a single wave of extracellular Ca^{2+} influx that ultimately promotes calcineurin activation, NFAT dephosphorylation and nuclear translocation. Apart from describing a novel signaling ability of CD14, this model raises a number of interesting questions about the regulation of this pathway at the molecular level. For example, how does CD14 transduce the LPS recognition signal intracellularly? In this regard it has to be noted that CD14 appears to signal NFAT activation through its GPI-anchor,

rather than by presenting LPS to an unknown transmembrane protein other that TLR4. Indeed, soluble CD14 (sCD14) restores sensitivity to low doses of LPS in CD14-deficient DCs in terms of proinflammatory cytokine production but not in terms of Ca^{2+}-NFAT signaling. Furthermore, disruption of lipid raft integrity with a cholesterol-depleting agent abolishes the ability of wild-type DCs to induce a Ca^{2+} response to LPS. These observations strongly support the hypothesis that membrane-anchored CD14 that resides in lipid rafts directly promotes NFAT activation, although we can not formally exclude the involvement of additional transmembrane players. We propose that the clustering of CD14 induced by LPS promotes the aggregation and consequent activation of other lipid raft-associated signaling proteins, namely SFKs, which in turn activate PLCγ2. Consistent with this hypothesis is the recent finding that colloidal gold-induced cross-linking of the GPI-anchored receptor CD59 results in the transient lateral immobilization in lipid rafts of Lyn and Gα2, which triggers the activation of Lyn and, consequently, PLCγ2[6,7]. An additional feature of CD14-dependent signaling to NFAT that merits further investigation is the mechanism by which an increase of $[Ca^{2+}]i$ is obtained upon LPS stimulation in DCs. T cell receptor-induced calcineurin activation is typically operated through a two-step Ca^{2+} mobilization system called store-operated Ca^{2+} entry (SOCE). According to the SOCE paradigm, $I(3,4,5)P_3$ promotes the opening of specific ion channel receptors (IP3-R) localized on the endoplasmic reticulum (ER). Since the $[Ca^{2+}]$ in the ER is higher than in the cytosol, this results in a transient wave of Ca^{2+} mobilization

from the ER. The consequent depletion of the Ca^{2+} store is then sensed and communicated to the plasma membrane by means of the STIM-Orai1 system, where additional ion channels allow a second, sustained wave of Ca^{2+} influx[8]. Interestingly, LPS induces a single and transient influx of extracellular Ca^{2+}, with no contribution from intracellular Ca^{2+} stores, which is still sufficient to activate NFAT. This suggests that LPS-induced Ca^{2+} signaling in DCs does not rely on a classical SOCE mechanism, but that $I(3,4,5)P_3$ may trigger direct activation of functional plasma membrane IP3-Rs, as already observed in B cells[9].

The CD14-dependent NFAT activation induced by LPS plays a crucial function in regulating the life cycle of activated DCs, which undergo apoptosis shortly after stimulation. Stimulus-induced apoptosis of DCs represents a strategy to limit T cell activation in lymph nodes and to prevent an excessive immune response. We have shown that, upon DC stimulation with LPS, activated NFAT proteins (namely NFATc2 and NFATc3) promote the expression of several genes with a pro-apoptotic function that altogether induce cell death. Among these genes we identified Nur77 as an NFAT-dependent regulator of DC apoptosis. It has to be noted that, although NFAT activation is normally observed in TLR4-deficient DCs after LPS treatment, no appreciable gene expression occurs in these conditions, suggesting that cooperation with NF-κB and AP-1 is a pre-requisite for NFAT to exert its biological function. We also found that macrophages, which do not undergo LPS-induced apoptosis, do not show a Ca^{2+} response or activation of NFAT upon LPS stimulation. However,

pharmacological activation of NFAT is sufficient to induce the cell death of macrophages upon LPS treatment, further supporting a role for NFAT as a master regulator of the cell life cycle. Although we still do not know the point where the CD14-dependent pathway is blocked in macrophages, this differential signaling ability between DCs and macrophages provides a molecular explanation for their peculiar life cycle, which is in turn responsible for their diverse biological functions.

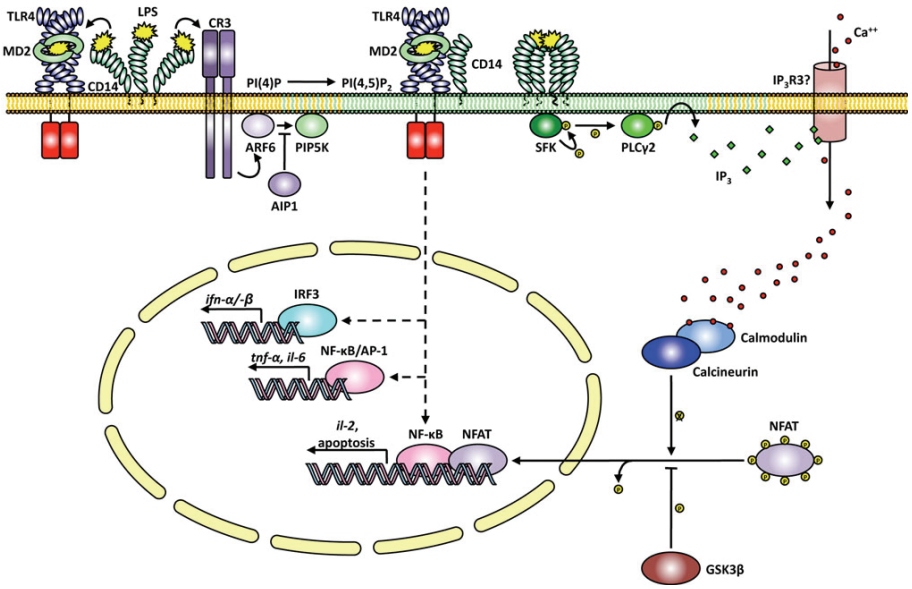

Fig. 1. CD14-dependent and TLR4-independent NFAT activation in dendritic cells. In addition to its role in LPS recognition and presentation to TLR4 and CR3 CD14 has autonomous signaling functions DCs. Upon LPS-induced clusterization, CD14 transiently recruits and activates a Src family kinase (SKF) member through an ill-defined mechanism that relies on CD14 GPI anchor and on its residency in lipid rafts. Active SFK then phosphorylates PLCγ2, which in turn catalyzes the hydrolisis of $PI(4,5)P_2$ into the second

messengers DAG and IP_3. Whereas the biological role of DAG in this system has not been investigated, it is likely to contribute to NF-κB activation through PKCs (not shown). On the other side, IP_3 directly triggers Ca^{2+} influx by acting on plasma membrane Ca^{2+} channel receptor (IP3R3?). The increased $[Ca^{2+}]_i$ stimulates activation of calcineurin, which dephosphorylates NFAT and promotes its nuclear translocation. Active NFAT cooperates with NF-κB to drive the expression of the genes coding for IL-2, several proapoptotic proteins as well as mPGES-1 (not shown). It has to be noted that, although LPS-induced activation of NFAT in DCs is TLR4-independent, no change in gene expression is observed in the absence of TLR4, which is therefore required for full transcriptional activity of NFAT through activation of NF-κB.

The CD14-NFAT pathway regulates pro-inflammatory functions of DCs

The identification and characterization of the CD14-NFAT pathway led us to discovery another function with important pharmacological applications, *i.e.* the production of PGE_2 by DCs through the regulation of mPGES-1 expression. PGE_2 is a crucial mediator of inflammation that regulates biological processes as diverse as tissue swelling, inflammatory pain and fever. Therefore, the biosynthesis of this molecule (mainly through inhibition of COX-2) is targeted by non-steroidal anti-inflammatory drugs (NSAIDs). Recent clinical studies have reported that widely used COX-2 inhibitors have serious secondary effects due to the systemic blockade of this enzyme, which plays important roles also in homeostatic conditions.

Microsomal PGE synthase-1 (mPGES-1) has emerged as a more selective and safer target for inhibition of PGE_2 synthesis, but in order to minimize the toxicity of such treatment it is necessary to limit the action of the drug to relevant PGE_2-producing cell types. Our data, based on a model of inflammation induced by LPS in vivo, clearly point to a major role of DCs in the production of PGE_2 and, therefore in the regulation of tissue edema formation. In conditions in which DCs were either absent or genetically impeded in their CD14-NFAT pathway, no significant LPS-induced inflammation was observed. Importantly, local administration of NFAT inhibitors reduced mPGES-1 mRNA induction by LPS, revealing a novel possible usage of such molecules as anti-inflammatory drugs in specific pathological settings. However, since NFAT inhibitors are immunosuppressive and not devoid of severe secondary effects, we propose that their usage should be limited to local and time-limited treatments. It should be noted that the full therapeutical importance of our discoveries will be fully understandable only when strategies to target NFAT inhibitors specifically to DCs will be devised.

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LETTERS

CD14 regulates the dendritic cell life cycle after LPS exposure through NFAT activation

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Toll-like receptors (TLRs) are the best characterized pattern recognition receptors[1]. Individual TLRs recruit diverse combinations of adaptor proteins, triggering signal transduction pathways and leading to the activation of various transcription factors, including nuclear factor κB, activation protein 1 and interferon regulatory factors[2]. Interleukin-2 is one of the molecules produced by mouse dendritic cells after stimulation by different pattern recognition receptor agonists[3–6]. By analogy with the events after T-cell receptor engagement leading to interleukin-2 production, it is therefore plausible that the stimulation of TLRs on dendritic cells may lead to activation of the Ca^{2+}/calcineurin and NFAT (nuclear factor of activated T cells) pathway. Here we show that mouse dendritic cell stimulation with lipopolysaccharide (LPS) induces Src-family kinase and phospholipase Cγ2 activation, influx of extracellular Ca^{2+} and calcineurin-dependent nuclear NFAT translocation. The initiation of this pathway is independent of TLR4 engagement, and dependent exclusively on CD14. We also show that LPS-induced NFAT activation via CD14 is necessary to cause the apoptotic death of terminally differentiated dendritic cells, an event that is essential for maintaining self-tolerance and preventing autoimmunity[7,8]. Consequently, blocking this pathway *in vivo* causes prolonged dendritic cell survival and an increase in T-cell priming capability. Our findings reveal novel aspects of molecular signalling triggered by LPS in dendritic cells, and identify a new role for CD14: the regulation of the dendritic cell life cycle through NFAT activation. Given the involvement of CD14 in disease, including sepsis and chronic heart failure[9,10], the discovery of signal transduction pathways activated exclusively via CD14 is an important step towards the development of potential treatments involving interference with CD14 functions.

Wild-type LPS, termed smooth LPS (the form of LPS used here), comprises three covalently linked moieties: lipid A, rough core oligosaccharide and an O-antigenic side chain determining serotype specificity. The TLR4–MD2 complex and the CD14 co-receptor are necessary and sufficient for a full response to smooth LPS[11]. CD14 is expressed by both $CD8\alpha^+$ and $CD8\alpha^-$ mouse dendritic cells, although at lower levels than expressed by macrophages (Supplementary Fig. 1). We tested whether LPS-induced interleukin-2 (IL-2) production by dendritic cells was dependent on NFAT. Ca^{2+} mobilization is the first event in NFAT activation. We observed that LPS was able to induce Ca^{2+} flux in bone-marrow-derived dendritic cells (BMDCs) and in a homogeneous dendritic cell line, D1 cells[12] (Fig. 1a, Supplementary Fig. 2 and Supplementary Movies). Next, we investigated the features of Ca^{2+} mobilization by LPS in BMDCs in detail, by recording Ca^{2+} transients in individual cells. The intracellular calcium concentration ($[Ca^{2+}]_i$) was increased by LPS and ATP ($P < 0.05$ versus untreated cells, Fig. 1b). Experiments in Ca^{2+}-free medium demonstrated that the increase in $[Ca^{2+}]_i$ induced by LPS was due to the influx of extracellular Ca^{2+} (Fig. 1c, responding cells in Ca^{2+}-free medium less than 1%). To determine the molecular component of the LPS receptor responsible for Ca^{2+} flux directly, we measured Ca^{2+} influx in TLR4- and CD14-deficient BMDCs. Ca^{2+} transients were fully preserved in TLR4-deficient BMDCs ($43 \pm 6\%$ of cells responding, Fig. 1d), whereas they were completely abolished in CD14-deficient BMDCs ($\leq 1\%$ of cells responding, Fig. 1d). To confirm that TLR4 is not involved in this process, Ca^{2+} mobilization was tested in each of the following mutant cell types: MyD88-, MD2 (also called LY96)- and TRIF (also called TICAM1)-deficient, and MyD88/TRIF- and TLR4/TLR2-double-deficient BMDCs. In all of these mutant cells Ca^{2+} mobilization was fully preserved (Supplementary Fig. 3), confirming that CD14 was the receptor responsible for Ca^{2+} influxes after LPS activation. Also, there was no Ca^{2+} mobilization in BMDCs in response to taxol (responding cells $\leq 1\%$), a selective TLR4 agonist[13] (Fig. 1d); this is further confirmation of the involvement of CD14. We next conducted various tests to ensure that the results obtained concerning Ca^{2+} transients could not have been due to the presence of contaminants in our LPS preparations (see Supplementary Fig. 4).

CD14 is a glycosylphosphatidylinositol-anchored receptor (GPI-AR). Cross-linking of GPI-ARs, such as CD59 (ref. 14), triggers the activation of the intracellular inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) Ca^{2+} pathway through the association with lipid rafts and Src kinase activation[15]. Therefore, we investigated whether CD14 membrane localization, presence of lipid rafts and Src kinase activation were conditions required to obtain Ca^{2+} mobilization. As shown in Fig. 1e, soluble CD14 did not restore extracellular Ca^{2+} influx in CD14-deficient dendritic cells, and disruption of lipid rafts by cholesterol depletion totally impaired Ca^{2+} mobilization. Thus, consistent with previous results[16], CD14 localization in lipid rafts[17] seems to be required for LPS-induced Ca^{2+} mobilization. Moreover, Src kinase inhibition completely abolished Ca^{2+} mobilization (Fig. 1e), indicating direct involvement of Src kinases in this process.

The induction of Ca^{2+} transients by LPS was also fully dependent on phospholipase C (PLC)-γ2 (responding PLC-γ2-deficient BMDCs $\leq 1\%$; Fig. 1e). Consistent with these results, PLC-γ2 activation was observed in wild-type but not in CD14-deficient BMDCs on LPS exposure (Supplementary Fig. 5).

We then analysed NFAT activation after LPS treatment in wild-type and CD14-deficient BMDCs. As shown in Fig. 2a, NFATc2

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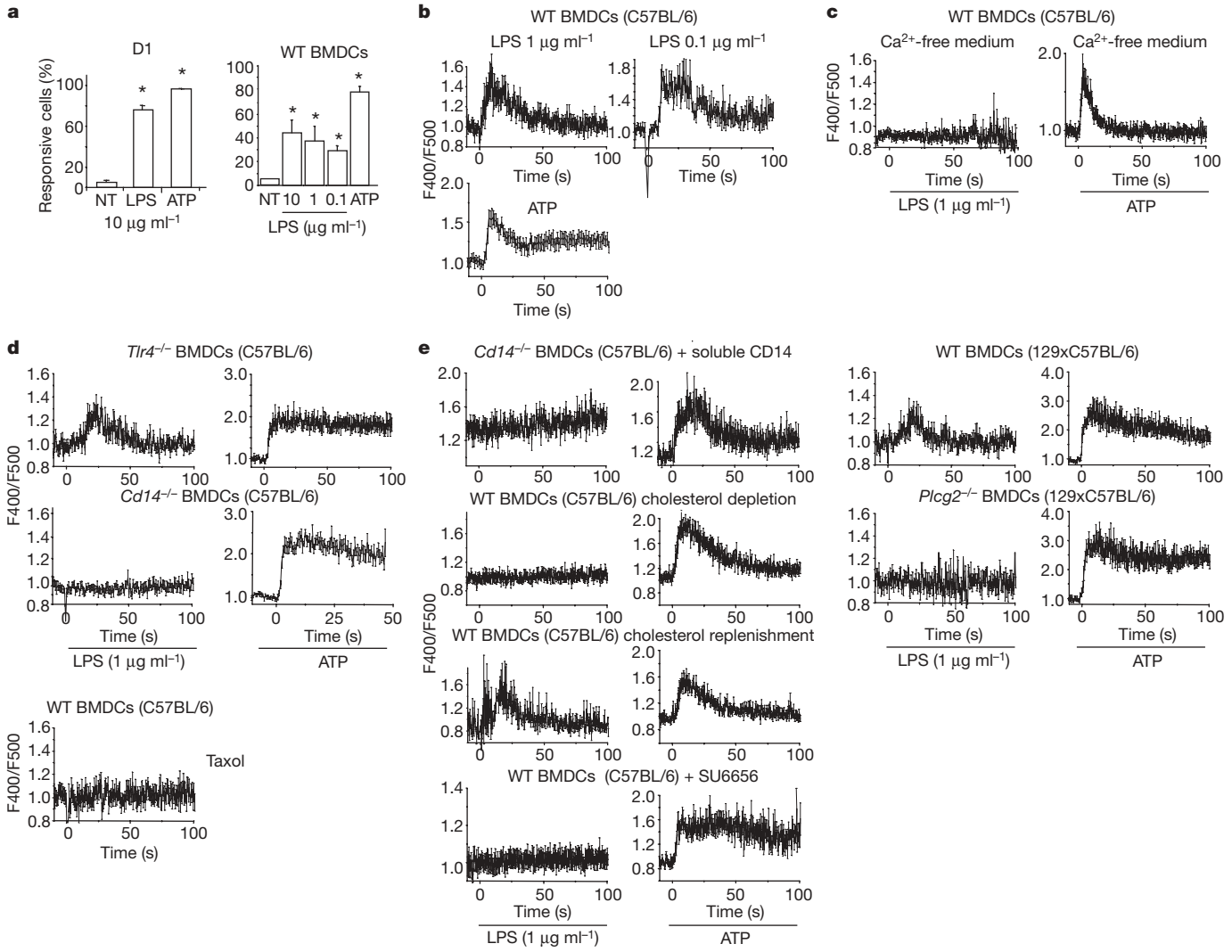

Figure 1 | CD14-dependent Ca^{2+} mobilization in dendritic cells following LPS treatment. **a**, Percentage of D1 cells and BMDCs showing Ca^{2+} mobilization in the absence (NT) and presence of LPS or ATP. Asterisk, $P < 0.05$ versus NT. **b–e**, Ca^{2+} transients in wild-type and mutant BMDCs and wild-type BMDCs in the presence of the Src kinase inhibitor (SU6656, 260 nM) or soluble CD14 (2 μg ml^{-1}). In **e** Ca^{2+} transients were recorded after cholesterol depletion and replenishment. Point 0 indicates the time of stimulus administration. Means and s.d. for a minimum of 30 cells are shown. Experiments were repeated at least three times. The ratio of fluorescence emissions at 400 nm/40 nm band-pass to those at 500 nm/20 nm band-pass was recorded (F400/F500) and used as an index of $[Ca^{2+}]_i$.

nuclear translocation occurred in wild-type but not in CD14-deficient BMDCs. In agreement with the Ca^{2+} mobilization studies, NFAT was translocated to the nucleus in wild-type, TLR4-, MD2- and MyD88/TRIF-deficient BMDCs but not in CD14- and PLC-γ2-deficient BMDCs or in wild-type BMDCs stimulated with LPS in the presence of the Src kinase inhibitor (Supplementary Fig. 6). These observations demonstrate that CD14 is directly responsible for activating, in dendritic cells, a TLR4-independent, Src-kinase- and PLC-γ2-dependent transduction pathway leading to NFAT activation. This is in addition to CD14 enhancing cellular responses to low doses of LPS[18,19] and being required for the LPS-induced recruitment of TRAM and TRIF[20].

We observed that, diversely from macrophages[21] (Fig. 2b), the production of tumour-necrosis factor (TNF)-α and IL-6 by dendritic cells after LPS stimulation was TRIF-independent (Fig. 2b) and, consequently, it was also CD14-independent[11,20], at least at high LPS doses (Fig. 2b). It is therefore possible that the selective engagement of TLR4 without CD14 activation allows the production of NF-κB-dependent and NFAT-independent cytokines, such as TNF-α[22] and IL-6[23], whereas it selectively prevents the production of cytokines, such as IL-2, that are transcriptionally dependent on NFAT, in addition to NF-κB and activation protein 1 (AP1).

We analysed the behaviour of CD14-deficient BMDCs after LPS stimulation and of wild-type BMDCs after activation with the TLR4-selective stimulus taxol to confirm the existence of a CD14-specific pathway. IL-2 production was impaired after the stimulation of CD14-deficient BMDCs with various concentrations of LPS, including very high concentrations at which the production of TNF-α and IL-6 was largely preserved (Fig. 2c). Similarly, the stimulation of wild-type BMDCs with taxol induced the production of TNF-α and IL-6, but not IL-2 (Fig. 2c). Nevertheless, coupling TLR4 stimulation with Ca^{2+} mobilization, using thapsigargin, restored IL-2 production (Fig. 2d and Supplementary Fig. 8). Similar results were obtained *in vivo* (Supplementary Fig. 8).

We used various inhibitors blocking the NFAT pathway to confirm CD14-dependent activation of the Ca^{2+}/calcineurin pathway induced by LPS. In particular, inhibition of the influx of external Ca^{2+} by the Ca^{2+} chelator EGTA selectively impaired IL-2 production but did not downregulate TNF-α or IL-6 synthesis (Supplementary Fig. 9a). This was also observed in the presence of thapsigargin (Supplementary Fig. 9b). Moreover, inhibition of Src kinases, PLC-γ2 and calcineurin downregulated IL-2 production, although TNF-α and IL-6 production was unaffected (Supplementary Fig. 9a, c). We also used the VIVIT peptide[24] to

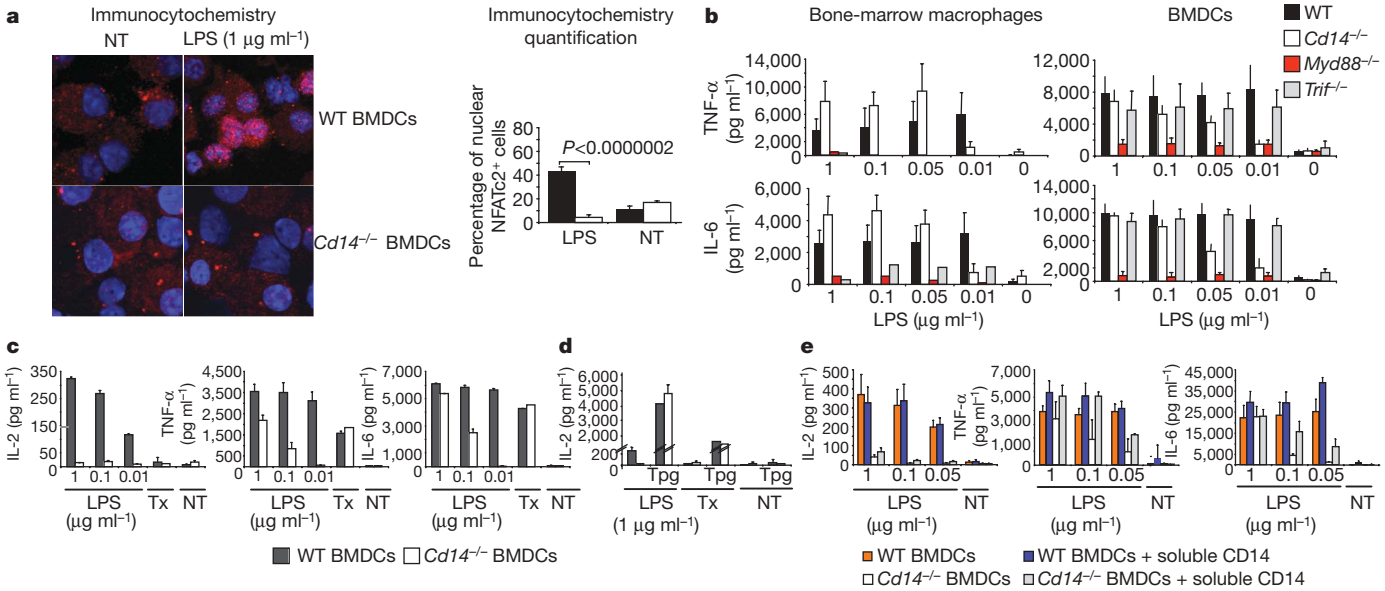

Figure 2 | CD14-dependent activation of NFAT in BMDCs after LPS treatment. **a**, NFATc2 translocation before (NT) and after LPS challenge for 2 h. Blue, DAPI; red, anti-NFATc2; original magnification, ×630. Percentages represent means and standard error of five fields (~50 cells each) in two experiments. **b**, LPS-induced TNF-α and IL-6 production by bone-marrow macrophages and BMDCs. **c**, LPS- or taxol (Tx, 100 μM)-induced cytokine production by BMDCs. **d**, LPS- or taxol-induced IL-2 production by BMDCs with and without thapsigargin (Tpg, 50 nM). **e**, LPS-induced cytokine production by BMDCs with and without soluble CD14 (2 μg ml^{-1}). **b**, **c**, **d**, **e** are representative of three experiments. Error bars in **b**–**e** represent standard errors.

inhibit NFAT proteins and obtained similar results (Supplementary Fig. 10).

In agreement with the Ca^{2+} mobilization data, addition of soluble CD14 to CD14-deficient BMDCs restored TNF-α production, as already observed in macrophages[20], but not IL-2 production at either low or high LPS concentrations (Fig. 2e). This observation supports the notion that a membrane localization of CD14 is required to activate the Ca^{2+} pathway.

As NFAT proteins are major regulators of many biological processes, we investigated the physiological role of CD14-mediated NFAT activation in dendritic cells, in addition to IL-2 production. We performed a kinetic microarray analysis to identify genes modulated specifically by NFAT in LPS-treated dendritic cells (see Supplementary Fig. 11 for details). From this analysis we hypothesized that LPS-activated dendritic cells in conditions allowing NFAT activation were exhausted cells with little or falling gene transcription and protein translation; in contrast, dendritic cells activated with LPS in conditions inhibiting NFAT activation were transcriptionally and translationally active, presumably not exhausted and not undergoing apoptosis (see Supplementary Table 1 for details).

To test whether CD14-mediated activation of the NFAT pathway was required to induce apoptotic death of terminally differentiated

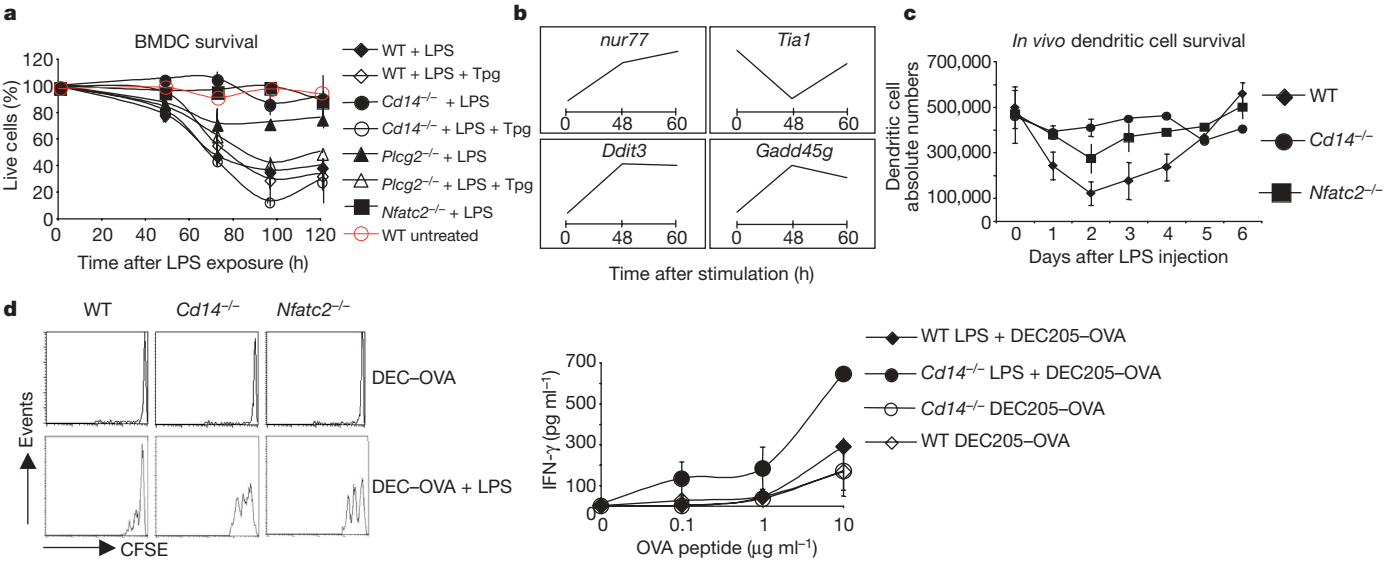

Figure 3 | Ca^{2+}-NFAT-mediated regulation of dendritic cell death. **a**, BMDC survival after incubation with LPS (1 μg ml^{-1}, $P < 0.00001$, wild-type versus mutant BMDCs) or LPS plus thapsigargin (50 nM). Survival of unstimulated cells kept in culture with granulocyte–macrophage colony-stimulating factor (GM-CSF) is also shown. **b**, Apoptosis genes regulated by NFAT after LPS stimulation. **c**, Absolute numbers of CD11c^{+} cells in spleens after intravenous LPS (1 μg g^{-1}) injection ($P \leq 0.0006$, wild-type versus mutant mice). Data are representative of two independent experiments (four mice per group). **d**, Left panel: antigen-specific T-cell proliferation *in vivo*. Right panel: IFN-γ production after *in vitro* re-stimulation. The experiment was repeated twice with similar results. Error bars in **a**, **c**, **d** represent standard errors.

cells, wild-type BMDCs and CD14-, NFATc2- and PLC-γ2-deficient BMDCs were activated with LPS and the percentage of living cells measured at various times thereafter. The c2 isoform of NFAT was chosen because it has a pro-apoptotic function in T and B cells[25]. Less than 30% of wild-type BMDCs survived after activation, whereas 70–100% of CD14-, NFATc2- and PLC-γ2-deficient BMDCs were still alive five days after exposure to LPS (Fig. 3a). CD14- and PLC-γ2-deficient BMDC death after LPS activation was restored by thapsigargin (Fig. 3a); thapsigargin alone had no effect (data not shown). Similar results were obtained using different LPS doses and LPS from different sources (Supplementary Fig. 12). A new microarray experiment performed at later time points (48 and 60 h) allowed us to identify four pro-apoptotic genes specifically modulated by NFAT: *nur77*, *Gadd45g*, *Ddit3* (also called *gadd153* and *CHOP-10*) and *Tia1* (Fig. 3b, see Supplementary Table 2 for details).

In vivo, splenic dendritic cell numbers declined quickly in wild-type C57BL/6 mice upon LPS administration, whereas absolute dendritic cell numbers remained almost constant in CD14-deficient and NFATc2-deficient mice after the same treatment (Fig. 3c). Although we cannot exclude an indirect effect *in vivo*, these findings provide further evidence that NFAT pathway activation via CD14 is required for dendritic cell apoptosis. The dendritic cell decline observed in NFATc2-deficient mice may have been the consequence of the effect of other NFAT isoforms with overlapping functions.

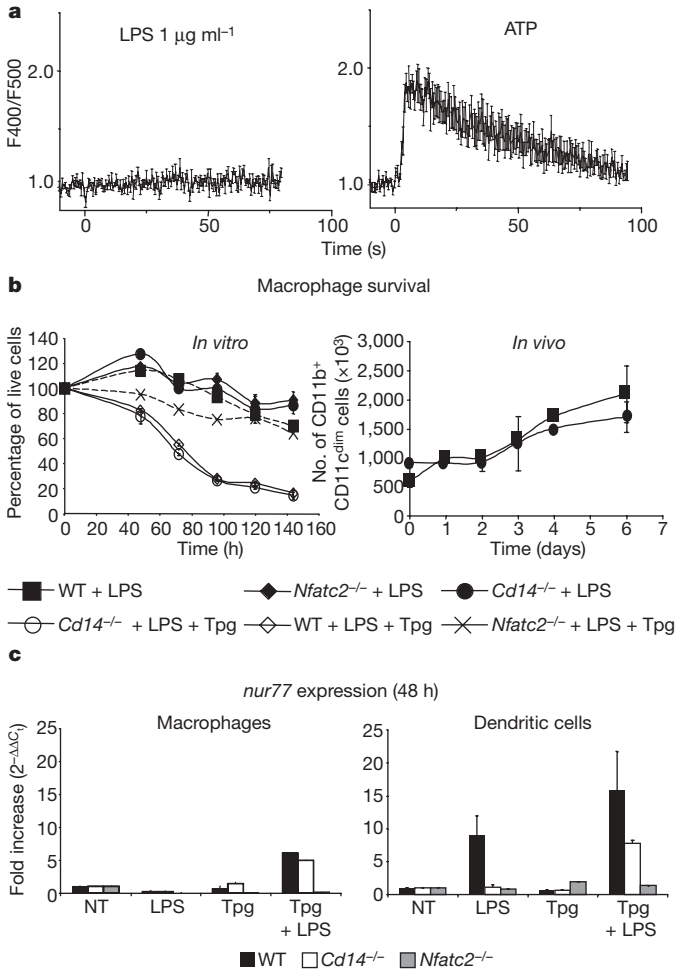

Figure 4 | Ca^{2+} mobilization and survival of macrophages after LPS treatment. **a**, Ca^{2+} transients in bone marrow macrophages. Point 0 indicates time of stimulus administration. Means and s.d. of 30 cells minimum are shown. Experiments were repeated at least three times. **b**, Left panel: survival of wild-type and mutant bone-marrow macrophages after incubation with LPS (1 μg ml^{-1}) or LPS plus thapsigargin (50 nM). Right panel: absolute numbers of $CD11b^{+}CD11c^{dim}$ cells in spleens of wild-type and CD14-deficient mice after intravenous LPS injection (30 μg). Data represent two independent experiments (four mice per group). **c**, Fold increase of *nur77* expression in stimulated versus unstimulated wild-type and mutant cells. Error bars in **b** and **c** represent standard errors.

METHODS SUMMARY

Calcium measurements. $[Ca^{2+}]_i$ was determined by a fluorometric ratio technique. Cells were loaded with 2 μM indo-1 (Molecular Probes) by incubation at 37 °C for 20 min. Cells were then washed three times with PBS to allow for intracellular de-esterification of indo-1. A direct optical microscope (Olympus, BX51) with a two-photon Ti-Sapphire laser source (720-nm wavelength; Mai Tai, SpectraPhysics) was used for indo-1 excitation. The fluorescence signals emitted by indo-1-loaded cells were digitized at 200 Hz and recorded every 0.5–0.8 s. The ratio of fluorescence emissions at 400 nm/40 nm band-pass to those at 500 nm/20 nm band-pass was recorded (R400/500) and used as an index of $[Ca^{2+}]_i$. Data were normalized to baseline. This approach overcame

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

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

Images of Ca^{2+} responses were measured by a laser-scanning confocal system using the membrane-permeable dye Fluo4-AM.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 17 March; accepted 28 April 2009.
Published online 14 June 2009.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank S. Akira, C. Kirschning and K. Miyake for mutant mice; M. Colonna for mutant mice and advice on PLC-γ2 activation; E. Serfling for mutant mice and advice on NFAT activation; A. Rao for the VIVIT peptide. We also thank Genopolis–Consorzio di Genomica Funzionale for microarray hybridization and data analysis and K. Mahnke for the anti-DEC205–OVA conjugate. This work was supported by grants from the CARIPLO Foundation, the European Commission 6th Framework Program (MUGEN and DC-THERA contracts), the European Commission 7th Framework Program (TOLERAGE and ENCITE contracts), the Associazione Italiana per la Ricerca sul Cancro (AIRC) and the and the Italian Ministry of Education and Research (COFIN).

Author Contributions F.G. conceived and oversaw the project and wrote the paper; I.Z. conceived the research and conducted most of the experiments with R.O.; M.C., M.C., M.R., G.C., B.C. and A.Z. helped with calcium experiments; G.C. and F.M. helped with experiments on D1 cells; M.F. helped with quantitative real-time PCR; A.E.R. performed the EMSA experiments; P.R.-C. provided advice.

Author Information All microarray data are available from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession code GSE15759. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to F.G. (francesca.granucci@unimib.it).

METHODS

Dendritic cells and macrophages. D1 cells and fresh bone marrow cells from C57BL/6 or mutant mice were cultured as previously described[29].

Mice and cells. C57BL/6 mice were purchased from Harlan. *Cd14*$^{-/-}$ mice were purchased from CNRS. OT-II mice were purchased from Charles River. *Tlr4*$^{-/-}$ mice were provided by S. Akira. *Plcg2*$^{-/-}$ 129×C57BL/6 and wild-type 129×C57BL/6 mice were provided by M. Colonna. TLR2/TLR4-double-deficient mice were provided by C. Kirschning. NFATc2-deficient mice were provided by E. Serfling, and MD2-deficient mice were provided by K. Miyake. All animals were housed under pathogen-free conditions. All experiments were carried out in accordance with the relevant laws and institutional guidelines.

Antibodies and chemicals. All the antibodies used for FACS analysis were purchased from BD Biosciences. TLR4-grade LPSs (*Escherichia coli*, 055:B5 and *Salmonella Typhimurium* S-form) were purchased from Alexis Corporation; non-TLR4-grade LPS (*E. coli* O55:B5) was purchased from Sigma. Recombinant soluble CD14 was purchased from CellSciences and Src inhibitor SU6656 from Sigma. Tacrolimus/FK506 (Fujisawa Pharmaceutical) was used at a concentration of 10 ng ml^{-1}.

Partial depletion and replenishment of cholesterol in the plasma membrane was performed according to ref. 14.

Indo1-AM and Fluo4-AM (Molecular Probes) were dissolved in DMSO. Stock solutions were diluted in Tyrode solution (154 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES-NaOH, 5.5 mM D-glucose, adjusted to pH 7.35). The concentration of DMSO in the medium did not exceed 0.1%.

Western blot analysis. Wild-type and *Cd14*$^{-/-}$ BMDCs were stimulated for the indicated times with LPS (1 μg ml^{-1}) and lysed in the presence of Protein Inhibitor Cocktail (Sigma) and Phosphatase Inhibitor Cocktails (Sigma). Proteins from cell lysates were separated by standard SDS–PAGE and analysed by immunoblotting with antibodies specific for phosphorylated PLC-γ2 (from Cell Signaling) and for β-actin.

NFAT activation. Electrophoretic mobility shift assay (EMSA). ^{32}P-labelled DNA oligonucleotide probes were incubated for binding with 5 mg of nuclear extracts[30] for 20 min at room temperature in a buffer containing 5% glycerol, 50 mM NaCl, 20 mM Tris pH 7.9, 0.5 mM EDTA, 5 mM MgCl, 1 mM dithiothreitol, 100 ng ml^{-1} poly(dI-dC), and 50 ng ml^{-1} bovine serum albumin (BSA) in a final volume of 15 ml. The protein–DNA complexes were then separated on a 5% polyacrylamide gel (29:1 acrylamide/bisacrylamide ratio) and were visualized by autoradiography. The sequences of the oligonucleotide probes (sense strand) used in the gel shift assay were as follows: NFATc consensus site, 5′-GCCCAAAGAGGAAAATTTGTTTCATACAG-3′; NFATc mutant site, 5′-GCCCAAAGcttAAAATTTGTTTCATACAG-3′; Oct consensus site, 5′-TCGTCAAACTCTGCTAATTAGCAATGCTGAGAAA-3′.

Immunocytochemistry. Cells were fixed in formaldehyde and spun onto glass slides (2×10^5 cells per slide in 200 μl) at 300 r.p.m. for 5 min. Cells were then permeabilized in chilled methanol and washed extensively with PBS. Fc block antibody (CD16/CD32, BD Pharmingen), rabbit anti-mouse NFATc2 (1:250, ImmunoGlobe), rabbit anti-mouse NFATc3 (1:250, Santacruz) and donkey anti-rabbit Alexa Fluor 555 antibodies (1:500, Molecular Probes) diluted in PBS plus 0.1% BSA were added and incubated at room temperature. DAPI (1:1,000, Sigma Aldrich) was added and the samples incubated for 5 min at room temperature.

IL-2, TNF-α and IL-6 measurements. ELISAs were performed with the DuoSet kits (R & D).

Plasmids and retroviral infection of dendritic cells. VIVIT–GFP DNA was inserted into the PINCO retroviral vector[31], using the HindIII and NotI restriction sites. Standard molecular biology methods were used for cDNA cloning. All the reagents used were from Invitrogen.

The production of high-titre vectors and the D1 infection protocol have been described elsewhere[31]. Transduction efficiency was evaluated by FACS analysis and was generally around 30%. GFP$^+$ cells were sorted and cultured as previously described[31].

***In vivo* production of IL-2 and TNF-α by spleen cells.** Aliquots of 10 μg of LPS or 10 μg of LPS and 5 mM Tpg (in a final volume of 50 μl) were injected into mouse spleens. After 3 h, spleens were removed and single-cell suspensions prepared. The cells suspensions were incubated with brefeldin A (10 μg ml^{-1}, Sigma-Aldrich) for 5 h. The cells were fixed using BD Phosflow Lyse/Fix buffer (BD Biosciences), permeabilized with BD Phosflow Perm Buffer III (BD Biosciences) and stained with PE-labelled anti-CD11c, APC-labelled anti-IL-2 and anti-TNF-α monoclonal antibodies or APC-labelled isotype control antibody. Cells were then analysed on a FACScalibur (Becton Dickinson).

Microarray experiment. Total RNA was extracted by the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol Invitrogen) followed by a Qiagen RNeasy clean-up procedure. Total RNA integrity was assessed with a Agilent Bioanalyser and the RNA Integrity Number (RIN) was calculated. Only high-quality RNA preparations, with RIN greater than 8.5, were used for microarray analysis.

Three micrograms of total RNA was used for cRNA target preparation according to the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix) using the one cycle target labelling kit and according to the manufacturer's instructions. Ten micrograms of biotinylated cRNA was hybridized to the Affymetryx GeneChip Mouse Genome 430A 2.0 arrays.

Bioconductor[32] was used for most data handling. The Guanine Cytosine Robust Multi-array Analysis GCRMA[33] method was used to calculate probe set intensity. The normalization method applied was the quantile.

***In vitro* survival assay.** Cells were seeded in 96-well plates at a concentration of 0.5×10^6 cells ml^{-1} in 100 μl of complete IMDM plus GM-CSF (for BMDCs) or M-CSF (for bone-marrow macrophages) in the presence or absence of LPS (1 μg ml^{-1}) and/or thapsigargin (50 nM). After 24 h, 100 μl of complete IMDM was added to the culture. Survival of cells was measured using the CellTiter-Blue Cell Viability Assay (Promega).

***In vivo* survival assay.** Four-to-six-week-old mice, showing an average of 90 million total spleen cells, were injected intravenously with 1 μg g^{-1} of LPS. At various times thereafter, spleens were collected, single-cell suspensions produced and total spleen cells counted. Absolute numbers of dendritic cells were determined by FACS analysis using CD11c–APC-conjugated, CD8α–PE-conjugated, and CD11b–FITC-conjugated antibodies.

T-cell activation. Mice were injected intravenously with LPS (1 μg g^{-1}) and anti-DEC205–OVA complex (10 μg) and 8 h later received CFSE-labelled CD4$^+$ T cells (4×10^6) from OT-II mice. T-cell proliferation was measured in the spleen after 48 h. One week after immunization total CD4$^+$ T cells were recovered and re-stimulated *in vitro* with OVA peptide in the presence of splenic APCs. IFN-γ production was measured by ELISA after 48 h of culture.

Real-time quantitative PCR. Total RNA was extracted from 10^6 cells using the TRIZOL reagent according to the recommended procedure (Gibco-BRL). Single-strand cDNA was synthesized using High-capacity cDNA Reverse Transcription Kits (Perkin-Elmer, Applied Biosystem Division). The NanoDrop (TermoScientific) was used to titre mRNA and amplification was performed using the Power Sybr Green PCR Master Mix (Perkin Elmer).

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

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…e così siamo davvero arrivati alla fine ed è tempo di ringraziare i miei compagni d'avventura...

Un grazie a Francesca, per tutte le possibilità offerte e per avermi permesso di crescere.

Grazie agli amici del lab per le infinite giornate, belle e meno belle, che abbiamo passato insieme e che porterò nel cuore. Ognuno di voi mi ha lasciato qualcosa e vorrei che a ciascuno restasse un pezzetto di me. Cibi e Aparnuzza, se guardando fuori dalla finestra un cielo grigio e senza sole vi capiterà di sentirvi un po' tristi, pensate al vostro carebear e subito comparirà un arcobaleno… Achille, resterai il mio primo nome in rubrica così ti farò un sacco di chiamate sbagliate. Mi raccomando i biglietti di Vasco! Tati come farò a non ingozzarmi a pranzo senza di te? Roby, passerò a trovarvi ma voglio i tuoi biscotti. Irene, in bocca al lupo e spacca tutto!!! Grazie a Matteo e alla Civi per tutte le perle di saggezza.

Simo, Pinco Panco ti ha lasciato la sua eredità e sai che non è cosa da poco… Franka, ti voglio tanto bene e ti prometto che almeno una volta mangeremo insieme! Oh ragazze… avete pianto per tutti e per tutto, se non lo fate anche per me mi incazzo.

Grazie di cuore, Ivan. Questi anni non li scorderò perché li ho condivisi con un amico vero…