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TOLL-LIKE RECEPTOR ACTIVATION INDUCED CHANGES IN DENDRITIC CELLS

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ABSTRACT

Dendritic cells (DC) are professional antigen-presenting cells that act as a “bridge” between innate and adaptive immunity by the induction and subsequent orchestration of immune responses. The ligation of Toll-like receptors (TLR) and other innate receptors on DC determines their immune-stimulating capacity. In the studies included in this thesis, TLR activation of DC and the different aspects of such activation were investigated. In paper I, we explored how low physiological temperatures, commonly found in the skin where DC reside, affect DC activation and function. We found that several cellular functions, including macropinocytosis, phagocytosis, podosome formation, migration, and antigen processing, were similar for unstimulated DC at 28°C and 37°C. However, when DC were stimulated with the TLR agonist LPS at 28°C the kinetics of macropinocytosis and TNF production were delayed. These altered responses are most likely explained by the observed delay in the kinetics of TLR signalling, e.g., via the MAPK signalling pathway at 28°C. In addition, other functions of DC were more severely affected by the low temperature, including a reduction in NO production, CD40 receptor upregulation, and degradation of the extracellular matrix by podosomes. Also, the capacity of DC to activate T-cells was reduced after TLR activation at 28°C. These data provide new insights into an area of DC biology with potential relevance for vaccine development.

Cellular migration involves a series of events including the formation of podosomes, which are highly dynamic actin-filament scaffolds. In paper II, we examined the role of the actin-severing and capping protein gelsolin for podosome formation and function in DC. For this purpose, DC from mice deficient in gelsolin were used. In contrast to what was previously shown for osteoclasts, we found that DC form podosomes independently of gelsolin. Moreover, the formation and disassembly dynamics of podosomes are normal in DC deficient in gelsolin, as is their matrix-degrading function. Furthermore, we found that gelsolin is not required for TLR4-induced podosome disassembly. The actin cytoskeleton of podosomes involved in DC extracellular matrix degradation thus appears to be regulated in a different manner to the cytoskeleton in osteoclast podosomes that mediate bone resorption.

In order to ingest particulate material via phagocytosis, for example apoptotic cells and microbes, DC depend on rearrangement of the actin cytoskeleton. It is known that upon pathogen recognition by TLR, DC undergo rapid actin cytoskeleton rearrangements. However, most studies on TLR stimulation and phagocytosis have focused on posttranscriptional effects, i.e., the upregulation of receptors involved in phagocytosis, rather than how the process of phagocytosis is affected directly after TLR activation. In paper III, we report that the stimulation of DC using soluble TLR ligands increased their capacity to phagocytose various substrates within minutes. These included polystyrene beads, sheep red blood cells, and apoptotic lymphoma B cells. We also found that signalling through both of the TLR4 adaptor molecules, MyD88 and TRIF, was necessary for optimal LPS-stimulated phagocytosis. Furthermore, we confirmed that stimulated phagocytic uptake proceeds independently of gene transcription, as actinomycin D, which blocks gene transcription, had no effect on the stimulated uptake. In summary, our data suggest that soluble TLR ligands induce enhanced phagocytic uptake, proximal to gene transcription. Thus, our study provides new information about the role of TLR engagement in modulating the phagocytic capacity of DC.

LIST OF PUBLICATIONS

This thesis is based on the following papers. The individual papers are referred to in the text by Roman numerals.

- I. **Hammarfjord O.**, and R. P. A. Wallin. Dendritic cell function at low physiological temperature.
Journal of Leukocyte Biology. 2010 vol. 88 (4) pp. 747-56.
- II. **Hammarfjord O.**, H. Falet , C. Gurniak, J. H. Hartwig, and R. P. A. Wallin.
Gelsolin independent podosome formation and function in dendritic cells.
PLoS ONE. 2011 vol. 6 (7) pp. e21615.
- III. **Hammarfjord O.**, P. Chen, and R. P. A. Wallin. Toll-like receptor signalling stimulates phagocytosis in dendritic cells.
Manuscript.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
DAMP	Danger associated molecular patterns
DC	Dendritic cell
DMA	Dimethyl amelioride
DTT	Dithiothreitol
GM-CSF	Granulocyte macrophage-colony stimulating factor
HEL	Hen egg lysozyme
HEV	High endothelial venules
IFN	Interferon
ICAM-1	Intercellular adhesions molecule-1
IRF3	Interferon-regulatory factor 3
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MyD88	Myeloid differentiation primary response gene (88)
NaN ₃	Sodium azide
NF- κ B	Nuclear factor-kappa B
NO	Nitric oxide
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PRR	Pathogen recognition receptor
PFA	Paraformaldehyde
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SRBC	Sheep red blood cell
TEM	Transmission electron microscopy
TIR	Toll/interleukin-1 receptor-like domain
TNF	Tumour necrosis factor
TLR	Toll-like receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein-inducing interferon
VCAM	Vascular cell adhesion molecule

GENERAL INTRODUCTION

The immune system is a remarkable defence mechanism found in all animals and it is constantly challenged by a myriad of surrounding potentially pathogenic microorganisms. Upon recognition of microorganisms the immune system can make rapid, highly specific, and often protective response against pathogens. Dendritic cells (DC) serve to orchestrate a repertoire of immune responses, linking innate and adaptive immunity [1-3]. The cells of the innate immune system, e.g., DC, neutrophils, and macrophages, detect conserved features of microbes using germ-line encoded pattern recognition receptors (PRR), such as the toll-like receptors (TLR), thus enabling these cells to respond rapidly but with limited specificity. In contrast, cells of the adaptive immune system, i.e., B and T cells, which generate receptors for antigen recognition *de novo*, respond later but with high specificity. In addition to recognize and respond to foreign molecules, the immune system also learns how to be tolerant to self. There is evidence supporting that DC are important in controlling inflammation-induced immunopathology through the generation of antigen-specific regulatory T cells. This includes the induction of IL-10 or TGF- β producing regulatory T cells that dampening immune effector mechanisms [4, 5]. Thus, DC partake in the induction of effective immunity against invading pathogens, as well as in preventing excessive inflammation and tissue pathology. Having a central role in orchestrating immune responses, DC are considered as potential targets for therapeutic interventions for a wide array of diseases [6]. Hence, discovering more about their biology is key. The aim of my thesis work was to increase the understanding of some aspects of DC biology, with a focus on innate activation and the subsequent effects of such activation. To better understand the concepts discussed in this thesis, and as an attempt to set the data presented herein in relation to previous studies, a brief overview of the field of study is presented below. For a more comprehensive description of the immune system I recommend *Janeway's immunobiology* [7].

DENDRITIC CELL BIOLOGY

The first observation of DC was made in the late 1880s by Paul Langerhans as he mistakenly identified these cells, residing in the epidermis, as cells of neurological origin and named them Langerhans cells [8, 9]. Dendritic cells as we recognize them today, however, were discovered in the early 1970s by Ralph Steinman, who showed that the DC are effective accessory cells involved in the priming of the adaptive immune response [10, 11]. It was not realized until later that the Langerhans cells present in skin are a distinct DC subpopulation [12, 13]. In many aspects, the Langerhans cells have become the archetypical DC. In fact, the DC life cycle that is most often described, also in this thesis, is often referred to as the “Langerhans cell paradigm” [14-16] (Figure 1). Dendritic cells in peripheral tissues, e.g., skin, express cellular receptors that allow the identification of microbes. In response to microbial stimuli DC undergo a process of cellular activation leading to cellular differentiation, a process termed maturation [17-19]. Upon maturation, DC transiently increase their capacity to internalize antigens, which is followed by increased migration and enhanced T cell stimulatory capacity [19, 20]. The maturing DC migrate from the periphery, via the lymphatics into the T cell-rich areas of lymph nodes, where they activate naïve T cells and direct the functional differentiation of antigen-specific effector T cells of various types (e.g. Th1, Th2, Th17 cells).

Since the first evidence of the capacity of DC to induce immunity, was found, numerous studies using a wide array of experimental approaches have supported the notion of a pivotal role of DC in the initiation of adaptive immunity. However, the unique capacity of DC to activate naïve T cells has been challenged by studies which demonstrated that also other cell types such as macrophages, osteoclast, mast cells, and basophils also have the capacity to induce T-cell responses under certain conditions [21-27]. Although other cell types might have such a capacity to stimulate T cells, the consensus is that DC are unique in their capacity to prime naïve T cells and initiate primary T cell-mediated immune responses [16].

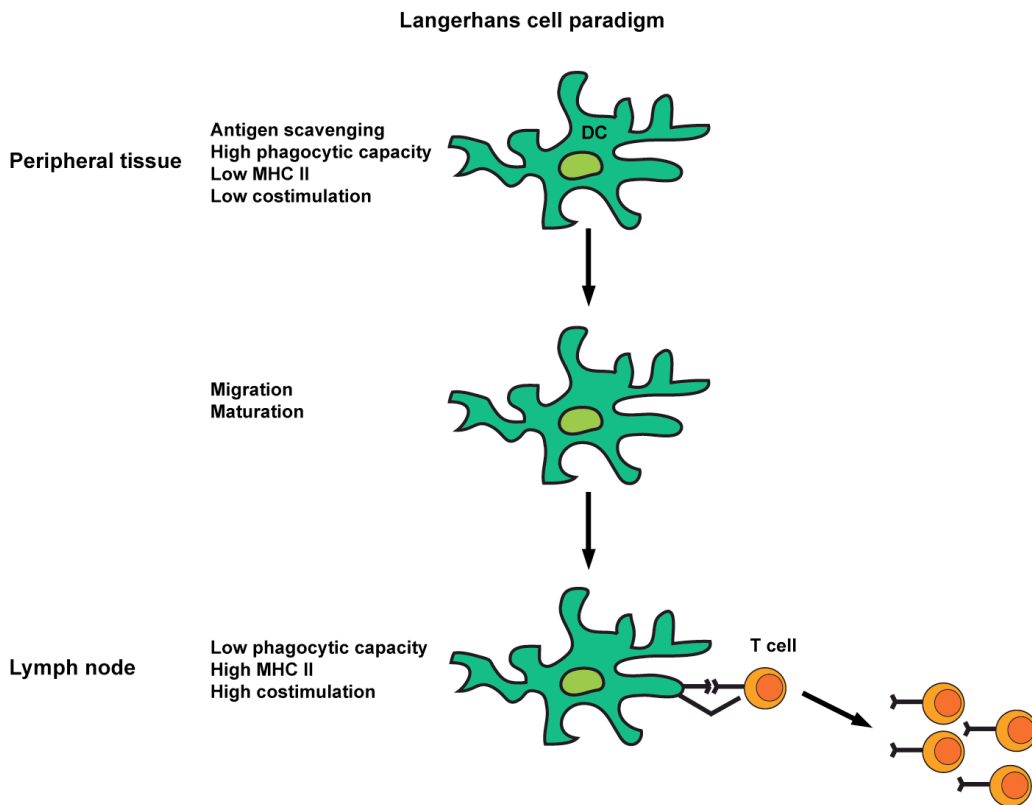


Figure 1. Schematic picture describing the “Langerhans cell paradigm”.

Dendritic cells originate from haematopoietic bone marrow progenitors and are widely distributed throughout the body. Facultative DC progenitors are not restricted to the bone marrow but can be found in numerous locations in the body, e.g., the thymus, most visceral organs, and in the circulation [28-30], as reviewed by Alvarez et al. [31]. In the early studies by Steinman and others during the late 1970s and during the 1980s, DC were isolated from many different tissues, such as blood and peripheral lymphoid organs, using different techniques. However, it was difficult to obtain large yields of DC and due to the experimental limitations of the time, it was hard to exclude the possibility that other cell types were responsible for the immune stimulatory capacity of DC. When an *in vitro* system that allows the generation of large numbers of DC was described, using the granulocyte-macrophage colony-stimulating factor (GM-CSF) with or without IL-4, the field of DC biology underwent a rapid expansion [32, 33]. This new technique was pivotal since it made it easy to generate a large number of immature primary DC that could be investigated in much greater detail.

Are DC superior to other APC in the activation of naïve T cells?

Early data suggested that DC were cells with a superior capacity to stimulate both CD4⁺ and CD8⁺ T-cell proliferation [34-38], compared to other antigen-presenting cells (APC) such as B cells and macrophages [11, 39, 40]. This notion was further solidified in the early 2000s, when Jung and colleagues studied the role of DC in initiating immunity in mice expressing the simian diphtheria toxin receptor (DTR) in DC under the control of the CD11c promoter (CD11c-DTR). This enables the “selective” depletion of DC when injecting the diphtheria toxin [41]. The authors used this technique to show that mice lacking CD11c⁺ cells were unable to mount CD8⁺ T-cell responses towards the intracellular bacterium *Listeria monocytogenes* or the malaria parasite *Plasmodium yoelii* [41]. In the years that followed, these data were further strengthened by several other studies. By using this technique, it was also shown that DC are important in induction of the immune response when infected with *Mycobacterium tuberculosis*, experimental cerebral malaria, and Herpes simplex virus [42-44]. It was also shown that DC are important for secondary immune responses towards *L. monocytogenes*, Vesicular stomatitis virus, and Influenza virus [45]. When considering data obtained using the CD11c-DTR experimental model it is important to mention that several cell types other than DC express low levels of CD11c, e.g., NK cells and some macrophages, which can also lead to the depletion of these cells. Therefore, caution is needed when interpreting these results. However, the wealth of data from both *in vivo* and *in vitro* studies, using isolation techniques, *in vitro* derived DC, and the CD11c-DTR-depletion system, collectively points to the fact that DC have key roles in immune regulation, both in the case of infection and during steady state homeostasis.

Dendritic cell expression of PRR

In peripheral tissues, such as the skin, Langerhans cells and dermal DC reside in an immature state, probing their surroundings using PRR, which recognize pathogen-associated molecular patterns (PAMP) and danger-associated molecular patterns (DAMP). Innate immune cells, such as the DC, use a limited numbers of germ-line encoded receptors specific for conserved molecular structures expressed on microbes to recognize invading pathogens, whereas the adaptive immune system uses randomly

generated, highly specific receptors that are clonally expressed to fight infection [46, 47]. The PRR consist of a growing number of receptor families, such as the membrane bound Toll-like receptors (TLR) and C-type lectin receptors (CLR), and the cytoplasmic NOD-like receptors (NLR) and the RIG-like receptors (RLR). Of note, there is emerging evidence to suggest that cross-talk between PPR families is key in shaping pathogen-specific responses [48]. Dendritic cells uses their TLR to sense their surroundings when they patrol the barrier surfaces of the body in search of invading microbes [49]. The TLR are the most frequently studied family of the PRR and they are also the focus of the studies herein.

Toll-like receptors and signalling

The TLR are a class of membrane spanning receptors that recognize conserved non-self molecules derived from microbes such as viruses and bacteria. In the mid-1990s, Jules Hoffmann and co-workers showed that the “toll” gene was involved in the immune response against fungi in fruit flies [50]. Subsequent studies by Medzhitov and Janeway in mice showed that TLR signalling induced the activation of genes involved in the adaptive immunity [51]. In a study the year after, in 1998, Bruce Beutler and colleagues showed for the first time that a mutation in the TLR4 gene, render mice (C3h/HeJ mice) unresponsive to the bacterial product lipopolysaccharid (LPS) [52]. This showed that mammalian cells have germ-line encoded receptors that recognize non-self microbially derived products. In 1989 Janeway had already hypothesized that because of the randomly generated antigen recognition receptors used by the adaptive immune system it could not reliably distinguish between self and non-self. Hence, Janeway hypothesized that the adaptive immune cells must be instructed of the origin of the antigen in order to elicit an appropriate immune response to clear infections and at the same time protect from autoimmunity [53]. He argued that this instruction might be provided by the innate immune system, which at that time was relatively understudied. Since then much data have supported his hypothesis [54]. To date, 13 different TLR receptors have been observed in mice and 10 in humans, many of which are expressed by DC [55]. The localization of TLR seems to differ depending of which molecules they recognize: TLR1, 2, 4, 5, and 6 are all present on the surfaces of the cells, recognizing molecules on the surfaces of the microbes, whereas TLR3, 7, 8, and 9 all reside intracellularly on membranes, recognizing nucleic acids (Figure 2). Emerging

evidence suggests that the localization and trafficking of TLR are key for the initiation of adequate responses to microbes, and is reviewed elsewhere [56].

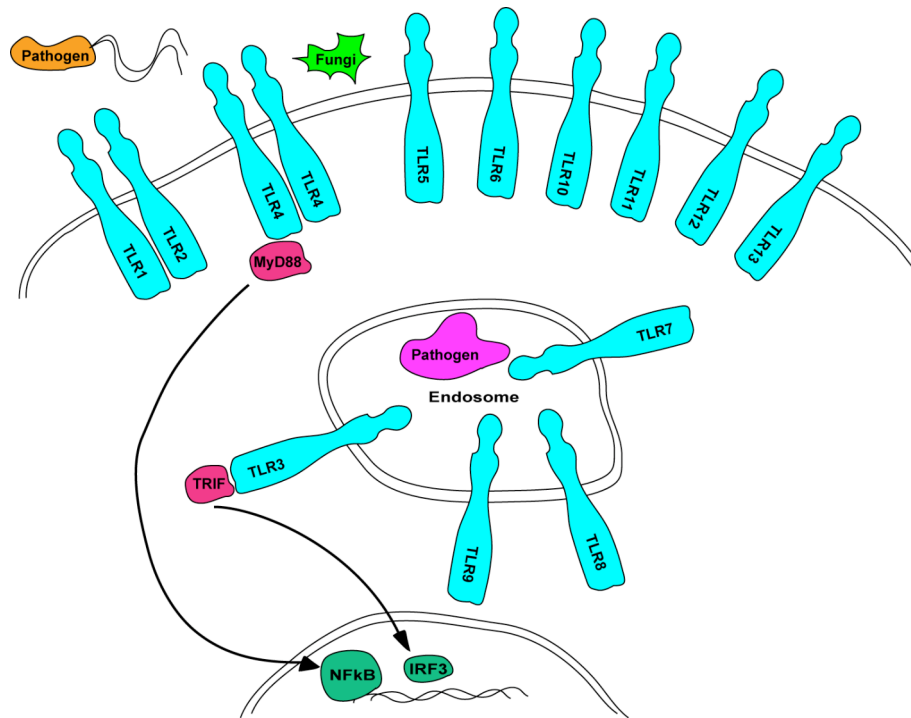


Figure 2. Schematic picture describing the localization of TLR.

At the molecular level, TLR activation can trigger two distinct, but to some degree overlapping, intracellular signalling pathways through the recruitment of Toll/interleukin-1 receptor-like domain (TIR)-containing adaptor molecules to the TIR domain of the TLR. Binding of the adaptor protein MyD88 leads to downstream signalling events that activate nuclear factor-kappa B (NF- κ B), leading to the transcription of genes encoding pro-inflammatory cytokines and chemokines. The TIR-domain-containing adaptor protein-inducing interferon (TRIF)-dependent pathway leads to the activation of genes involved in the production of IFN- β [57-59]. Toll-like receptor 4 is the only TLR that signals via both MyD88 and TRIF. Most other TLR only signal via MyD88, with one exception: TLR3, which utilizes TRIF signalling to activate NF- κ B and interferon-regulatory factor 3 (IRF3) [56, 60]. Interestingly, it has recently been shown that signalling through MyD88 and TRIF pathways downstream of TLR4 is spatially and temporally separated [57]. The activation of TLR4 first induces TIR domain-containing adaptor protein (TIRAP)/Mal-MyD88 signalling at the plasma membrane to initiate NF- κ B and it is then endocytosed to activate TRIF-related adaptor molecule- (TRAM)-TRIF signalling from early endosomes to initiate IRF3 [57].

Consequences of TLR ligation for DC function

Our understanding of how microbial and inflammatory stimuli affect and regulate the function of DC is continuously developing, as reviewed in [61-63]. At the site of infection DC start to produce inflammatory cytokines, e.g., TNF and IL-1 β , and participate in recruiting other immune cells to the site of infection by producing chemokines [64]. It is now clear that TLR signalling not only induces the production of cytokines, chemokines, and co-stimulatory molecules, but it also leads to the activation of a numerous of changes in the cytoskeleton and vacuolar system, and also protein degradation. The first studies to examine the effects of TLR stimulation in DC with LPS on DC functions such as antigen capture and presentation were performed in the late 1990s [65, 66]. It has since been shown that in DC, endocytic activity is transiently increased upon TLR stimulation [67, 68]. Furthermore, West et al. showed that the simultaneous exposure to antigen and LPS enhanced antigen uptake and the subsequent processing of the antigen acquired for both MHC class I and class II peptide presentation [68]. Thus, DC have the capacity to perform a whole set of complex functions immediatly after TLR activation. It was later shown by Blander et al. that the presence of TLR ligands in the phagosome affects phagosome maturation and subsequent antigen processing and presentation [69, 70]. Of note, however, this finding has been the subject of debate [71-74]. It is reasonable to suggest that from an immunological perspective it would be beneficial if TLR signalling from the phagosome would affect the maturation of the phagosome, and thus the subsequent antigen processing and presentation. In particularly, since antigen uptake by DC can be quite unspecific [68] (paper III). Moreover, it is clear that phagosomes are cellular compartments from where TLR signalling also occure. Interestingly, data published earlier this year suggest that TLR signalling from phagosomes can lead to the recruitment of mitochondria via TRAF6 and the subsequent induction of mitochondrial reactive oxygen species (mROS) production, which increase the intraphagosomal killing of the bacterium *Salmonella typhimurium* [75]. Collectively, these findings further solidify the notion that intraphagosomal TLR activation might affect the maturation and function of the phagosome.

As mentioned earlier, the capacity of DC to ingest extracellular material is key for efficient intracellular antigen processing, the loading of antigens onto MHC molecules, and presentation to T cells. Dendritic cells use different endocytic pathways for antigen uptake, i.e., phagocytosis, macropinocytosis, and receptor-mediated endocytosis. Phagocytosis and macropinocytosis are two distinct endocytic processes, which are utilized by DC to ingest various antigens [76, 77]. In general, DC internalize small soluble extracellular antigens via macropinocytosis, including viruses, proteins, and fragments from bacteria and apoptotic cells [78-82], whereas the uptake of particulate material larger than 0.5 μm occurs via phagocytosis [76] (Figure 3).

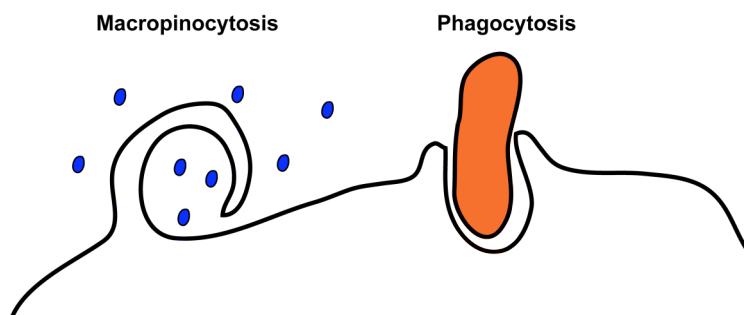


Figure 3. Schematic figure showing antigen uptake via macropinocytosis and phagocytosis.

Macropinosomes form as a result of plasma membrane ruffles that curve into open cups that then close and fuse, thereby trapping extracellular material [77, 83]. Thus, macropinocytosis proceeds independently of the material ingested particles. In contrast, phagosome formation is initiated and shaped by the particles they ingest [77]. At the molecular level, signalling in phagocytosis is localized in well defined short-range and medium-range membrane regions close to the particles that are internalized, whereas signalling for the regulation of macropinocytosis seems to be distributed more randomly [77]. Furthermore, phagocytosis can be subdivided into three distinct subtypes, i.e., “zipper phagocytosis”, “triggered phagocytosis”, and “sinking phagocytosis”. Despite the different spatial requisites for inducing signals that regulate the processes of macropinocytosis and phagocytosis, they share common signalling pathways, all of which are affected by TLR signalling [84].

Toll-like receptor signalling initiates a process of morphological and functional maturation, leading to changes in the chemokine receptor profile that initiate migration from peripheral tissues towards the draining lymph nodes, where the DC activate the adaptive arm of the immune system [61, 85, 86]. During maturation, DC upregulate the chemokine receptor CCR7, in turn making them responsive to CCL19 and CCL21, which drives their migration to the draining lymph node T cell areas where antigens are presented to T cells [87-90]. Complementary roles in DC migration have also been suggested for CCR8-CCL1, CXCR4-CXCL12, and S1P-S1P1/3 [91-93]. However, CCR7 seem to be most pivotal [94]. In the absence of CCR7 DC fail to reach the lymph nodes, and DC in the spleen remain located in the marginal zone outside of the white pulp and T cell areas [95, 96]. Exactly how DC execute this complex journey from peripheral tissues to the T cell areas of lymph nodes is not fully understood. It has been shown that endothelial cells of lymph vessels upregulate adhesion molecules, e.g., ICAM-1 and VCAM-1, E-selectin, and chemokines, e.g., CCL5, CCL20, and CXCL5, in response to cytokine stimulation [97]. Moreover, it was shown that neutralizing antibodies to ICAM-1 and VCAM-1 hindered DC adhesion to and migration through the endothelium of lymph vessels both *in vitro* and *in vivo* [97]. Along these lines, it was also shown that mice deficient in ICAM-1 have impaired trafficking of Langerhans cells to the draining lymph nodes [98]. In contrast, earlier data by Erdmann et al. suggests that Langerhans cell migration into lymph nodes is not impaired in mice deficient in selectin ligands [99]. Moreover, it was recently been shown that DC deficient in all integrin heterodimers migrate to the lymph node in an unimpaired manner [100]. Thus, it is believed that DC can migrate from peripheral tissues into lymph nodes independently of integrins [31], suggesting that more of an amoeboid type of migration is utilized [100, 101]. An important feature of cellular movement and navigation through dense tissues is the capacity to degrade extracellular matrix. It has been speculated that specialized cellular protrusions, the so-called podosomes are of importance in this process. Podosomes are highly dynamic, large actin dense adhesion structures (Figure 4) that are found in a variety of cell types, such as osteoclasts, macrophages, and DC, all of which needs to degrade extracellular matrixes [102, 103]. In Rous sarcoma virus transformed cells, where podosomes were first described, the podosomes were found on the ventral surface [104-106]. Podosomes have since then been linked to different functions such as resorption of bone and trans-cellular

migration [107, 108]. Osteoclasts use their podosomes, for bone degradation [107, 109]. In DC, however, podosomes thought to be used to enable migration through dense tissues [110]. This process involves matrix metalloproteinases (MMP), which are concentrated at the podosomes in order to degrade the connective tissue matrix [110-112]. It has been suggested that podosomes act as pathfinders in DC and enable degradation of the extracellular matrix at the front of the moving cell to facilitate movement in dense connective tissues [110].

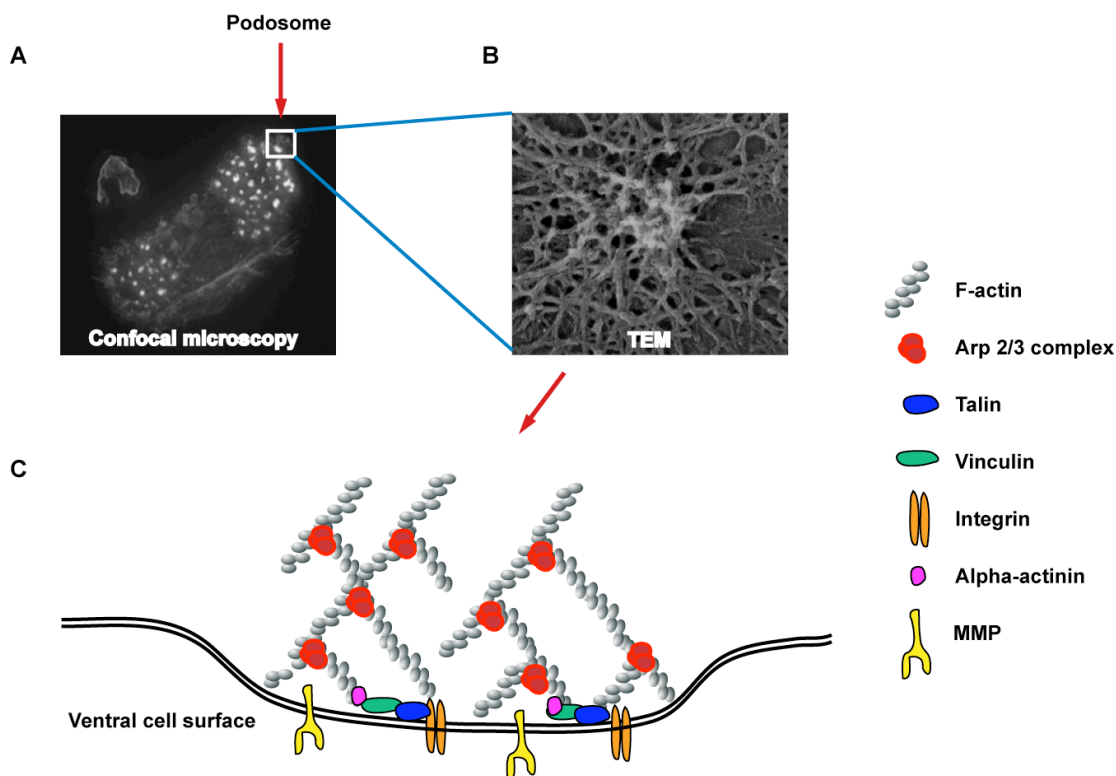


Figure 4. (A) Confocal microscopy picture of phalloidin-stained podosomes. (B) Transmission electron-microscopy (TEM) high-resolution picture of a single podosome. (C) Schematic figure of the actin cytoskeleton and some of the actin associated proteins, involved in the podosome structure.

On their way to the lymph nodes DC process the antigens acquired for peptide loading onto MHC class I and class II molecules and for the subsequent presentation to CD8⁺ and CD4⁺ T cells, respectively [16, 19, 113]. The ingested antigen is degraded and either loaded onto MHC class II molecules in specialized lysosomal compartments [114-117], or can be transferred into the MHC class I presentation pathway through a process referred to as cross-presentation [78, 118, 119]. The MHC class I molecules are synthesized *de novo* and, after peptide loading in the endoplasmic reticulum, MHC-I/peptide complexes are transported to the cell membrane. In contrast, newly

synthesized MHC class II molecules are transported to endolysosomal compartments where the peptides are loaded, followed by the transportation to the cell membrane. Toll-like receptor stimulation increases the efficiency of antigen processing and presentation for both the MHC class I and class II presentation pathways. Upon TLR activation both class I and class II molecules are stabilized on the cell surface, whereas in the absence of TLR activation MHC/peptide complexes are rapidly internalized and degraded [65, 120-122]. Furthermore, the generation of peptides for MHC class I and class II presentation is further enhanced by altered proteasome activity and lysosomal processing capacity, respectively [123-125]. On their way to the lymph nodes, TLR-activated DC also starts to upregulate co-stimulatory molecules including CD80 and CD86, which are important for the activation of naïve T cells [126]. Toll-like receptor activation also triggers the induction of the co-stimulatory molecule, CD40, on DC, which interacts with CD40L on helper T cells. The CD40-CD40L interaction leads to the further upregulation of co-stimulatory molecules and the production of cytokines by DC, which are key for the induction of adaptive immunity [61, 127]. Thus, when DC arrive in the lymph node they are ready for the activation of T cells.

INTRODUCTION TO THE WORK IN THIS THESIS

In order to give an overview of the work I undertook for my thesis, in this section I will present the studies included. My thesis is composed of three studies that focused on different aspects of DC activation and the consequent downstream effects of such activation. Briefly, in **paper I**, the effect of low physiological temperature on key DC functions associated with both innate and adaptive immunity, was explored. In **paper II**, podosome formation and function in DC derived from mice lacking the actin-severing and capping protein gelsolin, were analysed. In **paper III**, the effect of soluble TLR ligands, such as LPS, on the phagocytic capacity of DC, was investigated. In the following sections, I will start with an overview of the three papers included in the thesis, and explain the rationale and relevance of these projects. Then, the results will be presented and discussed in relation to existing literature in this field of research. Finally, I will describe the materials and methods used in these studies. For detailed descriptions of the studies on which my thesis is based, please read the papers that are included as appendices at the end of the thesis.

BACKGROUND TO THE PAPERS

Dendritic cells are widely distributed throughout most tissues and have important functions in both immunity and tolerance; they are also considered candidate targets in new immunotherapy protocols [128]. However, DC in different locations of the body operate under various conditions and the route of administration of a DC-based vaccination is often via intra-dermal or subcutaneous injections in sites that have significantly lower temperatures than the core temperature. In the skin, the temperature can be as low as 26°C at the extremities at an ambient temperature of 20°C [129-131]. This fact is often ignored and most studies on DC biology so far have only been performed at 37°C or at fever-range temperatures [132-135]. To the best of our knowledge, no studies have thus far examined how low physiological temperatures affect DC antigen handling and immune stimulatory capacities. This prompted us to examine some key DC functions at a low physiological temperature of 28°C (paper I). Although we found that several DC functions are intact at this low temperature, we also found that several functions were affected by low physiological temperature. This includes NO production, podosome activity, and the capacity of DC, activated at 28°C, to elicit T cell proliferation. We observed that several DC functions were slightly delayed but otherwise they were relatively unaffected by low physiological temperatures, whereas other functions important for activation of the adaptive immunity were impaired. Collectively, our study demonstrates that temperature changes within the physiological range have the capacity to modulate DC functions.

In order to activate T cells in the lymph nodes, DC migrate from peripheral tissues to the T cell areas of the draining lymph nodes. It is believed that podosomes facilitate migration through dense tissues and enable cells to penetrate barriers such as basement membranes and the endothelium [111]. However, their exact function in DC remains elusive. Several proteins involved in actin cytoskeleton remodelling have been suggested to be important for podosome dynamics and function [107, 136-140]. Since it was shown that the actin-severing and capping protein gelsolin is key for podosome formation and function in osteoclasts [107], gelsolin is commonly mentioned in the literature as an essential protein for podosome function [141-146]. Furthermore, in a paper by Hu and colleagues, a theoretical model for the regulation of podosome

dynamics that is dependent on the severing of actin filaments by gelsolin was presented [146]. Gelsolin was found to localize to podosomes already in the early studies of these structures, and gelsolin was also found in podosomes expressed by DC [110, 147]. However, it was reported that functions dependent on actin cytoskeleton rearrangement in DC seem to be independent of gelsolin [148]. This paper also reported that “podosome-like” structures were present in DC lacking gelsolin, but these structures were however not further investigated. Therefore, we investigated whether or not functional podosomes form in gelsolin-deficient DC. The actin-rich structures observed by West et al. [148], were indeed confirmed to be podosomes. Furthermore, we show that the dynamics and functions of the podosomes in DC are regulated independently of gelsolin.

Phagocytosis is an important function of DC for sampling antigens for subsequent antigen presentation to T cells. Upon the recognition of microbial danger products by TLR the cytoskeleton of DC rapidly starts to reorganize, leading to the dissolution of podosomes, increased ruffling, and increased uptake of extracellular bulk fluids via macropinocytosis [68]. The studies of the effects of TLR activation on phagocytosis, however, show somewhat contradictory results. It was shown that macrophages phagocytosed TLR4 ligand-expressing *Neisseria meningitidis* in an TLR4 independent manner [149]. Moreover, phagocytic uptake of TLR2-stimulating *Zymosan* proceeded normally in TLR2- or MyD88-deficient macrophages [150]. In contrast, when RAW264.7 cells were stimulated with LPS, for 24h before adding *Escherichia coli*, the phagocytic uptake was stimulated in a TLR4-dependent but MyD88-independent manner [151]. Along these lines, it was shown that TLR ligands stimulate the phagocytic uptake of *E. coli* and *Staphylococcus aureus*, while having negligible effects on the phagocytic uptake of latex beads [152, 153]. Although, many studies have been performed to explore the consequences of TLR activation and phagocytosis, these studies were all carried out after several hours of pre-activation. Thus, these studies mainly reflect the results of the induction of gene programmes and the subsequent synthesis of proteins involved in phagocytosis, e.g., upregulation of phagocytic receptors, rather than the immediate consequences of pathogen recognition. However, the process of phagocytosis is likely to be influenced within minutes of TLR ligation and an immediate increase in antigen uptake might be beneficial to the host both in terms of limiting the infection as well as in boosting antigen processing and presentation. In the case of bacterial infection, the active portion of TLR2 binding

lipoprotein when anchored in the bacterial membrane, remain unreachable for the ligation to TLR2 and, similarly, the TLR4-binding portion of LPS, i.e., lipid A, is integrated in the lipid bi-layer of Gram-negative bacteria, remaining inaccessible for TLR4 ligation. Consequently, lipoprotein and LPS ligation to the respective TLR most likely occurs when released due to disruption of the bacterial membrane. Thus, it may be of relevance to examine TLR ligation with soluble TLR ligands. Herein we study how soluble TLR ligands affect the phagocytic machinery in DC, proximal to gene transcription.

PRESENTATION OF THE PAPERS

Paper I: DC function at low physiological temperatures

The temperature of the body is tightly regulated. It is known that an increased body temperature modulates a wide array of immune functions. However, how physiological temperatures below the core temperature affect cells of the immune system remains unclear. In order to investigate whether or not a low physiological temperature affects the innate functions of DC we started by examining the capacity of DC to respond to TLR activation. Although we observed a delay in activation of the ERK and p38 MAPK pathways downstream of TLR activation at 28°C compared to 37°C, the DC also responded to TLR stimulation at 28°C. Therefore, we investigated whether or not cellular processes, which are dependent on TLR signalling, were affected by delayed phosphorylation and activation. The results showed a delayed, but not impaired, TNF secretion and macropinocytic uptake in response to TLR stimulation. Our cytokine data are along the lines of some previous studies, which showed that temperature modulates cytokine production and secretion in different cell types [154]. However, published data on cytokine secretion are somewhat contradictory, where some studies showed increased cytokine production whereas others showed decreased cytokine production. For example, Yan et al. showed that a temperature within the fever-range (39.5°C) promoted TLR4 expression and MAPK signalling in DC leading to the increased production of IL-6, IL-10, and IL-12 [155]. In contrast, studies by Hagiwara et al. on RAW264-7 cells, suggested that the cytokine release of HMGB1, IL-1 β , IL-6, and TNF was reduced following LPS stimulation at 40°C and 34°C compared to 37°C [156]. In study by Kirkley et al., who also used RAW264-7 cells, it was shown that TNF secretion was increased at 31°C compared to 37°C, while TNF secretion was decreased at 39°C. However, in the same study, IL-6 secretion was decreased at 31°C compared to 37°C and remained unchanged at 39°C. Moreover, the authors showed that IL-10 secretion was decreased on either side of 37°C, thus suggesting that temperature alterations might have different effects on cytokine secretion [157]. In addition, it was also shown that fever-range thermal conditions differentially regulate the cytokine production in immature or mature murine bone marrow-derived DC, suggesting that fever might have a direct regulatory capacity on DC [135]. In the present study, we observed a delayed TNF production but the levels of secreted TNF were similar to

those cells kept at 37°C, 8 hours after LPS activation. If cytokine secretion in our study had been measured only at one time point, i.e., at 4 h, 8 h, or at 18 h after TLR activation, the interpretation of the results would have been quite different depending on which time point was analysed (Figure 5). This may explain the varying results in the abovementioned studies, and it stresses the importance of performing adequate kinetic measurements.

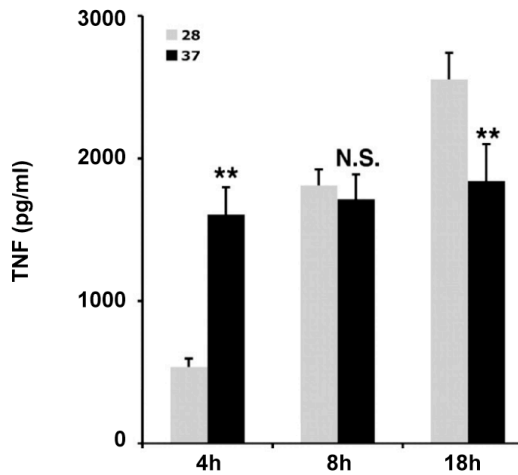


Figure 5. Adapted from Figure 3, paper I. Kinetics of TNF production after TLR activation at 28°C and 37°C.

Next, the capacity of DC to ingest antigens via phagocytosis and macropinocytosis was assessed at 28°C and 37°C. We found that phagocytosis of opsonized sheep red blood cells (SRBC) by DC was unaffected at 28°C compared to phagocytosis at 37°C. Moreover, although delayed, the TLR stimulated dextran uptake via macropinocytosis reached similar levels at 28°C and 37°C. However, the total amount of material ingested by the DC did not appear to decrease at the lower physiological temperature. The results of studies on how different physiological temperatures affect antigen uptake via phagocytosis are similar to the results of studies on cytokine secretion also somewhat contradictory. On the one hand it was shown that murine peritoneal macrophages increase their phagocytic uptake by 40% when incubated at 39°C compare to when kept at 37°C; and on the other hand, when incubated at 41°C the levels of uptake decreased [158, 159]. Collectively, this suggests that even small differences in temperature could have significant effects on phagocytosis. In contrast to this, Utoh and Harasaki observed no significant alterations in the phagocytosis of opsonized latex beads by human neutrophils of were to be found in the range of 25-

42°C [160]. We found that antigen uptake is unaffected by low physiological temperature. This might appear to be rather surprising from a biochemical point of view, since membrane fluidity could be affected by a decrease in temperature. However, from an immunological perspective, our results might be less surprising since 28°C is a common temperature in parts of the skin, thus, if the endocytic capacity was compromised at this temperature one could envisage that subsequent functions of DC, important in the induction of adaptive immunity, would be impaired.

Dendritic cells produce NO during the early defense against pathogens after TLR activation [161, 162]. If low physiological temperatures affects NO production remain unclear. Interestingly, we observed that production of the NO metabolite nitrite after TLR stimulation was severely impaired at 28°C (Figure 6). One could speculate that lower temperature might modulate and hinder NO production in order to ensure appropriate immune activation.

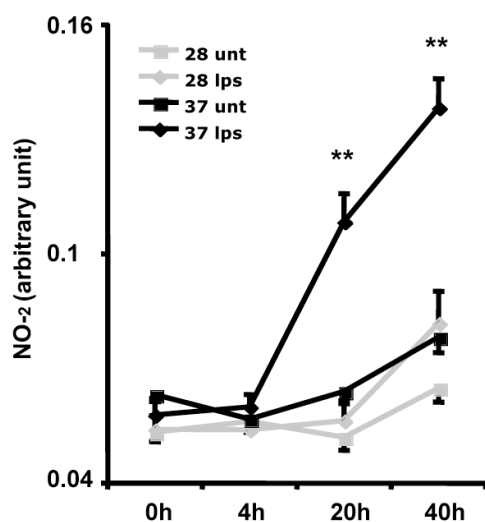


Figure 6. Adapted from Figure 3, paper I. NO production after the indicated time points at 28°C or 37°C, LPS treated and untreated.

The migration and homing of lymphocytes and DC to lymphoid organs are key for the induction of immunity and tolerance. Fever-range temperatures have been shown to increase the homing of circulating lymphocytes to lymph nodes by increasing L-selectin-dependent adhesive interactions between lymphocytes and high endothelial venules (HEV) [163, 164]. Chen and colleagues recently showed that fever-range temperatures increase the display of intercellular adhesions molecule 1 (ICAM-1) and

CCL21 specifically in HEV. This suggests that HEV are sensitive to thermal changes and that increase in temperature intensifies immune surveillance by increasing lymphocyte trafficking to lymphoid organs during inflammation [165]. Along these lines, it was also shown that DC migration, similar to lymphocyte migration, is stimulated by fever-range hyperthermia [132]. Hence, it is not surprising that treatment at fever-range temperature enhances both humoral and cellular immune response [164, 166]. Although, it has been shown that hypothermia leads to a decrease in lymphocyte circulation [167], no studies have to my knowledge addressed the effects of low physiological temperature on DC. It has, however, been shown that neutrophils display a significant impairment of migration at 29°C *in vitro* [168]. We observed that 28°C did not affect DC migration through uncoated transwell filters and podosome formation was not affected. However, we also found that the capacity of podosomes to degrade the matrix and the ability of DC to migrate through Matrigel-coated transwell-membranes were impaired at 28°C compared to at 37°C. These data suggest that an increase in temperature in peripheral tissues, as a consequence of inflammation, might enhance the ability of DC to migrate to lymphoid organs.

We next examined the effect of 28°C on the antigen uptake and processing capacity of DC by studying the presentation of the HEL (46-61) epitope on H-2 I-A^k using the specific antibody C3H4 [169]. We found no inhibitory effect of low temperature on the generation of I-A^k/HEL (46-61) complexes in unstimulated DC, suggesting that steady-state antigen processing and presentation, which are important for the maintenance of peripheral tolerance [2]. A consistent trend of higher generation of the HEL (46-61) epitope was, however, observed at 37°C after LPS stimulation in five experiments, although this trend did not reach statistical significance ($p>0.05$). When studying the surface molecule expression of co-stimulatory molecules, we observed that the upregulation of CD40 was reduced when the DC were activated and incubated at 28°C, but that this upregulation was rescued when the DC were moved to 37°C after 4 h of activation at 28°C. This experimental setup was used to mimic the local increase in temperature during inflammation, or when DC migrates to deeper tissues with a higher temperature, i.e., from the skin to draining lymph nodes. Because the CD40-CD40L interaction is important in the activation of naïve T cells, the lack of upregulation of CD40 at a low temperature could prevent inappropriate naïve T-cell activation by DC in the skin.

These temperature shift experiments imply that the inhibitory effect of low temperature can be reversed if the temperature is increased. However, the capacity of DC that had taken up antigens and been activated by TLR at 28°C and then shifted to 37°C after 4 hours to activate T cells was decreased. This observation was also confirmed using an allogenic setting, suggesting that temperature modulations of the ability of DC to activate T cells are not only dependent on antigen processing and presentation. Thus, other factors important for the T cell-stimulatory function of activated DC are suppressed when DC are activated at 28°C. The proliferation of T cells has been shown to be sensitive to lower 29-33°C temperatures, however, effector cytolytic activity of T cells is markedly insensitive to temperature changes. Hence, it has been speculated that fever or local tissue inflammation might act to temporarily ablate the cooler portions of normal thermal gradients, thereby selectively amplifying the emergence of T cell immunity in peripheral tissues [170]. Collectively, our results suggest that differences in temperature need to be taken into account when developing immunotherapeutic strategies that target DC at different locations. In particular, DC that have taken up antigens and been activated at 28°C have an impaired ability to activate T cells.

Paper II: Gelsolin-independent podosome formation in DC

The actin-severing and capping protein gelsolin is an actin-cytoskeleton-regulating protein that has been reported to localize to podosomes in several cell types. It has also been reported to be important for the formation and function of podosomes in osteoclasts [107, 147]. Moreover, in a paper by Hu et al., a theoretical model for podosome regulation was postulated in which gelsolin played a central role [146]. Thus, gelsolin is often mentioned in the literature as an essential protein for podosome formation [143, 144, 171-174]. In a previous paper by West et al. podosome-like structures were observed in gelsolin-deficient DC; however, these structures were not characterized [148]. Thus, we decided to study these podosome-like structures in DC in order to clarify whether or not they were indeed podosomes and, if so, whether or not they were functional. In contrast to what was previously shown in osteoclasts, we concluded in **paper II** that gelsolin is dispensable for podosome formation and function in DC, suggesting that podosomes are differentially regulated in DC and osteoclasts.

In the paper by West et al. actin was stained using phalloidin in gelsolin-deficient DC and showed structures that resembled podosomes [148]. We confirmed that these structures were indeed podosomes by using an antibody specific for the podosome-associated protein vinculin [173]. The number of cells presenting podosome structures was quantified and no difference was found between gelsolin-deficient and wild-type DC. Furthermore, the podosomes were visualized using transmission electron microscopy (TEM). The podosomes were clearly identifiable in both the wild-type and the gelsolin-deficient DC. After having established that the formation of podosomes in DC is independent of gelsolin, we continued these studies by investigating the dynamics of podosome assembly and disassembly in gelsolin-deficient DC. Dendritic cells were transduced using a retroviral vector coding for GFP-actin, after which the cells were studied using live-cell imaging. The quantitative analysis of the life-time of podosomes in DC revealed that the dynamics of podosome assembly and disassembly in DC are unaffected by a lack of gelsolin. In DC, it was shown that podosomes are negatively regulated by signalling downstream of TLR. Hence, the activation of DC with soluble TLR ligands leads to a rapid and transient disassembly of podosomes and the concomitant loss of migratory capacity [68, 112]. One could speculate that this transient halt in migration might serve to prevent DC from migrating from the site of infection for a short period and to focus them on acquiring antigens via

macropinocytosis and/or phagocytosis, which are boosted upon TRL activation [68] (**paper III**), (Figure 7).

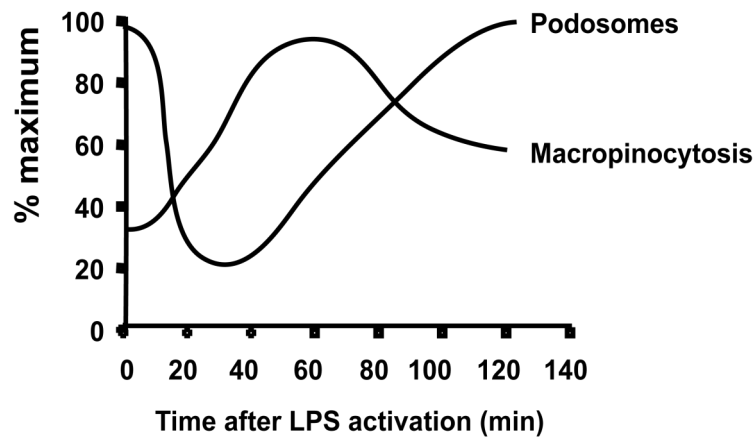


Figure 7. Adapted and modified from West et al. [68]. Transient boost in macropinocytic uptake after LPS stimulation and concomitant podosome disassembly.

Podosome dynamics were further studied by quantifying the percentage of wild-type and gelsolin-deficient DC that exhibited podosomes after LPS stimulation. The cells were stimulated for 20 min, as this is the peak time-point when most cells present no podosomes. No difference in podosome disassembly was found in the presence or absence of gelsolin. Thus, the actin severing activity of gelsolin is dispensable in podosome disassembly induced by TLR signalling.

Finally, the functionality of podosomes in the absence of gelsolin was investigated. In order to address this, the capacity of DC to degrade extracellular matrix was quantified. Glass cover-slips were coated with Oregon-green gelatin, after which DC were seeded on top and incubated overnight before analysis. Fluorescence microscopy was used to quantitatively analyse the total area of degraded gelatin (Figure 8).

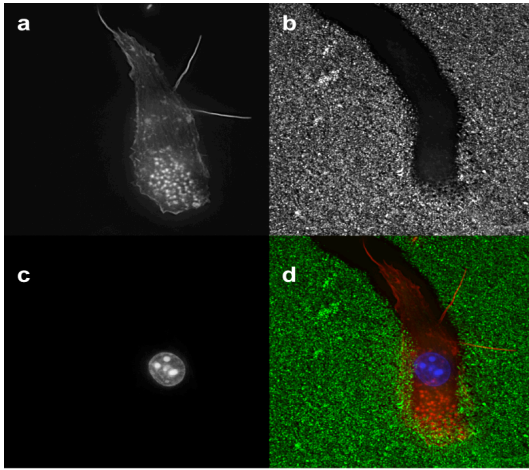


Figure 8. a) Actin stained with phalloidin, b) Oregon-green gelatin, c) nuclei stained with dapi, d) over-laying picture showing how the DC have degraded an area of gelatin. The area of degraded gelatin could then be measured and quantified.

Gelsolin-deficient DC were found to be able to degrade the gelatin as efficiently as wild-type DC. In **paper I**, we showed that the migration of DC migration in a transwell system correlates well with the matrix degrading activity of their podosomes. To further investigate the functionality of DC deficient in gelsolin, we asked whether or not gelsolin is required for this capacity to degrade extracellular matrix in order to migrate through dense tissue matrixes. In order to address this, a transwell migration assay, where the membranes were coated with a dense layer of Matrigel, was used. We found that gelsolin-deficient DC and wild-type DC migrate equally well from the upper chamber with the Matrigel-coated membrane to the lower chamber.

In conclusion, the actin-severing and capping protein gelsolin is dispensable for podosome formation, dynamics, and function in DC. Our data suggest that podosomes in different cell types may be differentially regulated. It is possible that rather than being one structure found in many cell types, as described in the current literature, podosomes could actually be a family of structures with many similarities but also many differences. Thus, the data obtained on podosomes from experiments on one cell type may not necessarily apply for podosomes in all other cell types.

Paper III: Toll-like receptor stimulated phagocytosis in dendritic cells

It has previously been shown that TLR activation with soluble LPS leads to a rapid and transient increase in the capacity of DC to ingest extracellular fluids via macropinocytosis [68]. Whether or not the stimulation of DC using soluble TLR ligands also affects the uptake of particulate material via phagocytosis has not yet been shown. The studies on TLR and their role in phagocytosis have so far examined whether or not TLR ligands affect the phagocytic capacity post protein synthesis, i.e., several hours after pathogen recognition. It is possible that when a pathogen is to be ingested, it would be favourable for the host cell to increase its phagocytic activity immediately after pathogen recognition. However, most studies on TLR activation and phagocytosis have studied phagosome maturation or phagocytic capacity after a period of 12 to 24 h post-TLR activation [69, 70, 73, 149-153].

It is well known that many of the molecules involved in cytoskeletal reorganization are activated upon TLR activation. More recently, a quantitative examination of the macrophage phosphoproteome also showed profound global changes in the phosphoregulation in cells downstream of TLR activation [84, 175]. In a study by Weintz et al., 1850 phosphoproteins with 6956 phosphorylation sites were identified. It was shown that TLR activation caused major dynamic changes in the phosphoproteome, with about 24% being upregulated and 9% being downregulated, identifying the cytoskeleton as a major hotspot of LPS regulated phosphorylation [84]. These studies further solidify previous speculations that TLR activation could affect the processes of phagocytosis [176-178].

In order to investigate whether or not TLR engagement could affect phagocytosis in DC, the phagocytic capacity of DC stimulated with the TLR4 agonist LPS for 30 min was studied. We found that LPS stimulation results in a significant transient increase in DC that ingests polystyrene beads shortly after activation. In order to confirm that the beads associated with DC were indeed internalized, the actin polymerization inhibitor cytochalasin D was used, and showed that the association with three or more beads represents a reliable measure of internalization; therefore, three beads were therefore set as a cut-off level for phagocytosis in the following experiments. Moreover, the inhibitor dimethyl amelioride (DMA), which specifically blocks macropinocytosis [179], was used to exclude the possibility that the beads were taken up via

macropinocytosis and not phagocytosis. Similar results to those found in previous studies [180, 181], were obtained, where the uptake of dextran was markedly decreased in the presence of DMA whereas the uptake of beads was not affected, demonstrating the functionality of DMA. Thus, we concluded that the LPS-stimulated uptake of polystyrene beads by DC was indeed mediated via phagocytosis. Internalization of the beads was further confirmed using immunofluorescence microscopy (Figure 9). We also confirmed that the stimulation of DC with LPS for 20 minutes, results in uptake of significantly more beads. We, thus, conclude that stimulation of DC with LPS leads to enhanced phagocytic uptake of 1 μ m polystyrene beads early (20-30 min) on after stimulation.

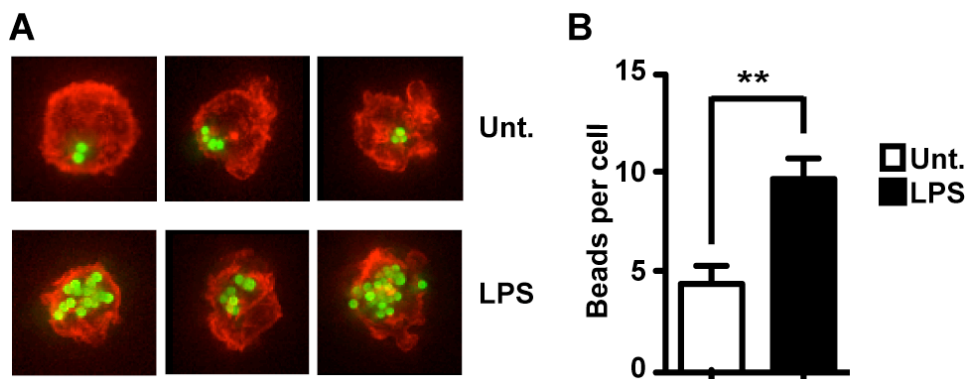


Figure 9. Adapted from Figure 1, paper III. Microscopy analysis of the capacity of DC to phagocytose polystyrene beads. *A*, Immunofluorescence images of Alexa 647-stained untreated or LPS-activated DC that had phagocytosed polystyrene beads. *B*, Quantification of the numbers of beads ingested by untreated (open bars) or LPS-treated (black bars) DC.

Next, we studied whether also other TLR ligands could stimulate phagocytosis. Indeed, all four TLR ligands studied, TLR1/2, TLR4, TLR6/2, and TLR9 ligands, were all capable of inducing increased phagocytic uptake in DC. Since LPS was the most effective stimulus for inducing enhanced phagocytosis, all subsequent assays were performed using LPS as the model TLR ligand. Next, we studied the kinetics of LPS-stimulated phagocytosis in DC. When studying the kinetics of phagocytosis, we found that the stimulation of DC was rapid; 10 min after LPS stimulation a significant increase in phagocytosis was observed. This suggests that the rapid and transient boost in phagocytic capacity most likely precedes gene transcription. This was confirmed by studying phagocytic uptake in the presence of actinomycin D, which blocks gene

transcription [182]. Thus, we concluded that the TLR-stimulated boost in phagocytic uptake precedes the effects of gene transcription.

The ligation of TLR4 results in a trans-membrane signalling transduction, which activates intracellular signalling via TIRAP-MyD88-dependent and TRAM-TRIF-dependent pathways [183, 184]. It has previously been shown that TLR4 activation has the capacity to regulate gene programmes involved in phagocytosis, independently of MyD88 [151]. However, since TLR4 signalling downstream of both MyD88 and TRIF is complex and to some extent overlaps, studies on MyD88-deficient cells alone, such as those of Kong et al. [151], may not reveal alterations in the process of phagocytosis of pathogens expressing TLR4 ligands. Hence, the participation of TLR4, MyD88, and TRIF on the LPS-stimulated phagocytosis were investigated using mice deficient in either MyD88 or TRIF, as well as in a mouse strain deficient in both MyD88 and TRIF, i.e., a “double knock-out” mice. First, we confirmed that the LPS-stimulated uptake only depended on TLR4 by studying the uptake in DC derived from mice deficient in TLR4. Next, we observed that in DC deficient in MyD88 or TRIF the stimulation of phagocytosis was only partially decreased, while in DC deficient in both MyD88 and TRIF the stimulation of phagocytic uptake was completely ablated. Thus, TLR4 signalling through both adaptor molecules, MyD88 and TRIF, was required for the optimal stimulation of phagocytosis proximal of protein synthesis.

In order to address whether or not TLR activation could boost the phagocytic uptake of larger and, to some extent, more physiological substrates, we used SRBC labelled with PKH26 or apoptotic cells labelled with CFSE. Of note, all extracellular SRBC were removed by lysis before analysis to ensure that only internalized SRBC were counted. The number of DC that phagocytosed SRBC upon LPS stimulation was significantly increased. Along these lines, we observed that TLR4-stimulated DC more efficiently phagocytosed apoptotic A20 cells. Thus, TLR4 activation has the capacity to augment the phagocytic uptake of cellular substrates. The target substrates studied above all share the common feature of being relatively weak inducers of phagocytosis, although one could argue that apoptotic cells trigger phagocytosis comparatively well. In order to examine whether or not TLR activation also has the capacity to stimulate the uptake of opsonized particles, which are potent inducers of phagocytosis, we used anti-SRBC antisera. We found that the effect of stimulating phagocytic uptake with TLR4 activation, as seen when studying the non-opsonized SRBC, decreased with increasing

concentrations of anti-SRBC antisera, suggesting that the TLR stimulation of phagocytosis is more prominent when the target substrates are poor inducers of phagocytosis. Similarly, it was previously shown that LPS is unable to augment the phagocytic uptake of particles opsonized with IgG [185]. Thus, considering all of the above, it is reasonable to suggest that TLR signalling does not further increase Fc-receptor-mediated phagocytosis. In summary, we provide evidence to show that soluble TLR ligands have the capacity to rapidly boost the phagocytosis process in DC, proximal to gene transcription.

MATERIALS AND METHODS

A brief description of the methods used in these studies is provided below. For a more detailed description, please see the “Materials and methods” section in each of the papers included in the thesis.

Mice and bone marrow-derived DC

In these studies, we chose to use DC derived from murine bone marrow using the GM-CSF, initially described by Inaba et al. [32]. This pivotal discovery provided a robust experimental system that has been of tremendous importance for the studies of DC biology. Furthermore, this system has the advantage of generating large numbers of DC, beneficial for the experimental setup used in our studies. In addition, it is still the most frequently used technique for generating primary DC, thus, enabling us to readily compare the data obtained to data from other studies within this field. More recently, it was been shown that the DC derived using this system seem to be equivalent to certain DC that emerge *in vivo*, in particular those that emerge upon inflammation [186]. Retrospectively, the Flt3-ligand-derived DC could also have been used for these studies as the DC derived using the Flt3-ligand might better represent the DC found at steady-state; however, these cell cultures are less homogenous and give rise to two DC subsets: CD24^{low}CD11b^{high} and CD24^{high}CD11b^{low} [186].

The mice used were six to ten weeks old, breed and housed under specific pathogen-free conditions. In **paper I**, C57BL/6 (B6) and C3H/He mice, were used and housed at the Department of Microbiology Tumour Biology and Cell Biology Animal Facility, Karolinska Institutet. In **paper II**, B6 and gelsolin knock-out mice with a B6 background were housed in the animal facility at the Translational Medicine Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School or at the Institute of Genetics, University of Bonn. In **paper III**, B6 and B6.TLR4^{-/-}, B6.MyD88^{-/-}, B6.TRIF^{-/-}, and B6.MyD88^{-/-}TRIF^{-/-} mice were bred and housed at the Department of Microbiology, Tumour Biology, and Cell Biology Animal Facility, Karolinska Institutet or at the Immunobiology Laboratory, London Research Institute, Lincoln’s Inn Fields Laboratories, London. The DC were generated from murine bone

marrow from femur and tibia as previously described [32], and also described in the papers.

Analysis of TNF and NO secretion (paper I)

In **paper I**, the capacity of DC to secrete TNF and NO in response to LPS stimulation at 28°C and 37°C was evaluated using an enzyme linked immunosorbent assay (ELISA) and Griess assay, respectively.

Internalization assays (paper I and paper III)

In both **papers I** and **III**, we studied the uptake of particles, including polystyrene beads and SRBC, via phagocytosis and the uptake of fluorescein isothiocyanate (FITC)-dextran via macropinocytosis. The TLR4 ligand LPS was used throughout **paper I** as the model TLR ligand, whereas, in **paper III**, other TLR ligands other than LPS (TLR1/2, TLR2/6 and TLR9) were also used. In order to study phagocytosis, after the DC were stimulated with TLR ligands, 1 µm yellow/green fluorescent polystyrene beads were added to each well at a bead to DC ratio of 40-50:1 and incubated for 30 minutes. For the studies on macropinocytosis, 70-kDa FITC-dextran was used and added to the cells for 20 minutes. The uptake of FITC-dextran is commonly used to investigate uptake via macropinocytosis [187-189]. The cells were washed before staining on ice with anti-CD11c mAb, followed by an additional washing step. Polystyrene bead uptake by the DC was quantified by flow cytometry using a FACSCalibur cytometer and CellQuest software. In **paper III** a variety of compounds that inhibit various molecular processes were used to determine the phagocytic uptake process. Moreover, dimethylamelioidide was used to inhibit macropinocytosis, specifically. The inhibitors were added to the cells 30 minutes before TLR stimulation.

To study the phagocytic uptake of cells we used non-opsonized or opsonized SRBC or apoptotic cells. The SRBC were stained using PKH-27; and for opsonization the SRBC were incubated with an anti-SRBC antisera. The DC were harvested and transferred to a 96-well plate as described above. The SRBC were added to the DC at an SRBC to DC ratio of 20-30:1 and incubated for 30-45 min, after which the cells were spun down and any extracellular SRBC were lysed with RBC lysis buffer. The cells were then

washed twice before staining with anti-CD11c mAb and incubated on ice, after which the cells were washed extensively. Phagocytosis was quantified using flow-cytometry as described above. In order to study the uptake of apoptotic cells, the A20 cell line, an Fas apoptosis sensitive B cell lymphoma [190, 191], was used. These cells were first stained with CFSE after which FasL mAb was added to the cells to induce apoptosis. The cells were incubated for 8 h, washed twice, added to the DC at a apoptotic cell to DC ratio of 20:1 and incubated for 30-45 min. The DC were then stained, washed and analysed by flow cytometry, as described above.

Immunofluorescence microscopy analysis of phagocytosis (paper III)

For the immunofluorescence microscopy, DC were processed in a similar way to the bead uptake assay described above. After washing in PBS the cells were fixed with 4% PFA in PBS for 15 min. The cells were then washed again with PBS and permeabilized using Triton X-100 in TBS. The actin cytoskeleton was stained with Alexa 647 phalloidin. The cells were then washed in TRIS buffered saline (TBS), resuspended in ProLong Gold anti-fade mounting medium containing DAPI on glass slides, and overlaid with glass coverslips. The cells were then allowed to set at room temperature in the dark overnight. Cells were imaged with a Delta Vision Spectris microscope, and the image stacks were deconvolved with the Delta Vision SoftWorks software. The deconvolved stacks of images taken through the cells were then further analysed with ImageJ software.

Western blot analysis (paper I and paper II)

In **paper I** and **paper II** Western blot analysis was performed in order to study phosphorylation status of ERK and MAPKAP2 (**paper I**) Western blotting was also used to confirm the presence of gelsolin in wild-type DC as well as absence of gelsolin in gelsolin-deficient DC (**paper II**).

In order to detect gelsolin, day 6 DC derived from wild-type or gelsolin-deficient mice were analysed. The DC were, without prior treatment, prepared in sample buffer, separated on gel, transferred to membranes, and stained with anti-gelsolin antisera. To assess the activation status of the ERK and P38 MAPK pathways, DC were activated

with LPS for the indicated time points at 37°C or 28°C. The cells were then prepared in sample buffer, separated on gel, transferred to membranes, and stained anti-phospho-ERK or anti-phospho-MAPKAPK2 antisera. Equal loading was confirmed by actin staining. The chemiluminescent signals were captured with a digital LAS 4000 system (Fuji Film Life Science), and the signal intensity was analysed with ImageJ software (<http://rsbweb.nih.gov/ij/>).

Podosome visualization and evaluation (paper I and paper II)

In **paper I**, sterilized glass coverslips were placed in bottom of each well of 24 well tissue culture plates, and cell suspensions containing day 6 DC were added. The cells were then incubated at 37°C or 28°C after which the cells were fixed for 15 min using pre-warmed (37°C) 4% PFA. The actin cytoskeleton was stained with phalloidin followed by a step of extensive washing, using PBS. In **paper II**, the podosomes were visualized by vinculin staining using an FITC anti-vinculin antibody followed by staining of the actin cytoskeleton with TRITC-phalloidin. The staining procedures were followed by extensive washing using PBS. The coverslips were then mounted with ProLong Gold antifade containing DAPI. The morphology of the actin cytoskeleton and the podosome structures were observed using a Delta Vision Spectris microscope and analysed with ImageJ software. The percentage of DC that exhibited podosome structures was calculated. To visualize and evaluate the capacity of podosomes to assemble and disassemble in **paper II**, DC from gelsolin-deficient and wild-type mice were transduced with a GFP-actin encoding retrovirus. The DC were then seeded in glass-bottom petri dishes and monitored using a DeltaVision Spectris live cell imaging system. The podosome lifetime was quantified from the videos obtained using the MTrackJ analysis plugin for ImageJ software (<http://imagej.nih.gov/ij/>).

Extra-cellular matrix (ECM)-degrading capacity (paper I and paper II)

The capacity of podosomes to degrade extracellular matrix was evaluated using a model in which degradation of fluorescent gelatin is measured and quantified. This approach has previously been described [192], although some modifications were made for the present study. Ethanol-sterilized coverslips were coated with Oregon-green gelatin. After an extensive washing procedure to remove PFA, used to stabilize the

gelatin, a day 6 DC cell suspension was added onto the gelatin-coated coverslips and incubated overnight before fixing. Then the cells were stained with phalloidin and mounted as described above. Images were acquired using a Delta Vision Spectris microscope. In **paper II**, the percentage of the image area that lacked a fluorescent signal from the Oregon-green gelatin, i.e., degradation as result of podosome function was analysed and quantified using ImageJ software.

Transmission electron microscopy of the podosome ultra-structure (paper I)

In **paper II**, the ultra-structure of the podosomes expressed in gelsolin-deficient DC compared to the wild-type was assessed using transmission electron microscopy (TEM). The cytoskeleton connected to the ventral cell surface membrane of the DC was prepared as previously described [193], with some modifications. In brief, ventral membrane preparations were made by mechanical fragmentation of the cells ("unroofing") followed by fixation. The samples were rapidly frozen on a helium-cooled block, freeze-dried, rotary shadowed with either platinum to tantalum-tungsten, and stabilized with 5 nm of carbon without rotation. The preparations were picked up on grids and viewed in the EM at 80 kV.

Migration assay (paper I and paper II)

In order to study migration, two transwell systems were used in **paper I**. Transwell membrane inserts were coated with a thin layer of Matrigel, just covering the filter area, or they were left untreated. In the uncoated transwell system the DC could freely migrate from the insert to the lower chamber, whereas in the Matrigel-coated system, the cells needed to degrade and penetrate the Matrigel in order to reach the lower chamber. Thus, this system provides a functional readout of the collagenolytic activity of the cells, followed by migration. The cells were harvested and seeded into the transwell inserts, and then incubated for the indicated times. The cells that had migrated to the lower chamber were counted in Bürker-chambers using trypan blue exclusion. In **paper II**, only the Matrigel coated system was used, and the cells counted Bürker-chambers and flow-cytometry. The cells were stained with anti-CD11c mAb and analysed by flow cytometry using a FACSCalibur cytometer and CellQuest software.

Evaluation of the cell surface expression, of DC maturation markers (paper I)

The DC were activated with LPS as described above and incubated overnight at 37°C or 28°C. The Fc receptors were blocked using 2.4G2 Fc blocking antibody before staining for the flow cytometric analysis. The cells were stained with anti-CD40 PE, anti-CD86 FITC and anti-CD11c Alexa 647. All stainings were performed on ice, after which the cells were washed and analysed by flow cytometry using a FACSCalibur cytometer and CellQuest software.

Antigen processing and presentation (paper I)

To measure antigen processing and the subsequent loading of peptides onto MHC class II at different temperatures, Hen egg lysozyme (HEL) and DC from C3H/HeN mice (I-A^k) were used. Different concentrations of HEL were used and the DC were either activated using LPS or they were left untreated. The DC were then incubated overnight at 37°C or 28°C, after which the cell surface expression of I-A^k/HEL (46–61) peptide was measured by staining with Alexa 647-conjugated C4H3 mAb, which recognizes this peptide-MHC class II complex [169]. The DC were analysed using a FACSCalibur cytometer and CellQuest software.

T-cell proliferation assay (paper I)

To study whether or not low physiological temperature affects the capacity of DC to induce T cell proliferation, the DC were incubated with ovalbumin (OVA) in the presence of LPS at 28°C or 37°C for 4 hours. The DC were then washed and seeded into 96-well plates at different concentrations in triplicate. The T cell receptor-transgenic OT-II T cells were added to the wells, and the cells were incubated at 37°C for 72 hours. Importantly, the T cells were not exposed to lower temperatures. Allogenic stimulation was performed using bone marrow derived DC from C57BL/6 mice and C3H/HeN spleen CD4⁺ T cells. The allogenic DC were seeded into 96-well plates in triplicates. The DC were stimulated with LPS or left untreated for 4 hours at 28°C or 37°C and then, the DC were then washed extensively, after which purified CD4⁺ T cells were added, and incubated at 37°C for 72 hours. Then, [³H]thymidine was added to each well of the T-cell cultures during the last 16 h. The cells were harvested

with a TOMTEC cell harvester onto filters, and scintillation sheets were added. The T cell proliferation was then measured using a β -counter.

Ethics statement

All animal experiments were performed in accordance with national and institutional guidelines. In **papers I-III** the experiments were approved by the Stockholm Animal Ethical Committee North (Stockholms Norra Djurförsöksetiska Nämnd), application numbers; N269/07 and N64/11. In **paper II**, the wild-type and gelsolin knock-out mice were bred and used in accordance with the guidelines at the Translational Medicine Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA, or at the Institute of Genetics, University of Bonn, Bonn, Germany. Moreover, in **paper III**, the MyD88 and TRIF knock-out mice were bred and used in accordance with the guidelines at the Immunobiology Laboratory, London Research Institute, Lincoln's Inn Fields Laboratories, London.

Statistical analysis

In **paper I**, the statistical analysis was performed using the single factor ANOVA test plugin in Microsoft Excel. In **Papers II and III**, the statistical analysis was performed using Graphpad Prism v.5 software and the two-tailed non-parametric Mann-Whitney test.

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