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In 2014 Maksim Baranov graduated from Master's program in Molecular Mechanisms of Disease (MMD) at the Radboud University of Nijmegen, the Netherlands. During his Master's training in the department of Tumor Immunology, in 2013, Maksim identified a new mechanism in dendritic cells, and how they utilize their actin cytoskeleton to probe for antigen across epithelial barriers. This led to two first-author publications (Baranov, et al. (2014) J. Cell Sci. 127, 1052; Baranov, et al. (2014) Commun. Integr. Biol. 7: e29084). In 2014-2018 Maksim worked on his PhD training in the group of Geert van den Bosqart researching how dendritic cells and other phagocytes of the immune system process ingested microbial pathogens in phagosomes. The key research objective was to understand how a specific class of lipids – phosphoinositides – drive formation and maturation of phagosomes over time. Maksim discovered the key adapter protein – SWAP70 – responsible for the specific tethering, stabilization and organization of the F-actin cytoskeleton on newly formed phagosomes containing microbial pathogens. These new data revealed that SWAP70 can anchor F-actin to phagosomes which is used for creation of pulling forces required for pathogen engulfment. These findings were published in (Baranov, et al. (2016) Cell Reports 17, 1518; Baranov, et al. (2017) Small GTPases 10, 1). In 2018 Maksim concluded his PhD research by publishing a study revealing an important role of PIKfyve kinase in producing PI(3,5)P, driving phagosomal maturation leading to presentation in MHC-class II (Baranov, et al. (2019) Science 11, 160).
Ins and outs of antigen processing by dendritic cells:
from antigen sampling, uptake, degradation to presentation.

Maksim Baranov
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Ins and outs of antigen processing by dendritic cells: from antigen sampling, uptake, degradation to presentation.

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Maksim Baranov

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Promotoren:
Prof. dr. G. van den Bogaart (RUG)
Prof. dr. C.G. Figdor

Manuscriptcommissie:
Prof. dr. R.E. Brock (voorzitter)
Prof. dr. J. Borst (UvA)
Dr. L. Albertazzi (TU Eindhoven)
CONTENTS

Chapter 1 Introduction 7

2 Chasing Uptake: Super-Resolution Microscopy in Endocytosis and Phagocytosis 23

3 SWAP70 Organizes the Actin Cytoskeleton and Is Essential for Phagocytosis 43

4 SWAP70 is a universal GEF-like adaptor for tethering actin to phagosomes 73

5 The phosphoinositide kinase PIKfyve promotes cathepsin-S mediated MHC class II antigen presentation 89

6 Podosomes of dendritic cells facilitate antigen sampling 129

7 Reaching for far-flung antigen: How solid-core podosomes of dendritic cells transform into protrusive structures 155

8 General discussion and future perspectives 163

APPENDIX Nederlandse samenvatting 183
Acknowledgments
Curriculum Vitae
Publications, Grants & Awards
Introduction
Chapter 1

Brief overview of membrane trafficking

Coordinated vesicular trafficking is necessary for internal communication between organelles, for targeting of proteins, lipids and other biomolecules to their cellular destinations, and for regulating organelar morphology and the spatiotemporal organization of cells (Fig. 1). Many cell types have specialized membrane trafficking, such as for the release of neurotransmitter at the chemical synapse between neurons [1], the delivery of granzyme B and perforin by natural killer cells and cytolytic T cells at the immunological synapse with target cells [2, 3]. Membrane trafficking is responsible for orchestrating endosomal and phagosomal maturation and for transporting cargoes and molecules from within the cell to be secreted or presented on the cell surface.

The protein machinery responsible for membrane trafficking is well understood. Cage proteins such as clathrin, caveolin, COP-I and COP-II [4-6] are responsible for the formation of trafficking vesicles at the donor compartment or at the plasma membrane for endocytosis. Some cell types are also capable of uptake of large material by phagocytosis, which does not rely on specific cage proteins, but can depend on the actin cytoskeleton. Cage proteins can interact directly with cargo molecules, or indirectly via so-called adapter proteins [5]. Many trafficking vesicles and whole organelles are transported within the cell by the microtubular cytoskeleton. The molecular identity of organelles is governed by several factors, including small GTPases of the RAB family, N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) [7] and phosphoinositides [8, 9]. Combinations of these factors determine the site of membrane fission and fusion and are recognized by tethering factors, which are large multiprotein complexes that can span over long distances up to 200 nm [10, 11]. SNARE proteins are the engines of membrane fusion, and the interactions of cognate SNAREs present in both the donor and acceptor membrane drive membrane fusion. The lipid membrane composition of organelles itself is a dynamic factor governing trafficking with many trafficking proteins being recruited to specific lipid species [9, 12]. Membrane trafficking is a large area of research focusing on resolving the mechanisms and pathways of intracellular transport.

In our work we focused on the aspects of membrane trafficking by dendritic cells (DCs) – important phagocytes of the innate immunity actively engaged into antigen sampling via endocytosis or phagocytosis, antigen degradation and presentation. Cancer-antigens and pathogens can be engulfed by DCs into a vacuole by endocytosis or phagocytosis (for small solutes and larger particles (> 0.5 µm), respectively). In our work we aim to focus on events guiding endo/phagosomal remodeling during early moments of internalization and later phagosomal maturation. Then, we focus on an important class of lipid species at the vacuolar membrane upon antigen internalization – phosphoinositides. We aim to understand the importance of some phosphoinositide species phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2) and phosphatidylinositol (3,5)-bisphosphate (PI(3,5)P2) in early and late maturation events leading to antigen presentation by human and mouse.
DCs. Finally, we discuss a potentially new mechanism of antigen sampling by human and mouse DCs via protrusive podosomes.

Role of phagocytes in immune response

The immune system of higher organisms has been evolving to attack malicious cancerous cells or pathogens encountered by the body. The central mechanism of antigen uptake by immune cells in multicellular organisms appeared initially in lower unicellular organisms and are used for nutrient uptake (well studied in the protozoan amoebae *Entamoeba histolytica*, *Amoeba proteus* and *Dictyostelium discoideum* [14-17]) which evolved into endocytic and phagocytic pathways in higher species [18].

Cargo of smaller size is internalized via endocytosis and can utilize clathrin coated pits or caveolin for formation of endocytic vesicles. Cellular drinking is called pinocytosis and used for non-receptor dependent internalization of liquids. Larger solid components are internalized via phagocytosis, a term first coined by Élie Metchnikoff [19], and currently defined as a clathrin-independent uptake mechanism of particles > 0.5 µm in diameter requiring F-actin polymerization. Phagocytes can be subdivided into professional (macrophages, DCs, neutrophils) and non-professional cell
types (epithelial cells and fibroblast) [20-23]. Endocytosis and phagocytosis are important steps towards constituting the first line of immune defense – called innate immunity – involving antigen recognition, killing and degradation of infectious microbes. Immune phagocytes continuously patrol the body for potential infections, this leads to formation of unique niches were phagocytes may reside. The major phagocytic leukocytes that provide innate immunity are granulocytes, macrophages and DCs.

Granulocytes, which include neutrophils, are believed to be the most efficient phagocytes capable of rapid and productive phagocytosis for intracellular killing of the pathogens or being able to kill pathogens via extracellular mechanisms [24]. Macrophages mainly play homeostatic roles and are tuned for killing pathogens and apoptotic cells, although they can also present peptides derived from ingested antigens in major-histocompatibility complex to T-cells. Tissue-resident macrophages are distributed within the most tissues of the body where they can be either replenished from blood recruited monocytes or accrued from embryonic sources in the tissue [25, 26]. Tissue-resident macrophages can be classified into inflammatory M1 and anti-inflammatory M2 phenotypes [26]. It has been shown that anti-inflammatory macrophages are engaged into a vigorous pinocytosis while pro-inflammatory macrophages are virtually inactive [27].

DCs create the link between innate and adaptive immunity. DCs orchestrate immunity against a wide range of pathogens as well as tolerance to self and harmless environmental pathogens [28]. DCs do not only patrol for pathogens but also are highly specialized antigen-presenting cells that internalize exogenous peptides derived from cancer cells or pathogens and present them within MHC-class I or II on the DC’s surface. Antigen presented on the surface of MHC-I can trigger adaptive immune system via activation of cytolytic CD8+ T-cells, whereas antigen presented within MHC-II can trigger helper CD4+ T-helper cells [29, 30]. Antigen processing and presentation between DCs and macrophages has differences and similarities [31]. Macrophages are tuned for faster antigen uptake leading to degradation and clearance of pathogens. DCs are known for higher activity of NOX2 associated with higher levels of ROS leading to alkalinized pH in their endo-/phagosomes. This leads to a delay in antigen processing due to inhibition of proteases in suboptimal pH or inhibition of lysosomal thiol reductases due to excess of ROS. This causes a delay in antigen processing, resulting in more diverse repertoire of antigens for presentation and allows for sustained presentation of antigens following DC's migration to lymph nodes which can take days, but is necessary for encountering naive T-cells by DCs [32-40]. The recent work suggests that monocyte-derived DCs (moDC) and monocyte-derived macrophages can cross-present efficiently exclusively through vacuolar pathway, but only moDCs specialize on the producing of co-stimulatory signals for activation of effector cytotoxic CD8+ T-cells [41].

DCs consist of multiple subsets [22, 29, 30, 42] showing high inter individual variation and tissue specialization [19]. Understanding the properties of different DC subsets is a current focus for
optimizing anti-tumor DC-based immunotherapies [26]. Depending on the lineage origin, type of transcription factors and surface markers expressed and cell specialization, naturally occurring DCs currently are classified into the following subsets: plasmacytoid DC (pDC), myeloid/conventional DC1 (cDC1) and myeloid/conventional DC2 (cDC2) [43]. DCs can also originate from monocytes and have a phenotype that can be placed between cDC2 and macrophages. Monocyte-derived DCs (moDCs) were initially used in immunotherapies until it was understood that they do not efficiently migrate to the lymph nodes [24] in contrast to other subtypes of DCs such as plasmacytoid (pDCs) and myeloid DCs (mDC). In our work, we mainly used primary human moDCs as it allows us to acquire substantial quantities of cells from healthy donor blood ex vivo from CD14$^+$ blood monocytes with granulocyte monocyte colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) [25, 44], and they can be transfected with plasmids and siRNA with high efficiency.

Bridging receptors and ligands as a first step towards phagocytosis

Co-evolution of phagotrophic protozoans and bacteria led to the emergence of phagocytic receptors binding pathogen-derived ligands [45]. Phagocytic receptors fall into two main categories: integrins [46-48] and lectins [44]. Phagocytosis in innate immunity is triggered through recognition of antigen ligands by the pathogen recognition receptors (PRR) that recognize pathogen-associated molecular patterns (PAMPs) [14, 31, 49, 50] and scavenger receptors that recognize signs of cell stress called danger-associated molecular patterns (DAMPs) [51] (apoptotic cell recognition is beyond the scope of this thesis). The best characterized PRRs are Toll-like receptors (TLRs) (Fig. 2) which are type I transmembrane receptors involved in recognition of a variety of microbial pathogens, orchestrating innate immune responses [52]. DCs that encounter pathogens [28] via PRRs [18] can engulf them via endocytosis or phagocytosis [53]. Phagocytosis is utilized for clearance of infections such as bacteria (~0.5–3 µm) or yeast (~3–4 µm) [53] or even larger apoptotic cells (~5-50 µm) [47, 53]. Pathogens can be directly ingested by PRRs or by scavenger receptors after opsonization [54]. PRRs (Fig. 2) can directly recognize ligands on the surface of a pathogen [49]. Pathogens can be also decorated by serum proteins called opsonins, which can be serum-derived antibodies recognized by Fc-receptors (FcR) (Fig. 2) or components of the complement system such as C3bi recognized by complement receptors (CR) (Fig. 2) [55]. Some pathogens developed efficient mechanisms of interfering with opsonization [56, 57], or avoiding the complement pathway by inactivating C4b [58]. In contrast, some pathogens hijack receptors for their survival and for instance the intracellular pathogen, *Mycobacterium tuberculosis* triggers scavenger receptor overexpression on the cell surface of macrophages to foster continuous uptake of the bacteria [59].

Pathogenic ligands engaging PRRs and scavenger receptors not only trigger cellular programs facilitating antigen uptake, but also downstream signaling cascades that lead to expression of costimulatory molecules, cytokines and chemokines. Although the uptake and killing of ingested microbes by DCs contributes to innate immunity [60], this eventually triggers the second line of
defense called adaptive immunity. Adaptive immunity is activated when antigens are presented by phagocytes to T lymphocytes.

In our studies we researched the roles of integrins, FcR, TLR2 and Dectin-1 receptors into phagosomal formation as well as mannose receptor antigen uptake of OVA-antigens.

---

**BACTERIA**

- Lipopolysaccharide (LPS)
- Endogenous peptidoglycan (PGN)
- Lipoprotein (lipoprotein)
- Fucoidin
- Lipopolysaccharide (LPS)

**VIRUS**

- gp130
- sDNA
- scDNA

**PARASITE**

- GPI anchor

**YEAST**

- Mannan (β-glucan)

---

**Pathogen recognition (PRR) and Fc receptors (FcR)**

- TLR
- CLR
- NLR

**Receptors and ligands**

- TLR (Toll-like receptor)
- CLR (complement receptor)
- NLR (Nod-like receptor)
- Fc (Fc receptor)

---

**Fig. 2:** Antigen recognition via receptors expressed by DCs. Top panel: Molecular structures of the different microorganisms called pathogen associated molecular pattern (PAMPs) that can be recognized by phagocytes. Bottom panel: strategic surface/intracellular localization and molecular targets of non-opsonic pathogen recognition receptors (PRR) (CLR, NLR, RLR, TLR; see figure for explanations) and opsonic Fc (FcR) and complement (CR) receptors (adapted from Rossana Zaru [52]).
Fig. 3: Schematic signaling programs initiated by TLRs, opsonic receptors (e.g., FcR) or direct phagocytic cargo binding by cell-surface integrins. Left-hand panel: Upon recognition of microbial ligands, TLRs initiate signaling via cytoplasmic tails by direct recruitment of adaptor molecules such as TRIF or MYD88 and by secondary adaptors such as TIRAP (for MYD88) and TRAM (for TRIF). Adaptor molecules can initiate the following pathways: MAP kinase (JNK, p38 and ERK), NFκB and IRF. Translocation of transcription factors leads to different immune response programs such as altered cytokine or chemokine production, expression of anti-microbial components and enhancing the antigen uptake and presentation. Middle panel: Antigens on the surface of pathogens or tumor cells can be opsonised via immunoglobulins (antibodies) of the humoral immune defense. The antibody Fc-region of the heavy chain can be recognized by Fc-receptors (FcR) triggering phosphorylation of the cytoplasmic tail, leading to the docking of Syk, activating multiple targets including phosphoinositide 3-kinase (PI3K). PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃) from phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) and creates a platform for Rac recruitment. Rac requires active guanine nucleotide exchange (GEFs) to trigger WAVE activation. After that, Cdc42 GTPase can be recruited to phago/endosomes and activate WASP. WAVE and WASP are essential for Arp2/3 activation orchestrating F-actin nucleation and branching, triggering membrane rearrangements and providing pulling forces for endo/phagocytosis. Syk and PI3K can also trigger phospholipase C by (PLCγ) recruitment, which activates downstream kinases or leads to Ca²⁺ release from the endoplasmic reticulum (ER), triggering calcium-dependent fusion of endomembranes with endo/phagosomes. Right-hand panel: Interaction of integrins (e.g., αMβ2 integrin recognizing C3bi opsonins or fibrinogen) with extracellular targets can lead to recruitment of focal adhesion kinase (FAK) and scaffold proteins, such as talin, kindlin, ILK, PINCH, parvin and paxillin, leading to actin polymerization. Syk activating signals from other receptors (FcR) can contribute to integrin-mediated phagocytosis.

Phagocytic cup formation for antigen uptake

The engagement of pathogens by DCs leads to phagocytic cup formation for antigen uptake. The first moments of particle engulfment rely on the deformations of the plasma membrane of the DC and on the formation of pseudopod-like extensions leading to the creation of the phagocytic cup [61-63]. This leads to engulfment of the microbe, eventually followed by the closure of the growing cup triggering movement of the phagosome into an intracellular space. During microbial cargo engagement, the membrane receptors undergo rapid rearrangements for creating necessary mechanical forces for cargo uptake [8, 64, 65].

The lipid content at the plasma membrane of the DC interface with the pathogen can drive internalization events. In has been shown that an increase of anionic lipids such as phosphatidylserine (PtdSer) in the membrane may drive a spontaneous curvature promoting endocytosis [66]. The membrane composition is also important for phagocytosis, as it undergoes multiple changes during microbe uptake [12]. Phagosomal lipid composition might differ between opsonic and non-opsonic type of receptors engaged with phagocytic cargo. One factor contributing to this change in lipid composition is the focal fusion of intracellular vesicles at the phagocytic cup, which provides a source of membrane for internalization of large cargo, although the source of these vesicles is still debated and might be either of recycling or late endosomal or even lysosomal origin [67]. A second factor contribution to the changing lipid composition are phosphoinositide kinases and phosphatases, and for instance phosphatidylinositol 3-kinase is essential for large cargo ingestion (≥ 0.5 µm) [68] but is dispensable for smaller particles [69, 70]. The dynamic changes of phosphoinositide content on phagosomes and pinosomes are well-described [9]. Many pathogens developed virulence factors targeting phosphatidylinositol metabolism in the host [71].
Another important component of phagocytic cup formation is F-actin polymerization driving the formation of membranous extensions of the phagocytic cup. The cascade of actin polymerization into filamentous F-actin can differ depending on the type of receptor involved and is tightly linked to phosphoinositide metabolism. F-actin polymerization is also targeted by pathogens that developed specific toxins interfering with F-actin polymerization pathways for enhancing intracellular invasion [72]. The phagosomal phosphoinositide content and F-actin rearrangements in relation to antigen uptake and maturation are summarized in (Fig. 4).

Fig. 4: Scheme of phosphoinositide metabolism during phagocytosis (based on [9, 72]). From left- to right-hand panel: Upon recognition of phagocytic cargo (e.g. via engagement of FcR or C-type lectin receptors), the concentration of P(4,5)P₂, present in the inner monolayer of the plasmalemma (PM) will increase at the base of the phagocytic cup and triggers initial steps of Rac1 and Cdc42 activation. Receptor clustering at the target interface can lead to a burst of tyrosine kinase activity, activating PI3K and displacing P(4,5)P₂ with P(3,4,5)P₃ at the basis of the phagocytic cup. This leads to further activation of lipid kinases and recruitment of adaptors and GEFs capable of activating Rac, promoting the further remodeling of the cytoskeleton, eventually driving F-actin-dependent pseudopod extension engulfing the prey. Vesicle secretion at the phagocytic cup provides the material for membrane extension. Shortly before closure of the phagocytic cup, the phagosome will acquire P(3,4,5)P₃, a signaling lipid capable of recruiting the F-actin adaptor SWAP70 which further anchors F-actin to the phagosomal membrane. Lastly, after cup sealing and complete internalization, the phagosome is detached from the PM via various lipid phosphatases and GAPs. Early endo/phagosomes are characterized by the presence of P(3)P guiding assembly of the NADPH oxidase NOX2 and acquisition of early markers such as EEA1 and Rab5. Endo/phagosomal PI3P is converted into P(3,4,5)P₃ by the kinase PIKfyve, which is a necessary step for cation channel TRPM1-activation promoting fusion with lysosomes and acidification.

NOX2 is a large multiprotein complex which transfers electrons from the cytosolic electron donor NADPH to O₂, thereby generating reactive oxygen species (ROS) which create a microbicidal environment within the phagosome [73] and control the activation status of proteases [42]. Phagosomal acidification is triggered by v-ATPase recruitment (reviewed [74]). Pro-inflammatory M1 and anti-inflammatory M2 macrophages have significant differences in NOX2 recruitment and phagosomal acidification rates [75]. The M1 phenotype is promoted in a pro-inflammatory environment such as upon infection, i.e. the presence of TLR stimuli or interferon(INF)-γ secretion. M1 macrophages are destined to quickly clear infections and have an enhanced antigen presentation. The M2 phenotype serves homeostatic function such as apoptotic cell clearance inflammation suppression, tissue repair and immunity to helminths [75, 76]. The differentiation of
M2 phenotype macrophages is triggered via activation of the STAT6 signaling pathway through interleukin(IL)-4 and IL-13.

**Phago/endosomal maturation**

Similarly to the endocytic pathway, phagosomal maturation can be divided into early, late and lysosomal stages. Rab GTPases facilitate this maturation process. Rab5 is a key marker of early phagocytosis that precedes the late Rab7-positive stage. Rab5 recruits endosomal early antigen 1 (EEA1) and Vps34 type III phosphatidylinositol 3-kinase, which generates PI(3)P. Later Rab5 is replaced by Rab7 in a process that requires recruitment of Rab7-GEFs [77], but the precise mechanism of this process is not completely clear [61]. Rab7 can recruit many effectors that guide vesicular transport over microtubulae in both antero- and retrograde directions. PI(3)P can also recruit PIKfyve via its FYVE-domain, which leads to production of PI(3,5)P₂, that activates the calcium-permeable cation channel TRPML1 and plays an essential role in phago/endosomal acidification and transport. Phago/lysosomal maturation is dependent on the presence of LAMP1 and 2, as it has been shown that in knock-outs lacking LAMP1 and 2, the Rab5 positive phagosomes fail to recruit Rab7 and fuse with lysosomes [78]. It was shown that individual phagosomes in DCs and monocytes behave autonomously both in terms of cargo degradation and antigen presentation. It was shown that IgG-opsonization cause faster degradation and presentation of the cargo as opposed to phagosomes with non-opsonised cargo from the same cell [30].

Phagosomal maturation can utilize components of the autophagy machinery. Such phagosomes are called LAPosomes and they may mediate the fusion with lysosomes [79]. A hallmark of LAPosomes is the direct recruitment of the key autophagosomal regulator LC3 to the phagosome. It is believed that the autophagy pathway may mediate antigen presentation but is not universally required for phagosomal maturation [80]. Phagocytic uptake of fungal pathogens via Dectin-1 was shown to cause LC3 recruitment to the phagosome and this was linked to fungal antigens presentation on the surface of MHCII but not MHC1 [81], although mechanistic details of the LAPosome pathway can differ between bacterial, fungal and parasitic microorganisms [82]. On the other hand, autophagy can also prevent immune detection and facilitate pathogen survival, and for instance some microorganisms can escape phagosomes and reside in autophagocytic compartments [83].

**Medical conditions and phagocytosis**

Several mutations in phagosomal/endosomal proteins are related to human disease. X-linked Wiskott–Aldrich syndrome, caused by mutations in the protein WASP is characterized by an inability to trigger F-actin polymerization at the phagocytic cup. This disease is associated with eczema, low platelet count and immune deficiency [62, 63, 84]. In chronic obstructive pulmonary disease (COPD), macrophages are characterized by a significantly reduced phagocytic capacity of bacteria and apoptotic cells [85]. Mutations in synaptotagmin-11 (Syt11) were found to be associated with impaired cytokine secretion and phagocytosis in microglia, contributing to the
development of Parkinson disease and schizophrenia [86]. Retinal pigment epithelium (RPE) cells are cells supporting homeostasis of the retina within the eye by phagocytosing photoreceptor outer segments (POS), and this is important for photoreceptor renewal and regeneration of photobleached visual pigments. It is suggested that deregulation of this process may result in a disease age-related macular degeneration (AMD) which can manifest into blindness [87]. Fc-independent phagocytosis of platelets by macrophages and neutrophils was shown to be crucial at thrombocytopenia prevention [88].

Phagocytosis is an important target in medicine development, as nano-particle drug carriers can be ingested by cells to combat a range of diseases such as HIV [89], tuberculosis [90] and cancer [91, 92]. A challenge is the controlled targeting of such drug carriers to affected cell types, as a possible culprit is that nano-particles can be cleared via phagocytosis as foreign particles [93, 94]. Overall, in recent years nano-particle therapy approaches have experienced unprecedented growth. Understanding the mechanisms of their uptake and processing may provide new ways to improve these therapies and allow insights in the adverse effects of nano-particle formulations, such as toxicity or inefficiency at triggering immune responses.

Podosomal protrusions: novel role in antigen uptake
Podosomes are mechanosensitive structures found in many cells types. In microscopy experiments in DCs and macrophages, podosomes were identified as solid cores of F-actin surrounded by a ring of adaptor proteins [95]. However, when placed on soft 3D surfaces, podosomes were shown to have the ability to protrude and degrade extracellular matrix [96]. The function of such protrusions was not completely understood. We utilized confocal and EM microscopy to demonstrate that DCs retain endocytic activity at the tip of the podosomal protrusions allowing them to sample antigens through endothelial and epithelial membranes [97, 98].

Fig. 5: Human monocyte derived DCs can form solid-core podosomes when cultured on stiff substrates. Top right panel: podosomes consist of a solid F-actin core surrounded by a ring of adhesion proteins (vinculin, talin, paxillin(pink)) and integ-
grins (blue) anchoring F-actin filaments to the plasma membrane. When encountering certain physiological cues or permissive substrate stiffness, podosomes can protrude and facilitate antigen sampling through epithelial and endothelial barriers. Antigen recognition receptors such as C-type lectin receptors (CLR; blue) can migrate to the tip of the protrusions and acquire antigens.

**Scope of this thesis**
The work outlined in this thesis is dedicated to understanding the routes of antigen uptake and processing by DCs with the emphasis on the role of a particular class of signaling lipids – phosphoinositides – that tightly control phago/endosomal life from uptake to degradation as well as subsequent antigen presentation on the DC surface.

We first discuss the state-of-the-art knowledge on membrane trafficking in phagocytes based on super-resolution microscopy techniques (such as STED, PALM, STORM). In chapter 2 we provide an overview on breakthrough discoveries in membrane trafficking encompassing topics from organization of the plasma membrane to cytoskeleton arrangement and dynamics in endocytosis, phagocytosis and immunological synapse formation. We focus on identifying key areas in organellar trafficking where super-resolution microscopy is a required approach for addressing long-standing questions.

In chapter 3 we addressed the role of the understudied and short-lived PI(3,4)P₂ in antigen uptake via phagocytosis by DCs. Initial quantitative mass-spectrometry data on phosphoinositide-binding proteins published in [99] identified SWAP70 as a potential interaction partner of PI(3,4)P₂. We confirmed with imaging experiments that SWAP70 is a strong binder to PI(3,4)P₂ in phagocytic cups during early stages of phagocytosis. SWAP70 was not found in other F-actin-dense structures such as podosomes, confirming previous observations [100]. 3D super-resolution STED imaging revealed that SWAP70 aligns along parallel arches or rings of F-actin filaments together with the Rac1 GTPase at the phagocytic cup. Mutational analysis and knock-down of SWAP70 or pharmacologically-induced perturbation of PI(3,4)P₂ production in human moDCs revealed a key role for SWAP70 in Rac1 activation, F-actin formation and phagocytosis. Thus, we identified a novel PI(3,4)P₂ binder which as an adaptor for F-actin tethering on the phagosome during a short time frame of phagocytic cup formation necessary for successful antigen uptake.

In chapter 4 we extended our conclusions about the role of SWAP70, testing it not only in human monocyte-derived DCs, but also in other DC subsets such as pDCs and mDCs. We also confirmed the universal role of SWAP70 in phagocytosis in mouse bone-marrow derived DCs (BMDCs) subsets generated with FLT3 and GM-CSF and in the mouse macrophage cell line RAW264.7. We present additional data demonstrating the role of SWAP70 in controlling F-actin at the phagosome, but not at the immunological synapse where DCs come in contact with T-cells [101]. We further validated the role of SWAP70 by inhibiting its function with the small molecule inhibitor sanguinarine, strengthening our findings observed with SWAP70 knock-down and mutational analysis in chapter 3. Additionally, we observed that active SWAP70 does not only sustain F-actin
formation, but also blocks activation of the F-actin severing factor cofilin.

In chapter 5 we looked into later maturation events of phagosome transitioning from early to late phagosomes and we addressed the role of the phosphoinositide kinase PIKfyve in this process. PIKfyve is the only kinase in the cell capable of producing PI(3,5)P$_2$. This is another understudied phosphoinositide species which is crucial for converting late phagosomes into phagolysosomes characterized by low pH and elevated protease activity such as cathepsins. We showed that pharmacological inhibition of PIKfyve resulted in blockage of phagosomal maturation and led to the prolonged presence and elevated activity of NOX2. This was accompanied by higher levels of ROS which resulted in reduced activity of cystein cathepsins. We investigated how blockage of PIKfyve and lower levels of cathepsin activity affect antigen degradation and presentation at MHC class II. Additionally, we developed an alternative bio-orthogonal MHC class II presentation assay to trace viral hemagglutinin presentation within MHCI directly at the DC surface. Our experiments reveal an important role of PIKfyve in antigen degradation within phago/endolysosomes leading to antigen presentation within MCH class II triggering adaptive immunity.

In chapter 6 we identified a novel role of podosomes in antigen uptake. Podosomes are found in different cell types and are well-understood to perform mechanosensitive functions for guiding cell migration through the extracellular matrix and endo/epithelial barriers. Our study was inspired by observations that the dense cores of podosomes can form protrusive structures when encountering soft spots on substrates [80], raising the possibility of a potential role in antigen sampling. Indeed, our experiments revealed that DCs can utilize these podosome-derived protrusions for receptor-mediated endocytosis and thereby sample for antigens across extracellular matrix, trans-epithelial and trans-endothelial membranes.

In a short study presented in chapter 7, we further strengthen the protrusive nature of podosomes, and demonstrate the presence of various endocytic and exocytic markers at protrusive podosomes.

Finally, in chapter 8 we discuss our results and relate our observations to currently available data in the literature. We discuss possible new directions in phosphoinositide research with a focus on the possible applications of super-resolution microscopy and novel biorthogonal labeling techniques. Finally, findings outlined in this thesis are summarized in an overview figure allowing us to trace the unique and complex mechanisms of antigen uptake and processing by antigen-presenting cells before presentation to T-cells. These fundamental discoveries allow a further understanding of what routes of phosphoinositide metabolism are crucial for endo/phagocytic functions of DCs and might provide a mechanistic explanation for the findings from several clinical trials that inhibitors of phosphoinositide-related proteins such as apilimod or YM201636 have an overall toxic effect.
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Chasing Uptake: Super-Resolution Microscopy in Endocytosis and Phagocytosis
Abstract
Since their invention about two decades ago, super-resolution microscopes have become a method of choice in cell biology. Owing to a spatial resolution below 50 nm, smaller than the size of most organelles, and an order of magnitude better than the diffraction limit of conventional light microscopes, super-resolution microscopy is a powerful technique for resolving intracellular trafficking. In this review we discuss discoveries in endocytosis and phagocytosis that have been made possible by super-resolution microscopy – from uptake at the plasma membrane, endocytic coat formation, and cytoskeletal rearrangements to endosomal maturation. The detailed visualization of the diverse molecular assemblies that mediate endocytic uptake will provide a better understanding of how cells ingest extracellular material.

Highlights
• Super-resolution microscopy is widely available to most cell biologists and has become the method of choice for studying endocytic and phagocytic trafficking.
• Current live imaging techniques allow 3D super-resolution microscopy with a temporal resolution of seconds to visualize uptake and trafficking processes at the whole-cell level.
• Quantification strategies combined with super-resolution microscopy have enabled a quantitative understanding of receptor clustering, the appearance of signaling lipids, and the dynamics of F-actin assembly on endosomes and phagosomes.
• Super-resolution microscopy is often combined with techniques such as electron microscopy and TIRF to enable more accurate localization of endocytic proteins in relation to stage of uptake, membrane curvature, and protein–protein interactions.
• Although generally of lower spatial resolution compared with other super-resolution microscopy techniques, structured illumination microscopy has at present yielded most new insights in endocytic trafficking because it combines improved spatial resolution with a reasonably high temporal resolution and can be readily used for live cell imaging applications.

Outstanding Questions
• How are receptors organized in the membranes of organelles to facilitate sorting of cargo and trafficking proteins for different intracellular destinations?
• What is the precise role of the F-actin cytoskeleton for different types of endocytosis?
• What is the role of calcium for membrane fusion and fission in organelar trafficking? How is membrane fusion and fission coupled in organelar trafficking?
• How do membranes bend in endocytosis and organelar trafficking processes that do not involve known cage proteins?
• How do lipid turnover and transfer at membrane contact sites between endosomes, the ER, and the plasma membrane contribute to endosomal maturation?
• Nearly three-quarters of the 70 members of the Rab family in humans are involved in endocytic trafficking, and they play a role in endosomal maturation, coordination of vesicle
Chasing Uptake: Super-resolution Microscopy in Endocytosis and Phagocytosis

budding and fusion, effector recruitment, and coordination of endosomal signaling. What are their precise localizations, interactions, and stoichiometries?

Early Visualization of the Cellular Trafficking System
The mystery of how cells organize endocytic trafficking system has been extensively studied [1–4]. Endocytosis and phagocytosis (reviewed in [5]) are evolutionarily conserved mechanisms of internalization of solutes or small particles (endocytosis) or large particles sized above ~0.5 μm (phagocytosis). Insight into endocytic and phagocytic trafficking provides understanding of disease mechanisms because they are essential for many physiological processes, including nutrient uptake, immune clearance of pathogens, and the removal of apoptotic cells [3]. They are highly dynamic processes that begin with clustering of specialized membrane receptors at the site of uptake [6], followed by the assembly of coat proteins such as clathrin (endocytosis) or the engulfment of a particle by rapid actin rearrangements into protrusive pseudopodia (phagocytosis). Alternatively, many receptors are captured by pre-existing clathrin sites, thereby facilitating their uptake. Endocytosis and phagocytosis are orchestrated by the membrane composition, coat proteins, the F-actin (see Glossary) cytoskeleton, and membrane fission proteins such as dynamin [7]. After their formation, endosomes and phagosomes undergo a mechanistically similar maturation process in which they are transported via microtubules to the microtubule organizing center (MTOC) and gradually convert into late endo/phagosomes and finally into lysosomes [8].

Our current understanding of the nanoscale organization and morphology of endosomes and phagosomes stems mainly from electron microscopy (EM). From this technique, we know that cells are very crowded, containing organelles of sizes ranging from tens of nanometers for small trafficking vesicles to up to several micrometers for the nucleus and large phagosomes (Figure 1A). Moreover, many organelles have a highly complex and dynamic morphology, such as the heterogeneous network of recycling endosomes. However, EM can only be used on fixed or frozen material and cannot follow dynamic processes in living cells, making this technique unsuitable for research questions related to dynamic alterations and stoichiometries of molecular assemblies. Live cell imaging is feasible with optical microscopy, but, owing to their small size, individual organelles are often impossible to resolve with conventional optical microscopy. Starting about two decades ago, the development of super-resolution microscopy techniques, including stimulated emission depletion (STED) (Figure 1B), single-molecule localization microscopy (SMLM), structured illumination microscopy (SIM), and lattice light sheet microscopy (LLSM), is helping to overcome this problem (Box 1 for more information on each super-resolution technique) [9–16]. It is therefore no surprise that these techniques have provided crucial information on trafficking, particularly on exocytosis in neurons, neuroendocrine cells, and immune cells [14,17,18]. This review aims to provide an overview of the breakthrough discoveries that have been enabled by super-resolution microscopy specifically in endocytosis and phagocytosis.
Organization of Endocytic Receptors at the Plasma Membrane

The clustering of endocytic receptors in distinct regions of the plasma membrane allows the localized uptake of material. SIM microscopy revealed that receptor-mediated caveolar and clathrin-coated vesicular uptake and nonspecific endocytosis occur at separate locations on the plasma membrane [19–21]. The spatial clustering and segregation of these different forms of endocytosis likely enable more efficient uptake and might facilitate the differential sorting of cargoes into different types of endosomes. In addition, receptor clustering can promote downstream signaling, for instance by oligomerization of kinase domains. Indeed, a SIM microscopy study in human macrophages showed that the Fc receptor FcγRIIA must diffuse laterally to become activated upon clustering by multivalent targets [22]. SMLM revealed that such clusters are approximately ~70 nm in size and contain ~100 Fc receptors [23], and that they locate adjacent to clusters enriched in the inhibitor of phagocytosis signal regulatory protein α (SIRPα) [24]. SMLM microscopy revealed that two other endocytic receptors, DC-SIGN and the mannosereceptor CD206, both organize in similarly ~80 nm sized domains which, upon binding to fungal pathogens, merge into larger disc-shaped clusters of 150–175 nm in diameter, and this facilitates the ingestion of the pathogen [25–28] (Box 2). Toll-like receptor 4 (TLR4) is also present in nanodomains of 60–80 nm in size, and it was demonstrated by SMLM that its oligomeric state is controlled by its ligand endotoxin and its coreceptors MD2 and CD14 [29,30]. Finally, a SMLM study of endocytic cargo revealed that the vesicular acetylcholine transporter rapidly diffuses over the membrane until it is trapped for uptake by a membrane structure that contains clathrin and its adapter protein AP2 [31].

Role of Lipids in Receptor Clustering

The combination of SMLM and total internal reflection fluorescence (TIRF) microscopy enables the tracking of single molecules in time, and revealed that membrane clustering of glycosylphosphatidylinositol (GPI)-anchored receptors in the outer leaflet of the plasma membrane is promoted by transmembrane coupling to the long acyl-chain lipid phosphatidylserine in the inner leaflet [32,33]. Phosphatidylserine directly interacts with F-actin adaptors and thereby anchors the outer-leaflet receptors to the cortical actin cytoskeleton [32,33]. Several other lipid species can also contribute to receptor clustering. First, cholesterol-mediated lipid phase separation is involved in clustering of GPI-anchored proteins [33,34]. Second, domains with lactosylceramide (LacCer), a glycosphingolipid present in immune phagocytes that contributes to integrin-mediated phagocytosis, are separated from domains containing another abundant phosphatidylinositol 4-phosphate (PtdGlc) [35]. Finally, domain formation of the WNT receptor LNP6, clathrin, and its adaptor AP2 depend on coclustering with phosphatidylinositol 4,5-bisphosphate [P(4,5)P2] lipids [36]. Such clustering of phosphoinositides in membrane domains has been reported for several species. For instance, a SMLM study revealed that phosphatidylinositol 4-phosphate (P4P), phosphatidylinositol 4,5-bisphosphate [P(4,5)P2] and phosphatidylinositol 3,4,5-triphosphate [P(3,4,5)P3] are all present in domains of about 350–400 nm diameter in the plasma membrane of insulin-secreting INS-1 cells [37]. Membrane domains of phosphoinositides can be highly specific...
because plasma membrane domains containing \(\text{PI}(4,5)\text{P}_2\) can segregate from domains enriched in \(\text{PI}(3,4,5)\text{P}_3\) [38].

The lateral diffusion of uptake receptors can be obstructed by transmembrane proteins (so-called pickets), such as CD45 [22,39], that are immobilized by interactions with the cortical cytoskeleton. STED microscopy showed that the extracellular domain of CD45 can bind to hyaluronic acid which forms a pericellular coat and, together with the actin cytoskeleton, limits the mobility of phagocytic Fc receptors [22,39]. Such remodeling of the actin fence is necessary to enable Fc receptor clustering and the initiation of phagocytosis [22,39]. Remodeling of cortical actin is also essential for some forms of endocytosis [7] because a SIM study showed that actin rearrangements at the mammalian growth cone of neuronal axons by the F-actin bundling protein fascin are essential for endophilin-mediated endocytosis but not for clathrin-mediated endocytosis [20].

Thus, the clustering of endocytic receptors results from the interactions with various lipid species, the cortical cytoskeleton, and pericellular glycosylation. In this respect, it is similar to the clustering of proteins involved in exocytosis in distinct domains of the plasma membrane, particularly soluble...
N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) (reviewed in [17]). In the future, the increasing number of colors that can be simultaneously resolved by super-resolution microscopy [40] will facilitate the characterization of the different types of membrane domains enriched in endocytic and phagocytic receptors. A largely unexplored question is how receptors are precisely organized in intracellular membranes of organelles such as early endosomes, where STED revealed cholesterol-dependent clustering of SNARE proteins [41].

Endocytosis

Clathrin Coat Formation

Many super-resolution microscopy studies have aimed to resolve the localization, shape, and time-dependent localization of clathrin and clathrin-associated proteins (Figure 2) [42–44]. For instance, SMLM in combination with TIRF was used to visualize the different topologies of curved clathrin-coated pits and flat clathrin plaques [44]. SIM microscopy revealed that mature clathrin-coated pits assemble as rings of about ~100 nm in diameter that persist for seconds to minutes and are associated with the actin cytoskeleton [45]. Two correlative light-electron microscopy (CLEM) studies mapped the arrangement of multiple endocytic proteins during various assembly stages of the clathrin coat, and showed that many proteins are primarily located near the edge (e.g., FCHO1/2, Eps15, dynamin) or the center (epsin, CALM, receptor cargo) of the clathrin lattice (Figure 2) [46,47]. Pulse-chase approaches in yeast and mammalian cells also allowed the localization of endocytic proteins to be resolved during various stages of endocytosis, and revealed that the actin-recruiting protein N-WASP is organized radially in a ring-like pattern during the early stages of clathrin pit formation, and that interactions between the clathrin coat, N-BAR proteins, and phosphoinositides orchestrate F-actin positioning at the endocytic site (Figure 2) [48–51]. Nevertheless, the role of actin in clathrin-mediated endocytosis remains controversial. Although actin might only be necessary to limit the lateral movement of endocytic vesicles in yeast [48], F-actin is already present at the base of the forming clathrin-coated vesicle [49,51], and an F-actin peak can be observed following dynamin recruitment [7,52], arguing for a function in vesicle internalization. Accordingly, a high-throughput super-resolution SMLM study in yeast showed that WASP family proteins form a ring-shaped pattern on membranes for future F-actin nucleation, and that this drives membrane invagination [51]. The latter seems to be also the case in mammalian cells because a SIM microscopy study in murine fibroblasts showed that actin can accelerate endocytosis and that clathrin-coated pit internalization is aided by actin filaments in about half of the cases [45]. A combination of TIRF and CLEM was used to visualize how clathrin coat assembly produces membrane curvature, which is the rate-limiting step for vesicle formation (Figure 2) [47]. It was found that clathrin coat assembly is versatile and accommodates membrane bending concurrently with or after the assembly of the clathrin lattice, and this bending may be regulated by axillary proteins such as CALM [47].

The process following vesicle fission and shedding of the clathrin coat has been addressed by SMLM, where it was found in yeast that the levels of phosphatidylinositol 3-phosphate (PI3P) at
internalized endocytic vesicles increased 100-fold after clathrin coat shedding [53]. The yeast homolog of the early endosomal GTPase Rab5 (Vps21p) located to smaller endosomes (~80 nm-sized) at the initial stages of PI3P production, whereas the homolog of late endosomal Rab7 (Ypt7p) bound to larger endosomes (~170 nm) with more saturated PI3P levels [53]. Despite this increase in PI3P levels following endocytosis and during endosomal maturation, PI3P may already play a role during the uptake itself and in recruitment of endocytic cargo. SMLM on cultured mouse cortical neurons showed that the PI3 kinases ATM and ATR play a role in synaptic vesicle recycling and colocalize with the clathrin adaptor protein AP2, with ATR being recruited to nascent inhibitory (VGAT) and ATM to excitatory (VGLUT) vesicles [54]. Moreover, LLSM in SUM159 breast cancer cells expressing probes that bind clathrin and phosphoinositides revealed a new cascade of phosphoinositide lipids after fission of clathrin-coated vesicles. A pool of PI3P was already present before phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2) and Rab5, and this pool was distinct from the PI3P pool of early endosomes [55].

Fig. 2: Coupling Membrane Curvature to Nanoscale Localization of Endocytic Proteins. (Upper panel) Electron microscopy (EM) [46,47], atomic force microscopy (AFM) [47], or polarized total internal reflection fluorescence microscopy (pol-TIRF) [47] have been used to determine the curvature of endocytic membranes. (Lower panel) Super-resolution imaging using stimulated emission depletion (STED) [50], structured illumination microscopy (SIM) [46,50], or single-molecule localization microscopy (SMLM) [43,44,46] can be coupled to the membrane curvatures determined with the methods from the upper panel. This allowed the positions of endocytic proteins (EPS, CALM, BAR-proteins, dynamin, F-actin) to be mapped during various stages of endocytosis [46,47,50].

**Caveolin Coat Formation**

Super-resolution microscopy has also allowed new insights into another endocytic coat: caveolin. Although the structural organization of caveolar structures was already well understood from EM [56,57], super-resolution studies offered the possibility to image caveolae under various physiological challenges. For instance, SMLM revealed that caveolae under osmotic stress flatten and increase by ~30% in volume [58]. Although SIM and SMLM showed that caveolin oligomers
can assemble in structures with different shapes (~60–80 nm diameter) [59], individual caveolae might be able to cluster in much larger (~300 nm) ribbon-like assemblies [45]. The shape and size of these caveolar structures depend on the isoform of caveolin because flask-shaped invaginating caveola are fourfold more densely packed with caveolin-1 than are flat caveola, and the density of caveolin-1 is sensitive to osmotic tensions [60]. Super-resolution microscopy also revealed signaling-mediated receptor rearrangements at caveolae, as shown by SIM for the purinergic receptor P2X7R in osteoblasts [61], by CLEM and STED for the Ca\(^{2+}\) release channel ryanodine receptor at the sarcoplasmic reticulum in muscles [62,63], and by STED for the inward rectifier potassium channel Kir2.1 in human cardiac ventricles [64].

**Noncanonical Endocytosis**

Endocytosis can also occur in unconventional pathways that do not depend on clathrin and caveolin. Endophilins are members of the BAR domain superfamily and are involved in noncanonical endocytosis in neuronal [20] and nonneuronal cells [65]. In the axon growth tips (cones) of neurons, SMLM revealed a novel form of clathrin-independent endocytosis that relies on endophilin-3 and dynamin-1, and that correlates with fascin-1-dependent F-actin bundling at the endocytic sites, and F-actin was required for retrograde trafficking of the presynaptic proteins VAMP2, SCAMP1, SV2, and synaptophysin [20]. Endophilins-A1 and A2 interact with several endocytic proteins, including dynamin, synaptojanin, and lamellipodin, and also with endocytic cargo proteins via their N-BAR or SH3 domains, leading to uptake of G protein-coupled protein receptors, receptor tyrosine kinases, and the interleukin-2 receptor [65]. Moreover, SIM revealed that endophilin-A2 reshapes membranes and, together with F-actin and dynamin, contributes to membrane scission during clathrin-independent uptake of Shiga and cholera toxins [66].

Endocytosis can be coupled to exocytosis, and this enables the rapid recycling of exocytic trafficking proteins and cellular homeostasis. STED revealed that hemifused intermediates (in which the membrane outer leaflet, but not the inner leaflet, is fused) and sometimes fully fused vesicles are able to fluctuate back to the fully separated or hemifused state [67]. This reversible transition likely resulted from a competition between membrane fusion and Ca\(^{2+}/\)dynamin dependent membrane fission [67]. Other studies provided evidence for a role of dynamin in this coupling of endocytosis with exocytosis. By SIM, it was shown that dynamin controls fusion pore kinetics, promoting closure of the fusion pore, vesicle detachment from the plasmalemma, and internalization [68]. STED microscopy showed that dynamin in conjunction with the F-actin cytoskeleton led to the formation of transient fusion pores with sizes ranging up to 490 nm [69], much larger than was previously thought (<5 nm) [70,71].

**Multivesicular Body Formation**

Early endosomes mature to form multivesicular bodies where the membrane is bulged into the lumen of the endosomes by the endosomal sorting complex required for transport (ESCRT) complex. This results in the formation of intraluminal vesicles which play a role in the degradation
of integral membrane proteins [72]. By LLSM, it was shown that ESCRT-III subunits polymerize rapidly on yeast endosomes, together with the recruitment of at least two hexamers of the AAA ATPase Vps4 [73]. Intraluminal vesicle formation depended on ATPase activity of Vps4 and was associated with a continuous and stochastic exchange of Vps4 and ESCRT-III components [73].

**Phagocytosis**
After a cell encounters a particle, a phagocytic synapse is established by formation of F-actin rich filopodia-like extensions from the membrane which engulf the particle [4]. This structure is called the phagocytic cup and creates a physical barrier for organizing the signaling cascade induced by target binding [22]. Arp2/3 complex-mediated F-actin nucleation and branching at the nascent phagosome is triggered by the GTPases Rac1, Rac2, and Cdc42, although the contribution of this actin remodeling depends on the type of phagocytosis. In case of Fc receptor-mediated phagocytosis, but not for complement C3bi receptor-mediated phagocytosis, a super-resolution microscopy study showed that branching of F-actin within lamellipodia depends on Arp2/3 [74]. By SIM on macrophages derived from Cdc42 knockout mice, it was shown that Cdc42 is dispensable for the formation of filopodia (finger-like protrusions) upon uptake of yeast particles, but is necessary for the subsequent formation of lamellipodia (the F-actin meshwork at the leading edge of a migrating cell) with parallel actin bundles during cell spreading [75].

STED microscopy was used to resolve the organization of Rac1 and actin during phagocytosis of yeast cells by dendritic cells [76]. It was found that F-actin, Rac1, and their binding protein SWAP70 align in parallel arches and sometimes in concentric rings on the surface of phagosomes [76]. In this case SWAP70 acted as an adapter, tethering the F-actin cytoskeleton to the phagocytic surface by interacting with Rac1 and phosphatidylinositol (3,4)-bisphosphate. Although structures such as actin rings or radial actin bundles for parasite internalization into host cells can already be visible by conventional confocal microscopy [77], the increased resolution of SMLM allowed the organization of such structures to be resolved and addressed the bundling of actin filaments, such as for the more accurate measurements of the dimensions of the 100 nm-sized F-actin ring during *Salmonella typhimurium* phagocytosis [78]. Although the nature of these actin rings is still unknown, they could be mediated by septins [79]. Septins control receptor and phospholipid dynamics and can form ring-like structures by bundling actin via anillin and myosin binding at the site of pathogen invasion in mammalian cells [79], structures that are reminiscent of the structures observed during phagocytosis [76]. Moreover, SMLM revealed that septins can form cables with a thickness of 20–30 nm in complex with Cdc42 and Gic1 in vitro [80]. However, in cells, high-resolution microscopy data on septin collars are only available for prokaryotes and budding yeast, where septins are involved in cell division and budding [80], and it would be interesting to resolve potential septin rings in phagocytosing cells by super-resolution microscopy.

**Future Technical Improvements Needed**
Super-resolution microscopy has led to important new insights in endocytic trafficking, but some
technical improvements are highly desirable.

**Improving Spatial Resolution**

Most of the current super-resolution microscopy techniques that are widely used by biologists offer a spatial resolution between 20–60 nm lateral and about 100 nm axial, but this axial resolution is still too low to resolve complex organelle morphologies and individual endosomes. Isotropic imaging, with identical resolution in all dimensions, would be beneficial because it allows the shape and localization of 3D cellular structures to be resolved. To address this problem, new microscopy developments are increasingly improving the spatial resolution, such as MINFLUX that allows 1 nm lateral and axial resolution in live cells [81]. However, at such high microscope resolutions the size of the fluorescent probes becomes the limiting factor (Figure 3). Antibodies are several nm in size, and, although this has a negligible influence in conventional optical microscopy, it is becoming a major problem in super-resolution microscopy because it limits the spatial resolution (Figure 3) [82]. The same holds true for the ~3 nm-sized fluorescent proteins and protein tags that can specifically bind to organic fluorophores (e.g., HaloTag [11]). Nanobodies, that contain only a single antigen-binding fragment, may provide a partial solution to this problem because they are about twofold smaller than a full-size antibody, and the creation of a library of nanobodies with high specificities for mouse and rabbit IgGs is an important development [83].

Alternative approaches to replace the large fluorescent proteins are fluorescent non-natural amino acids (reviewed in [21,84,85]). The need for smaller probes has become evident in super-resolution imaging studies showing that the glutamate receptor AMPAR clusters in dynamic and transient membrane domains, instead of in the more stable domains previously observed by antibody-based labeling [86–88]. However, even organic fluorophores have a minimum physical size limit and likely cannot be made much smaller, particularly for the conjugated ring systems needed for red fluorescent dyes. Therefore, in the long run, the size of the fluorophores will become limiting for the spatial resolution of fluorescence microscopy, and other (nonfluorescent) methods would need to be developed to reach even higher spatial resolutions.

**Improving Spectral Resolution**

Most current super-resolution microscopy techniques have typically only two colors that can be discerned simultaneously. One of the reasons for this is that, in SMLM and STED, one channel often requires more than one spectral input – the first for image acquisition and the second for fluorophore switching (localization techniques in SMLM) or because of the doughnut shape of the emission depletion beam (STED). However, resolving the nanostructure of complex molecular assemblies, such as protein complexes or organelles, requires more colors. Moreover, multiple colors would allow functional probes in super-resolution microscopy, such as ratiometric pH probes [89] or fluorescence resonance energy transfer (FRET) sensors for GTPases [90]. Three- and four-color STED microscopes have been developed, based on adjustable laser intensities [40] and spectral unmixing of fluorescence emission wavelengths and lifetimes [91]. SMLM can also be performed with multiple colors, for instance by careful selection of dyes and excitation schemes
or by sequential exchange of antibodies or probes \([93,94]\). These new techniques will be important for the endocytic trafficking field because they will, for instance, enable the different types of early, recycling, and late endosomes and lysosomes to be resolved.

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**Fig. 3**: Spatial Scale of Phagosomes, Clathrin-Coated Vesicles, Coat Proteins, Uptake Receptors, and Probes Commonly Used in Fluorescence Super-Resolution Microscopy (SRM). All molecules and models are displayed to scale. Structures are from the Protein Data Bank (PDB): 1xi4, 3zys, 6d8c, 3j2u, 1igt, 3mkq, 1fdl, 3ogo, 5dty, 3kzy, 3s05, 2cl8, 3wjj, 3vq2, 1a1o, 3qxa. For comparison, the diffraction-limited point spread function of confocal microscopy (blue mesh) and an example of the 50 nm resolution attainable by SRM (blue ball) are shown. Abbreviations: \(B.\) taurus, \(Bos\) taurus; \(D.\) melanogaster, \(Drosophila\) melanogaster; \(G.\) gallus, \(Gallus\) gallus; \(H.\) sapiens, \(Homo\) sapiens; \(M.\) musculus, \(Mus\) musculus; \(S.\) cerevisiae, \(Saccharomyces\) cerevisiae.

**Improving Temporal Resolution**

The low speed of image acquisition is a third limitation of current super-resolution techniques because this precludes the visualization of endo/phagosomal maturation and cytoskeletal...
transport (Figure 4). One issue that is apparent from this review is that most of the results discussed here have been obtained with SIM, even though this technique generally has a lower spatial resolution than either SMLM or STED. Indeed, SIM offers the advantage that it combines improved spatial resolution with a reasonably high temporal resolution (seconds), and thereby allows the dynamics of spatiotemporal recruitments, stoichiometries, and interactions between proteins and organelles to be resolved. SMLM and STED are intrinsically slow techniques because SMLM requires the acquisition of many (thousands) of images, and laser scanning in STED requires a very small pixel step to attain high spatial resolution. SIM is faster, but still requires the acquisition of multiple optical planes, and the spatial resolution of this technique is typically relatively modest compared with STED and SMLM. LLSM combines fast imaging with a high lateral and axial spatial resolution, has the additional advantage of low light exposure [45], and enables imaging of up to six colors [95], but this approach is not yet broadly available. For events at the proximity of the microscopy coverslip, SIM can be combined with TIRF, thereby enabling both high spatial and temporal resolution [45]. Very recently, deconvolution approaches in combination with low photon dose and submillisecond exposure times have been developed to improve applications of SIM in live cell imaging [96]. Many other developments in super-resolution microscopy also aim to increase the speed of image acquisition, and live cell SMLM is possible now with ~10 s temporal resolution [97], for instance by ‘moving-window binning’ [98,99]. In SMLM, improved optics, more sensitive and faster cameras, and improved image processing algorithms [96] allow not only faster acquisition of individual frames but also higher labeling densities, reducing the number of frames necessary to compute a high-resolution image in SMLM [100]. There also are ongoing technological developments in STED, where the image acquisition rate was substantially improved by so-called parallelization, which involves scanning with optical lattices of the excitation and depletion beams [101]. All techniques will benefit from machine-learning approaches for image evaluation that allow improved resolution and prediction of structures [39,102]. These developments will allow fast events in endocytic trafficking to be resolved with not only high spatial but also temporal resolution [67–69].

\[ \text{Fig. 4: Temporal Resolution for Imaging of Intracellular Trafficking. (A) Confocal live cell imaging showing intracellular transport of phagosomes containing yeast particles. Individual phagosomes (labeled with stars) are transported by} \]

\[ \text{microtubules from the cell periphery towards the cell center over minutes after uptake. Scale bar, 10} \]
Concluding Remarks

In conclusion, super-resolution microscopy techniques have enabled the visualization of endosomal structures with nanoscale resolution, allowing membrane curvature and stages of endosome and phagosome formation to be linked with protein and lipid contents. There are still longstanding questions that will be addressable by these new techniques (see Outstanding Questions). Ongoing technological developments in super-resolution microscopy will lead to further improved spatial, temporal, and spectral resolution, enabling more applications in endocytic trafficking. Given the central role of membrane trafficking in eukaryotic cells, this will not only allow fundamental longstanding questions in cell biology to be resolved but will also lead to new understanding of the development and progression of diseases.

Box 1. Super-Resolution Microscopy Techniques

Several super-resolution microscopy techniques are now available with spatial resolutions well below the diffraction limit of conventional light microscopy. Total internal reflection fluorescence (TIRF) microscopy uses the evanescent wave of reflected light at the interface between the microscope cover glass and the specimen for excitation of fluorophores that are within ~100 nm distance of the cover glass. TIRF thereby has a high axial (z) resolution, whereas the lateral (x,y) resolution remains diffraction-limited. TIRF is often combined with other super-resolution microscopy techniques to achieve super-resolution in all dimensions. Stimulated emission depletion (STED) microscopy uses two laser beams of different wavelengths, one surrounding the other and creating a ‘donut’ [16]. The outer beam brings fluorescent molecules into a dark state of emission depletion, leaving only the fluorescent molecules in the center to actively emit photons, thereby effectively reducing the size of the excitation spot to below the diffraction limit. A second de-excitation pattern can be superimposed on the first ‘donut’ for combined lateral and axial super-resolution [103]. The family of single-molecule localization microscopy (SMLM) techniques uses a completely different approach and differentiates fluorophores using time [104]. The principle is to sequentially image frames with limited numbers of molecules and fit the intensity profiles of individual fluorophores. This allows the positions of these fluorophores to be obtained with localization accuracies better than the diffraction limit. By recording a large number of frames, positional information of numerous fluorophores can be combined to reconstruct a high-resolution image. High 3D resolution can be obtained by deforming the point spread function with an asymmetric lens or a deformable mirror in the emission light path. SMLM includes a range of techniques differing in the types of fluorophores used and in the methods for visualizing individual fluorophores, but the best known are photoactivatable localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). In structured illumination microscopy (SIM), super-resolution is obtained using an overlapping pattern of excitation beams, followed by
computational deconvolution. For lattice light sheet microscopy (LLSM), a planar illumination array is created by multiple beams with tightly controlled spacing oriented perpendicular to the optical light path of the emission [10]. This list is not complete, and there are other techniques for imaging below diffraction-limited resolution, for instance based on mathematical reconstruction [105,106]. Finally, fluorescence (super-resolution) microscopy can be combined with EM, which facilitates determination of the cellular localization of fluorescently labeled molecules by a technique called correlative light and electron microscopy (CLEM). Several recent reviews describe in detail the technical breakthroughs of super-resolution microscopy [9–12,16,107].

Box 2. Pathogen Entry

Several super-resolution microscopy studies have explored the nanostructures of the interactions of pathogens with host cells. SIM revealed that neutrophils prestore Zn\(^{2+}\) in lysosomes and azurophilic granules, and utilize Zn\(^{2+}\)-toxicity for combating streptococcal infections [108]. Another SIM study on phagosomes formed in mouse macrophages in response to Candida albicans infection showed enriched SECS and inositol (1,4,5)-trisphosphate receptor (InsP3R) colocalization at phagosomes, and this enhanced phagocytosis and promoted antifungal signaling events [109]. Moreover, by SMLM, it was revealed how Salmonella enterica employs its virulence factors SifA and SseJ for endosomal tubule formation, and can thereby survive the acidic vacuoles of host cells [110]. It was found that SseJ hijacks the host motor protein kinesin for force-driven invasion along microtubules [110]. SMLM was also used to show that Salmonella typhimurium is surrounded by a heterogeneous coat of ubiquitin in the cytosol of host cells, with different densities of ubiquitination and of the E3 ubiquitin ligase [111]. Knockdown experiments of the host deubiquitinase OTULIN resulted in increased methionine-ubiquitin observable by SMLM microscopy, and this in turn promoted NF-κB signaling, leading to restriction of bacterial proliferation [111]. A SIM study also revealed nonhomogeneous methionine-linked ubiquitination at the surface of S. typhimurium, and reported a role for the ubiquitin ligase LUBAC in restricting bacterial proliferation through xenophagy and NF-κB activation [112]. Thus, super-resolution microscopy has made it possible to resolve how host ubiquitination limits the growth of S. typhimurium. By contrast, Shigella flexneri, another intracellular pathogen, expresses a protein called IpaH1.4 to negate the effect of LUBAC and promote its escape into the cytosol for survival [112]. Super-resolution microscopy has also revealed roles of ESCRT in assembly of viral particles, especially for HIV-1, as recently reviewed elsewhere [113].

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Glossary

- **BAR domain**: named after three proteins that contain this domain – bin/amphiphysin/rvs. BAR domains facilitate membrane curvature and/or bind to curved membranes.
- **Correlative light-electron microscopy (CLEM)**: overlapping fluorescence and electron microscopy images of the same specimen.
- **Electron microscopy (EM)**: a microscopy technique that uses electron beams of much shorter wavelength than visible light, allowing the ultrastructure of frozen or chemically fixed samples to be resolved.
- **F-actin**: filamentous actin, a component of the cytoskeleton.
- **Fluorescence resonance energy transfer (FRET)**: enables the detection of small changes (nm) in distance between two fluorophores.
- **Glycosylphosphatidylinositol (GPI)**: a glycolipid that can be posttranslationally coupled to the C-terminus of proteins.
- **HaloTag**: 33 kDa tag derived from the bacterial enzyme haloalkane dehalogenase that can bind fluorescent dyes via a fluochloroalkane linker.
- **IgG**: immunoglobulin G, a type of antibody that can be fluorescently labeled, allowing the location of specific proteins to be visualized.
- **Microtubule organizing center (MTOC)**: a cellular structure from which microtubules emerge.
- **Soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE)**: a family of proteins that facilitate intracellular vesicle docking and fusion.

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CHAPTER 3

SWAP70 Organizes the Actin Cytoskeleton and Is Essential for Phagocytosis
Abstract
Actin plays a critical role during the early stages of pathogenic microbe internalization by immune cells. In this study, we identified a key mechanism of actin filament tethering and stabilization to the surface of phagosomes in human dendritic cells. We found that the actin-binding protein SWAP70 is specifically recruited to nascent phagosomes by binding to the lipid phosphatidylinositol (3,4)-bisphosphate. Multicolor super-resolution stimulated emission depletion (STED) microscopy revealed that the actin cage surrounding early phagosomes is formed by multiple concentric rings containing SWAP70. SWAP70 co-localized with and stimulated activation of RAC1, a known activator of actin polymerization, on phagosomes. Genetic ablation of SWAP70 impaired actin polymerization around phagosomes and resulted in a phagocytic defect. These data show a key role for SWAP70 as a scaffold for tethering the peripheral actin cage to phagosomes.

Introduction
Phagocytosis is an evolutionarily conserved mechanism by which immune cells take up foreign particles for degradation, such as microbial pathogens and tumor cells. Phagocytosis, thereby, plays an essential role in the defense against disease. Actin executes a pivotal function in phagocytosis with its precise role depending on the stimuli and receptors involved (Freeman and Grinstein, 2014; May et al., 2000; Rohatgi et al., 2001; Swanson, 2008). F-actin-rich protrusions facilitate the initial capture of phagocytic targets. Actin is polymerized around the nascent phagosome forming a cage that helps wrapping of the membrane for particle engulfment and membrane fission. The actin cytoskeleton also exerts physical forces for pulling the pathogen inside the cell from the outside environment (May et al., 2000). Although the precise course of actin cytoskeleton assembly varies for different receptors (Allen and Aderem, 1996; Caron and Hall, 1998; Kuiper et al., 2008), actin polymerization is mediated by small GTPases of the RHO family: CDC42, RHOA, and RAC1 (Caron and Hall, 1998; May et al., 2000; Swanson, 2008). Within minutes after internalization, actin is depolymerized, a step that is required for the unhindered fusion of the maturing phagosome with endomembranes (Ferrari et al., 1999; Liebl and Griffiths, 2009). The maturation process that succeeds phagosome formation can be accompanied by successive waves of actin polymerization and depolymerization, which can facilitate or impede additional fusion events with endolysosomal compartments (Anes et al., 2003; Greenberg et al., 1991; Kjeken et al., 2004; Yam and Theriot, 2004). Despite phagocytosis being a well-studied phenomenon, the molecular mechanisms for F-actin cage tethering to the phagocytic surface are not clear. The structure of the phagosomal actin cage has also not been resolved, as F-actin is densely packed around the phagosome, and there is a lack of high resolution data.

In this study, we report that the actin cage in dendritic cells is connected to phagosomes by the 69 kDa scaffolding protein SWAP70. SWAP70 is expressed in many cell types, including mast cells (Gross et al., 2002), fibroblasts (Shinohara et al., 2002), dendritic cells (Oberbanscheidt et al., 2007), monocytes, and macrophages (Hilpela et al., 2003). In these cells, the absence of SWAP70 leads to defects in actin polymerization and impairs cell migration, adhesion, polarization, and
SWAP70 ORGANIZES THE ACTIN CYTOSKELETON AND IS ESSENTIAL FOR PHAGOCYTOSIS

morphology Bahaie et al., 2012; Chopin et al., 2010; Garbe et al., 2012; Murugan et al., 2008; Ocana-Morgner et al., 2011; Pearce et al., 2006; Ripich and Jessberger, 2011; Shinohara et al., 2002; Sivalenka and Jessberger, 2004). SWAP70 can directly bind, bundle, and stabilize actin filaments by means of its C-terminal actin binding domain (Chacon-Martinez et al., 2013; Gomez-Cambronero, 2012; Hilpela et al., 2003; Ihara et al., 2006; Murugan et al., 2008; Pearce et al., 2011; Shinohara et al., 2002). SWAP70 regulates cellular actin dynamics and organization via activation of RHOA and RAC1 (Dwyer et al., 2015; Oberbanscheidt et al., 2007; Ocana-Morgner et al., 2009; Ocana-Morgner et al., 2011; Shinohara et al., 2002; Sivalenka and Jessberger, 2004; Sivalenka et al., 2008). Binding of SWAP70 to phosphoinositides is required for this activation of RHO-GTPases (Murugan et al., 2008; Shinohara et al., 2002). We found that SWAP70 was transiently recruited to nascent phagosomes in human monocyte-derived dendritic cells. SWAP70 remained associated to these phagosomes shortly after phagocytic cup closure by specific binding of its Pleckstrin homology (PH-) domain to phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P$_2$) and to a lesser extent to phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P$_3$). Knockdown of SWAP70 by small interfering RNA (siRNA) significantly decreased RAC1 activation, actin polymerization, and phagocytic capability. Multicolor super-resolution STED microscopy showed that SWAP70 overlapped with RAC1 and aligned with parallel F-actin filaments and concentric rings surrounding the phagosome. These F-actin structures were not observable upon siRNA knockdown of SWAP70. Our findings show that SWAP70 is a scaffolding protein that promotes particle internalization by enabling the formation of the phagosomal actin cage.

Results

SWAP70 Aligns with F-Actin Filaments on the Surface of Early Phagosomes

Because SWAP70 can bind directly to F-actin via its C-terminal region (Chacon-Martinez et al., 2013; Hilpela et al., 2003; Ihara et al., 2006) and can regulate cellular actin dynamics and organization via activation of RHOA and RAC1 (Dwyer et al., 2015; Oberbanscheidt et al., 2007; Ocana-Morgner et al., 2009; Ocana-Morgner et al., 2011; Shinohara et al., 2002; Sivalenka and Jessberger, 2004; Sivalenka et al., 2008), we hypothesized that SWAP70 would have an important role in the formation of the phagocytic actin cage. To prove this hypothesis, we first investigated whether SWAP70 is recruited to phagosomes. Dendritic cells derived from monocytes isolated from blood of healthy volunteers were pulsed with zymosan particles, a cell wall preparation of the yeast Saccharomyces cerevisiae. Zymosan signals through a number of pattern recognition receptors, including Toll-like receptor 2 and the C-type lectin CLEC7A (Dectin-1). Zymosan is taken up by CLEC7A, which is the main phagocytic receptor for live fungi (Goodridge et al., 2009). Cells were immunolabeled for SWAP70 and F-actin was stained with fluorescently labeled phalloidin. A substantial portion of SWAP70 localized to F-actin-rich phagosomes that often (35%–70%, depending on the donor) were positive for SWAP70 (Figures 1A and 1B). Live cell experiments with dendritic cells overexpressing SWAP70 fused to GFP revealed transient recruitment of SWAP70 to phagosomes simultaneously with the F-actin binding probe LifeAct-RFP (Riedl et al., 2008) (Figures 1C and 1D; Movie S1). We performed three-dimensional super-resolution stimulated emission depletion (STED) microscopy to visualize
the ultrastructure of SWAP70 on the surface of the phagosomes. The cores of the zymosan particles were visualized by their auto-fluorescence in the GFP channel. Filamentous structures of SWAP70 were distinguishable by STED but not by conventional confocal microscopy (Figure 1E). Interestingly, we frequently observed that SWAP70 was arranged in parallel arched structures that aligned with the membrane of the phagosome (Figure 1F; Movies S2, S3, and S4). We also often observed concentric ring-like structures of SWAP70 (Figure 1G; Movies S5 and S6). This observation raised the possibility that SWAP70 would align with the F-actin filaments previously described to form around the phagocytic cup following uptake of non-opsonized zymosan (Goodridge et al., 2012; Huang et al., 2014; Liebl and Griffiths, 2009). We performed multi-color STED microscopy to address this possibility and observed perfect alignment of the F-actin filaments with SWAP70 (Figures 1H–1J; Movie S7). These results suggest a role for SWAP70 in the organization and/or formation of peripheral actin filaments on the phagosome. We next investigated when and under what conditions SWAP70 was recruited to phagosomes.

The recruitment of SWAP70 to phagosomes was not dependent on the type of phagocytic receptor, as it was broadly observed for different phagocytic cargoes, such as IgG-opsonized and naked latex beads (taken up by integrin αMβ2) (Freeman and Grinstein, 2014) (Figures 2A and S1A). Remarkably, the recruitment of SWAP70 to phagosomes was much stronger than to other actin-rich structures, such as podosomes (>5-fold based on fluorescence intensity; Figure 2B). SWAP70 has been shown to locate to podosomes in mouse dendritic cells, but its role there is not clear, as it is not required for podosome formation or turnover (Goetz and Jessberger, 2013). Although SWAP70 has been detected in nuclear fractions of B cells (Borggrefe et al., 1999) and mouse dendritic cells (Ocana-Morgner et al., 2013), we did not detect endogenous for overexpressed SWAP70 in the nucleus of human monocyte-derived dendritic cells. In order to determine when SWAP70 was recruited to phagosomes, dendritic cells were pulsed with fluorescein isothiocyanate (FITC)-labeled zymosan followed by immunolabeling with an antibody directed against FITC (i.e., without permeabilization). Zymosan particles that were completely taken up by the dendritic cells are inaccessible to antibody, allowing for selective labeling of free zymosan and nascent phagocytic cups. SWAP70 was recruited to sealed phagosomes (i.e., no anti-FITC labeling) and to nascent cups (Figure 2C; Movie S8). SWAP70 was only transiently recruited to phagosomes, as the total fraction of all SWAP70-positive phagosomes readily decreased in time (Figure 2D). As expected, the fraction of nascent cups compared to total phagosomes also readily decreased in time as more and more zymosan particles were fully internalized (Figure 2E). Approximately 50% of all nascent cups were positive for SWAP70 at all time points tested (Figure 2F). SWAP70 resided on phagosomes for 1–3 min after zymosan uptake based on the time-lapse imaging of dendritic cells expressing SWAP70-GFP, although we sometimes observed considerable longer residence times (up to 10–15 min; Figure 2G). We performed co-immunofluorescence labeling experiments to determine whether SWAP70 was still present on early phagosomes defined by the presence of the early endosomal protein EEA1 (Figures S1B and S1C). We could not find a single phagosome positive for both EEA1 and SWAP70, indicating that SWAP70 dissociated from the phagosomes.
prior to EEA1 recruitment. We also observed complete exclusion of SWAP70 with the late endosomal/lysosomal protein LAMP1 (Figures S1D and S1E). In agreement with these findings, live cell imaging revealed that SWAP70-GFP was recruited 3 min prior to mRFP-tagged RAB5A (Vonderheit and Helenius, 2005), an early endosomal small GTPase that binds EEA1 (Figures 2H and 2I; Movie S9). These results mirror the transient recruitment of SWAP70 to macropinosomes in mouse dendritic cells prior to RAB5A (Oberbanscheidt et al., 2007). Thus, in most cases, SWAP70 is already recruited to the nascent cup during phagosome formation and disappears within minutes after cup closure prior to the recruitment of EEA1.
Phagosomal Recruitment of SWAP70 Is Promoted by PI(3,4)P₂

The phosphoinositide composition of phagosomes changes progressively during phagosome formation and maturation. Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂), which is the predominant phosphoinositide at the plasma membrane (van den Bogaart and ter Beest, 2014), converts into PI(3,4,5)P₃ by the action of PI3-kinases (Greenberg and Grinstein, 2002; Gu et al., 2003; Hoppe and Swanson, 2004; Terebiznik et al., 2002). PI(3,4,5)P₃ is then dephosphorylated by SHIP1 (INPP5D) and SHIP2 (INPPL1), yielding PI(3,4)P₂ (Brooks et al., 2010; Drobek et al., 2015; Fuhler et al., 2012), which in turn is converted into PI(3)P by PI 4-phosphatases. Both SHIP1 and 2 are expressed by human monocyte-derived dendritic cells (Figure S2A). As EEA1 is known to be recruited to early phagosomes by binding to phosphatidylinositol 3-phosphate (PI(3)P) (Levin et al., 2015; Simonsen et al., 1998), we speculated that SWAP70 is recruited to the phagosome through its known binding partners PI(3,4)P₂ and PI(3,4,5)P₃ (Hilpela et al., 2003; Shinohara et al., 2002). To assess this, we overexpressed in dendritic cells GFP-tagged probes with specificity for several phosphoinositide species: the PLCδ1 (PLCD1) PH-domain specific for PI(4,5)P₂ (Lemmon et al., 1997; Stauffer et al., 1998), the PH-domain of AKT specific for PI(3,4,5)P₃ and less for PI(3,4)P₂ (Klippel et al., 1997; Rosen et al., 2012), the PH-domain of TAPP2 (PLEKHA2) specific for PI(3,4)P₂ (Marshall et al., 2002), the PX domain of p40phox (NCF4) specific for PI(3)P (Kanai et al., 2001), and the N-terminal sequence of MCOLN1 specific for PI(3,5)P₂ (Li et al., 2013). We then tested the co-localization of endogenous SWAP70 (i.e., by immunostaining) with these phosphoinositide-probes (Figures 3A and 3B). Of all probes, co-localization of SWAP70 on phagosomes was strongest with the probe sensing PI(3,4)P₂ (70% of all PI(3,4)P₂ positive phagosomes). Live cell imaging of dendritic cells co-expressing the GFP-tagged probes together with SWAP70 fused to mCherry showed simultaneous recruitment of SWAP70 and the probe for PI(3,4)P₂ to phagosomes (Figures S2B and S2C; Movie S10). The probes for PI(4,5)P₂ and PI(3,4,5)P₃ were mostly present on phagosomes before SWAP70-mCherry was recruited and the probe for PI(3)P was recruited later than SWAP70-mCherry (Figures S2D–S2I; Movies S11, S12, and S13). Experiments with co-expression of the mCherry-tagged PH-domain of SWAP70 (amino acid residues 210–306) with our GFP-tagged phosphoinositide probes demonstrated that PI(3,4)P₂ binding was mediated by the PH-domain of SWAP70 (Figures 3C, S3A, and S3B). Phagosome binding was not observed for the PH-domain carrying mutations R223E and R224E (Figure S3C), which is unable to bind to phosphoinositides in vitro (Hilpela et al., 2003).

To further validate the role of PI(3,4)P₂ in SWAP70 recruitment, we cultured dendritic cells in the presence of the specific SHIP1 inhibitor 3-a-aminocholestane (3AC) or the specific SHIP2 inhibitor AS1949490 (Brooks et al., 2010). We selected concentrations of 25 and 50 µM for 3AC and 10 and 25 µM for AS1949490; just below and above the concentrations where cellular viability and phagocytosis capacity were affected (Figures 3D, 3E, S3D, and S3E). These concentrations of 3AC
and AS1949490 resulted in a lower recruitment of the PI[3,4]P₂ probe to phagosomes compared to the solvent controls, indicating a reduced presence of PI[3,4]P₂ at the phagosomes (Figures S3F–S3H). We also observed 50% reduction of phagosomes containing SWAP70 with 3AC or AS1949490 (Figures 3F–3H), supporting a role for PI[3,4]P₂ in phagosomal recruitment of SWAP70. This reduced recruitment of SWAP70 was not due to a general trafficking defect, because the presence of gp91phox (CYBB, an integral membrane component of the NADPH oxidase NOX2) at the phagosome was not affected by 3AC nor AS1949490 (Figures S3I–S3K).

![Fig. 2: SWAP70 Is Recruited to Nascent Phagosomes.](image)

(A) Human dendritic cells were pulsed with a combination of FITC-labeled zymosan (zymosan-FITC; green in merge) and latex beads followed by immunostaining for SWAP70 (magenta). SWAP70 was recruited to both zymosan (pink arrowheads) and latex beads (yellow arrowhead). BF, bright field. See also Figure S1A. (B) Maximum intensity z projection of zymosan-pulsed dendritic cells immunostained for SWAP70 (magenta) and F-actin (green). F-actin and SWAP70 are shown in the fire look-up-table to emphasize the low recruitment of SWAP70 to podosomes (podosome region encircled in yellow) compared to phagosomes (pink arrowheads). (C) Identification of nascent cups by labeling of zymosan-FITC (green) with an antibody (blue; anti-FITC) in absence of permeabilization. Only free zymosan particles (yellow arrowhead) and nascent cups (pink) were accessible to anti-FITC labeling. Insert: magnification.
of a nascent cup. See also Movie S8. (D–F) Phagosomes in dendritic cells from three different donors over time of uptake (70 cells/donor/time point) were analyzed for the fraction of SWAP70-positive phagosomes (D), the fraction of nascent cups to total phagosomes (E), and the fraction of SWAP70-positive nascent cups to all SWAP70-positive phagosomes (F). (G) Residence time of SWAP70-GFP on phagosomes quantified based on live cell imaging (150 phagosomes). (H) Live cell imaging of dendritic cells expressing SWAP70-GFP (green in merge) and Rab5A-RFP (magenta). The inset shows a time series during zymosan uptake (arrowhead). See also Figures S1B–S1E and Movie S9. (I) Quantification from (H). The histogram shows the time difference of peak recruitment of SWAP70-GFP and Rab5A-RFP based on fluorescence intensity (50 phagosomes). Negative values indicate that Rab5A was recruited prior to SWAP70 and positive values later than SWAP70. Scale bars, 10 µm.

PI(3,4)P₂ is not the only factor recruiting SWAP70 to the phagosome, as the PH-domain alone showed considerably higher cytoplasmic background compared to endogenous SWAP70 (Figure S3a) and to overexpressed full-length SWAP70 fused to GFP (Figure S4). This is also indicated by the finding that a GFP-tagged full-length SWAP70 mutant unable to bind to phosphoinositides (R223E and R224E (Hilpela et al., 2003)) was still recruited to phagosomes, albeit less efficiently than wild-type SWAP70 (Figure S4). We tested GFP-tagged N- and C-terminal truncation mutants of SWAP70 to test which domains contribute to SWAP70 binding to phagosomes. Truncation mutants of SWAP70 lacking its N-terminal fragment containing the EF-hand motif or its C-terminal fragment containing the putative Dbl-homology domain (DH) (Shinohara et al., 2002) and actin-binding domain were recruited to phagosomes less efficiently compared to full-length SWAP70 (Figure S4), indicating that not only the PH-domain but also the N- and C-terminal regions contribute to phagosomal recruitment of SWAP70. These results compare well with the reported domain requirement for binding of SWAP70 to macropinosomes (Oberbanscheidt et al., 2007), except that the PH-domain alone is recruited to phagosomes (Figure S3a, S4) but not to macropinosomes (Oberbanscheidt et al., 2007). Possibly, phagosomes have prolonged presence and/or higher levels of PI(3,4)P₂ than macropinosomes. F-actin is not a major contributor for phagosomal SWAP70 recruitment, as mutants lacking part of or the entire C-terminal actin-binding domain (Ihara et al., 2006) were recruited to phagosomes with similar efficiency as full-length SWAP70 (Figure S4).

SWAP70 Controls Phagocytosis via F-Actin Polymerization

We then addressed the functional role of SWAP70 in phagocytosis. We first evaluated how siRNA knockdown of SWAP70 (80% knockdown efficiency; Figure 4A) affected uptake of FITC-labeled zymosan by flow cytometry (Figure S5A). For all donors tested, SWAP70 knockdown reduced the phagocytosis capacity of dendritic cells. The total fraction of cells that ingested zymosan was significantly reduced upon SWAP70 knockdown (Figures 4B and S5B). In this fraction of cells that took up zymosan, the total uptake was also significantly reduced (Figures 4C, S5C, and S5D). Lower zymosan uptake upon SWAP70 knockdown was also apparent from confocal imaging (Figures 4D and 4E). Zymosan uptake could be rescued by combining SWAP70 knockdown with overexpression of SWAP70-GFP with altered codon usage to protect it from siRNA targeting (Figures 4F, S5E, and S5F). These results contrast a study where TAT-mediated transduction of human granulocytes with a dominant-negative form of SWAP70 did not (or only somewhat) affect phagocytosis of gonococci via CEACAM3 (Schmitter et al., 2007). We also tested the effect of SWAP70 knockdown
on pinocytosis, because SWAP70 is recruited to pinosomes (Oberbanscheidt et al., 2007) and S1P-induced endocytosis is reduced in dendritic cells from SWAP70−/− mice (Ocana-Morgner et al., 2011). Similar to our findings with zymosan particles, we observed a significant reduction (25%) in uptake of BSA labeled with Alexa Fluor 488 (Figure S5G).

\[ \text{SWAP70 ORGANIZES THE ACTIN CYTOSKELETON AND IS ESSENTIAL FOR PHAGOCYTOSIS} \]

Fig. 3: Phagosomal SWAP70 Is Mainly Recruited by PI(3,4)P₂. (A) Confocal images of zymosan-pulsed dendritic cells expressing GFP-tagged phosphoinositide-probes (PI probe; green in merge) and immunolabeled for SWAP70 (magenta). The following PI probes were used: the PH-domain of PLCδ1 for PI(4,5)P₂, the PH-domain of AKT for PI(3,4,5)P₃, the PH-domain of TAPP2 for PI(3,4)P₂, the PH-domain of NCF4 for PI(3)P, and the N-terminal sequence of MCOLN1 for PI(3,5)P₂. Yellow arrowheads, phagosomes positive for SWAP70; BF, bright field. See also Figure S2 and Movies S10, S11, S12, and S13. (B) The percentages of phagosomes positive for the phosphoinositide biomarkers and endogenous SWAP70 (mean ± SEM for three donors; >60 phagosomes/condition). (C) Same as (B) but now with the mCherry-tagged PH-domain of SWAP70 (PH-SWAP70; mean ± SEM for three donors). See also Figures S3A–S3C. (D) Phagocytosis capacity with the SHIP1 inhibitor 3AC by flow cytometry. MFI, mean fluorescence intensity of the zymosan-FITC signal (mean ± SEM for three donors). EtOH, ethanol solvent control. See also Figure S3D. (E) Same as (D) but now for the SHIP2 inhibitor AS1949490. DMSO, solvent control. See also Figure S3E. (F) Confocal micrographs of dendritic cells pulsed with zymosan in the presence or absence of 25 µM 3AC. Yellow arrowheads, phagosomes positive for SWAP70. (G and H) Quantification of SWAP70-positive phagosomes.
In our flow cytometry experiments, we stained F-actin with fluorescently labeled phalloidin. This allowed quantification of the amount of F-actin per cell. Knockdown of SWAP70 led to a small (10%–20%) but significant reduction of F-actin (Figures 4G and S5H). This result is in line with the reduced F-actin levels in macrophages from SWAP70−/− mice (Chacon-Martinez et al., 2013). Confocal imaging showed that the percentage of F-actin-positive phagosomes was reduced by 50% upon SWAP70 knockdown and the phalloidin intensity at these F-actin-positive phagosomes was also reduced by 50% (Figures 4H and 4I). We no longer observed the parallel arches or rings of F-actin on the surface of the phagosomes by STED microscopy upon SWAP70 knockdown, whereas these structures were readily observable in the non-targeting siRNA control (Figures S6A and S6B). Phagosomes of SWAP70 knockdown dendritic cells still contained PI(4,5)P₂, PI(3,4,5)P₃, and PI(3,4)P₂, based on experiments with the GFP-tagged phosphoinositide probes (Figure S6C). Thus, knockdown of SWAP70 leads to a phagocytotic defect and reduced actin filament formation around phagosomes. Our data suggest that SWAP70 acts as a scaffolding protein, coordinating the tethering and stabilization of actin filaments on the phagosome surface. We then investigated the role of RAC1 in this process.

**SWAP70 Promotes Phagosomal RAC1 Activity**

SWAP70 can bind to RHO-GTPases, including the activated form of RAC1 (Ihara et al., 2006; Oberbanscheidt et al., 2007; Ocana-Morgner et al., 2009; Sivalenka and Jessberger, 2004). RAC1 activity is promoted by its binding to SWAP70 (Dwyer et al., 2015; Murugan et al., 2008; Shinohara et al., 2002; Sivalenka et al., 2008). RAC1 stimulates actin polymerization on phagosomes by Arp2/3 through activation of WAVE (Swanson, 2008). In its inactive state, RAC1 is associated to RHO-GDI that keeps the protein in the cytosol. Dissociation of RHO-GDI from RAC1 results in membrane association of RAC1 via insertion of its geranyl-geranyl moiety (Bustelo et al., 2007; van de Donk et al., 2005). RAC1 is transiently recruited to phagosomes and disappears within minutes after phagocytic cup closure (Hoppe and Swanson, 2004; Oberbanscheidt et al., 2007). By confocal microscopy, we estimated that the majority (60%–90%, depending on the donor) of SWAP70-positive zymosan phagosomes contained RAC1 and 70% of these also contained F-actin (Figures 5A and 5B). RAC1 not only localized to phagosomes but also to the plasma membrane, as reported previously for lymphocytes (Cernuda-Morollón et al., 2010). In live cell imaging experiments, overexpressed RAC1 fused to RFP (Hoppe and Swanson, 2004) also localized to phagosomes simultaneously with SWAP70-GFP (Figures 5C and 5D; Movie S14). By multi-color three-dimensional STED microscopy, we observed strong overlap between RAC1 and SWAP70 on the surface of phagosomes (Figure 5E; Movie S15). As expected, based on the high overlap between SWAP70 and F-actin, RAC1 also overlapped with F-actin on the surface of phagosomes (Figure 5F; Movie S16).

> Fig. 4: SWAP70 Regulates Phagocytosis and Phagosomal F-Actin. (A) Knockdown of dendritic cells with control (black, siControl) or SWAP70 siRNA (orange, siSWAP70) quantified by western blot (mean ± SEM). GAPDH, loading control. (B)
SWAP70 ORGANIZES THE ACTIN CYTOSKELETON AND IS ESSENTIAL FOR PHAGOCYTOSIS

Dendritic cells with siSWAP70 were pulsed with FITC-labeled zymosan. Zymosan-positive cells were quantified by FACS after 30 and 60 min for at least four donors. See also Figures S5A and S5B. (C) The total zymosan-FITC signal for the zymosan-positive cells from (B). MFI, mean fluorescence intensity. See also Figures S5C and S5D. (D) Confocal micrographs of FITC-labeled zymosan (red) pulsed dendritic cells with siControl (left) or siSWAP70 (right). F-actin was stained with phalloidin (cyan). Yellow arrowheads, F-actin positive phagosomes. Scale bar, 20 µm. (E) Quantification of phagocytosis from (D) (mean ± SEM; >40 cells/donor/condition). (F) Rescue experiment of phagocytosis for dendritic cells with siSWAP70 and SWAP70-GFP (individual donors shown). See also Figures S5E and S5F. (G) The MFI of phalloidin labeling in the zymosan-positive and -negative populations of cells from (B). (H) Percentage of phalloidin-positive phagosomes from (D). (I) Quantification of the phalloidin signals of F-actin-positive phagosomes from (D). See also Figures S5G, S5H, and S6.

We then decided to test how knockdown of SWAP70 would affect RAC1 activation by measuring activated (GTP-bound) RAC1 by a colorimetric assay. Although such global methods are not optimal to detect localized changes, SWAP70 knockdown reduced the zymosan-induced activation of RAC1 almost completely (Figure 5G). The recruitment of total RAC1 to phagosomes...
was also reduced upon SWAP70 knockdown by 60% (Figure 5H). We showed that phagosomal F-actin was also reduced upon SWAP70 knockdown (Figures 4H and 4I). The residual phagosomes that contained RAC1 in the SWAP70 knockdown samples had similar levels of F-actin as the RAC1-positive phagosomes in the control samples (Figure 5I). This indicates that SWAP70 affects both phagosomal RAC1 and F-actin equally. The residual RAC1 and F-actin recruitment could be due to functional redundancy (i.e., another scaffolding protein) and/or to residual SWAP70. Our results are reminiscent of the reduced levels of activated RAC1 in Mast cells isolated from SWAP70−/− mice (Sivalenka and Jessberger, 2004) and of the reduced RAC1 recruitment to actin-rich areas in dendritic cells from SWAP70−/− mice (Ocana-Morgner et al., 2011). Thus, SWAP70 promotes the presence of F-actin and RAC1 on phagosomes.

Discussion

SWAP70 is a multitasking protein, harboring multiple domains that can bind to DNA, phosphoinositides, RHO-GTPases, and F-actin. SWAP70 is implicated in many diseases, including cancer (Chiyomaru et al., 2011; Fukui et al., 2007; Murugan et al., 2008; Shu et al., 2013), various autoimmune diseases (Bahaie et al., 2012; Biswas et al., 2012; Erdag et al., 2012; Turkoglu et al., 2014; Vural et al., 2009), and HIV (Kimbara et al., 2006). In this study, we show that SWAP70 is recruited to the surface of the nascent phagocytic cup in dendritic cells. SWAP70 remains associated with phagosomes for 1–3 min but up to 15 min after cup closure, reminiscent of the transient localization of SWAP70 to early macropinosomes in mouse dendritic cells and NIH/3T3 cells (Oberbanscheidt et al., 2007). Knockdown of SWAP70 reduces phagosomal F-actin and RAC1 and impairs phagocytosis. SWAP70 localization to phagosomes was much stronger than to other F-actin-rich structures such as podosomes (Goetz and Jessberger, 2013). Our results indicate that SWAP70 is recruited to phagosomes via association of its PH-domain to PI(3,4)P₂. This confirms previous phosphoinositide dot-blot results indicating preferential binding of SWAP70 to PI(3,4)P₂, regulates the actin cytoskeleton for cell protrusion (Hilpela et al., 2003). SWAP70 was also identified as a PI(3,4)P₂ binding protein in a quantitative phosphoinositide-binding proteomics study in HeLa cells (Jungmichel et al., 2014). Finally, binding of SWAP70 to PI(3,4)P₂ was observed in competitive pull-down experiments, although in this case SWAP70 bound stronger to PI(3,4,5)P₃ (Shinohara et al., 2002). Thus, it seems reasonable to conclude that the PH-domain of SWAP70 preferentially binds to PI(3,4)P₂, although it may also bind to PI(3,4,5)P₃.

Phosphoinositides recruit factors promoting the polymerization and tethering of actin fibers to phagosomes (Levin et al., 2015). Especially, PI(4,5)P₂ is well-understood in these processes via the ERM proteins, CDC42 and WASP (Bretscher et al., 2002; Hoppe and Swanson, 2004; Rohatgi et al., 2001; Yonemura et al., 2002). PI(3,4)P₂ has not yet been described to play a role in phagosomal actin and was long believed to be merely a transition product in the conversion from PI(3,4,5)P₃ to PI(3)P (Li and Marshall, 2015). In fact, the action of SHIP1 was even reported to negatively affect both CR3 and Fcγ receptor-mediated phagocytosis, as it decreased levels of PI(3,4,5)P₃ on the nascent phagosome (Horan et al., 2007). However, it is now increasingly clear that PI(3,4)P₂ is a signaling
SWAP70 ORGANIZES THE ACTIN CYTOSKELETON AND IS ESSENTIAL FOR PHAGOCYTOSIS

phosphoinositide itself with unique roles in phagocytosis and pinocytosis (Li and Marshall, 2015; Maekawa et al., 2014; Welliver and Swanson, 2012). Several proteins specifically bind to Phosphatidylinositol 3,4-bisphosphate ($\text{PI(3,4)P}_2$), such as TAPP1 (PLEKHA1) or TAPP2 (PLEKHA2), which are both involved in actin remodeling via their interaction with syntrophins (Hogan et al., 2004). Phosphatidylinositol 3-phosphate ($\text{PI(3)P}$) is the main phosphoinositide of early endosomes/phagosomes, and the RAB5A effector protein EEA1 is recruited to PI(3)P by binding of its FYVE-domain (Levin et al., 2015; Simonsen et al., 1998). The finding that SWAP70 does not bind to PI(3)P is in accordance with our observations that SWAP70 is recruited to phagosomes prior to EEA1 and RAB5A and with the reported finding that SWAP70 recruitment to macropinosomes occurs prior to RAB5A (Oberbanscheidt et al., 2007).

Our super-resolution STED-microscopy showed that SWAP70 aligned with F-actin and RAC1 on the surface of phagosomes, forming parallel arches and rings. Upon knockdown of SWAP70, these structures were no longer observable. The binding of the C-terminal domain of SWAP70 to non-muscle F-actin is well-established (Gomez-Cambronero, 2012; Hilpela et al., 2003; Ihara et al., 2006; Murugan et al., 2008; Shinohara et al., 2002). SWAP70 can oligomerize and thereby bundle actin filaments in both a parallel and anti-parallel fashion and this delays dilution-induced F-actin depolymerization (Chacon-Martínez et al., 2013). This bundling of actin by SWAP70 likely explains the filamentous-like organization of SWAP70 on the phagosome surface revealed by STED. We also observed overlap between SWAP70 and RAC1 and impairment of RAC1 activation upon SWAP70 knockdown. Although SWAP70 was initially shown to be a guanine nucleotide exchange factor (GEF) for RAC1 (Shinohara et al., 2002), this is no longer believed (Oberbanscheidt et al., 2007) because mammalian SWAP70 (and also plant SWAP70) (Yamaguchi et al., 2012) binds to activated RHOA and RAC1, but not to GDP-bound or nucleotide-free RAC (Ihara et al., 2006; Murugan et al., 2008; Oberbanscheidt et al., 2007; Ocana-Morgner et al., 2009). Likely, RAC1 is activated by GEFs such as DOCK180 (DOCK1) and VAV (Rossman et al., 2005; Swanson, 2008) and stabilized in its active form by SWAP70. Our findings are in accordance with the reduced activation of RAC1 and decreased recruitment to actin-rich cellular regions in SWAP70−/− mice (Shinohara et al., 2002; Sivalenka et al., 2008; Ocana-Morgner et al., 2011). Moreover, the aberrant activation of RAC seen in Kaposi’s sarcoma requires SWAP70 (Dwyer et al., 2015). Our data and these studies all support a role for SWAP70 in regulation of RHO-GTPases and hence we conclude that SWAP70 does not only bind and stabilize the phagocytic actin cage, but also promotes their formation via binding-activated RAC1 (Figure 5J).

What is the sequence of events of SWAP70, RAC1, and F-actin recruitment to the phagocytic cup? Our data show that phosphoinositide binding by the PH-domain of SWAP70 clearly contributes to specific phagosomal recruitment. This result corroborates previous findings that a SWAP70 mutant impaired in Phosphatidylinositol 3,4,5-trisphosphate ($\text{PI(3,4,5)P}_3$), binding failed to locate to F-actin rich plasma membrane ruffles (Fukui et al., 2007; Shinohara et al., 2002; Wakamatsu et al., 2006). The binding of SWAP70 to RAC1 likely also contributes to phagosomal recruitment, as our data show that phagosomal recruitment is supported by both the N-terminal fragment (containing the EF-hand motif) and the
putative Dbl-homology domain (DH) (Shinohara et al., 2002) of SWAP70, but not by its C-terminal actin-binding region, similar to previous findings with macropinosomes (Oberbanscheidt et al., 2007). In accordance with this notion, RAC1 is recruited to the extending pseudopods of the nascent cup of phagosomes (Hoppe and Swanson, 2004), and SWAP70 is recruited after RAC1 to macropinosomes (Oberbanscheidt et al., 2007). However, we did not observe such a clear time-dependency for phagosomes in dendritic cells and instead saw simultaneous recruitment of SWAP70 and RAC1 and reduced RAC1 recruitment upon SWAP70 knockdown. Because SWAP70 binds to activated RAC1 (Ihara et al., 2006; Murugan et al., 2008; Ocana-Morgner et al., 2009), it may well be that SWAP70 and RAC1 are recruited to nascent phagosomes by a positive feedback loop, where binding of SWAP70 to PI(3,4)P$_2$ results in recruitment of activated RAC1 and F-actin that in turn recruits more SWAP70 (Figure 5J). PI(3,4)P$_2$ may also directly recruit RAC1 itself and promote RAC-GEF activation, as is well-established for PI(3,4,5)P$_3$ (Fleming et al., 2000; Hill and Welch, 2006; Missy et al., 1998). The notion that SWAP70 is recruited to phagosomes by multiple binding partners is supported by the finding that the N-terminal region, the PH-domain, and the putative Dbl-homology domain (DH) all contribute to binding of SWAP70 to phagosomes. Such a synergistic engagement of multiple binding partners is called coincidence detection (Carlton and Cullen, 2005) and is a well-understood mechanism to achieve highly restricted localizations as the one we report here for SWAP70 at phagosomes.

Our data show that SWAP70 plays a role in the assembly of the F-actin cage on phagosomes that persists well after cup-closure and depends on PI(3,4)P$_2$. This is not only a novel role for PI(3,4)P$_2$ in the organization of actin on phagosomes, but it also refutes the long-standing belief that F-actin on early phagosomes is primarily depending on PI(4,5)P$_2$ and PI(3,4,5)P$_3$, but not on other 3-phosphoinositides (Bretscher et al., 2002; Hoppe and Swanson, 2004; Rohatgi et al., 2001; Yonemura et al., 2002). Likely, the formation of an F-actin cage by SWAP70 promotes the remodeling of the membrane and provides a mechanic pulling force for ingestion of the phagocytic load (May et al., 2000). F-actin surrounding newly internalized phagosomes is known to rapidly depolymerize upon the conversion of PI(4,5)P$_2$ to PI(3,4,5)P$_3$, which leaves the phagosome membrane available to fuse with endosomes and lysosomes (May et al., 2000; Schlam et al., 2015). Retaining the F-actin cage on the phagocytic surface by SWAP70 likely delays this phagosomal maturation, which could well explain the maturation defects and reduced T cell activation observed in SWAP70-deficient dendritic cells (Ocana-Morgner et al., 2009; Ocana-Morgner et al., 2013). Thereby, SWAP70 could potentially lead to preservation or rerouting of the phagocytic cargo for specific processing of antigen for naive T cell activation.

> Fig. 5: SWAP70 Promotes Phagosomal RAC1 Activity. (A) Confocal micrographs and quantification of RAC1 (green in merge) and SWAP70 (magenta) positive phagosomes for seven donors (100 cells/donor). Ave., average ± SEM. (B) Venn diagram showing phagosomal distribution of RAC1, F-actin, and SWAP70. (C) Live cell imaging of dendritic cells expressing SWAP70-GFP (green in merge) and RAC1-RFP (magenta). The inset shows a time series during zymosan uptake. BF, bright field. See also Movie S14. (D) Quantification from (C). The histogram shows the time difference of peak recruitment of SWAP70-GFP and RAC1-RFP based on fluorescence intensities (88 phagosomes). Negative values indicate that RAC1 was recruited prior to SWAP70 and positive values later than SWAP70. (E and F) Multicolor 3D-STED microscopy of zymosan-pulsed dendritic cells immunostained for RAC1 (green) and SWAP70 (magenta; E) or F-actin (F). Left: cross-section and orthogonal sections
SWAP70 Organizes the Actin Cytoskeleton and Is Essential for Phagocytosis

3

(indicated by dashed yellow lines). Middle: maximum intensity height maps. Right: maximum intensity surface projection. Yellow arrowheads, overlap of RAC1 with SWAP70 (E) or F-actin (F) on the surface of the phagosomes. See also Movies S15 and S16. (G) RAC1 activation upon control (siControl) and SWAP70 siRNA (siSWAP70) after zymosan addition by G-LISA assay.
(Abs, absorbance units at 490 nm; mean ± SEM). The background (t = 0) is from cells without zymosan. (H) RAC1-positive phagosomes upon siControl and siSWAP70 counted by microscopy (>20 cells/donor/condition analyzed; individual donors shown). (I) Phagosomes positive for F-actin and/or RAC1 with siControl or siSWAP70. (J) Model of F-actin cage formation by SWAP70. SWAP70 is recruited to phagosomes by coincidence detection. Scale bars: 10 µm (A and C), 2 µm (E and F).

Material and Methods
Antibodies and Reagent
The following primary antibodies were used: rabbit polyclonal anti-SWAP70 (Novus Biologicals; cat. no NBP1-82979) at 1:200 dilution (v/v) for immunofluorescence (IF) and 1:500 for western blot; mouse IgG1 anti-EEA1 (BD Biosciences; cat. no. 610457) at 1:100; mouse IgG1 anti-LAMP1 (Biolegend; 328601) at 1:200; mouse monoclonal anti-RAC1 (Cell Biolabs; 240106) at 1:100; mouse IgG1 anti-FITC Alexa Fluor 647 (Jackson Immunoresearch; 200-602-037) at 1:200; rabbit polyclonal anti-actin (Sigma; A5060) at 1:100; and mouse IgG1 anti-CYBB (MBL; D162-3) at 1:200. The following secondary antibodies were used: goat-anti-mouse Alexa Fluor 488 (Life Technologies; A11029); goat-anti-rabbit Alexa Fluor 568 or 647 (Life Technologies; A11036 and A21245); goat-anti-mouse STAR635 (Abberior; 2-0002-002-0); sheep-anti-mouse KK114 (Abberior Red); and goat-anti-rabbit Alexa Fluor 633-labeled phalloidin was from ThermoFisher (A22284) and used at 1:200 (v/v). mCLING labeled with ATTO647N was from Synaptic Systems (710006AT1) and used at 1:200 (v/v). The RAC1 activation G-LISA kit was from Cytoskeleton (BK128-S).

Cells and Transfection
Buffy coats from healthy donors (consent obtained and according to national ethics guidelines and approved by the institutional review board [Radboud UMC]) were used to isolate peripheral monocytes as described (de Vries et al., 2002). Constructs for full-length human SWAP70, the PH-domain (residues 210–306), the PH-domain and full-length SWAP70 with R223E and R224E mutations, and C- and N-terminal truncations (residues 205–585, 1–313, 1–525, and 1–572) were generated as codon-optimized synthetic genes (Genscript) in the EcoRI/BamHI sites of pEGFP-C1 and pmCherry-C1 (Clontech). RABSA-mRFP was a gift from Ari Helenius (Addgene plasmid #14437) (Vonderheit and Helenius, 2005). YFP-RAC1 was a gift from Joel Swanson (Addgene plasmid #11391) (Hoppe and Swanson, 2004) and was re-cloned into mCherry-C1. LifeAct-RFP was a gift from Michael Sixt (Max Planck Institute of Biochemistry, Martinsried). GFP-C1-PLCD1-PH was a gift from Tobias Meyer (Addgene plasmid #21179) (Stauffer et al., 1998). The GFP-tagged PH-domain of AKT is described (de Keijzer et al., 2011). NCF4-PX-EGFP was a gift from Michael Yaffe (Addgene plasmid #19010) (Kanai et al., 2001). The GFP-tagged residues 179–311 of TAPP2 were described (de Keijzer et al., 2011; Haugh et al., 2000). Mouse MCOLN1 residues 1–68 were generated as a synthetic gene in the Xhol/BamHI sites of pEGFP-C1. Transfection was performed as described (Baranov et al., 2014; Dingjan et al., 2016) and zymosan uptake and imaging experiments were performed 8–12 hr post-transfection.
Microscopy
Samples were imaged with a Leica SP8 confocal microscope fitted with a 63x 1.2 NA water immersion objective. For live cell microscopy, cells were imaged 3–5 hr post-transfection with IgG-opsonized zymosan at 1:10 cell-to-particle ratio. Imaging was performed in Hank’s balanced salt solution (HBSS) (ThermoFisher, 14025050) supplemented with 10 mM HEPES at pH 7.4. Samples were imaged with a Leica DMI6000 epi-fluorescence microscope fitted with a 63x 1.4 NA oil immersion objective, a metal halide EL6000 lamp for excitation, a DFC365FX CCD camera, and GFP and DsRed filter sets (all from Leica). Focus was kept stable with the adaptive focus control from Leica. The STED microscope was from Abberior Instruments. Emission of two fluorescent markers (excitation [Ex.]: 560/640 nm, emission [Em.]: 580–630 nm/640–720 nm) was depleted with a single STED-laser operating at 775 nm with 800 picosecond (ps) long pulses at 40 MHz. The STED-laser point-spread-function was shaped by a spatial light modulator. STED-laser power in the backfocal-plane was 180 mW, with 65% of that power used for the 2D-donut and 35% for the 3D-donut. Zymosan particles were detected by excitation at 485 nm and collecting autofluorescence at 500–550 nm. Excitation power of the three excitation lasers 485 nm/560 nm/640 nm were 4 mW/10 mW/8 mW in the back-focal-plane of an 1003 1.4 NA oil immersion objective (Olympus).

Fluorescence-Activated Cell Sorting
For fluorescence-activated cell sorting (FACS), cells were incubated with FITC-labeled zymosan at 1:10 cell-to-zymosan ratio or 100 mg ml⁻¹ BSA-AF488 for 5 min to 2 hr. Incubation was 1 hr for the uptake experiments with 3AC and AS1949490. Cells were fixed in 4% PFA (w/v) and F-actin was labeled with Alexa Fluor 633 phalloidin in S-PBS (see the Supplemental Information). Data were acquired on a FACS-Calibur cytometer (BD Biosciences).

Statistical Analysis
All data were analyzed using Student’s two-tailed paired t tests. A p value < 0.05 was regarded as statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).
References
SWAP70 ORGANIZES THE ACTIN CYTOSKELETON AND IS ESSENTIAL FOR PHAGOCYTOSIS

3-kinase interact with Rac1 GTPase and stimulate GDP dissociation. J. Biol. Chem. 273, 30279–30286.


SWAP70 ORGANIZES THE ACTIN CYTOSKELETON AND IS ESSENTIAL FOR PHAGOCYTOSIS

754–760.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and sixteen movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.10.021

Fig. S1: related to Figure 2. Phagosomal SWAP70 recruitment occurs prior to EEA1 and is independent of the type of antigen. (a) Confocal micrographs of dendritic cells pulsed with uncoated latex beads (top panels) or IgG-opsonized latex beads (bottom) immunostained for SWAP70 (magenta in merge) and F-actin (green). Pink arrowheads indicate double-positive phagosomes. Blue: DAPI. BF: bright field. (b–c) Dendritic cells pulsed with zymosan and immunostained for SWAP70 (magenta), EEA1 (green) and F-actin (cyan) (b) and quantification (c; >40 cells/condition for 3 donors; mean ± SEM). Arrowheads indicate SWAP70 (pink) and EEA1 (yellow) positive phagosomes. (d–e) Same as panels b–c but now for LAMP1 (green). No double-positive phagosomes containing both SWAP70 and EEA1 or LAMP1 were observed. Scale bars: 10 μm.
SWAP70 ORGANIZES THE ACTIN CYTOSKELETON AND IS ESSENTIAL FOR PHAGOCYTOSIS

^ Fig. S2: related to Figure 3. PI(3,4)P₂ mediates SWAP70 recruitment to the phagosome. (a) SHIP1 (INPP5D) and 2 (INPPL1) expression by PCR on cDNA from dendritic cells. The position of the forward (F) and reverse (R) primers as well as the expected fragment sizes are indicated. (b) Live cell imaging of dendritic cells pulsed with zymosan (arrowhead) and expressing a GFP-tagged PI(3,4)P₂-binding probe (the PH-domain of TAPP2 (PLEKHA2); green in merge) and SWAP70-mCherry (magenta). The inset shows a time series during zymosan uptake. See also Movie S10. BF: bright field. (c) Quantification from b. The histogram shows the time difference of peak recruitment of SWAP70-mCherry and the PI probe based on fluorescence intensities (>10 phagosomes). Negative values indicate that the PI probe was recruited prior to and positive values later than SWAP70-mCherry. (d–e) Same as b–c, but now for a PI(3,4,5)P₃-binding probe (PH-domain of AKT). See also Movie S11. (f–g)
Same as b–c, but now for a PI(4,5)P2-binding probe (PH-domain of PLCδ1 (PLCD1)). See also Movie S12. (h–i) Same as b–c, but now for a PI(3)P-binding probe (PX-domain of NCF4 (p40phox)). See also Movie S13. Scale bars: 10 μm.

Fig. S3: related to Figure 3. PH-domain mediates SWAP70 recruitment to the phagosome by PI(3,4)P2. (a) Confocal images of zymosan-pulsed dendritic cells co-expressing GFP-tagged phosphoinositide probes (PI probe; green in merge) with the mCherry tagged PH-domain of SWAP70 (PH-SWAP70; magenta). Yellow arrowheads indicate PH-SWAP70-positive...
phagosomes. BF: bright field. (b) Pearson correlation coefficients for the PI probes and PH-SWAP70 from panel a (mean ± SEM; >25 phagosomes/condition for at least 3 donors). (c) Confocal image of zymosan-pulsed dendritic cells expressing GFP-tagged PH-SWAP70 (magenta) carrying mutations R223E and R224E. Arrowheads: phagosomes positive for F-actin (green). No recruitment of mutant PH-SWAP70 to phagosomes was observed. (d) Cell viability of dendritic cells by the MTT assay for different concentrations of the SHIP1 (INPP5D) inhibitor 3AC (mean ± SEM for 3 donors). EtOH: ethanol solvent control. (e) Confocal image of zymosan-pulsed (magenta) dendritic cells expressing the PI(3,4)P₂ probe (green) in presence or absence of 25 µM 3AC. DAPI is blue in merge. (g) Quantification of phagosomes positive for the PI(3,4)P₂ probe for the 3AC concentrations indicated (individual donors shown; >30 phagosomes/donor/condition; mean ± SEM). (h) Same as panel g, but now for AS1949490. (i) Confocal image of dendritic cells pulsed with zymosan (yellow) and immunolabeled for SWAP70 (green) and CYBB (gp91<sub>phox</sub>; magenta). Cells were treated with 10 µM AS1949490. DAPI is blue in merge. (j) Quantification of gp91<sub>phox</sub>-positive phagosomes for the 3AC concentrations indicated (individual donors shown; >80 phagosomes/donor/condition; mean ± SEM). (k) Same as panel j, but now for AS1949490. Scale bars: 10 µm (a, c, f) and 20 µm (i).

Fig. S4: related to Figure 3; Phagosomal SWAP70 recruitment by phosphoinositides and RAC1 but not or less by F-actin. (a) Representative confocal micrographs of zymosan-pulsed dendritic cells overexpressing full-length or mutant SWAP70 N-terminally fused to GFP (magenta in merge). F-actin was labeled with phalloidin (green). Yellow arrowheads indicate F-actin-rich phagosomes. The graphs show the intensity plot profiles as indicated. The position of phagosomes is indicated by the yellow shaded areas. FL: full-length SWAP70 (residues 1–585); PH: PH-domain of SWAP70 (209–306); ΔAB1: C-terminal truncation mutant of SWAP70 with part of its actin-binding (AB) domain removed (1–572); ΔAB2: C-terminal truncation mutant of SWAP70 with its entire AB domain removed (1–525); ΔDH, ΔAB: C-terminal truncation mutant of SWAP70 with its putative Dbl-homology (DH) domain (Shinohara, et al. (2002) Nature. 416, 759-763) and AB domain removed (1–313); ΔEF: N-terminal truncation mutant of SWAP70 with its EF-hand motif removed (205–585); FL (R223E, R224E): full-length SWAP70 carrying mutations R223E and R224E. BF: bright field. Scale bars: 10 µm (b) Domain topologies of the tested mutants and summary of their phagosomal recruitment as judged from the confocal images. Representative images for the PH-domain of SWAP70 (PH-SWAP70) carrying mutations R223E and R224E are in figure S3c. –: no association; +: some association; ++: regular association.
Fig. S5 related to Figure 4. SWAP70 regulates phagocytosis via F-actin. (a) FACS plot of dendritic cells transfected with control (siControl) or SWAP70 siRNA (siSWAP70). Cells were pulsed with FITC-labeled zymosan (zymosan-FITC) for 60 min, fixed and stained with phalloidin conjugated to Alexa Fluor 633 (phalloidin-AF633). (b) Percentage of zymosan-phagocytosing cells relative to total cells for a representative donor with siControl (black) or siSWAP70 (orange). (c) Mean fluorescence intensity (MFI) of zymosan-FITC for the zymosan-positive cells from panel b. (d) Fluorescence distribution of the zymosan-FITC signal of zymosan-positive cells for a representative donor 60 min after uptake. (e) SWAP70 knockdown efficiency for the rescue experiments. siRNA was transfected simultaneously with plasmid encoding SWAP70-GFP and knock-down levels were determined 24 h post-transfection by Western blot (mean ± SEM for 3 donors). GAPDH: loading control. (f) Epi-fluorescence microscopy image of rescue experiment. Note that the cell expressing SWAP70-GFP (green) contains more zymosan particles (arrowheads) than the surrounding cells without rescue. Scale bar, 10 μm. (g) Uptake of BSA labeled with Alexa Fluor 488 (BSA-AF488) by FACS with siControl or siSWAP70 for 3 different donors. Cells were pulsed with BSA for 60 min. (h) Fluorescence distribution of phalloidin-AF633 staining in siControl or siSWAP70 for a representative donor 15 min after zymosan uptake.
Fig. S6 related to Figure 4. SWAP70 knockdown affects the phagosomal F-actin cage, phosphoinositides are still present. (a) Super-resolution multicolor 3D-STED microscopy of dendritic cells with siRNA knockdown of SWAP70. Cells were pulsed with zymosan (blue in merge) and immunostained for Factin (green) and SWAP70 (magenta; the SWAP70 signal is absent due to the knockdown). Shown are crosssections (left), orthogonal sections (middle; indicated by the dashed yellow lines) and a depth-encoded maximum intensity height map of the F-actin (right) for 4 representative phagosomes. Note the absence of parallel arches or ring-like structures of F-actin on the surface of the phagosomes. (b) Same as a, but now with non-targeting siRNA control. Arrowhead: ring-like structure of F-actin on the surface of the phagosome (depicted in the inset). (c) GFP-tagged phosphoinositide probes (PI probe-GFP; green in merge) were co-expressed with non-targeting siRNA (siControl) or SWAP70 siRNA (siSWAP70) in zymosan-pulsed dendritic cells. The PI probes for PI(4,5)P\(_2\) (PH-domain of PLCδ1 (PLCD1)), PI(3,4,5)P\(_3\) (PH-domain of AKT) and PI(3,4)P\(_2\) (PH-domain of TAPP2 (PLEKHA2)) were used. Left: representative confocal images (from 3–5 donors/condition). Arrowheads: PI probe-positive phagosomes. BF: bright field. Right: representative Western blots and quantifications of knockdown efficiencies (mean ± SEM). GAPDH: loading control. Scale bars: 2 µm (a, b), 10 µm (c).
Supplemental Experimental Procedures

Particle preparation and phagocytosis assays
IgG-coated latex beads were produced by incubating 0.3% streptavidin bead suspension with 1.3 mg ml\(^{-1}\) biotin-SP (long spacer) Chrompure human IgG (Jackson ImmunoResearch; 009-060-003) for 30 min at 37 °C followed by extensive washing. The beads were added at a 1:10 cell-to-beads ratio. IgG opsonized zymosan particles were produced by incubating 20 mg/ml zymosan suspension with an equal volume of Opsonizing solution from ThermoFisher (Z2850) for 60 min at 37 °C followed by extensive washing. FITC-labeled zymosan was purchased from ThermoFisher (Z2841). Unlabeled zymosan was from Sigma (Z4250-1G). The same zymosan was labeled with Alexa fluor 633 maleimide (Life Technologies) by incubating 16.4 mg ml\(^{-1}\) with 77 μM in 0.2 M Na-carbonate at pH 9.0 for 1 hour followed by vigorous washing. BSA Alexa fluor 488 conjugate was from ThermoFisher (cat. No. A13100). For the SHIP1 (INPP5D) or SHIP2 (INPPL1) inhibition, cells were incubated with zymosan at 1:10 cell-to-particles and either with 3AC (Echelon; B-0341; 10–200 μM in ethanol) or AS1949490 (Echelon; B-0342; 1.25–200 μM in DMSO).

Cell viability assay
For cell viability, 0.66 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma M2128-1G) was applied to the cells together with 3AC or AS1949490 for 2 h. The MTT reaction was stopped with lysis buffer containing 90% isopropanol, 0.0125% SDS and 0.04 M HCl. Absorbance was measured at 595 nm to determine cell death.

siRNA knock-down
Knockdown in human dendritic cells was performed as described (Dingjan et al., 2016), except that 3 day differentiated monocytes were used for transfection and samples were used 24-72 h post-transfection. A mix of 3 siRNAs was used (Life Technologies): GCCUU CAGAC UCAAG UGGAA CUUCA, AAAGA AGCUG GAGAU GGCAA CUAAU, and CAGAA GAGAU UGAAU ACCUG CUUAA. Control samples were transfected with irrelevant ON-TARGET plus non-targeting (NT) siRNA (Dharmacon). For the rescue experiments and the combination of siRNA with phosphoinositide probes, day 6 differentiated monocytes were simultaneously transfected with siRNA and plasmid DNA and used 24 h post-transfection. For all experiments, only samples with knockdown levels above 70% (72 h post-transfection) or 40% (24 h) were used.

Immunofluorescence
For immunofluorescence, cells were incubated with zymosan at 1:10 cell-to-zymosan ratio in serum-free medium for 15 to 60 minutes. The cells were subsequently fixed with 4% PFA in PBS and permeabilized in SPBA (0.1% Saponin, 0.5% BSA and 0.01% NaN, in PBS) for 5 min. Primary and secondary antibodies were incubated in S-PBA. Cells were embedded in DAPI containing mounting medium containing 0.01% (v/v) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid and 68% (v/v) glycerol in 200 mM sodium phosphate buffer at pH 7.5. In order to label the phagocytic cups, FITC-labeled zymosan was used and samples were labeled with an antibody
directed against FITC prior to permeabilization.

**PCR**

The total mRNA from day 6 differentiated monocytes was isolated with the Quick-RNA MiniPrep kit (Zymo Research, R1055) and reversely transcribed into cDNA. SHIP1 (INPP5D) mRNA was amplified by PCR with 3 forward primers (nucleotides 128–147: GCGTG CTGTA TCGGA ATTGC; 354–372: AAGTG TCGTG TCTCC ACCC; 433–454: TTTTC AAACG AGAAT CCCCG AG) and 3 reverse primers (232–211: TGGTG AAGAA CCTCA TGGAG AC; 452–430: CGGGG ATTCT GTTCT GAAAA AGG; 579–559: GCCGA GCTGA GTGCT TAAAT A). SHIP2 (INPPL1) mRNA was amplified with one forward primer (198–217: GCACA CGTAT CGCAT TCTGC) and reverse primer (364–344: CTGC GTCCT CAGGAA).

**Supplemental References**

SWAP70 is a universal GEF-like adaptor for tethering actin to phagosomes.
Abstract
We recently identified a key role for SWAP70 as the tethering factor stabilizing F-actin filaments on the surface of phagosomes in human dendritic cells by interacting both with Rho-family GTPases and the lipid phosphatidylinositol (3,4)-bisphosphate. In this study, we aimed to investigate whether this role of SWAP70 was general among immune phagocytes. Our data reveal that SWAP70 is recruited to early phagosomes of macrophages and dendritic cells from both human and mouse. The putative inhibitor of SWAP70 sanguinarine blocked phagocytosis and F-actin polymerization, supporting a key role for SWAP70 in phagocytosis as demonstrated previously with knock-down. Moreover, SWAP70 was recently shown to sequester the F-actin severing protein cofilin and we investigated this relationship in phagocytosis. Our data show an increased activation of cellular cofilin upon siRNA knockdown of SWAP70. Finally, we explored whether SWAP70 would be recruited to the immune synapse between dendritic cells and T cells required for antigen presentation, as the formation of such synapses depends on F-actin. However, we observed that SWAP70 was depleted at immune synapses and specifically was recruited to phagosomes. Our data support an essential and specific role for SWAP70 in tethering and stabilizing F-actin to the phagosomal surface in a wide range of phagocytes.

Introduction
Actin has a well-understood role in the phagocytosis of microbes and tumor cells by immune cells (24, 40, 50, 55). Although the precise course of F-actin formation depends on the phagocytic receptors engaged (4, 13, 37), F-actin filaments are generally assembled early during phagocytosis at the site of phagosome formation and F-actin depolymerizes within several minutes after sealing of the phagosome (6, 21, 38). We recently showed an essential role for the adaptor protein SWAP70 in phagocytosis by human dendritic cells derived from blood-isolated monocytes (6). In our study, we demonstrated that SWAP70 is transiently recruited to phagosomes ingested by different phagocytic receptors (6). This phagosomal recruitment of SWAP70 coincided with the presence of F-actin on the phagosomal surface, and occurred before recruitment of the early endosomal small GTPase Rab5 (6, 42). SWAP70 is well known to bind to and stabilize F-actin with its C-terminal region (14, 30, 34, 35, 41, 46, 51). Using 3-dimensional multicolor super-resolution STED microscopy, we showed that SWAP70 organizes F-actin as parallel fibers or concentric rings on the surface of phagosomes (6). In our previous study (6), we also showed that the specific recruitment of SWAP70 to phagosomes is driven by its binding to the small Rho-family GTPase Rac1 (35, 42, 45, 54) and to phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P₂) (34, 51). Our data indicated that SWAP70 binds to and stabilizes activated Rac1 at the phagosomal surface (6), although it is most likely not a guanine exchange factor (GEF) itself (42). We also showed that SWAP70 was not or less recruited to other F-actin rich cellular regions (6), such as podosomes which are involved in cell migration and trans-epithelial antigen sampling (7, 8).

In this study, we further explored the role of SWAP70 in phagocytosis. First, we aimed to determine whether the role for SWAP70 in phagocytosis was only present in human dendritic cells or was also
Results

SWAP70 is recruited to early phagosomes in murine dendritic cells and the macrophage cell line RAW264.7

In our recent study (6), we demonstrated that SWAP70 is essential for phagocytosis by human dendritic cells derived from blood-isolated monocytes (monocyte-derived dendritic cells; moDCs). To determine how general this mechanism is among other phagocytes of the immune system and other species, we first tested for the expression of SWAP70 in mouse dendritic cells and human monocyte-derived macrophages. We also tested for the expression of SWAP70 in the murine macrophage cell line RAW264.7, which is a widely-studied model system for phagocytosis. Mouse dendritic cells were derived from bone marrow stem cells (bone marrow-derived dendritic cells; BMDC) by culturing with granulocyte-macrophage colony-stimulating factor (GM-CSF) (39, 42) and human macrophages were derived from blood monocytes by culturing with macrophage colony-stimulating factor (M-CSF). Human and mouse SWAP70 amino acid sequences are 95% identical and could both be recognized by our polyclonal antibody by Western blot (Fig. 1A). SWAP70 was expressed both in mouse BMDCs and RAW264.7 macrophages, as has been reported previously (42-45, 20, 29). SWAP70 was expressed in mouse BMDCs at slightly lower levels than in moDCs, whereas the expression of SWAP70 was higher both in human macrophages and the mouse RAW264.7 macrophage cell line (Fig. 1B). Expression levels of SWAP70 did not notably change upon exposure of the cells to the fungal adjuvant zymosan (prepared from Saccharomyces cerevisiae). Additionally, we tested SWAP70 expression in the naturally occurring myeloid (mDC; CD1c-positive) and plasmacytoid (pDC; BDCA4-positive) dendritic cell subsets observable in other immune phagocytes and other species. Our data show specific recruitment of SWAP70 to phagosomes in murine primary immune cells as well as the murine macrophage cell line RAW264.7. Second, we explored how phagocytosis and F-actin polymerization would be affected by the putative SWAP70 inhibitor sanguinarine (15, 26). Corroborating our previous findings that siRNA knockdown of SWAP70 results in phagocytic defects (6), we found that sanguinarine blocked phagocytosis in human dendritic cells. Third, we investigated the link between SWAP70 and coflin in phagocytosis. Coflin is an F-actin severing factor (18) that is sequestered by SWAP70 and this in turn interferes with its F-actin depolymerizing activity (14). In line with this, we observed increased activation of cellular coflin upon knockdown of SWAP70 by siRNA. Finally, we addressed the possibility whether SWAP70 would be recruited to the immune synapses of dendritic cells with T cells required for antigen presentation. Similar to phagocytosis, the formation of these immunological synapses relies on cellular rearrangements of the F-actin cytoskeleton, local turnover of phosphoinositides and activation of Rho-GTPases (2, 9, 10, 22, 53, 58), and all these factors could potentially recruit SWAP70 to the synapse. However, we observed no enrichment but rather a depletion of SWAP70 at the immune synapse between dendritic cells with T cells, both with a murine antigen specific immune synapse model and with the human allogenic mixed leukocyte reaction. Overall, these data support a broad role for SWAP70 as a specific tether and stabilizing factor of the phagosomal F-actin cage.
isolated from human blood (Fig. 1A). Whereas SWAP70 was expressed by mDCs, we observed almost no expression of SWAP70 in pDCs (Fig. 1B). This low expression of SWAP70 in pDCs could relate to their low phagocytic ability (36, 49). Stimulation of the mDCs with the bacterial adjuvant lipopolysaccharide (LPS) did not result in a significantly altered expression of SWAP70.

In the next step, we examined whether SWAP70 would be present on phagosomes of mouse BMDCs and RAW264.7 macrophages. In these experiments, we tested BMDCs differentiated both with GM-CSF or with FMS-like tyrosine kinase-3 ligand (FLT3L). Culturing in the presence of FLT3L results mainly in mixed populations of pDCs and conventional dendritic cells (cDCs), compared with the more heterogeneous mixture of macrophages and dendritic cells present in GM-CSF-differentiated BMDCs (32, 33, 39). Similar to our previous study (6), we used zymosan particles as phagocytic cargo and selectively searched for early phagosomes containing semi-internalized particles (i.e. nascent phagocytic cups). We loaded the cells with zymosan-FITC and immunolabeled with an anti-FITC antibody in absence of permeabilization, resulting in exclusive labeling of partially internalized and uninternalized zymosan-FITC particles (Fig. 1C; blue). In all cell types, endogenous SWAP70 was recruited in a polarized fashion to phagosomes and very often was found at nascent phagocytic cups. In GM-CSF differentiated dendritic cells and RAW264.7 macrophages, on average about ~10% of all phagosomes was positive for SWAP70 and about ~35% of all SWAP70-positive phagosomes were phagocytic cups (Fig. 1D), which resembles our previous observations with human moDCs (6). Compared to GM-CSF-differentiated dendritic cells and RAW264.7 macrophages, SWAP70 was present at significantly more nascent cups for FLT3L-differentiated dendritic cells, and this probably relates to a lower phagocytic capability and/or altered kinetics of uptake. In the same set of experiments, we compared the phagocytic ability of BMDCs and RAW264.7 macrophages by counting the fully internalized zymosan particles in those cells. Overall, FLT3L-differentiated BMDCs and RAW264.7 were phagocytosing much less zymosan per cell compared with GM-CSF-differentiated BMDCs (Fig. 1E). RAW264.7 is an Abelson murine leukemia virus-induced tumor cell line cultured for over 4 decades (47) and perhaps has lower phagocytic capability than primary phagocytes. FLT3L-differentiated BMDCs are substantially smaller (~30 µm diameter) compared with GM-CSF-differentiated BMDCs (~70 µm diameter) and perhaps their small cell size limits uptake of zymosan particles (average zymosan particle is ~3–5 µm in diameter).

In moDCs, we previously showed by live cell imaging transient recruitment of SWAP70 to early phagosomes concomitantly with F-actin (6). We made similar observations for RAW264.7 macrophages transiently co-expressing SWAP70 fused to GFP (SWAP70-GFP) together with the F-actin binding probe LifeAct fused to RFP (LifeAct-RFP), and observed simultaneous recruitment of both proteins to phagosomes engulfing zymosan (Fig. 2A, Movie S1). We also performed immunolabeling experiments for Rac1 to determine whether Rac1 was present at SWAP70 and F-actin-rich phagosomes in RAW264.7 macrophages, GM-CSF-differentiated BMDCs and FLT3L-differentiated BMDCs (Fig. 2B). In all 3 cell types, we frequently observed the co-localization of Rac1
to SWAP70 and F-actin-positive phagosomes (Fig. 2C), very similar to our previous observations with moDCs (6). These data show that SWAP70 is not only recruited to phagosomes in human dendritic cells, but also to phagosomes in murine dendritic cells and macrophages.

The putative inhibitor of SWAP70 sanguinarine blocks F-actin polymerization and phagocytosis

In our recent study, we demonstrated that siRNA knockdown of SWAP70 in human moDCs results in a defect in F-actin polymerization and phagocytosis in human moDCs (6). In the current study, we aimed to extent these findings and tested whether pharmacological inhibition of SWAP70 with the putative SWAP70 inhibitor sanguinarine (15, 26) would result in a similar functional outcome and block phagocytosis and F-actin polymerization. Sanguinarine is a quaternary ammonium
compound produced by a variety of plants and an inhibitor of the Na+/K+-ATPase. Although not directly proven, sanguinarine is proposed to inhibit SWAP70 action based on the findings that it can reverse the oncogenic transforming phenotypes of mouse embryo fibroblasts (MEFs) and the COS-7 fibroblast cell line expressing wild-type or an oncogenic mutant of SWAP70 (15, 26).

We tested the effect of sanguinarine on cell viability, phagocytosis and F-actin polymerization. moDCs were pre-cultured for 1 hr with sanguinarine before the 1 hr incubation with zymosan-FITC and phagocytic kinetic was measured by flow cytometry and confocal microscopy. Under these experimental conditions, sanguinarine did not affect viability as measured by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and co-staining with 7-aminoactinomycin D (7AAD; Fig. 3A). MTT is a substrate for mitochondrial reductases and allows to measure metabolic activity. 7AAD is a DNA intercalating dye that labels necrotic cells. However, we observed a significant increase in staining with the phosphatidylserine-binding probe Annexin-V at the highest concentration of sanguinarine tested (24 µm), suggesting that some moDCs entered the early apoptotic state (Fig. 3A). We then tested the effect of sanguinarine on phagocytosis by flow cytometry (Fig. 3B–D) and confocal microscopy (Fig. 3E–G). For all donors tested, the fraction of moDCs that took up zymosan-FITC was significantly reduced by sanguinarine and this zymosan-positive fraction took up considerably less particles than without sanguinarine (Fig. 3C–F). Similar phagocytic defects caused by sanguinarine were observed for neutrophils (1). Moreover, immunofluorescence microscopy revealed a reduced recruitment of SWAP70 to phagosomes upon sanguinarine treatment (Fig. 3G), in line with a role for SWAP70 in phagocytosis. We also investigated the effect of sanguinarine on the cellular content of F-actin filaments by staining the cells with the F-actin binding probe phalloidin conjugated to Alexa fluor 647. Using both flow cytometry and confocal microscopy, the phalloidin intensity was reduced by sanguinarine in a dose dependent manner (Fig. 3H–J). Thus, sanguinarine blocked phagocytosis and caused a defect in F-actin polymerization. These results mirror our previous observations with siRNA knockdown of SWAP70 in moDCs (6).

SWAP70 inhibits cofilin activity in dendritic cells

In the next set of experiments, we explored the link between SWAP70 and cofilin in phagocytosis. Recently, SWAP70 was described to directly regulate cofilin activity in vitro (14). The role of cofilin as a severing factor of filamentous actin is well-established (18) although its role in phagocytosis is still controversial (24). Cofilin is tightly spatiotemporally regulated by direct binding to regulatory proteins, phosphorylation and subcellular localization (23, 28, 57). Cofilin is also negatively regulated by binding to phosphatidylinositol (4,5)-bisphosphate (11). SWAP70 is reported to directly sequester cofilin and thereby interferes with its F-actin depolymerizing activity (14). In line with this finding, we observed that siRNA knockdown of SWAP70 in moDCs (75% knockdown efficiency by Western blot; Fig. 4A) resulted in a small (~15%) but significant decrease in the cellular content of inactive cofilin (Serine 3 phosphorylation (5, 56, 60)) (Fig. 4B–C). However, the detection of phosphorylated cofilin by Western blot is a global method that potentially underestimates
local effects at the phagosomal surface. Indeed, immunolabeling showed the enrichment of the inactive form of coflin at actin-rich phagosomes (Fig. 4D), suggesting that coflin was locally sequestered at phagosomes. We were unable to perform co-labeling experiments of inactive coflin together with SWAP70 due to the incompatibilities of the antibodies (both same host species and isotype). Thus, SWAP70 depletion not only leads to a reduced activation of Rac1 at the phagosomal surface as we have shown previously (6), but also to increased activation of coflin. Both these effects likely contribute to the formation and stabilization of F-actin at the phagosomal surface by SWAP70.

Fig. 2: SWAP70 in murine cells localizes on the phagosomal surface with F-actin and Rac1. (A) Live cell epi-fluorescence imaging of RAW264.7 macrophages expressing SWAP70-GFP (green in merge) and LifeAct-mRFP (magenta). The inset shows a time series during zymosan uptake (yellow square). See also Movie S1. BF: bright-field. Scale bar: 10 µm. (B) Confocal
micrographs of zymosan-pulsed GMCSF-differentiated BMDCs, FLT3L-differentiated BMDCs and RAW264.7 macrophages with immunolabeling for SWAP70 (red in merge) and Rac1 (green). F-actin was labeled with phalloidin (magenta). Yellow arrowheads: phagosomes positive for SWAP70, Rac1 and F-actin. Scale bars: 5 µm. (C) Venn diagrams and bar graphs from panel B showing phagosomal distributions of Rac1, F-actin and SWAP70 (N=3; >100 cells per cell type). Statistical significance was assessed for triplicates of the experiment using 1-way ANOVA with post hoc Tukey.

SWAP70 is not enriched at the immunological synapse between dendritic cells and T cells

The phagosome is not the only F-actin-rich structure in dendritic cells and macrophages. We recently showed that although SWAP70 is present at actin-dense mechanosensitive structures called podosomes (31), it is much more enriched at phagosomes (6). This is in line with the findings that phagocytosis depends on SWAP70 (6), whereas podosome formation and turnover do not depend on SWAP70 (31). Another F-actin-rich region is the immunological synapse formed between dendritic cells and T cells. The immunological synapse is a highly organized tight cellular interface that provides a platform for the presentation of antigen in major histocompatibility class I and II complexes (MHC class I and II) on the surface of the dendritic cell/macrophage to receptors on the surface of the T cell (58). In T cells, the actin cytoskeleton is well-understood to play an important role in synapse formation, and retrograde transport over F-actin filaments mediates the centripetal motion of T cell receptors and adhesion molecules to the synapse (48). F-actin also plays a key role at the dendritic cell-side of the immunological synapse, because it provides a mechanical counter force that keeps the immunological synapse in a more disorganized state with less clustering of T cell receptors (12, 25, 58). Here, the actin cytoskeleton of the dendritic cell polarizes toward the immunological synapse and this polarization is required for complete T cell activation (2, 3).

We tested whether SWAP70 would be enriched at the immunological synapse between dendritic cells and T cells with 2 model systems for immunological synapse formation. First, we performed a mixed leukocyte reaction, where human moDCs were co-incubated with leukocytes isolated from blood of a different donor. The C-type lectin DC-SIGN was used for labeling the dendritic cells, while the T cells were recognized by labeling for CD3. We did not observe clear localization of SWAP70 to the immunological synapse (Fig. 5A–B). When we included zymosan particles during the dendritic cell incubation with T cells, we observed massive enrichment of SWAP70 to phagosomes, but not to the immunological synapse (Fig. 5B). Second, we tested the recruitment of SWAP70 to an antigen-specific immunological synapse in mouse BMDCs. In these experiments, mouse FLT3L-differentiated BMDCs depleted of pDCs (B220Ccells) were used as these better resemble the conventional resident dendritic cells than GM-CSF-differentiated BMDCs (33,59). The BMDCs were activated in presence of the adjuvants LPS and 5’-C-phosphate-G-3’ (CpG) and preincubated with a peptide contained in the model antigen ovalbumin (OVA, residues 257–264). These dendritic cells can present the OVA\textsubscript{257–264} peptide in MHC class I to CD8\textsuperscript{+} cytotoxic T cells isolated from OT-I mice which express a T cell receptor specific for this peptide (17). We observed almost complete exclusion of SWAP70 from the immunological synapse, while SWAP70
was detectable in the F-actin-rich region facing away from the immunological synapse (Fig. 5C). Interestingly, in these FLT3L-differentiated BMDCs, SWAP70 was recruited to phagosomes containing latex beads of comparable size to T-cells (~6 µm diameter) (Fig. 5D). These data show that SWAP70 is not enriched at the immunological synapse between dendritic cells and T cells, but is specifically recruited to phagosomes.

Fig. 3: Putative SWAP70 inhibitor sanguinarine blocks phagocytosis and impairs F-actin polymerization in human moDC. (A) Viability tests of human moDCs by the MTT assay (Abs: absorbance units at 592 nm) and by flow cytometry with AnnexinV and 7AAD staining for the sanguinarine concentrations indicated (0-concentration: methanol control; MeOH). (B–D) MoDCs were pretreated with sanguinarine at the concentrations indicated and stimulated with zymosan-FITC for 1 hour. Shown are the flow cytometry histograms (panel B), the percentages of zymosan-positive cells (panel C; right from the gray dashed lines in panel B), and the mean fluorescence index (MFI) of zymosan-FITC (panel D). (E–G) Similar to panels B–D, but now phagocytic capability was determined by confocal microscopy. (E) representative confocal micrographs of moDCs with internalized zymosan-FITC (green in merge), immunolabeling for SWAP70 (red) and F-actin staining (magenta).
BF: bright-field. Scale bar, 10 µm. F: Phagocytic index calculated as the number of internalized zymosan per cell normalized to the condition without sanguinarine (N=3 donors, >90 cells/condition/donor). (G) Quantification of SWAP70-positive phagosomes per cell. H–I. Same as panels B and D, but now for the phalloidin Alexa Fluor 647 signal (phalloidin-647; F-actin) instead of zymosan-FITC. J. Same as panel G, but now for phalloidin-647-positive phagosomes. Statistical significance was assessed using one-way ANOVA with post hoc Tukey.

Discussion

In this study, we demonstrate that SWAP70 is recruited to phagosomes in murine dendritic cells and macrophages. This adds to our previous finding of transient recruitment of SWAP70 to phagosomes in human dendritic cells, and supports that SWAP70 could potentially be a general marker of early phagocytosis. We previously showed that phagosomal recruitment of SWAP70 is highly specific, and its recruitment to other F-actin-rich regions such as podosomes is much less compared with phagosomes (6). In the present study, we show that SWAP70 is not enriched at the immunological synapse, even though F-actin has a role in synapse formation and organization (2, 3, 12, 25, 5).

SWAP70 co-localized at the phagosome with the Rho-GTPase Rac1 and F-actin, and this strengthens our previous conclusion that SWAP70 specifically tethers Factin at the phagosome via Rac1 and PtdIns(3,4)P₂ (6). Since we did not observe enrichment of SWAP70 to the immunological synapse, it seems logical that one or both of these factors are not sufficiently present at the synapse.
Finally, we showed that SWAP70 promotes the inactivation of cofilin, likely by sequestering the inactive form of cofilin on the phagosomal surface as has been shown in vitro (14). As cofilin is a severing factor of filamentous Factin (18), this likely contributes to the stabilization of F-actin on the phagosomal surface by SWAP70 (6).

Our findings are of particular interest given the increasing evidence that SWAP70 has a potential role in cancer. SWAP70 is targeted by microRNAs in prostate cancers, and silencing of SWAP70 exhibited anti-tumor effects (16). In mouse embryo fibroblasts (MEFs), SWAP70 was necessary for acquiring an oncogenic phenotype upon v-Src transformation (27, 41) as well as for their spontaneous transformation (15). NIH3T3 cells and MEFs overexpressing SWAP70 bearing naturally occurring mutations exhibited increased growth rates and skewed signaling pathways (26, 52). Our data now show that besides its previously described role in promoting oncogenesis,
SWAP70 could also be required for the clearance of cancer material by phagocytes of the immune system. All these studies put SWAP70 forward as an interesting potential target for future anti-cancer therapeutic intervention.

Material and Methods

Animals

Wild-type female C57Bl/6JRccHsd mice were purchased from Charles River (Sulzfeld, Germany). For experiments including immune synapses of murine cells, the C57BL/6-Tg(TcraTcrb)1100Mjb/J/Thy1 strain (OT-I) was used. Mice were housed at the Central Animal Laboratory of the Radboud University Medical Center, Nijmegen. All animal experiments were performed under approval by the Animal Experimental Committee of the Radboud University Medical Center, and in accordance with European, national and institutional guidelines.

Cells

BMDCs were generated from 7–11 weeks old mice. Briefly, bone marrow cells were isolated and cultured in RPMI 1640 medium (Gibco), supplemented with 10% fetal calf serum (FCS), 1% L-alanyl-L-glutamine, 0.1% β-mercapto-ethanol and 0.5% antibiotics/antimycotics (AA, Gibco). For differentiation, this complete medium was supplemented with either 20 ng/ml GM-CSF or 200 ng/ml FLT3L (both from Peprotech) on the day of culture onset (Day 0). In both cases cells were used on day 8 of culture. MoDCs were generated as described (7), human macrophages were generated from blood-derived adherent monocytes by culturing in presence of recombinant human M-CSF (40 ng/ml) (R&D Systems; 216-MC-005) for 6 d. The fully differentiated human moDCs and macrophages were validated by comparative expression analysis of CD14, CD163 and CD206 with flow cytometry. Primary mDCs and pDCs were isolated from donor’s blood by magnetic bead separation with CD1c+ (Miltenyi Biotec; 130–090–506) and BDCA4+ (Miltenyi Biotec; 130–090–532) isolation kits. SiRNA knockdown procedures are described (6, 19). For live cell microscopy of RAW264.7 macrophages, cells were transfected with Fugene HD (Promega; Lot 0000027400; REF E231) following the manufacturer’s guidelines. The SWAP70-GFP and LifeAct-RFP plasmids and imaging protocols have been described (6). Cells were pulsed with IgG-opsonized zymosan as described (6). Day 8 FLT3L-differentiated BMDCs (depleted of B220+ cells with mouse CD45R MicroBeads, Miltenyi Biotec; 130–049–501) and activated for 4 h with 1 µg/ml LPS and 1 µg/ml CpG-B 1826) were pulsed with 6.0 µm-sized Polybead Carboxylate beads (Polysciences; Cat. No. 17141) for 1 h before fixation and staining.

Immunological synapse models

For the mixed leukocyte reaction, moDCs cultured on coverslips (50,000 cells) were first activated with LPS (1 µg/ml) for 3 h in complete in RPMI 1640 medium containing 10% FCS, 1% L-alanyl-L-glutamine and AA in a humidified, 5% CO2-containing atmosphere. MoDCs were then co-cultured for another 3 h with peripheral leukocytes (100,000 cells) that were directly isolated from blood of a different donor. Zymosan was added at a 1:10 cell-to-particle ratio during the final 1 hour
of incubation. For the OVA-specific immunological synapses, day 8 FLT3L-differentiated BMDCs (depleted of B220+ cells) were activated for 4 h with LPS and CpG-B. The OVA-derived 257–264 (SIINFEKL) antigenic peptide was added during the last 30 min of incubation. OT-I splenocytes were isolated from a 6 month old OT-I male mouse with a CD8a+ T Cell Isolation Kit (Miltenyi Biotec; 130–104–075). CD8a+ T cells were labeled with CFSE (ThermoFisher; C34554) for 10 min at RT and washed with MACS buffer. They were then resuspended in BMDC medium at a ratio of 2 OT-I cells to 1 FLT3L-differentiated cell. Synapses were allowed to form by culturing for 3 h. Cells were fixed for 20 min in 4% PFA and stained for SWAP70 and F-actin as described (6).

Immunoblotting, flow cytometry, MTT and AnnexinV-7AAD viability assays
Detailed immunoblotting procedures are described. Cofilin was immunolabeled with a rabbit monoclonal IgG anti-phospho cofilin (Ser3) (Cell Signaling; 3313P; used at 1:500). Immunolabeling protocols, FACS and MTT assays were performed as described (6, 19). Cells were treated with sanguinarine at 2, 6, 12, 24 μM concentrations in RPMI medium from a 1 mM stock in methanol similar to as described for the SHIP inhibitors (6). 2.4% methanol in RPMI was used as a solvent control (i.e., equivalent to the methanol content at the highest concentration of sanguinarine). AnnexinV-FITC (BD Biosciences; 56419) and 7AAD (eBioscience; 00–6993–50) viability tests were done according to the manufacturer’s guidelines. In short, drug-treated cells were stained on ice for 10 min in the presence of 1.5 mM CaCl2.

Microscopy
Protocols for immunofluorescence labeling and staining of the phagocytic cup with zymosan-FITC are described (6). The following primary antibodies were used: rabbit polyclonal anti-SWAP70 (Novus Biologicals; cat. no NB1–82979) at 1:200 dilution (v/v) for IF and 1:500 for Western blot; mouse monoclonal anti-Rac1 (Cell Biolabs; 240106) at 1:100; mouse IgG1 anti-FITC Alexa fluor 647 (Jackson ImmunoResearch; 200–602–037) at 1:200; mouse IgG2b anti-human DC-SIGN (BD PharMingen;551186; clone DCN46) at 1:100; mouse IgG2a kappa anti-CD3 (BioXcell; clone OKT3) at 500 ng/ml. Alexa fluor 633 labeled phalloidin was from ThermoFisher (A22884) and used at 1:200 (v/v). The following secondary antibodies were used: goat-anti-mouse Alexa fluor 488 (Life Technologies; A11029); goat-anti-rabbit Alexa fluor 568 or 647 (Life Technologies; A11036 and A21245). Samples were imaged on a Leica SP8 confocal microscope as described (6), or on a Zeiss LSM880 confocal microscope with 405 nm and 561 nm diode lasers, a multi-line argon laser and a 633 nm laser for excitation and a 63 x 1.40 NA oil immersion objective. Live cell microscopy was performed on a Leica DMi6000 epi-fluorescence microscope as described (6).

Statistics
All data were analyzed for a minimum of 3 independent donors/repeats using 1-way ANOVA (with post hoc Tukey) or with Student’s 2-tailed paired t tests (*P < 0.05; **P < 0.01; ***P < 0.001; ns: not significant). Data are plotted as average of SEM.
References

SWAP70 IS A UNIVERSAL GEF-LIKE ADAPTOR FOR TETHERING ACTIN TO PHAGOSOMES

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The Phosphoinositide Kinase PIKfyve Promotes Cathepsin-S-Mediated Major Histocompatibility Complex Class II Antigen Presentation
Abstract
Antigen presentation to T cells in major histocompatibility complex class II (MHC class II) requires the conversion of early endo/phagosomes into lysosomes by a process called maturation. Maturation is driven by the phosphoinositide kinase PIKfyve. Blocking PIKfyve activity by small molecule inhibitors caused a delay in the conversion of phagosomes into lysosomes and in phagosomal acidification, whereas production of reactive oxygen species (ROS) increased. Elevated ROS resulted in reduced activity of cathepsin S and B, but not X, causing a proteolytic defect of MHC class II chaperone invariant chain Ii processing. We developed a novel universal MHC class II presentation assay based on a bio-orthogonal “clickable” antigen and showed that MHC class II presentation was disrupted by the inhibition of PIKfyve, which in turn resulted in reduced activation of CD4+ T cells. Our results demonstrate a key role of PIKfyve in the processing and presentation of antigens, which should be taken into consideration when targeting PIKfyve in autoimmune disease and cancer.

Introduction
Endocytosis and phagocytosis of microbial pathogens, virus-infected cells, and cancer cells is essential for their immune clearance by phagocytes of the immune system (Fairn and Grinstein, 2012; Neefjes et al., 2017). In addition, dendritic cells present peptides derived from ingested antigens on major histocompatibility complex (MHC) classes I and II to T lymphocytes and are thereby responsible for the initiation of adaptive immune responses (Banchereau and Steinman, 1998). There are various types of endocytosis and phagocytosis, which differ in the receptors engaged, the role of the cytoskeleton, cage proteins, and lipid composition (Fairn and Grinstein, 2012; Levin et al., 2015; Nunes et al., 2013). Following uptake, endosomes and phagosomes undergo a transition from early to late endo/phagosomes and eventually to lysosomes. This process is mediated by membrane remodeling called maturation, where endosomes and phagosomes fuse with other endosomes and lysosomes and recruit cytosolic proteins to their membranes, such as Rab-GTPases and tethering factors (Egami, 2016; Fairn and Grinstein, 2012; Flannagan et al., 2012; Naüfer et al., 2012; Neefjes et al., 2017; Urpe-Querol and Rosales, 2017; Vieira et al., 2003). Early endosomes and phagosomes are only mildly acidic or even basic (Flannagan et al., 2009; Mantegazza et al., 2008; Savina et al., 2006), whereas lysosomes and phagolysosomes are very acidic due to activation of vacuolar proton pumps (pH < 5; Trombetta et al., 2003) and have increased presence and activity of metabolic enzymes for the degradation of ingested material (Kinchen and Ravichandran, 2008).

The MHC class II loading of proteolytically derived peptides occurs within specialized lysosome-like compartments called MHC class II compartments (MIIC) (Amigorena et al., 1994; Calafat et al., 1994; Castellino and Germain, 1995; Sadegh-Nasser, 2016; Tulp et al., 1994; West et al., 1994). Within these acidic luminal compartments, the non-polymorphic chain of MHC class II is proteolytically activated by its cleavage, reducing it to the 24-amino acid class II-associated Ii peptide (CLIP) (Jasanoff et al., 1999). Ii is a chaperone carrying sorting signals on its cytoplasmic
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... tail and interacting with MHC class II thereby controlling its trafficking from the endoplasmic reticulum to the trans-Golgi network (TGN), endosomal compartments, and eventually to the cell surface (Jones et al., 1978; Teyton et al., 1990). It prevents premature antigen loading into the cleft of MHC class II, whereas CLIP can be exchanged in MIIC for an antigen peptide by the chaperone human leukocyte antigen (HLA)-DM. The protease cathepsin S (CatS) plays an essential role for Ii processing to CLIP and for the proteolytic processing of ingested antigen (Honey and Rudensky, 2003; Riese et al., 1996; Villadangos and Ploegh, 2000), and only three cathepsins (CatB, CatH, and CatS) suffice for generating essential immunodominant epitopes in vitro (Kim et al., 2014a).

A central hallmark of endo/phagosomal maturation is the progressive change in the content of phosphoinositide lipids on the endosomal and phagosomal membrane (Levin et al., 2015; Shisheva, 2001). These phosphoinositides are important for endo/phagosomal maturation, as they are major determinants of organellar identity and form anchor points for numerous proteins involved in membrane trafficking or cytoskeletal tethering (Baranov et al., 2016, 2017; Levin et al., 2015). Within minutes after uptake, phosphatidylinositol 4,5-bisphosphate, which is the main phosphoinositide species at the plasma membrane, is converted into phosphatidylinositol 3-phosphate (PI(3)P) by various kinases and phosphatases. PI(3)P is the main phosphoinositide species at early endosomes and phagosomes and resides there for 5–10 min (Ellson et al., 2001a; Fratti et al., 2001; Gillooly et al., 2000; Vieira et al., 2003) before the acidification of these organelles (Naufer et al., 2018). The maturation of early into late endo/phagosomes and lysosomes requires the conversion of PI(3)P into phosphatidylinositol 3,5-bisphosphate (PI(3,5)P) (Ho et al., 2012; Sbrissa et al., 2002). PI(3,5)P is a low-abundance phosphoinositide amounting to 0.04%–0.1% of the total phosphoinositide pool (Ho et al., 2012; McCartney et al., 2014). Phosphoinositide 5-kinase (PIKfyve; yeast ortholog Fab1p) is the sole enzyme capable of producing PI(3,5)P from PI(3)P, which it targets via its FYVE domain (Ho et al., 2012; Sbrissa et al., 2002; Shisheva et al., 2015). Blocking of PIKfyve activity delays phagosomal maturation, leading to the buildup of cellular PI(3)P and a drastic reduction of cellular levels of PI(3,5)P (Hazeki et al., 2012; Kim et al., 2014b). Given the late endosome/lysosome-like nature of MIIC, a role of PI(3,5)P and PIKfyve in MHC class II presentation can be expected, but has not been shown.

PIKfyve can be inhibited by two small molecule inhibitors: apilimod (STA-5,326) (Cai et al., 2013) and YM201636 (Hazeki et al., 2012; Jefferies et al., 2008). Apilimod was originally developed to treat inflammatory diseases (Burakoff et al., 2006; Krausz et al., 2012; Sands et al., 2010; Wada et al., 2007, 2012) and was later found to specifically target and inhibit PIKfyve (Cai et al., 2013). YM201636 is a specific antagonist of PIKfyve (Jefferies et al., 2008), and although it has not been tested in clinical trials, it is commonly used to inhibit PIKfyve (Compton et al., 2016; Gomez et al., 2018; Hazeki et al., 2012; Ikonomov et al., 2009; Kerr et al., 2010; Kim et al., 2014b; Krishna et al., 2016; Sbrissa et al., 2012). Pharmacological inhibition of PIKfyve or expression of a dominant negative form of PIKfyve (Ikonomov et al., 2001; Jefferies et al., 2008; Shisheva, 2001) causes the formation of enlarged (“foam-like”) vacuoles (Cai et al., 2013; Dong et al., 2010; Jefferies et al., 2008; Min et al.,
2014), likely because of osmotic differences caused by the reduced activity of PI(3,5) P2-dependent cation channels such as the lysosomal cation channel TRPML1/MCOLN1 (Compton et al., 2016). These enlarged vacuoles are likely endosomes that cannot be reformed into lysosomes (Bissig et al., 2017), and their formation can be used as a readout of depletion of cellular PI(3,5)P2 (Kim et al., 2014b).

Given the role of late endosome/lysosome-related MIIC in the proteolytic processing of antigen and subsequent MHC class II loading, we hypothesized that interfering with PIKfyve activity would inhibit the degradation and presentation of antigen by dendritic cells. To address this hypothesis, we studied the effects of PIKfyve inhibition by apilimod and YM201636 on endo/phagosomal maturation, protease activity, and antigen presentation. As reported (Cai et al., 2013, 2014), PIKfyve inhibition blocked interleukin (IL)-12 secretion in monocyte-derived dendritic cells. PIKfyve inhibition also affected the maturation of phagosomes, with impaired acidification and lower recruitment of the lysosomal proteins lysosome-associated membrane protein 1 (LAMP1) and mannose 6-phosphate receptor (M6PR) to phagosomes, whereas the early phagosomal markers PI(3)P and EEA1 were more abundant (Compton et al., 2016; Dayam et al., 2017; Dove et al., 2009; Gayle et al., 2017a; Gomez et al., 2018; Hazeki et al., 2012; Ikonomov et al., 2009; Kerr et al., 2010; Kim et al., 2014b; Krishna et al., 2016; Sbrissa et al., 2007, 2012). Moreover, inhibition of PIKfyve selectively blocked the activity of cathepsin B and S, and this resulted in an increased presence of Ii-bound MHC class II within the cell, but not on the cell surface. PIKfyve inhibitors caused prolonged presence of the NADPH oxidase NOX2, which is present on early, but not late, endosomes and phagosomes (Dingjan et al., 2017a, 2017b). This resulted in increased reactive oxygen species (ROS) production, leading to lower activity of CatS. We show that the impaired processing of Ii and MHC class II trafficking defects upon blockage of endo/phagosomal maturation with PIKfyve inhibitors results in lower MHC class II antigen presentation. This was demonstrated with a novel assay based on a viral antigen carrying an unnatural amino acid amendable to bio-orthogonal labeling of the MHC-class-II-presented epitope. The combined effects of reduced proteolytic processing and reduced MHC class II presentation upon blocking PIKfyve result in an impaired activation of antigen-specific T cells. These results show a key role for PIKfyve in T cell activation, and this should be taken into consideration for the use of PIKfyve-targeting drugs in clinical trials.

Results

PIKfyve Inhibition Delays Phagosomal Maturation

We started by determining whether PIKfyve would localize to antigen-containing compartments. To visualize the localization of PIKfyve in live cells, we transfected dendritic cells differentiated from human-blood derived monocytes with plasmids coding for GFP-tagged PIKfyve (PIKfyve-GFP) (Figure 1A; Video S1). The actin cytoskeleton was visualized by simultaneous transfection with a plasmid coding for the F-actin-binding probe LifeAct fused to the red-shifted fluorescent protein mRFPruby (Riedl et al., 2008). Late endo/phagosomes and lysosomes were identified
with LAMP1 fused to red fluorescent protein (RFP) (Figure 1B; Video S2) (Sherer et al., 2003). The dendritic cells were pulsed with the model pathogen zymosan, relatively large yeast-derived particles (4–5 µm diameter) that allow for the unequivocal assignment of phagosomal localization of candidate proteins by microscopy and result in an increased expression of PIKfyve (Figure S1A). We observed that PIKfyve-GFP was recruited within 2 min after the formation of the phagosomal F-actin cytoskeleton (Figure 1A), which we recently showed is formed at the nascent cup of emerging phagosomes by tethering to phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂) and is subsequently removed after closure of the phagocytic cup (Baranov et al., 2016). At later time points after uptake (>7 min), the signal of PIKfyve-GFP gradually decreased at the phagosomes, whereas the signal of LAMP1-RFP increased (Figure 1B). We also immunostained zymosan-pulsed dendritic cells expressing PIKfyve-GFP for endogenous LAMP1 as well as for the early endosomal marker EEA1 (Figure 1C). EEA1 is known to bind to early endo/phagosomes by interactions of its FYVE domain with PI(3)P (Ho et al., 2012; Sbrissa et al., 2002), which is the substrate for PIKfyve. PIKfyve-GFP was partially co-residing with EEA1 on phagosomes, whereas overlap with LAMP1 was lower (Figure 1D). Together, these results show that PIKfyve is recruited to phagosomes within minutes after their formation and remains present at early phagosomes before their conversion into late phagosomes.
GFP and EEA1 double-positive phagosomes. (D) Quantification of (C) (150 phagosomes per donor; three donors). Scale bars, 10 μm. See also Figure S1.

Next, we validated the reported inhibition of endosome and phagosome maturation by blockage of PIKfyve activity with apilimod and YM201636 (Compton et al., 2016; Dayam et al., 2017; Dove et al., 2009; Gayle et al., 2017a; Gomez et al., 2018; Hazeki et al., 2012; Ikonomov et al., 2009; Kerr et al., 2010; Kim et al., 2014b; Krishna et al., 2016; Sbrissa et al., 2007, 2012). We selected concentrations of 200 nM apilimod (Cai et al., 2013; Compton et al., 2016; Terajima et al., 2016; Wada et al., 2012; Wong et al., 2017) and 4 µM YM201636 (Currinn et al., 2016; Ikonomov et al., 2009), which did not significantly affect the viability of the dendritic cells, although metabolic activity was somewhat affected by apilimod (by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay; Baranov et al., 2016; Baranov et al., 2017; Dingjan et al., 2016) and YM201636 treatment non-significantly increased the fraction of early apoptotic cells (by annexin V staining; Figures S1B–S1D). These concentrations were used in previous studies and resulted in inhibition of IL-12 production by the dendritic cells (Cai et al., 2013; Wada et al., 2012) (Figure S1E). Treatment of dendritic cells with apilimod or YM201636 resulted in massive cellular vacuolization (Figure S1F), a well-known effect of PIKfyve inhibition (Cai et al., 2013; Dayam et al., 2017; Krishna et al., 2016). This vacuolization was also observed when a dominant negative form of PIKfyve was expressed (PIKfyve-GFP with K1831E [Cai et al., 2013; Ikonomov et al., 2001; Sbrissa et al., 2000]) (Figure S1G) and with small interfering RNA (siRNA) knockdown (Figure S1H). Moreover, treatment of dendritic cells by apilimod or YM201636 compromised phagocytosis of the zymosan particles as well as endocytosis of fluorescently labeled albumin by almost half (Figures S1I and S1J). Previously reported PIKfyve perturbations led to inhibition of IgG-latex bead uptake by neutrophils (Dayam et al., 2017) and mouse RAW 264.7 macrophages (Kim et al., 2014b) and to lower *Escherichia coli* uptake by RAW 264.7 macrophages (Wong et al., 2017). To account for this reduced antigen uptake, we present our results relative to the total number of phagosomes per cell. Both apilimod and YM201636 treatment resulted in blockage of phagosome maturation, as apparent from a reduced fraction of LAMP1 and increased fractions of EEA1-positive (Figures 2A and 2B) and PI(3)P-positive phagosomes (Figures 2C and 2D). In addition, phagosomal recruitment of M6PR, another late endosomal marker that is involved in trafficking from the TGN to lysosomes, was also reduced upon apilimod and YM201636 treatment (Figures S1K and S1L). To exclude off-target effects of the drugs, we performed siRNA knockdown of PIKfyve. Even though we only reached a 50% knockdown efficiency of PIKfyve by qPCR (Figure S1M), this led to a reduced recruitment of LAMP1 to phagosomes (Figure S1N) similar to the small molecule inhibitors. Finally, acidification of the phagosomal lumen was reduced by apilimod or YM201636 treatment, as shown by experiments with zymosan conjugated to pH-Rodo, a pH-sensitive dye with increased fluorescence at low pH (Figures 2E and 2F). Similar blockage of acidification was observed with PIKfyve siRNA knockdown (Figure 2G).

**Cathepsin B and S Activities and Proteolytic Activation of MHC Class II Are Reduced upon PIKfyve Inhibition**
Phagosomal maturation and acidification are necessary to trigger the activity of hydrolases involved in the degradation of ingested antigen, and thereby for deriving antigenic peptides for presentation in MHC class II (Blum et al., 2013; ten Broeke et al., 2013). A major class of proteases are cysteine cathepsins, named after the catalytic cysteine located within their active sites (Verma et al., 2016) and consisting of 11 family members present in endosomes, phagosomes, and lysosomes (Turk et al., 2012). CatS is predominantly expressed by professional antigen-presenting cells, such as dendritic cells and B cells (Blum et al., 2013) and, unlike other cathepsins, is active not only in acidic but also in neutral and slightly alkaline environments (Jancic et al., 2007; Kirschke et al., 1989). As we observed that inhibition of PIKfyve by apilimod or YM201636 impaired lysosomal acidification and affected trafficking to late endosomes/lysosomes, we hypothesized that this would alter the activity of cathepsins. We applied the activity-based probe BMV109 to living cells before lysis (Verdoes et al., 2013). The BMV109 probe results in covalent attachment of a fluorophore to the catalytic sites of active cysteine cathepsins (Verdoes et al., 2013), thereby allowing for the quantitative assessment of their activities by in-gel fluorescence (Figure S2A). We compared unstimulated and zymosan-pulsed dendritic cells treated with apilimod or YM201636 (Figure 3A). Analysis of protein levels by western blot showed that PIKfyve inhibitors did not cause changes of total cathepsin levels (Figure S2A). For unstimulated cells, we observed reduced cathepsin B and S activities by treatment with apilimod or YM201636, whereas the activity of cathepsin X was not altered (Figures 3B, 3C, and S2B). However, stimulation of the cells with zymosan diminished these effects, and significant differences in cathepsin activity could no longer be detected after 1-hr incubation. Immunolabeling of endogenous CatS in phagocytic dendritic cells was visible at the core of the zymosan particles (Figure 3D). Microscopy experiments with the activity-based probe BMV109 confirmed that this is the site of cathepsin activity (Figures S2C and S2D). BMV109 signal became apparent at phagosomes 30–60 min after zymosan uptake and was present in LAMP1-positive phagosomes (Figure S2C). Immunofluorescence microscopy revealed that the majority of CatS-positive phagosomes are also LAMP1 positive (Figure S2E). Treatment with apilimod or YM201636 reduced the signals of both CatS immunostaining (Figures 3D–3E) and BMV109 (Figures S2D and S2F) at the phagosomes, indicating that trafficking of CatS to phagosomes is reduced by PIKfyve inhibitors. To determine whether the reduced CatS activity was solely attributable to its reduced recruitment to phagosomes upon PIKfyve inhibition, we performed experiments with BMV109 in lysates of dendritic cells (Figure S3A). For some conditions, apilimod and YM201636 still reduced the signal of BMV109 for CatS, whereas the signals for cathepsins B and X were unaltered (Figures S3B and S3C). We conclude that the reduced CatS activity upon PIKfyve inhibition is not solely due to altered trafficking, because CatS activity is also reduced in cell lysates.
positive phagosomes. (D) Quantification of (C) normalized to the total number of phagosomes per cell (~116 phagosomes per condition per donor; mean ± SEM of three donors). (E) Confocal imaging of dendritic cells treated with DMSO, apilimod, or YM201636 for 3 hr before addition of pHrodo-labeled zymosan 1 hr before live imaging. The color intensity of pHrodo (magenta) scales with acidic pH. Cyan in merge, Hoechst. (F) Quantification of (E); MFI, mean fluorescence intensity (mean ± SEM for three donors, ~1,000 phagosomes per condition per donor). (G) Same as (F), but now with siPIKfyve (~400 phagosomes per condition per donor; see also Figure S1M for knockdown levels). Scale bars: 10 µm. *P < 0.05, **P < 0.01, ***P < 0.001. See also Figure S1.

CatS is expressed at high levels by dendritic cells and has a strong immunological relevance, because it controls the generation of CLIP (Honey and Rudensky, 2003; Riese et al., 1996; Villadangos and Ploegh, 2000). Within endolysosomes, CLIP is derived from the invariant chain II via the intermediate fragments lip23 and lip10 (Amigorena et al., 1995; Neefjes and Ploegh, 1992; Villadangos et al., 2000). The final processing of lip10 into CLIP is mediated by CatS. The inhibition or loss of CatS in mice (Nakagawa et al., 1999; Shi et al., 1999) causes accumulation of lip10-bound MHC class II in endo/lysosomal compartments, impeding MHC class II trafficking to the cell surface (Brachet et al., 1997; Riese and Chapman, 2000). As inhibition of PIKfyve results in reduced activity of CatS, we hypothesized that apilimod and YM201636 would cause a defect of II processing and MHC class II trafficking. To address this hypothesis, we first assessed the effects of apilimod or YM201636 treatment on plasma membrane localization of MHC class II. Flow cytometry experiments with an antibody recognizing the extracellular domain of MHC class II revealed that apilimod or YM201636 treatment caused a small (10%–20%) but consistent reduction of the presence of MHC class II at the plasma membrane (Figures 4A and 4B). In contrast, total cellular levels of MHC class II, measured by immunolabeling in the presence of a detergent, were unaltered or even (inconsistently) increased (Figure 4C). Similar observations were made with dendritic cells.
derived from mouse bone marrow (Figures S4A–S4C). We also performed flow cytometry experiments with an antibody recognizing Ii or CLIP-bound to αβMHC class II (clone CerCLIP; Denzin et al., 1994), revealing that the presence of this inactive form of MHC class II was also reduced at the plasma membrane, whereas the total cellular levels were increased (Figures S4D–S4F). The defect in processing of CLIP upon PIKfyve inhibition was also observed by western blot with mouse bone-marrow-derived dendritic cells using an antibody recognizing the lip10 fragment (Figures S4G and S4H). Here, we used the CatS inhibitor LHVS (morpholinurea-leucine-homophenylalanin-vinylsulfone-phenyl) as a positive control (Palmer et al., 1995; Riese et al., 1998). A direct inhibition of CatS with LHVS also had a similar effect on MHC class II trafficking as PIKfyve inhibition with apilimod or YM201636 (Figures S4I and S4J). Microscopy imaging of phagocytic cells overexpressing fluorescently tagged MHC class II (Zwart et al., 2005) showed that MHC class II is recruited to phagosomes after zymosan uptake (Video S3). Immunofluorescence microscopy showed that treatment with PIKfyve inhibitors showed increased accumulation of endogenous MHC class II on the phagosomes (Figures 4D and 4E), suggesting a trafficking defect. Together, these results show that PIKfyve supports CatS activity and thereby regulates the proteolytic activation of MHC class II.

The Inactivation of Cathepsin S upon PIKfyve Inhibition Is due to Oxidative Modifications

In dendritic cells, NOX2 produces large amounts of ROS within the lumen of endosomes and phagosomes, and this ROS production is sustained for hours after uptake (Dingjan et al., 2016, 2017a, 2017b; Jancic et al., 2007; Joffre et al., 2012; Kotsias et al., 2013; Mantegazza et al., 2008; Nunes et al., 2013; Savina et al., 2006; Vulcano et al., 2004). ROS alter the processing of the ingested antigen for MHC classes I and II presentation (Hari et al., 2015; Mantegazza et al., 2008; Rybicka et al., 2012), with one of the mechanisms being the oxidative modification of a cysteine located within the catalytic site of CatS (Allan et al., 2014; Hari et al., 2015; Jancic et al., 2007; Kanai et al., 2001; Rybicka et al., 2012). In line with this, treatment of cells with the membrane-permeable organometallic compound phenylarsine oxide, which modifies cysteines, resulted in an inhibition of CatS, but not or less of cathepsins B and X (Figures 5A and 5B), indicating that the activity of CatS is particularly sensitive to cysteine modifications. We recently showed that NOX2 is already present at nascent phagosomes, because the catalytic subunit gp91phox of NOX2 is co-invaginated from the plasma membrane together with the antigen during phagocytosis (Dingjan et al., 2017a). Moreover, NOX2 requires the presence of PIP3, and/or PI(3)P for its activity and is active on early phagosomes (Anderson et al., 2010; Ellson et al., 2001b; Groemping and Rittinger, 2005; Kanai et al., 2001). We also showed that even though NOX2 resides in lysosomal compartments in naive dendritic cells, it is gradually removed from phagosomes during their conversion into LAMP1-positive phagolysosomes (Dingjan et al., 2017a, 2017b). As our data show that blockage of PIKfyve led to an accumulation of PI(3)P on phagosomes (Figures 2C and 2D) and delayed the conversion of early into late endo/phagosomes, we hypothesized that the reduced CatS activity was caused by an increased ROS production resulting from a prolonged presence and increased activity of
NOX2. The presence of phagosomal NOX2 upon PIKfyve inhibition by apilimod and YM201636 was assessed using immunofluorescence staining for its main catalytic subunit gp91phox (Dingjan et al., 2017a, 2017b). Treatment with apilimod and YM201636 resulted in a prolonged presence of gp91phox at zymosan-containing phagosomes compared with the DMSO control (Figures 5C and SD). We also measured ROS production in cells pulsed for 1 hr with zymosan using two different assays. First, apilimod treatment resulted in an increased extracellular presence of H$_2$O$_2$ as measured with the Amplex Red assay (Dingjan et al., 2016) (Figure 5E). Second, intra-phagosomal ROS production was measured with zymosan particles conjugated to the ROS-sensitive probe OxyBURST (Dingjan et al., 2017a), and this also showed an increased ROS production upon PIKfyve inhibition with apilimod or YM201636 (Figures 5F and 5G). Our data suggest that CatS is more sensitive to oxidative modifications than other cathepsins, and the increased ROS production upon PIKfyve inhibition can thereby explain the reduced activity of CatS (but not B and X) in cell lysates (Figure S3). Together, we conclude that PIKfyve inhibition results in an increased production of ROS within endo/phagosomes and that this leads to a reduced activity of CatS, which is more prone to oxidative modifications than cathepsins B and X.

PIKfyve Inhibition Impairs MHC Class II Presentation

In the final set of experiments, we tested whether inhibition of PIKfyve would lead to impaired MHC class II antigen presentation. We first performed a classical T cell activation assay with T cells isolated from transgenic OT-II mice (Barnden et al., 1998). CD4$^+$ T cells from these mice carry a T cell receptor specifically recognizing ovalbumin residues 323–339 (OVA$_{323-339}$) presented on H-2IAb MHC class II. Apilimod treatment resulted in a 25% reduction of production of interferon (IFN)$\gamma$ by the OT-II T cells over time, whereas YM201636 even completely blocked IFN$\gamma$ release (Figures S5A and S5B). However, the long incubation times with apilimod or YM201636 required for T cell activation, reduced the viabilities of both the dendritic cells and OT-II T cells by 20%–40% (Figures SSC and SSD). Moreover, as expected, surface presentation of MHC class II in those experiments was reduced by 10-30% upon PIKfyve inhibition (Figures SSE and SSF). Because of these effects, the impaired activation of T cells upon PIKfyve inhibition cannot be solely attributed to lower antigen presentation. Indeed, control experiments showed that both apilimod and especially YM201636 blocked T cell activation independent of antigen presentation, as these compounds resulted in reduced production of IFN$\gamma$ and lower surface expression of the activation markers CD25 and CD69 (Figure S6). Thus, although we observed a reduction of T cell activation by inhibition of PIKfyve, this is probably mainly caused by direct effects of apilimod and YM201636 on the T cells and the T cell activation assay does not allow to discern direct effects on MHC class II presentation.
To overcome this problem, we developed a new method allowing for direct visualization of MHC class II presentation. This is based on a recently developed assay for visualization of MHC class I presentation (Pawlak et al., 2015, 2016), and employs synthetic peptides as antigen carrying a non-natural amino acid amendable to bio-orthogonal ligation with fluorescent dyes. Based on the crystal structure of a complex of human HLA-DR1 MHC class II with influenza A virus hemagglutinin (HA) (strain A/Aichi/2/1968 H3N2) peptide (322–334 amino acid positions;
PKYVKQNTLKLAT; HA_{322-334} (Stern et al., 1994; Zavala-Ruiz et al., 2004), we synthesized a peptide consisting of the MHC class II epitope of influenza extended both N and C terminally with four natural amino acids (YGACPKYVKQNTLKLATGMRN; HA_{318-338}) (Figure S7, related to Table S1). This extended peptide needs to be processed and loaded by HLA-DM in MIIC to be presented on MHC class II. The central lysine (K326) was converted into a propargylglycine. Modeling indicated that the alkyne moiety of propargylglycine is solvent accessible when this peptide is bound to MHC class II (Figure 6A). This alkyne moiety can be conjugated to CalFluor-488, which increases the fluorescence signal by two orders of magnitude, resulting in a low signal-to-noise ratio (Shieh et al., 2015). Moreover, the alkyne moiety is only three carbon atoms in size and does not (or less) perturb the molecular mechanisms of antigen processing and presentation, in contrast to large fluorophores, and survives the harsh environment in lysosomes (Bakkum et al., 2018).

By incubating human dendritic cells with bio-orthogonal functionalized peptide HA_{318-338}, we could show increased MHC class II presentation over time for at least up to 5-hr incubation (Figures 6B–6D and S8). Moreover, no increase in CalFluor-488 signal was observed with negative control experiments with a wild-type HA_{318-338} peptide (without propargylglycine) or the HA_{322-334} epitope, which cannot be processed for HLA-DM loading onto MHC class II, or by proteolytically cleaving surface-exposed MHC class II with trypsin. Compared with the 5-hr time point, longer incubation (18 hr) led to reduction of the CalFluor-488 signal, suggesting that the peptide was internalized and/or degraded by the cells (Figure S8). When apilimod or YM201636 was present during the last 3 hr of 5- or 7-hr incubation with the bio-orthogonally functionalized peptide HA_{318-338}, the signals from CalFluor-488 were consistently reduced by 20%–80% (depending on the donor) (Figures 6C–6E), demonstrating that these drugs directly blocked presentation of the HA antigen. Thus using bio-orthogonal functionalized antigenic peptide, we could demonstrate that blockage of PIKfyve leads to impaired MHC class II antigen presentation.
Discussion

In this study, we studied the role of PIKfyve in MHC class II antigen presentation by dendritic cells. Our data confirm the key role of PIKfyve in phagosome maturation (Compton et al., 2016; Dayam et al., 2017; Dove et al., 2009; Gayle et al., 2017a; Gomez et al., 2018; Hazeki et al., 2012; Ikonomov et al., 2009; Kerr et al., 2010; Kim et al., 2014b; Krishna et al., 2016; Sbrissa et al., 2007, 2012) and treatment of dendritic cells with apilimod and YM201636 blocked recruitment of LAMP1, M6PR, and CatS to phagosomes. A still open question is via which mechanisms the reduced levels of cellular PI(3,5)P₂ cause these trafficking defects. Endosomal maturation via the conversion of Rab5- to Rab7-positive membranes by the endosomal tethering complexes CORVET (class C core vacuole/endosome tethering) and HOPS (homotypic fusion and protein sorting)
is well understood (Balderhaar and Ungermann, 2013). The role of PI(3,5)P$_2$ and downstream effectors of PI(3,5)P$_2$ in endosomal maturation is uncertain, and PI(3,5)P$_2$ might govern trafficking events via several possible mechanisms. The best characterized downstream effector of PI(3,5)P$_2$ is the cation channel TRPML1; its activity directly depends on PI(3,5)P$_2$. However, it is also not known if and how TRPML1 affects endo/phagosomal maturation. As TRPML1 triggers the efflux of Ca$^{2+}$ from lysosomes to the cytosol, one possibility is that it might be able to activate ALG-2, a dynein-interacting protein sensitive to Ca$^{2+}$. Dyneins promote retrograde vesicular migration along the tubular tracks, and this might somehow facilitate endosome-lysosome fusion (Li et al., 2016). Alternatively or additionally, the increased Ca$^{2+}$ efflux might directly promote intracellular fusion events via Ca$^{2+}$-sensing proteins (Hay, 2007). Another downstream effector of PI(3,5)P$_2$ is the V-ATPase, as yeast strains with mutations in the PIKfyve ortholog Fab1p showed impaired vacuolar acidification (Li et al., 2014), and this might explain the reduced acidification upon pharmacological inhibition or siRNA knockdown of PIKfyve.

Fig. 5: Pharmacological Inhibition of PIKfyve Promotes NOX2-Mediated ROS Production. (A) SDS-PAGE with in-gel fluorescence for the cathepsin-activity-based probe BMV109-Cy5 for dendritic cells pulsed with zymosan for 0.5 or 1 hr and untreated (0 hr). PAO, cysteine-modifying agent phenylarsine oxide; DMSO, vehicle control; GAPDH, loading control by western blot. (B) Quantification of (A) (individual donors shown; normalized to DMSO controls). (C) Confocal images of
representative dendritic cells pulsed with zymosan for 15, 30, or 120 min in the presence of apilimod (200 nM) or YM201636 (4 µM) for 3 hr and immunostained for gp91<sup>phox</sup>. Yellow arrowheads, gp91<sup>phox</sup>-positive phagosomes. Scale bar, 5 μm. (D) Quantification of (C); Gp91<sup>phox</sup>-positive phagosomes were counted and normalized to the total number of phagosomes per cell (mean ± SEM for three donors, 300 phagosomes per condition). (E) Extracellular H<sub>2</sub>O<sub>2</sub> measurements with Amplex Red assay for dendritic cells treated for 3 hr with apilimod and 1 hr with zymosan. (F and G) Phagosomal ROS production determined with zymosan labeled to both OxyBURST and Alexa Fluor 633 (AF633) and confocal live imaging. Cells were treated for 3 hr with apilimod (F) or YM201636 (G) and incubated for 1 hr with zymosan-OxyBURST/AF633. Signals from OxyBURST signal were normalized to that of AF633 (3,000 phagosomes per condition; individual donors shown). *P < 0.05, **P < 0.01, ***P < 0.001.

Our results showed that the blockage of phagosome maturation by PIKfyve inhibition resulted in reduced activity of CatS and B, but not of cathepsin X. Our data suggest that the reduced activity of CatS upon apilimod or YM201636 treatment might be attributed to both a reduced trafficking to phagosomes and an increased ROS production by NOX2. Our microscopy data show that CatS trafficking to LAMP1-positive phagosome relies on PIKfyve activity. Moreover, we showed that NOX2-produced ROS affect cysteine cathepsins, including CatS, because the ROS can modify cysteines within their catalytic cores (Allan et al., 2014; Hari et al., 2015; Jancic et al., 2007; Rybicka et al., 2012). The increased ROS production caused by a prolonged presence of NOX2 at phagosomes upon apilimod or YM201636 treatment might even be amplified by higher phagosomal levels of PI(3)P, which might enhance NOX2 activity through NOX2 subunit p40<sup>phox</sup> binding to PI(3)P by its PX domain (Anderson et al., 2010; Ellson et al., 2001b; Groomping and Rittinger, 2005; Kanai et al., 2001). An open question is why addition of zymosan reduced the inactivation of cathepsin B and S. As we also observe this effect in our lysate controls, perhaps the zymosan particles sequester ROS species and thereby prevent the oxidative modification of CatS. This suggests that the NOX2-mediated reduction of CatS activity will mainly have physiological relevance in the absence of phagocytic cargo, and thereby might be involved in the maintenance of self-tolerance (Steinman and Nussenzweig, 2002), for instance, for the induction of Th2 responses by immature dendritic cells (Na et al., 2016). In any case, our data show that PIKfyve-mediated phagosomal maturation affects the activity of cysteine cathepsins via NOX-produced ROS, and this can modulate the epitope repertoire for MHC presentation (Balce et al., 2011; Rybicka et al., 2010, 2012). Because NOX2 mediates the disulfide reduction of protein antigens (Ewanchuk and Yates, 2018), the effects of NOX2-produced ROS on antigen processing will be even more pronounced. Only linear peptide/protein stretches are accessible to proteases for cleavage, hence all inter- and intra-disulfide bonds have to be reduced before antigen processing (Bogunovic et al., 2010), as has been also demonstrated for MHC class II with model antigens (Jensen, 1991). γ-Interferon-inducible lysosomal thiol reductase activity can be inhibited by ROS, and this may cause different antigen processing patterns leading to altered repertoires of peptides for presentation, because some of the proteins cannot be unfolded and are therefore not accessible to proteinases for degradation (Ewanchuk and Yates, 2018).

In addition to the proteolytic processing of ingested antigens, CatS mediates the activation of MHC class II in MIIC via the processing of Ii to CLIP and is thereby required for antigen presentation (Nakagawa et al., 1999; Riese and Chapman, 2000; Shi et al., 1999). Our data show that inhibition
of PIKfyve by apilimod or YM201636 results in an accumulation of Iip10, the precursor of CLIP, and in reduced trafficking of MHC class II to the cell surface. This is in line with the finding that the inhibitor of PIKfyve AS2795440 caused a marked reduction of surface MHC class II expression on CD45R+B cells in anti-IgM-stimulated whole blood from rats (Terajima et al., 2016). These findings can be explained by the decreased activity of CatS, as incomplete invariant chain degradation is known to result in disturbance of MHC class II presentation and blockage of MHC class II transfer from endocytic compartments to the cell surface (Brachet et al., 1997; Riese and Chapman, 2000), as confirmed by our experiments with the CatS inhibitor LHVS. The proteolytic truncation of II to MHC-class-II-bound CLIP peptide is a prerequisite for antigen loading and thereby for MHC class II antigen presentation (Roche and Furuta, 2015), and surface trafficking of MHC class II is important for successful antigen presentation (Rocha and Neefjes, 2008). This might explain the reduced presentation of influenza virus HA, on HLA-DR1 by human dendritic cells upon YM201636 or apilimod treatment. Unsurprisingly, we also observed reduced activation of CD4+ T cells upon PIKfyve inhibition, although this is probably caused by the combined effects of (1) reduced uptake of antigen, (2) blocked production of IL-12/IL-23 and type I IFN (Cai et al., 2013, 2014), (3) reduced MHC class II presentation, (4) reduced cell viability of both the dendritic cells and T cells, and (5) other direct effects of apilimod and YM201636 on T cells.

Fig. 6: PIKfyve Inhibition Impairs MHC Class II Presentation. (A) Crystal structure of human HLA-DR1 (DRA, DRB1*0101; PDB: 1DLH) in complex with a virus HA (strain A/Aichi/2/1968 H3N2) peptide residues 322–334. The position of a clickable non-naturalized amino acid L-C-propargylglycine (pra) for bio-orthogonal labeling with CalFluor-488 is indicated (bright green); pockets for peptide binding at the HLA-DR1 cleft are highlighted in different colors. A scheme of the bio-orthogonal reaction is also shown. (B) Time line of stimulation with HA peptide residues 318–338 for MHC class II presentation by human dendritic cells. (C) Flow cytometry gating strategy for quantification of HLA-DR1 presented HA by labeling with CalFluor-488 as depicted in (A and B). Cell viability was assessed with fixable viability dye eFluo780. Surface levels of HLA-DR1 were assessed with HLA-DR-APC. (D) Quantification of HA-CalFluor-488 signals from (C) after 5 hr of stimulation with HA-peptide (bar graphs). Gray, non-specific background from CalFluo labeling of dendritic cells that were not incubated with HA-peptide; green, trypsin-positive control for HA removal from the cell surface; line graphs, individual donors with background subtracted and normalized to DMSO control. (E) The same as (D), but now for 7 hr of stimulation with HA.
peptide. *P < 0.05, **P < 0.01, ***P < 0.001. See also Figures S7 and S8 and Table S1.

To overcome this, we developed a new universal assay allowing for the direct visualization of MHC class II antigen presentation. This assay is an adaptation of a recent assay to measure antigen presentation on MHC class I (Pawlak et al., 2015, 2016) and is based on synthetic peptides containing an MHC class-II compatible epitope (in this case from HA residues 318–338 containing epitope residues 322–334). One residue on the epitope, which remains solvent exposed when bound to MHC class II, is converted to a non-natural propargylglycine, which contains an alkyne moiety of only three atoms in size. In contrast to large fluorescent labels, peptides carrying propargylglycine can still be proteolytically processed and loaded onto MHC class II. After fixation of the cells, the peptide bound to MHC class II at the surface of the dendritic cells can be labeled with CalFluor dyes that increase in fluorescence signal by orders of magnitude (Shieh et al., 2015). As previously shown for MHC class I (Pawlak et al., 2015, 2016), this signal increase allows for the detection of the limited amounts of MHC class-II-presented epitopes by flow cytometry. In contrast to assays that assess T cell priming and rely on co-stimulatory factors, such as the OT-II assay, the assay developed in this study provides a direct and quantitative readout of MHC class II presentation and is easily adaptable for different antigens and MHC haplotypes.

^ Fig. 7: Delayed Phagosomal Maturation Results in a Reduced MHC Class II Antigen Presentation. Model figure showing antigen uptake by a dendritic cell and subsequent processing within phagosomes by ROS and cathepsins. Upon phagosomal maturation, MHC class II bound to Ii is trafficked to the phagosome where cathepsin S is involved in the processing of Ii to CLIP, which can then be exchanged by an antigen to be presented by MHC class II on the cell surface. Inhibiting PIKfyve delays phagosomal maturation, and due to prolonged ROS formation, cathepsin S becomes oxidized and deactivated, thereby blocking CLIP-to-antigen exchange and eventually resulting in impaired antigen presentation.

In conclusion, we showed that the phagosomal maturation driven by phosphoinositide kinase PIKfyve is essential for MHC class II antigen presentation (Figure 7). Disruption of this process by the PIKfyve inhibitors apilimod and YM201636 delays the maturation process of the phagosome.
Delayed phagosomal maturation, in turn, results in elevated levels of ROS thereby deactivating CatS, which plays a key role in the proteolytic cleavage of Ii to CLIP and is essential for MHC class II antigen presentation. PIKfyve inhibition results in an incomplete processing of CLIP, and Ii-bound MHC class II is retained in phagosomes. Apilimod is proposed as a promising treatment for multiple variations of B cell non-Hodgkin lymphoma (NHL) due to its cytotoxic effect on B-NHL cancer cells lines (Gayle et al., 2017b) and was used in clinical trials for blocking IL-12/IL-23-mediated Th1/17 pro-inflammatory response in Crohn disease, psoriasis, and rheumatoid arthritis (Burakoff et al., 2006; Krausz et al., 2012; Sands et al., 2010; Wada et al., 2007, 2012). As concluded from NHL cancer cell line experiments, these cells have maximal sensitivity to apilimod, due to non-understood modulatory effects of PI(3,5)P2 on the transcription factor TFEB and apilimod leading to elevated expression of genes coding for lysosomal proteins, resulting in lysosomal swelling and disruption of lysosomal homeostasis and maturation, proliferation block, and cell death. In addition, PIKfyve might be a potential target for targeting solid tumors, as it plays a role in metastasis and promotes cancer cell migration and invasion together with the phosphoinositide phosphatase myotubularin-related protein 3 (MTMR3), which converts PI(3,5)P2 to PI(5)P (Oppelt et al., 2014). Depletion of PIKfyve and MTMR3, as well as inhibition of PIKfyve alone by YM201636, resulted in decreased in vitro migration of cancer cell lines of lung, rhabdomyosarcoma, and osteosarcoma origins (Oppelt et al., 2014), indicating that PIKfyve could be a novel therapeutic target in cancer migration. However, our results show that inhibition of PIKfyve impairs antigen presentation by dendritic cells and reduces activation of T cells. These effects on immune activation must be taken into account in clinical trials targeting PIKfyve.

Limitations of the Study

In this study, we addressed the role of phagosomal and endosomal maturation of MHC class II presentation with a focus on the phosphoinositide kinase PIKfyve. Most of our results are obtained using the small molecule inhibitors that are shown to be specific for PIKfyve: apilimod (Cai et al., 2013) and YM201636 (Jefferies et al., 2008). We confirmed that PIKfyve inhibition with these compounds was similar to PIKfyve siRNA knock down in primary human monocyte-derived dendritic cells, and both resulted in formation of enlarged endosomes, delayed phagosomal acidification, and delayed LAMP1 recruitment. However, the efficiency of our siRNA knockdown was incomplete, leading to less pronounced functional effects compared with the inhibitors. Our work could benefit from stable knockdown/out of PIKfyve to demonstrate that all experiments with drug inhibitors are comparable to knockout. However, the primary human monocyte derived dendritic cells used in our study are terminally differentiated and not amenable to CRISPR/Cas knockout.

A potential caveat of our study is that we only managed to immunolabel endogenous CatS, but not the other two cathepsins X and B. Therefore we cannot draw clear conclusions on the effects of PIKfyve inhibition on the trafficking of those cathepsin species.
We admit that MHC class II labeling with CalFluor-488 method has still some limitations, mainly a low signal-to-noise ratio, which is likely caused by a low number of MHC-class-II-presented epitopes. Additional optimizations of this method will be required in future.

In our live cell microscopy experiments we followed the trafficking of MHC class II to phagosomes, revealing that a large portion of it is recruited from the cell surface during formation of the phagosome. However, we only followed the fate of MHC class II during PIKfyve inhibition for a short time and never studied longer time points after zymosan uptake. It would be interesting to determine whether MHC class II also reaches phagosomes from an intracellular pool, as we recently showed for NOX2 (Dingjan et al., 2017a).

Our study raises the key open question as to which effectors of PI(3,5)P₂ mediate phagosomal maturation, as explained in the Discussion section. It would be interesting to identify those in the future.

Material and methods
Antibodies and reagents
The following antibodies were used: mouse monoclonal IgG1 anti-EEA1 at 1:100 (BD Biosciences; 610456 and 610457); rabbit polyclonal anti-LAMP-1 (CD107a) at 1:100 (Sigma; L1418); mouse monoclonal IgG1, κ anti-LAMP-1 (CD107a) at 1:100 (Biolegend; 328601, clone H4A3); mouse monoclonal IgG2a anti-M6PR at 1:50 in methanol fixed samples (Abcam; ab2733); rabbit polyclonal anti-GAPDH (14C10) at 1:1,000 (Cell Signaling; 2118); goat monoclonal IgG anti-Cathepsin X at 1:500 (R&D Systems; AF1033-SP); rabbit polyclonal anti-Cathepsin S at 1:250 (Novus Biologicals; NBP2-85807); mouse monoclonal IgG2a anti-Cathepsin B at 1:500 (Calbiochem; IM27L); mouse IgG2a,κ anti-HLA-DR, DP, DQ conjugated to FITC at 1:4 (BD Biosciences; clone Tü39) with isotype control mouse FITC-conjugated IgG2a,κ at 1:10 (BioLegend; 400208; clone MOPC-173); mouse monoclonal IgG1 anti-HLA-DR in complex with CLIP at 1:50 (Santa Cruz; sc-12725; clone CerCLIP.1); mouse IgG1, κ isotype control conjugated to APC at 1:10 (eBioscience; 17-4714-82); rat IgG2 anti-mouse CD74 at 1:500 (Biolegend; 151002); mouse monoclonal IgG1 anti-gp91phox at 1:1000 (MBL); mouse monoclonal IgG2a,κ anti-HLA-DR at 1:100 (BD Biosciences; clone G46-6, RUO); mouse monoclonal IgG2a,κ anti-human-HLA-DR at 1:100 (Biolegend; 307614; clone L243); rat IgG2b,κ anti-mouse I-A/I-E (MHC-II) conjugated to BV510 or PE at 1:200 (BioLegend; clone M5/114,15.2) with isotype control rat IgG2b,κ–BV510 or PE at 1:400 (BioLegend; 400645; clone RTK4530); anti-mouse CD69–PE from hamster at 1:100 (BD Biosciences; 553237; clone H1.2F3,RUO); anti-mouse CD25–APC from rat at 1:400 (eBioscience; 17-0251-82; clone PC61.5); OT-II were labeled with CellTrace Far Red labeling following the manufacturer’s guidelines (ThermoFisher; C34564). The following secondary antibodies were used at 1:400: donkey-anti-mouse IgG (H&L) Alexa Fluor 647 (ThermoFisher; A-31571); donkey-anti-rabbit IgG (H&L) Alexa Fluor 568 (ThermoFisher; A-10042); goat-anti-mouse IgG (H&L) Alexa Fluor 488 (ThermoFisher; A-11029); goat-anti-Rabbit IgG (H + L) labeled with IRDye 800CW at 1:5,000 (LI-COR; 926-32211); goat-anti-rat Alexa Fluor 680
Cell viability was assessed with the fixable viability dye eFluor450 (ThermoFisher; 65-0863-14) or eFluor780 (ThermoFisher; 65-0865-18) at 1:2,000 in PBS for 30 min on ice prior to 4% PFA fixation. The labeling for Flow cytometry was done as described (Baranov et al., 2016; Wimmers et al., 2016). All surface flow cytometry stainings were in PBA buffer (0.5% BSA and 0.01% NaN3 in PBS) and intracellular/total staining in PBA supplemented with 0.1% saponin as a detergent, all labeling was done for 10-30 min, PBS was used for washing. Annexin V-FITC (BD Biosciences; 556419) and 7AAD (eBioscience; 00–6993–50) labeling was performed as described (Baranov et al., 2017) on ice for 10 min in buffer containing 1.5 mM CaCl2. The MTT assay was performed as described (Baranov et al., 2016; Baranov et al., 2017) as follows: 0.66 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma M2128-1G) was in contact with the cells for 2 h before lysis with the following buffer: 90% isopropanol, 0.0125% SDS and 0.04 M HCl. Absorbance was analyzed at 595 nm.

Apilimod (STA-5326) was from Axon Medchem (ID 1369; CAS 541550-19-0) and MT-Diagnostics (USbiol-002800) and was used at 200 nM. YM201636 was from Calbiochem (ID 524611; CAS 371942-69-7) and used at 4 μM. Phenylarsine oxide (PAO) (at 1 μM) was incubated for 1 hour as described (Dingjan et al., 2016). LHVS (at 25 nM) was used as selective inhibitor for cathepsin S (provided by Dr. M. Bogyo, Stanford School of Medicine, CA, USA) (Koblansky et al., 2013). For control conditions, 1% DMSO was used (i.e., the highest vehicle content in all drug dilutions). In the cathepsin activity and confocal microscopy experiments, phagocytosis was induced using un-opsonized zymosan particles at a 1:10 and 1:5 cell-to-particle ratio respectively. For live cell microscopy, IgG-opsonized zymosan was used as described (Baranov et al., 2016). Briefly, 20 mg/ml zymosan suspension with an equal volume of opsonizing solution from ThermoFisher (Z2850) was incubated for 60 min at 37 °C and vigorously washed in PBS. BSA conjugated to Alexa fluor 488 was used at 10 μg/ml (ThermoFisher; A13100). Human IL12 was measured by ELISA using 96 flat bottom well plates (Microlon; 655092), coating antibody at 3 μg/ml (Pierce Endogen; M122), IL-12/IL-23 p40 detection antibody at 250 ng/ml (Thermo Scientific; M121B), Mouse IL12p70 (eBioscience; 88-7121-88) and INFγ (Invitrogen; 88-7314-88) were measured by ELISA following manufacturer’s kit guidelines. All flow cytometry experiments were done on a FACS-Calibur cytometer or a FACS Verse system (both BD Bioscience).

Animals
OVA-T cell receptor transgenic OT-I mice purchased from Charles River Laboratories (Sherbrooke, PQ) and OT-II mice purchased from Jackson Laboratories (Bar Harbor, ME) were held in a pathogen-free environment in Nijmegen Central Animal Laboratory, Netherlands, in accordance with institutional and European guidelines and approved by the Animal Experimental Committee (Radboud UMC). CD8+ T cells recognizing OVA257-264, SIINFEKL peptide or CD4+ T cells recognizing OVA323-339 Peptide presented on H-2IAb MHC II, were isolated from spleen as described (den Brok...
MHC-II-restricted OVA presentation to OT-II

For BMDCs differentiation, bone marrow cells of 7-11 weeks old mice was isolated and cultured in RPMI 1640 medium (Gibco) (10% fetal calf serum (FCS), 1% L-alanyl-L-glutamine, 0.1% β-mercaptoethanol and 0.5% antibiotics/anti-mycotics (AA, Gibco)) supplemented with 20 ng/ml GM-CSF (Peprotech). BMDCs were collected between day 6-7 for experiments (Baranov et al., 2017). Poly(lactic-co-glycolic acid) (PLGA) particles containing OVA were prepared as described (Dolen et al., 2016) and 200 μg of particles containing 10 μg OVA were used to stimulate 50,000 GM-CSF-cultured day 6-7 mouse BMDCs in 100 μl per well (96 well plate) starvation serum-free RPMI (2.1 mM ultra-glutamine and 50 μM β-mercaptoethanol) for 3 hours before drug addition to ensure comparable OVA-PLGA uptake. Next, free OVA-PLGA particles were washed away and the medium was changed to 200 μl per well complete RPMI (10% FBS, 2.1 mM ultra-glutamine and 50 μM β-mercaptoethanol) containing 100,000 OT-II pre-labeled with CellTrace Far Red (DMSO, Apilimod and YM201636 were added in concentrations mentioned above). The supernatants for ELISA and cells for flow cytometry were collected at 12, 24 and 48 hours and the cells were immediately pretreated with viability dye eFluor on ice as mentioned above, then fixed and antibody-labeled for flow cytometry. OT-I cells were isolated from splenocytes as described (Baranov et al., 2017) from a 6 month old OT-I male mouse with a CD8a+ T Cell Isolation Kit (Miltenyi Biotec; 130–104–075) and directly stimulated with OVA$_{257-264}$ peptide (SIINFEKL) at 1 μg/ml.

Receptor stimulation, RNA isolation, cDNA synthesis and qPCR

For stimulation of pathogen recognition receptors the following ligands were used: polyinosinic:polycytidylic acid (Poly:IC) (TLR3 agonist) at 100 μg/ml for 4 hours; lipopolysaccharides (LPS) (TLR4 agonist) at 1 μg/ml for 4 hours; resiquimod (R848) (TLR7 agonist) at 2.5 μg/ml for 4 hours; Pam(3)Cys (TLR2 agonist) at 5 μg/ml for 4 hours; zymosan (Dectin-1 and TLR2 agonist) at 2x10$^8$ particles/ml for 1 hour; peptidoglycan (PGN) (LTR2 agonist) at 10 μg/ml for 1 hour; OVA (CD206 mannose receptor agonist) at 1 μg/ml for 1 h. After stimulation, RNA isolation was performed from 1 million dendritic cells using Quick-RNA MiniPrep kit (ZymoResearch) in accordance with the manufacturer’s guidelines. For generating cDNA, master mix 1 (1 μl Random Hexamers (100 μM), 1.2 μl dNTPs (10 mM), RNA (1 μg) and H2O) was made. This mix was incubated at 65 °C for 5 minutes and directly chilled on ice. Mastermix 2 (4 μl 1st Buffer (5x), 2 μl DTT, 1μl RNAsin, 1 μl Reverse Transcriptase or H$_2$O) was added and the RT-program on a PCR machine was started. The RT-program consisted of the following steps: 10 min 25 °C, 50 min 37 °C, 15 min 70 °C, pause at 4 °C. After cDNA synthesis, qPCR was performed using 10 μl Sybr Green, 1.2 μl Primer Mix F + R (10 μM), 4.8 μl MQ and 4 μl cDNA (80 ng) dilution per well. The qPCR-program consisted of the following steps: 50 °C for 2 minutes, 95 °C for 10 minutes, 95 °C for 15 seconds, 60 °C for 1 minute and repeated 39 times. The results were analyzed using the Bio-RAD Prime PCR program. The primers that were used for qPCR of PIKfyve were 5’-TGTCT GTGCT TGATC CAAGT G-3’ and 5’-GCCAG GCCAA ATCAT CCTCT AA-3’.
siRNA knockdown assays

Post 72 hour knock-down in human dendritic cells was performed as described (Baranov et al., 2016). As control, siRNA irrelevant ON-TARGET plus non-targeting (NT) (Dharmacon) was used. The following cocktail of siRNAs was used for knockdown of PIKfyve (Invitrogen): 5’-GGAAA GGAAU UAGUC AACU GCUA-3’, 5’-GGAGA CCUCC GAGCU UGCUC AUAAU-3’, 5’-GAGGC CAGGG AGAAC AGCAG CCGUU-3’. Knockdown efficiency was measured with qPCR.

Microscopy and image processing

For the microscopy experiments, 100,000 dendritic cells were plated on 12 mm-diameter glass coverslips in serum free RPMI medium with 1% antibiotics/antimycotics and 2 mM ultra-glutamine and incubated at 37°C and 5% CO₂. Fixation in most cases was done with 4% PFA solution in PBS. For CatS immunostaining, we performed antigen retrieval according to the manufacturer’s protocol (R&D Systems; CTS013, CTS014, CTS015, CTS016). Fixation in 3% v/v glyoxal (Sigma-Aldrich #128465) for CatS and LAMP1 immunolabeling was done for 20 min as described (Richter and Revelo, 2018) and the solution mix was made with following ratios: 19.84 ml ddH₂O, 5.52 ml ethanol (absolute, for analysis), 2.18 ml glyoxal, 0.21 ml acetic acid, 1M NaOH was used to adjust pH to 4 or 5. Immunolabeling and blocking was performed with saponin permeabilization, as described (Baranov et al., 2016), in CLSM buffer (PBS with 20 mM glycine and 3% (w/v) BSA). Primary antibodies were incubated overnight, and secondary for 0.5-1h. In between, washing was done with PBS. Live cell imaging for BMV109 was done for 50,000 cells per well in μCLEAR F-bottom 96 well cell culture microplates (Greiner bio-one; 65500) in phenol red free and antibiotic free RPMI medium. The inhibitor and BMV109 concentrations and pipetting schemes were identical as for the in-gel fluorescence experiments. Samples were imaged with a Leica SP8 confocal laser scanning microscope with a 63x 1.20 NA water immersion objective (Leica HC PL APO 63x/1.20 W CORR CS2) or Leica DMI6000 epi-fluorescence microscope fitted with a 40×0.85 NA dry objective, a metal halide EL6000 lamp for excitation, a DFC365FX CCD camera, and GFP and DsRed filter sets (all from Leica). Images were analyzed with Fiji (ImageJ).

Solid-phase peptide synthesis

Peptides were synthesized on a pre-loaded Wang resin (0.05 mmol, 0.27-0.32 mmol/g; Nova Biochem) on a Liberty Blue CEM microwave-assisted peptide synthesizer. The synthesis was conducted via a standard Fmoc/tBu-protocol using the recommended coupling (5 eq. amino acids, 5 eq. DIC, 5 eq. OxymaPure in DMF, 1: 75 °C, 170 W, 15 s, 2: 90 °C, 30 W, 50 s) and deprotection methods (piperidine/DMF, 1:4, v/v, 1: 75 °C, 155W, 15 s, 2: 90 °C, 30W, 50 s). The following L-amino acid building blocks were used in the automated synthesis: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Pro-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH and Fmoc-Pra-OH (GL Biochem; ABCR). For all amino acids, except for arginine, single couplings were performed. Special care was taken for the incorporation of Cys, Pra and Arg residues. For cysteine and propargylglycine, the temperature of the microwave-assisted coupling
The Phosphoinositide Kinase PIKfyve Promotes Cathepsin-S-Mediated MHC-II Antigen Presentation

was reduced and the reaction time elongated (1: 25 °C, 0 W, 120 s; 2: 50 °C, 30 W, 480 s). Arginine was introduced by double coupling ((a) 1: 25 °C, 0 W, 1500 s, 2: 75 °C, 30 W, 120 s, (b) 1: 75 °C, 30 W, 300 s). After synthesis, the resin was filtered off, washed successively with DMF (5 times), methanol (5 times) and DCM (10 times) and dried in vacuo. Acidic cleavage from the resin was achieved by treatment with a mixture of trifluoroacetic acid (TFA) / triisopropylsilane / ethanedithiol / water (94:2.5:2.5:1, 5 ml, 2 h). The resin was extracted with additional TFA (5 ml), and the combined extracts were concentrated to 2 ml under a flow of nitrogen. The crude peptide was then precipitated in cold diethylether (10 ml) and isolated by centrifugation and decantation of the supernatant. The precipitate was washed twice with ice-cold diethylether and subsequently dissolved in 5 mL of water and then freeze-dried to give a fine white solid.

Peptide purification

Peptides were purified by preparative reversed-phase HPLC using a Pharmacia Äcta Basic 900 device (pump type P-900, variable wavelength detector UV-900) at flow rates of 10 ml/min, and a Macherey-Nagel Nucleodur 100-5-C18 ec, (250 mm by 21 mm, 5 μm) reversed-phase column. Linear gradients of water and acetonitrile or methanol (solvent A: water, 0.1 % TFA, solvent B: acetonitrile / water 4:1, 0.1 % TFA or methanol, 0.1 % TFA) over 30 min were used for purification.

Peptide characterization

Peptides were characterized by electrospray ionisation (ESI) and high-resolution (HR-MS-ESI) mass spectrometry on a Bruker maXis spectrometer (Billerica, USA). Analytical HPLC measurements were performed using a Thermo chromatography system (pumps UltiMate 3000, detector UltiMate 3000, autosampler UltiMate 3000 diode array) and an ACE Excel 2 C18 (2 μm, 2.1 x 100 mm) reversed-phase column. A linear gradient of water and methanol run (buffer A: water, 0.1 % TFA, buffer B: methanol, 0.85 % TFA) from 20-90 % buffer B over 15 min was used. Chromatograms were monitored at 220 nm wavelengths. The column was run at a controlled temperature of 50 °C.

Cell culture and transfection

Cultures of human monocyte-derived dendritic cells derived from peripheral blood monocytes (PBMCs) were obtained from buffy coats of healthy donors as described (Baranov et al., 2014; Baranov et al., 2016). Briefly, adherent monocytes isolated from the blood of healthy donors were in culture for 6 days in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Greiner Bio-one), 1 mM ultra-glutamine (BioWhittaker), antibiotics (100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 0.25 μg ml⁻¹ amphotericin B, Gibco), IL-4 (500 U ml⁻¹) and GM-CSF (800 U ml⁻¹). After that moDCs were detached from the plastic within 1h under cold PBS at 4 °C before freezing in liquid nitrogen in 10% DMSO and 40% FBS. Mouse GM-CSF differentiated BMDCs were generated as described (Baranov et al., 2017) and outlined above. The construct for full-length human PIKfyve was from Invivogen (cat nr puno1< hPIKFYVEb >) and subcloned in the EcoRI/BamHI sites of pEGFP-C1. pEGFP-HA-PIKfyve and pEGFP-HA-PIKfyve K1831E

111
within pEGFP-C2 were obtained from Assia Shisheva (Ikonomov et al., 2001). LifeAct-RFP was a gift from Michael Sixt (Max Planck Institute of Biochemistry, Martinsried, Germany) (Riedl et al., 2008). The mRFP-tagged LAMP1 is described (Sherer et al., 2003) and LAMP1-mGFP was a gift from Esteban Dell’Angelica (Addgene plasmid # 34831 (Falcon-Perez et al., 2005)). NCF4-PX-EGFP was a gift from Michael Yaffe (Addgene plasmid #19010, (Kanai et al., 2001)). The YFP was replaced by mCherry in HLA-DRA-IRES-HLA-DRB-YFP, which was a gift from Jacques Neefjes (Netherlands Cancer Institute, Amsterdam, Netherlands) (Zwart et al., 2005). Transfection was performed as described (Baranov et al., 2014; Baranov et al., 2016) with a Neon Transfection system (Invitrogen), a ratio of 3 μg of DNA per 1*10^6 cells was used, resuspended in 100 μl buffer R (Invitrogen) and electroporated (2 pulses of 40 ms, 1,000 V). After transfection, the cells were kept in antibiotic-free and serum-free RPMI medium (Invitrogen) for 3h before addition of full medium. Zymosan uptake and imaging experiments were performed 5–12 h post-transfection.

pHRodo zymosan
Dendritic cells (200,000 cells/well) were cultured in RPMI-1640 with 1% glutamine without phenol red in the presence of Apilimod or YM201636 for 3 h, in the final hour with drugs the cells were stimulated with 200 μl pHRodo Red Zymosan Bioparticles Conjugate for Phagocytosis (0.5 mg/ml) (ThermoFisher; P35364). Hoechst (5 μg/ml) was added 15 minutes before imaging. Samples were imaged with a Leica SP8 confocal laser scanning microscope with a 63x 1.20 NA water immersion objective (Leica HC PL APO 63x/1.20 W CORR CS2). Images were independently analyzed with Fiji (ImageJ) with identical treshholding parameters.

Cathepsin activity-based probe
Dendritic cells (300,000 cells/well) were cultured in RPMI-1640 with 1% ultra-glutamine (without Phenol Red) and incubated with different pharmacological treatments. Cells were stimulated using 15 μl unlabeled zymosan per well for 0, 0.5 or 1 h prior to lysis. Cathepsin probe BMV109-Cy5 at 1 μM (Verdoes et al., 2013) was added 0.5 h before lysis or in lysate control experiments probe BMV109-Cy5 was added immediately after lysis and incubated on ice for 1.5 h. In lysate control experiments probe BMV109-Cy5 was added immediately after lysis. Cells were centrifuged and washed with ice-cold PBS, lysed in hypotonic lysis buffer (50 mM PIPES pH 7.4, 10 mM KCl, 5 mM MgCl₂, 2 mM EDTA, 4 mM DTT, and 1% NP-40). The cells were cooled on ice for 10 minutes. The lysates were transferred to new tubes and BCA protein determination was performed (Micro BCA™ Protein Assay Kit, Thermo Scientific; 23235). The samples were resolved on 15% SDS-PAGE and in-gel fluorescence of BMV109-Cy5 of active cathepsins was tested with a TRIO+ Typhoon scanner (670BP-500PMT-Rex-ex-Focal plane 3x). The SDS gel was subsequently blotted and immunostained for GAPDH loading control. The membrane was incubated with secondary antibody coupled with IRDye 680 or 800 at a dilution of 1:5000 for 1 h. The membrane was washed three times with 0.05% Tween-20 for 10 minutes each and scanned with an Odyssey scanner (LI-COR). PAO was added to dendritic cells at 1 μM (Dingjan et al., 2016).
ROS measurements
ROS in apilimod treated dendritic cells was measured with the Amplex Red assay as described (Dingjan et al., 2016). Zymosan particles were co-labeled with ROS-sensor OxyBurst-Green H2DCFDA (ThermoFisher; D-2935) and Alexa-Fluor-633 C5-maleimide (A-20342, ThermoFisher) and live cell imaging was performed as described (Dingjan et al., 2017). For analysis, the OxyBurst fluorescence signal from each zymosan particle was divided by that of Alexa-Fluor-633.

HLA-DR1-restricted presentation of viral influenza HA
Peptides containing influenza A virus haemagglutinin (strain A/Aichi/2/1968 H3N2) peptide (HA) residues 322-334 with or without lysine 326 converted into L-propargylglycine were synthesized (as described in Solid-phase peptide synthesis section). 100,000 dendritic cells were pre-stimulated for 5 h in 100 μl serum-free RPMI with a water solution of HA-peptide at 5 μM final concentration. Apilimod or YM201636 were added 3 h prior to the end of the HA-stimulation. As a control, cells were incubated for 15 min at 37 °C with trypsin-containing buffer (NaCl 0.8% (w/v), KCl 0.04% (w/v), D-glucose 0.1% (w/v), NaHCO₃ 0.058% (w/v), EDTA 0.02% (w/v), trypsin 0.05% (w/v) (BD Difco 250; 215240)). Cell viability was traced with eFluor780 (ThermoFisher; 65- 0865-18) and cells were fixed in 4% PFA in PBS. At all steps before and during viability staining or click-labeling, the cells were washed at least 2 times with either PBS or HBS (N₂-bubbled, NaCl 150 mM, HEPES 20 mM, pH 7.4). Autofluorescence was reduced after 20 min by washing with quenching buffer (100 mM glycine, 100 mM NH₄Cl), surface presentation of HA was labeled for 1 h with CalFluor488 (Shieh et al., 2015) in HBS containing 1 mM CuSO₄, 0.5 mM THPTA (Tris(3-hydroxypropyltriazolylmethyl)amine), 5 mM sodium Lascorbate; 0.01% (w/v) BSA, aminoguanidine 2.2% (w/v). Finally, surface HLA-DR1 was labeled with mouse anti-human-HLA-DR conjugated to APC at 1:100 (BD Biosciences; 559866; clone G46-6, RUO) in PBA buffer (0.5% BSA and 0.01% NaN₃ in PBS).

Statistical Analysis
All data were analyzed using Student’s two-tailed paired t tests. A p value < 0.05 was regarded as statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).
References


Supplementary Figures

^Fig. S1: Characterization of PIKfyve inhibitors apilimod and YM201636. Related to Figure 2. (A) Expression of PIKfyve by qPCR in human monocyte-derived dendritic cells after 1 h stimulation with polyionosinic-polyctydilic acid (poly(I:C)) (TLR3 agonist); lipopolysaccharides (LPS) (TLR4 agonist); resiquimod (R848) (TLR7 agonist); Pam3Cys (TLR2 agonist); zymosan (Dectin-1 and TLR2 agonist); peptidoglycan (PGN) (TLR2 agonist); OVA (CD206 mannose receptor agonist). The expression levels were normalized to hydroxymethyl-bilane synthase (HMBS)(mean ± SEM for multiple donors). (B) Viability of human dendritic cells treated for 3 h with the indicated concentrations of apilimod or YM201636 by MTT assay (left) or fixable viability stain eFluo780 (right) (mean ± SEM for three donors). Black arrowheads: drug concentrations used throughout this study. DMSO: solvent control. (C) Representative flow cytometry dot-blots of dendritic cells stained with annexin-V (early
apoptotic cells) and 7ADD (necrotic cells) after 3 h treatment with apilimod (200 nM) or YM201636 (4 μM). (D) Quantification of panel C (individual donors shown). Left panel: quantification of sum of Q2 and Q3 population of annexin-V positive early apoptotic cells. Right panel: quantification of populations in Q1 and Q2 of 7AAD-positive necrotic cells. NS: not significant. (E) IL-12 production measured after 24 h LPS (1 μg/ml) stimulation by human monocyte-derived dendritic cells (left) or murine bone-marrow-derived dendritic cells (middle and right) in the absence or presence of apilimod or YM201636 (mean ± SEM for three donors or mice). (F) Confocal images of monocyte-derived human dendritic cells treated with DMSO, apilimod or YM201636 and immunostained for LAMP1 (magenta in merge). Cyan: DAPI. BF: bright-field. Yellow arrowheads: enlarged vacuoles. (G) Confocal images of representative dendritic cells expressing LAMP1-RFP with mouse wild-type or kinase-dead (K1831E) PIKfyve-GFP. Yellow arrowheads: enlarged vacuoles with the kinase-dead PIKfyve-GFP. (H) Representative images of moDC with siRNA knockdown of PIKfyve (siPIKfyve). siControl: non-targeting siRNA control. Yellow arrowheads: enlarged vacuoles. BF: bright-field. (I) Phagocytosis by dendritic cells measured with flow cytometry and FITC-labeled zymosan particles (zymosan-FITC) and DMSO, apilimod or YM201636 treatment. Cells were labeled with phalloidin-Alexa Fluor 647 (phal-AF647). Bar graphs show quantification of phagocytosing cells (Q2 population in representative scatter plots; mean ± SEM of 3 donors). (J) Same as panel I, but now for endocytosis of BSA-Alexa Fluor 488 (BSA-AF488) for 1 h. Representative histograms shown. (K) Confocal images of zymosan-pulsed dendritic cells immunolabeled for mannose-6-phosphate receptor (magenta in merge) and with 3 h treatment with DMSO (solvent control), apilimod or YM201636. Yellow arrowheads: phagosomes positive for mannose-6-phosphate receptor. (L) Graph: quantification of panel G for 3 donors (~300 phagosomes/condition/donor; mean ± SEM). (M) Quantification of siPIKfyve by qPCR (mean ± SEM of 3 donors). (N) Quantification of LAMP1-positive phagosomes as in main Fig. 2A-D, but now with siPIKfyve. Zymosan was added for 1 h before fixation (~160 phagosomes/condition/donor). Scale bars: 10 μm.
Figure S2: Control experiments for cathepsin activity and trafficking in human dendritic cells. Related to Figure 3. (A) Upper row: SDS-PAGE followed by in-gel fluorescence of human monocyte-derived dendritic cells treated with zymosan and activity-based probe BMV109-Cy5 (red bands in merge). Middle row: subsequent immunoblotting to PVDF and labeling with antibodies specific for cathepsins (Cat) X, B and S and GAPDH (green bands in merge). Lower row: merged images of BMV109 probe (red) and immunolabeled cathepsins (green) or GAPDH (green, only labeled on the bottom right blot). Every column of images is from a separate donor. Full SDS PAGE/PVDF blots are shown. (B) Quantification of CatS activity measured from panel A. Normalization of each condition is either to GAPDH or total CatS (individual donors shown; normalized to DMSO controls). (C) Confocal live cell imaging of representative dendritic cells expressing GFP-tagged LAMP1 (LAMP1-GFP; green in merge) and pulsed with BMV109 (red in merge) after 1h of zymosan stimulation. Yellow arrowheads: phagosomes.
positive for LAMP1-GFP and BMV109 signal. BF: bright-field. (D) Representative confocal live cell image of dendritic cells treated as in panel A (1h with zymosan). (E) Confocal image of zymosan-pulsed dendritic cell with immunolabeling for LAMP1 (magenta in merge) and cathepsin S (green). Blue: DAPI. Yellow arrowheads: phagosomes positive for LAMP1 and CatS. Orange arrowhead: phagosome with CatS only. (F) Quantification of panel D (~500 images per condition/donor; mean ± SEM of 3 donors). Scale bars: 10 μm.

Fig. S3: Cathepsin activity in lysate of human monocyte-derived dendritic cells. Related to Figure 3. (A) Zymosan pulsed dendritic cells were lysed and BMV109 probe was added to the cell lysates and incubated on ice for 1.5 h. Lysates were resolved with SDS-PAGE and in-gel fluorescence (right-hand scheme). GAPDH: loading control by Western blot. Only part of the SDS PAGE/PVDF blot is shown, the rest of the image carried no information. (B) Quantification of BMV109 signals from panel A (3 donors; individual donors shown; normalized to DMSO controls). (C) Same as panel B, but now BMV109 signal for CatS normalized to CatX and CatB.
Figure S4: Control experiments for cathepsin activity and trafficking in human and mouse dendritic cells. Related to Figure 4. (A) Gating strategy for flow cytometry experiments with mouse bone-marrow derived dendritic cells (BMDCs) where surface MHC-II (I-A/E(MHCII)-BV510) was accessed in non-permeabilized cells negative for the fixable viability dye eFluo780. Representative histograms show the geometric mean fluorescence intensities (gMFI). Cells were treated for 3 h with apilimod or YM201636; SSC: side scatter. FSC: forward scatter. Light blue: isotype control. (B) Quantification of viable eFluo780-negative cells and surface MHC-II signals from panel A normalized to DMSO solvent controls (individual mice shown; normalized to DMSO controls). (C) Same as panels A-B, but now in presence of detergent permeabilization for assessment of total cellular MHC-II. (D) Gating strategy for flow cytometry of human monocyte-derived dendritic cells (moDC) immunolabeled using an antibody recognizing CLIP-bound MHC class II (CLIP-MHCII-AF647) labeled with Alexa Fluor-647 (AF647). (E) Representative histograms and quantification of surface CLIP-bound MHC class II (CLIP-MHCII-AF647) with flow cytometry experiments of moDCs treated for 3h with apilimod (200 nM) or YM201636 (4 μM) (geometric mean fluorescence intensities (gMFI); individual donors shown; normalized to DMSO control). (F) Same as panel E, but now for total CLIP-bound MHC-II (Total CLIP-MHCIIAF647) with detergent permeabilization. (G) Representative Western blot of lysates from murine BMDCs labeled for CLIP. Lip10: 10 kDa precursor fragment of CLIP. LHVS: selective cathepsin S inhibitor. GAPDH: loading control. Only part of the SDS PAGE/PVDF blot is shown, the rest of the image carried no information. (H) Quantification of panel G normalized to GAPDH (BMDCs from different mice shown; normalized to DMSO control). (I-J) Same as D-F, but now using an antibody recognizing MHC-II (HLA-DR1) conjugated with APC and an extra condition with CatS inhibitor LHVS (25 nM) (individual donors shown; normalized to DMSO controls).
Figure S5. Control experiments for OT-II T cell priming experiments. Related to Figure 4. (A) Time line of murine OT-II T cell activation. Bone-marrow derived dendric cells (BMDC) were pre-incubated with PLGA-OVA particles for 3 hours. Subsequently, OVA_{323-339}-recognizing OT-II cells were added and the cells were exposed to apilimod or YM201636 for 12, 24 or 48 hours. (B) INFγ in supernatants by ELISA as indicated in panel A (mean from 3 independent experiments ± SEM). (C-E) Viability of experiment from panels A-B of OT-II cells (C) and BMDCs (D), and gating strategy (E). Viability was assessed with fixable viability dye eFluo405. OT-II cells were identified with OTII-CellTrace Far Red. SSC: side scatter; FSC: forward scatter (mean from 3 independent experiments ± SEM). (F) Surface expression of MHC class II (I-A/I-E-PE) for the experiments from panels A-E. gMFI: geometric mean fluorescence intensity. Representative histogram and quantifications are shown (normalized to DMSO control at 12 h; mean from 3 independent experiments ± SEM).

Figure S6. Apilimod and YM201636 impair T cell function. Related to Figure 4. (A) Scheme of experiment. OT-I T cells specifically recognizing ovalbumin residues 257-264 (SIINFEKL) in context of MHC-I were incubated with the epitope and PIKfyve inhibiting drugs apilimod or YM201636. (B) INFγ in the supernatants of the OT-I T cells from panel A. Note that YM201636 (but not or less apilimod) results in reduced production of INFγ. (C-F) Flow cytometry gating strategy and representative histograms (C) for T cell viability (D) and activation (E-F). Viability of OT-I T cells with fixable viability dye eFluo405 (D). Viable OT-I T cells were gated for early activation marker CD69-PE (E) or CD25-APC (F). SSC: side scatter; FSC: forward scatter; gMFI: geometric mean fluorescence intensity (mean from 3 independent experiments ± SEM).
The Phosphoinositide Kinase PIKfyve Promotes Cathepsin-S-Mediated MHC-II Antigen Presentation

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Fig. S7: Analytical HPLC profiles and mass spectrometry validation of synthesized peptides. Related to Figure 6. Analytical HPLC chromatograms of: (A) hemagglutinin (HA) (residues 318-338) long clickable, (B) HA(318-338) long non clickable, (C) HA(322-334) short non clickable, and (D) HA(318-338) long clickable. ESI and HR-ESI-MS spectra of (E) HA(318-338) long clickable m/z (ESI) = 775.3956 [M+3H]3+, 1162.5909 [M+2H]2+ and m/z (HR-ESI-MS) = calculated: 775.0610 [M+3H]3+, found: 775.0613 [M+3H]3+, (F) HA(318-338) long non clickable m/z (ESI) = 472.2 [M+5H]5+, 590.0 [M+4H]4+, 786.4 [M+3H]3+, 1079.0 [M+2H]2+ and m/z (HR-ESI-MS) = calculated: 1178.6168 [M+2H]2+, found: 1178.6183 [M+2H]2+, (G) HA(322-334) short non clickable m/z (ESI) = 376.7 [M+4H]4+, 501.9 [M+3H]3+, 752.4 [M+2H]2+ and m/z (HR-ESI-MS) = calculated: 752.4483 [M+2H]2+, found: 752.4489 [M+2H]2+ and (H) HA(322-334) short clickable m/z (ESI) = 490.9 [M+3H]3+, 735.9 [M+2H]2+ and m/z (HR-ESI-MS) = calculated: 735.9194 [M+2H]2+, found: 735.9198 [M+2H]2+.

^ Fig. S7: Analytical HPLC profiles and mass spectrometry validation of synthesized peptides. Related to Figure 6. Analytical HPLC chromatograms of: (A) hemagglutinin (HA) (residues 318-338) long clickable, (B) HA(318-338) long non clickable, (C) HA(322-334) short non clickable, and (D) HA(318-338) long clickable. ESI and HR-ESI-MS spectra of (E) HA(318-338) long clickable m/z (ESI) = 775.3956 [M+3H]3+, 1162.5909 [M+2H]2+ and m/z (HR-ESI-MS) = calculated: 775.0610 [M+3H]3+, found: 775.0613 [M+3H]3+, (F) HA(318-338) long non clickable m/z (ESI) = 472.2 [M+5H]5+, 590.0 [M+4H]4+, 786.4 [M+3H]3+, 1079.0 [M+2H]2+ and m/z (HR-ESI-MS) = calculated: 1178.6168 [M+2H]2+, found: 1178.6183 [M+2H]2+, (G) HA(322-334) short non clickable m/z (ESI) = 376.7 [M+4H]4+, 501.9 [M+3H]3+, 752.4 [M+2H]2+ and m/z (HR-ESI-MS) = calculated: 752.4483 [M+2H]2+, found: 752.4489 [M+2H]2+ and (H) HA(322-334) short clickable m/z (ESI) = 490.9 [M+3H]3+, 735.9 [M+2H]2+ and m/z (HR-ESI-MS) = calculated: 735.9194 [M+2H]2+, found: 735.9198 [M+2H]2+.
^ Fig. S8: Control experiments for bio-orthogonal labeling of MHC-II presented epitope. Related to Figure 6. (A) Human dendritic cells were incubated with hemagglutinin (HA) peptides carrying an unnatural propargylglycine amino acid amendable to bio-orthogonal labeling with CalFluor488. Left: flow cytometry dotblots with dendritic cells with (green) and without (background; black) HA peptide. SSC: side scatter. Right: gMFI (geometric mean fluorescence intensity) and population of HA-presenting cells incubated for the indicated times with long (residues 318-338) or short (residues 322-334) HA peptides with or without clickable propargylglycine amino acids. (B) The cells from panel A were tested for surface exposed MHC-II (HLA-DR) by flow cytometry. Representative histograms (left) and quantification by gMFI and population of HLA-DR-positive cells (right) are shown (mean from 5 donors ± SEM).

| HA(322-334)  | C_{98}H_{118}N_{18}O_{19}S_{2}  | 1503.8 [g/mol] | H-P-K-Y-V-K-G-N-T-L-K-L-A-T-OH |
| HA(322-334)  | C_{98}H_{118}N_{18}O_{19}S_{2}  | 1469.8 [g/mol] | H-P-K-Y-V-K-G-N-T-L-K-L-A-T-OH |

^ Table. S1: Related to Figure 6. Synthesized hemagglutinin (HA) peptides with and without propargylglycine.
Supplemental References


Podosomes of dendritic cells facilitate antigen sampling
Abstract

Dendritic cells sample the environment for antigens and play an important role in establishing the link between innate and acquired immunity. Dendritic cells contain mechanosensitive adhesive structures called podosomes that consist of an actin-rich core surrounded by integrins, adaptor proteins and actin network filaments. They facilitate cell migration via localized degradation of extracellular matrix. Here, we show that podosomes of human dendritic cells locate to spots of low physical resistance in the substrate (soft spots) where they can evolve into protrusive structures. Pathogen recognition receptors locate to these protrusive structures where they can trigger localized antigen uptake, processing and presentation to activate T-cells. Our data demonstrate a novel role in antigen sampling for the podosomes of dendritic cells.

Introduction

The main function of dendritic cells is antigen presentation. Immature dendritic cells are localized in the spleen and other non-lymphoid tissues and constantly sample tissue and blood for antigens by so-called pattern recognition receptors (PRRs) (Lipscomb and Masten, 2002; McGreal et al., 2005; Takeda and Akira 2005; Kaparakis et al., 2007). Dendritic cells express many different PRRs that recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide, from the outer membrane of gram-negative bacteria, as well as peptides of bacterial, fungal or viral origin. Binding of antigen to some PRRs, such as C-type lectins, can trigger uptake of antigen via receptor-mediated endocytosis or phagocytosis. Recognition of antigen results in maturation of dendritic cells and surface presentation of the antigen by major histocompatibility complex (MHC) molecules, upregulation of specific co-stimulatory molecules and finally migration to the lymph nodes, where dendritic cells prime T-cells (Banchereau and Steinman, 1998; McGreal et al., 2005). Because only dendritic cells can induce a primary immune response in resting naive T-lymphocytes, they are considered a crucial component that balances the innate and adaptive immune systems. For antigen sampling, immature dendritic cells need to migrate from their sites of origin (i.e. bone marrow) to specific sites of sampling activity (peripheral tissues), and for activation of T-cells, mature dendritic cells need to travel to the draining lymph nodes where they present antigen to T-cells.

Immature dendritic cells form particular cell–matrix contacts called podosomes that facilitate cell migration within peripheral tissues (reviewed in Buccione et al., 2004; Gimona et al., 2008; Linder et al., 2011; Murphy and Courtneidge, 2011; Schachtner et al., 2013b). Podosomes consist of an actin-rich core region surrounded by integrins from which anti-parallel actin filaments radiate (Schmidt et al., 2011; van den Dries et al., 2013b). Adaptor proteins such as vinculin, talin and paxillin connect the cortical actin cytoskeleton to the plasma membrane and to integrins, and are enriched around the actin-rich cores of podosomes. Unlike focal adhesions, podosomes contain the protein WASP that can recruit the actin nucleating complex Arp2/3 (Linder et al., 1999; Burns et al., 2001; Jones et al., 2002; Calle et al., 2006). Podosomes are points of concentrated release of the metalloproteinase MMP14 (also called MT1-MMP) and several other proteases that locally degrade the extracellular matrix.
matrix (ECM) (van Helden et al., 2006; Gimona et al., 2008; Linder et al., 2011; Murphy and Courtneidge, 2011). Protease release at podosomes promotes cell invasiveness and facilitates cell migration through endothelium, epithelium and connective tissue (Matías-Román et al., 2005; Carman et al., 2007; Linder, 2007; Schachtner et al., 2013b). In fact, podosomes are commonly located at the leading edge of migrating cells and their turnover is required for cell migration and passage through endothelium (Burns et al., 2001; Calle et al., 2006). WASP-deficient leukocytes do not form podosomes and chemotactic migration is severely compromised in these cells (Zicha et al., 1998; Jones et al., 2002; Dovas et al., 2009). Podosomes are mechanosensitive (Collin et al., 2008) as demonstrated by their selective localization on the edges of 3D-micropatterned substrates (van den Dries et al., 2012).

Podosomes are not only formed by dendritic cells (Burns et al., 2001) but also by many other types of adherent cells, such as smooth muscle cells, endothelial cells, megakaryocytes, fibroblasts, macrophages, osteoclasts, microglia and at the postsynaptic site of the neuromuscular junction (Buccione et al., 2004; van Helden et al., 2006; Proszynski et al., 2009; Linder et al., 2011; Schachtner et al., 2013a). Although podosomes are very similar to the well-studied invadopodia of cancer cells (Wolf and Friedl, 2009), which are involved in cancer metastasis (Sabeh et al., 2004; Bravo-Cordero et al., 2012), they are considered to differ from invadopodia in at least two ways. First, podosomes are very dynamic with lifetimes that can be as short as 1 to 12 min, whereas invadopodia can persist for hours (reviewed in Linder et al., 2011; Murphy and Courtneidge, 2011). Second, podosomes are smaller and measure only 0.5 to 2 μm in diameter and protrude only about 0.4 to 2 μm, whereas invadopodia can reach 8 μm in diameter and can protrude more than 5 μm from the cell surface into the extracellular environment (Linder et al., 2011; Murphy and Courtneidge, 2011). However, this limited protrusion depth of podosomes is contradicted in a recent study where it was shown that podosomes of dendritic cells could protrude more than 3 μm into the pores of polycarbonate filters (Gawden-Bone et al., 2010). Thus, the geometry of the substrate not only dictates the localization of podosomes (van den Dries et al., 2012), but might also affect the morphology of podosomes and allow podosomal protrusion into the extracellular environment.

The study by Gawden-Bone et al. (Gawden-Bone et al., 2010) demonstrated that when dendritic cells were cultured on filters, podosomes formed precisely on top of the pores and could extend into the lumen of the filter pores. Both the release of MMP14-containing vesicles and uptake of extracellular material occurred at the tips of these protrusive structures (Gawden-Bone et al., 2010). These results are interesting because dendritic cells are well known to be capable of sampling for antigen in the lumen of the lung, gut and small intestine by extending ‘protrusive dendrites’ through the epithelium of these organs (Rescigno et al., 2001; Niess et al., 2005; Chieppa et al., 2006; Vallon- Eberhard et al., 2006; Lelouard et al., 2012; Thornton et al., 2012; Farache et al., 2013; Shan et al., 2013; Strisciuglio et al., 2013). We speculated that this trans-epithelial antigen sampling might be related to the protrusive podosome-like structures first described by Gawden-Bone et al. (Gawden-Bone et al., 2010) and performed a molecular characterization of these actin-rich structures. Here,
we demonstrate that podosomes of human dendritic cells can evolve into protrusive structures that help the cell to sample for antigen from deeper within the substrate. A wide range of PRRs locate to these protrusive structures where they can trigger receptor-mediated uptake of antigen. Our results show a direct role for podosomes in antigen presentation, and this constitutes a new mechanism of how cells might be able to sense extracellular signals through physical barriers.

Results
Podosomes extend in soft spots of the substrate
When human dendritic cells are cultured on a glass substrate, they form clusters of podosomes (Fig. 1A) (Burns et al., 2001; van Helden et al., 2006). Podosomes on glass appear as dome-shaped actin-rich structures with an actin core of ~350 nm diameter and a height of ~500 nm, surrounded by a 250-nm wide ring of integrins and integrin-associated proteins, such as vinculin, talin and paxillin (Labernadie et al., 2010; Schmidt et al., 2011; Cox et al., 2011; van den Dries et al., 2012; van den Dries et al., 2013b). It has been recently reported that when dendritic cells are cultured on polycarbonate filters with pores of defined size, podosomes locate on top of the filter pores (Gawden-Bone et al., 2010). This alignment of the podosomes with the filter pores is likely related to the mechanosensitivity of podosomes that form around local deformations of the substrate (van den Dries et al., 2012). Indeed, when we cultured human dendritic cells for 1 hour on gelatin-soaked polycarbonate filters with 400-nm diameter pores, almost all podosomes formed directly on top of the filter pores (Fig. 1B–E). The actin-rich cores of these podosomes were surrounded by adaptor proteins, such as talin, vinculin and paxillin, similar to podosomes formed by cells on glass substrates (Linder et al., 2011). Optical z-sectioning by confocal microscopy revealed that these podosomes did not protrude into the lumen of the 400-nm-sized pores of the polycarbonate filters (less than 1 μm protrusion depth; Fig. 1B).

We speculated that a pore size of 400 nm might be too small to allow for protrusion of podosomes. To address this, we cultured dendritic cells on gelatin-impregnated filters with larger pores of 1 μm diameter, identical to those employed by Gawden-Bone et al. (Gawden-Bone et al., 2010). Indeed, as reported previously (Gawden-Bone et al., 2010), we observed clear protrusion of actin-rich structures into these 1-μm-sized pores (Fig. 2A) and regardless of whether the filters were impregnated with gelatin. The actin-rich core of these podosome-like structures could penetrate through the entire filter of ~10-μm thickness. The actin-rich cores were surrounded by adaptor proteins, such as vinculin, which was predominantly present at the base of the pores and did not seem to protrude into the 1-μm-sized pores. However, vinculin increasingly protruded into the lumen of the pores when cells were cultured on filters with larger pore sizes of 2 μm and 3 μm in diameter (Fig. 2B,C). On filters with these larger pore sizes, podosomes were not exclusively colocalized with the pores but were increasingly also present in between the pores (Fig. 2D). On filters with 3-μm-sized pores, small individual podosomes could be distinguished at the edges of the pores and the pore size was large enough to facilitate migration of the whole cell through the filter pores (Fig. 2C,E), as reported previously (Gawden-Bone et al., 2010). Thus, for cells cultured...
Podosomes of dendritic cells facilitate antigen sampling

on polycarbonate filters with increasing pore sizes from 400 nm to 3 µm, the morphology of the podosomes differs; in smaller pores, the actin core and, in larger pores, adaptor proteins, such as vinculin, can progressively protrude into the lumen of the pores.

Overall, a picture is emerging where the initial non-protrusive podosomes of dendritic cells search for soft spots of low physical resistance in the substrate (e.g. the filter pores). When such a place is found, podosomes become increasingly invasive and can protrude into the substrate by local degradation of extracellular matrix and by exerting mechanical forces (Gawden-Bone et al., 2010; Labernadie et al., 2010). This results in growth of a protrusive podosome-like structure and at some point (i.e. for pores above 3 µm in diameter; Gawden-Bone et al., 2010) facilitates transmigration of the cell through physical barriers. Dendritic cells and other blood leukocytes have to cross blood vessel and lymphatic endothelium in order to activate the immune system (Muller, 2011; Vestweber, 2012). Secretion of the metalloproteinase MMP14 and turnover of podosomes are well-known to be essential for this transendothelium migration (Matias-Roman et al., 2005; Calle et al., 2006; Carman et al., 2007). Indeed, dendritic cells can migrate through
monolayers formed by the endothelial cell line EA.hy926 (supplementary material Fig. S1A,B). Dendritic cells are also able to penetrate and pass monolayers formed by the epithelial cell line Caco-2 (supplementary material Fig. S1C–E) (Rescigno et al., 2001; Strisciuglio et al., 2013) and, for megakaryocytes, podosomes have also recently been shown to play a role in this epithelial penetration (Schachtner et al., 2013a). Thus, it is increasingly clear that podosomes facilitate cell migration across both endothelial and epithelial barriers.

Characterization of protrusive podosome-like structures
To further investigate the protrusive podosome-like structures on filter substrates, we performed immunostainings for typical podosomal marker proteins on dendritic cells cultured on filters with 1-µm pore sizes. Similar to podosomes of cells cultured on glass (Fig. 1A), the F-actin-rich core of protrusive podosome-like structures was surrounded by rings or clusters of talin, paxillin and the integrins ITGAM (a subunit of integrin αMβ2) and ITGB1 (integrin b1) (Fig. 3A). Interestingly, protrusive podosome-like structures often (for ~10–30% of all podosomes), but not always (for the remaining ~70–90% of podosomes), contained a ring of actin at the base of the protrusions, as could be observed with both phalloidin in fixed cells (Fig. 3B) and with the F-actin-binding probe LifeAct-GFP in live cells (supplementary material Fig. S2A,B). This is in clear contrast to podosomes formed by cells on glass substrate, which are generally considered to contain solid cores of actin (Fig. 1A). Integrins stimulate the production of phosphatidylinositol 4,5-bisphosphate (PIP$_2$), which stimulates WASP activity (Prehoda et al., 2000) and binds directly to talin and vinculin (Brakebusch and Faessler, 2003), and PIP$_2$ accumulates at and is essential for the formation of invadopodia of cancer cells (Yamaguchi et al., 2010). We therefore probed for PIP$_2$ by exogenously adding bacterially expressed pleckstrin homology domain of phospholipase C delta subunit fused to citrine (PLCδ-PH) (van den Bogaart et al., 2011). PLCδ-PH stained a ring surrounding the actin-rich core, demonstrating the presence of PIP$_2$ in this region (Fig. 3C). Podosome-like protrusive structures still depended on WASP (Linder et al., 1999; Burns et al., 2001; Jones et al., 2002; Calle et al., 2006), because treatment of the cells with wiskostatin, a selective and reversible inhibitor of WASP (Peterson et al., 2004), resulted in disassembly of the actin-rich protrusions at a similar rate to that of podosomes of cells cultured on glass (Fig. 3D,E) (Dovas et al., 2009; Tsujita et al., 2013).

Degradation of ECM occurs at podosomes and is dependent on the local release of metalloproteinases, such as MMP14 (Buccione et al., 2004; Gimona et al., 2008; Linder et al., 2011; Schachtner et al., 2013b). Indeed, degradation of ECM took place at protrusive podosome-like structures as apparent by the (~2-fold) local increased fluorescence intensity when the filters were impregnated with double-quenched FITC-labeled collagen in gelatin (Fig. 4A,B), in agreement with previous results (Gawden-Bone et al., 2010). We observed by immunocytochemistry that MMP14 was present in the protrusive podosome-like structures (formed on 1-µm-sized pores) and this is a clear difference from the (non-protrusive) podosomes of cells cultured on glass, where MMP14 was not enriched but rather seemed excluded from the actin-rich podosome cores (Wiesner et al., 2010) (Fig. 4C). MMP14-containing vesicles are known to traffic intracellularly.
Podosomes of dendritic cells facilitate antigen sampling on microtubules in a kinesin-mediated manner (Wiesner et al., 2010; Cornfine et al., 2011) and to travel via microtubules to the tip of invadopodia of cancer cells (Schoumacher et al., 2010). Our data suggest that MMP14-containing vesicles could also traffic via microtubules to the tip of protrusive podosome-like structures, because microtubules clearly penetrated the F-actin ring of these structures and extended into the lumen of the filter pores (Fig. 4D). This tubulin protrusion again is different from podosomes of cells cultured on glass, where the extension of the tubules into the podosomal cores was not observed.

^ Fig. 2: Podosome-like protrusive structures of dendritic cells. (A) Dendritic cells cultured on polycarbonate membrane filters impregnated with Alexa-Fluor-633-labeled gelatin (gray) with pore sizes of 1 µm. Cells were immunostained for vinculin (green) and actin was visualized with phalloidin (Phal, magenta) similar to in Fig. 1. Shown are confocal sections just above the surface of the filters. The yellow line indicates the position of the orthogonal views showing the protrusion of actin in the lumen of the pores. Yellow arrowheads depict randomly selected actin-rich cores. The red arrowhead indicates the approximate filter surface. The right-hand panel shows a schematic diagram of the protrusions (actin: magenta; vinculin: green). (B) Same as A, but now for filters with pore sizes of 2 µm and showing the protrusion of both the actin core and vinculin in the pores as indicated by the white arrows. The cells also formed podosomes that did not overlap with the pores as indicated by the yellow arrowheads. (C) Same as B, but now with pore sizes of 3 µm. (D) Quantification of the fraction of actin cores aligning with the filter pores from panels A–C (means ± s.d.). (E) Same as C, showing cell migration through the 3-µm pores in the filter (white arrows) and the formation of podosomes on both sides of the filter (yellow arrowheads). Confocal sections above, through and underneath the filter are shown. Scale bars: 5 µm.

The substrate influences the lifetime of podosomes, and periodic undulations of the actin-rich core are proposed to contribute to the protrusive and mechanosensitive properties of podosomes (Schachtner et al., 2013a; van den Dries et al., 2013a). Indeed, podosomes seem to become less dynamic and more stable when they become more invasive, because protrusive podosome-like structures have lifetimes that exceeded several hours compared to less than 1 h for podosomes on glass substrate. Moreover, the periodic undulations of the actin cores of protrusive podosome-like structures were reduced ~2-fold compared to podosomes of cells cultured on glass (supplementary material Fig. S2C–E; Movies 1, 2) (see also Labernadie et al., 2010; van den Dries et al., 2013a). Taken together, we conclude that when podosomes encounter spots of low physical resistance of the substrate (e.g. the filter pores), their morphology and protein composition change as they become increasingly more protrusive and less dynamic. We next
addressed the role of endocytosis at these protrusive podosome-like structures of dendritic cells.

Protrusive podosome-like structures are involved in antigen sampling
In a previous study (Gawden-Bone et al., 2010), uptake of extracellular material at protrusive podosome-like structures of dendritic cells was shown by electron microscopy. In accordance with this observation, we found that the coat protein clathrin localized to these protrusive structures (Fig. 5A–C). Given that it is well-established that dendritic cells can sample for antigen across epithelial membranes (Rescigno et al., 2001; Niess et al., 2005; Chieppa et al., 2006;
Podosomes of dendritic cells facilitate antigen sampling

Vallon-Eberhard et al., 2006; Lelouard et al., 2012; Thornton et al., 2012; Farache et al., 2013; Shan et al., 2013; Strisciuglio et al., 2013) and as confirmed by our observations with monolayers of the epithelial cell line Caco-2 (supplementary material Fig. S1E), we hypothesized that antigen recognition and uptake might occur at the protrusive podosome-derived structures. In order to test this possibility, we first determined whether PRRs involved in antigen uptake located to these protrusive structures. We cultured monocyte-derived dendritic cells on filters with 1-µm pore sizes for 1 hour and performed immunostaining for various PRRs (the C-type lectin family receptors DC-SIGN (also known as CD209), DCIR (also known as CLEC4A), dectin-1 (also known as CLEC7A) and the mannose receptor CD206 (also known as MRC1)). We also stained for the transferrin receptor CD71 (also known as TFRC) as a general endocytic receptor. All of these PRRs and CD71 localized to protrusive podosome-like structures, but not to non-protrusive podosomes on glass substrates (Fig. 5D–F). The presence of DC-SIGN and CD206 at these protrusive structures was confirmed by immunogold labeling followed by transmission electron microscopy (similar to Gawden-Bone et al., 2010), where we observed a ~3-fold (DC-SIGN) to ~9-fold (CD206) enrichment of gold beads at the protrusive structures compared to at the ventral membrane of the cells (i.e. membrane in contact with the filter surface) (Fig. 6). We subsequently determined the antigen sampling activity of these PRRs that localize to protrusive podosome-like structures.

We performed functional uptake experiments of quantum dots tethered to the HIV-1 envelope...
glycoprotein gp120 as a prototype antigen (Cambi et al., 2007); gp120 is a ligand for DC-SIGN (Geijtenbeek et al., 2000) and these gp120-coated quantum dots have a particle size of about 40 nm, which is comparable to the size of many viruses (Cambi et al., 2007). We also performed uptake experiments with OVA (ovalbumin), a well-characterized antigen and ligand for the mannose receptor CD206 (Burgdorf et al., 2006). Leakage experiments demonstrated that gelatin-impregnated filters were not or only poorly permeable to both OVA and gp120-quantum dots (Fig. 7A–C). We measured cellular uptake of gp120-quantum dots and of OVA through these gelatin-impregnated filters by fluorescence microscopy (Fig. 7D–F). Live-cell imaging showed that uptake of OVA through the filter occurred at the actin-rich protrusive structures (Fig. 7F). Uptake could be suppressed by the inhibitor of both clathrin-dependent (von Kleist et al., 2011) and independent (Dutta et al., 2012) endocytosis, Pitstop 2, indicating that uptake occurred via endocytosis (Fig. 7G).

Importantly, uptake of both gp120-quantum dots and OVA through the filters was promoted by the presence of protrusive podosome-like structures, because uptake was strongly reduced (1) when the dendritic cells were cultured on filters with a 400-nm pore size (where podosomes form but cannot protrude; Fig. 1B) (Fig. 8A,B) and (2) when CHO cells heterogeneously expressing recombinant DC-SIGN were used instead of dendritic cells (Fig. 8A). These CHO–DC-SIGN cells
Podosomes of dendritic cells facilitate antigen sampling. The antigen was not only taken up by the dendritic cells through the filters, but also subsequently processed by proteases, as apparent from the increase in fluorescence of double-quenched OVA (Fig. 8D,E). Degradation of this double-quenched OVA by proteases in endosomal/lysosomal compartments results in loss of fluorescent quenching and an increased fluorescence intensity. Control experiments with bafilomycin demonstrated that this processing was dependent on the acidification of endosomal/lysosomal compartments. Bafilomycin blocks the vesicular ATPase.
and thereby prevents the acidification required for activation of proteases. We performed experiments with dendritic cells isolated from mouse bone marrow (BMDCs) to determine whether dendritic cells that took up OVA antigen via podosomes could activate T-cells. Similar to human dendritic cells, these mouse BMDCs were able to form protrusive podosome-like structures on filters with 1-µm pore sizes and these protrusive structures contained vinculin, MMP14 and CD206 (the receptor for OVA antigen (Burgdorf et al., 2006) (supplementary material Fig. S3B). Finally, BMDCs that were loaded with OVA antigen via the filters could activate mouse hybridoma DO11.10 T-cells (supplementary material Fig. S3C). DO11.10 T-cells recognize OVA (323–339 epitope) bound to MHC class II and this results in an increased secretion of interleukin (IL)-2 (Shimonkevitz et al., 1983). However, owing to (1) the great sensitivity of T-cells, (2) the limited amount of dendritic cells we could culture on the filters (~103 cells) and (3) the inability to completely wash OVA from the gelatin coated filters (because of non-specific binding), we were unable to exclude the possibilities that antigen was directly taken up from the medium during the prolonged T-cell incubation step or that antigen leaked out of the cells or was regurgitated to be presented to the T-cells in trans rather than in cis. Nevertheless, co-localization experiments indicated that the antigen at least partly reached compartments containing MHC class II in human dendritic cells (Fig. 8F). We conclude that antigen uptake can occur at the protrusive structures of dendritic cells that contain podosomal elements and this antigen can be subsequently processed by dendritic cells, which might eventually result in T-cell activation.

Discussion

In this study, we demonstrate that podosomes can evolve into protrusive structures that can contribute to antigen sampling of dendritic cells. It is increasingly well established that podosomes respond to and sense the stiffness and geometry of the cellular substrate (Collin et al., 2008; Labernadie et al., 2010; van den Dries et al., 2012). Podosomes localize to spots of low physical resistance in the substrate. At these soft spots, podosomes exert physical forces (Labernadie et al., 2010) and locally degrade extracellular matrix through the concentrated release of metalloproteinases, such as MMP14 (Buccione et al., 2004; Gimona et al., 2008; West et al., 2008; Gawden-Bone et al., 2010; Linder et al., 2011; Schachtner et al., 2013b). This can result in remodeling of the ECM, and when or if pores are formed of sufficiently large size (>1 µm), podosomes become progressively protrusive and less dynamic and the morphology and protein composition of podosomes change. These findings are in agreement with the role of invasive podosome-like structures in the formation of transcellular pores in the endothelium (Carman et al., 2007).

At least at the base of protrusive podosome-like structures, actin forms a ring-shaped structure that aligns with the edges of the pore. This differs from (non-protrusive) podosomes on glass substrates, which are generally considered to consist of solid cores of actin, although recent stochastic optical reconstruction microscopy (STORM) super-resolution microscopy data from our group also seems to suggest in this case that there is an uneven distribution of actin in podosome
Podosomes of dendritic cells facilitate antigen sampling cores that cannot be resolved by conventional diffraction-limited microscopy (van den Dries et al., 2013b). Microtubules penetrate the actin ring of the protrusive podosome-like structures and likely facilitate delivery of MMP14-containing vesicles to the protrusive tips (Wiesner et al., 2010; Cornfine et al., 2011), similar to in the invadopodia of cancer cells where this is well established (Schoumacher et al., 2010). This again differs from podosomes on glass substrates where MMP14-containing vesicles do not seem to reach the core but only transiently contact the periphery of podosomes (Wiesner et al., 2010) and, there, perhaps the dense actin cores prevent the entry of microtubules.

Fig. 7. Antigen uptake by protrusive podosome-like structures. (A) Schematics of the control experiments for passive leakage of quantum dots or OVA–Alexa-Fluor-647 (OVA-647) through the filters. (B,C) Leakage assay of quantum dots (B) or OVA-647 (C) through filters with different pore sizes and with or without gelatin impregnation. (D) Scheme of the antigen uptake experiments. (E) Confocal images of dendritic cells cultured on gelatin-coated filters with 1-µm pore sizes. A suspension of quantum dots linked to gp120 (Qdot; left, magenta) or a solution of OVA-647 (right, magenta) was applied to the other side of the filter. The cells were stained with phalloidin–Alexa-Fluor-488 (Phal, green) and imaged after 1 h incubation (see Fig. 8A,B for quantification). (F) Live-cell imaging of dendritic cells transfected with LifeAct-GFP and cultured on filter. At time t=0, OVA-647 was applied to the other side of the filter. The inset shows the increase of OVA-647 fluorescence in time at the position of the three actin cores marked with orange arrowheads (i–iii). The full dataset is in supplementary material Movie 3. (G) Time course of OVA-647 uptake for dendritic cells on filter treated with 20 µM Pitstop 2 (black) or Pitstop 2 negative control (red) (± s.e.m. of three independent repeats). Scale bars: 10 µm (E), 2 µm (F).
Eventually, the combined effects of the mechanical forces exerted by the podosomes and the protease-mediated degradation of extracellular material can allow for the complete cell to migrate through physical barriers. Here, pores in the substrate need to exceed a threshold size (~3 µm) (Gawden-Bone et al., 2010), which is likely limited by the size of the nucleus (Wolf et al., 2013). Migration of dendritic cells and other leukocytes through ECM and across endothelial membranes of blood and lymph vessels is crucial for immune system function (Muller, 2011; Vestweber, 2012), and our data further support the well-established role of podosomes in this process (Zicha et al., 1998; Burns et al., 2001; Jones et al., 2002; Matiás-Román et al., 2005; Calle et al., 2006; Dovas et al., 2009).

In this study, we demonstrate a clear role of podosomes in antigen sampling of dendritic cells. We have shown that when podosomes become progressively protrusive, a variety of PRRs localize to these protrusive structures. These PRRs allow for receptor-mediated uptake of antigen from the...
protrusive tips (Gawden-Bone et al., 2010) and thereby can help the cell to sample for antigens from deeper within the substrate (i.e. from the other side of the polycarbonate filter). Antigen cannot only be taken up, but can also be subsequently processed and trafficked to MHC class II compartments and, after antigen presentation, this might result in T-cell activation. Interestingly, it has recently been reported that T-cells also employ protrusive podosome-like structures that contain T-cell receptors to probe for peptides presented in MHC (Sage et al., 2012), in clear analogy to our findings of antigen recognition by podosomes of dendritic cells.

A body of evidence suggests that dendritic cells can sample for antigen across lung, gut and small intestine epithelium (Rescigno et al., 2001; Niess et al., 2005; Chieppa et al., 2006; Vallon-Eberhard et al., 2006; Lelouard et al., 2012; Thornton et al., 2012; Farache et al., 2013; Shan et al., 2013; Strisciuglio et al., 2013). Here, dendritic cells can extend protrusive dendrites across the tight junctions of epithelial cells (Rescigno et al., 2001) or through trans-cellular pores in specialized microfold (M) cells (Lelouard et al., 2012); this facilitates the uptake of bacteria from the apical (lumen) side of the epithelial membrane. Our findings now suggest that these protrusive trans-epithelial dendrites might have evolved from podosomes and thus could provide a mechanistic explanation for the capability of dendritic cells to phagocytose intra-epithelial bacteria.

In summary, we have shown that podosomes can sample the extracellular environment for spots of low mechanical resistance and, once such a spot is identified, podosomes can become increasingly protrusive. These protrusive structures not only facilitate cellular migration through extracellular matrix and endothelial membranes but can also be engaged in receptor-mediated uptake of antigen. Thereby podosomes can effectively increase the search area that a dendritic cell samples for antigen. This localized uptake of antigen from podosome-derived protrusive structures constitutes a novel role for podosomes of dendritic cells. Understanding how dendritic cells and other monocytes sample for antigen in the complex physical environment of the human body is crucial for our understanding of immune function.

Material and Methods
Preparation of human dendritic cells
Dendritic cells were derived from peripheral blood monocytes isolated from a buffy coat (de Vries et al., 2002). Monocytes isolated from healthy blood donors (informed consent obtained) were cultured for 6 days in RPMI 1640 medium (Life Technologies) containing 10% fetal bovine serum (FBS, Greiner Bio-one), 1 mM ultra-glutamine (BioWhittaker), antibiotics (100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin and 0.25 µg ml⁻¹ amphotericin B, Gibco), IL-4 (500 U ml⁻¹) and GMCSF (800 U ml⁻¹) in a humidified, 5% CO₂-containing atmosphere.

Antibodies and reagents
The following primary antibodies were used for the immunofluorescence: mouse anti-vinculin (V9131, Sigma) at 1:200 dilution (v/v), mouse anti-talin (T3287, Sigma) at 1:100 (v/v), mouse anti-
paxillin (349 | MAB3060, Sigma) at 1:100 (v/v), mouse anti-CD11b (ITGAM or Bear-1) (IM2581, Coulter) at 1:200 (v/v), mouse anti-CD29 (or ITGB1) (clone ts2/16) at 1:200 (v/v), mouse anti-MMP14 (MAB3328, Bio Connect/MilliPore) at 1:100 (v/v), rat anti-tubulin (ab6161, Abcam) at 1:500 (v/v), mouse anti-human CD209 (or DC-SIGN) (551186, BD Bioscience) at 1:200 (v/v), mouse anti-human DCIR (DDX0180, Dendritics) at 1:200 (v/v), mouse anti-human dectin-1 (MAB1859, R&D Systems) at 1:100 (v/v), mouse anti-human CD206 (555953, BD Biosciences) at 1:250 (v/v), mouse antihuman CD71 (347510, SALK) at 1:50 (v/v), mouse anti-clathrin light chain (C1985, Sigma) at 1:100 (v/v) and mouse anti-human MHC class II (clone Q5/13; Quaranta et al., 1980) at 20 µg ml⁻¹. The following secondary antibodies were used: goat anti-mouse IgG (H+L) (A-11001), goat anti-rat IgG (H+L) (A-11006) and goat anti-rabbit IgG (H+L) (A-11008), all labeled with Alexa Fluor 488 dye (Invitrogen). Alexa-Fluor-488-, -546- and -633-conjugated phalloidin (Invitrogen) was used at dilutions of 1:200 (v/v) to stain F-actin. The quantum dot streptavidin conjugate (655 nm) was from Invitrogen (Q10121MP). Biotin rgp120 (HIV-1 III B) was purchased from ImmunoDiagnostics.

Gelatin impregnated filters
Gelatin was labeled with Alexa Fluor 633 by incubating 2% (w/v) gelatin from porcine skin in PBS with 100 µg ml⁻¹ Alexa Fluor 633 succinimidyl ester (Invitrogen) for 30 min at room temperature. Unbound dye was removed by methanol precipitation of the gelatin. Hydrophilic polycarbonate membrane filters with different pore sizes (0.4, 1, 2 and 3 µm and 13 mm in diameter) (PCT0413100, PCT1025100, PCT2013100, PCT3013100; all from Sterlitech, Kent, WA) were washed first in 70% and then in 96% ethanol at room temperature. A 5-µl droplet of 2.5% gelatin in PBS was placed on top of a parafilm sheet (sterilized with ethanol) and a filter together with a coverslip was positioned on top of the gelatin solution. After 10 min of incubation, the filters were detached from the coverslips and subsequently transferred into a 24-well plate and kept in PBS until needed for cell culturing.

DQ-FITC-conjugated collagen (Invitrogen) was used for detection of ECM degradation. Human immature dendritic cells were grown for 1 h on filters prepared with 2.5 µg ml⁻¹ DQ-collagen (type I from bovine skin conjugated with fluorescein) in 1% gelatin in PBS. Degraded collagen was visualized after fixation and staining for F-actin with phalloidin–Alexa-Fluor-633.

Immunofluorescence
Cells were cultured on filters at , ~200,000 cells per filter (only several 1000 cells adhered) for 1 to 4 h and fixed for 15 min in 4% PFA in PBS at room temperature. Cells were then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min and blocked with CLSM-buffer [PBS with 20 mM glycine and 3% (w/v) BSA] for 30 min. For immunostaining, the cells were incubated with primary antibodies diluted in CLSM overnight at room temperature. Subsequently, the cells were washed with PBS and incubated with Alexa-Fluor-488-labeled secondary antibodies and Alexa-Fluor-546-conjugated phalloidin for 30 min. Finally, cells were washed with PBS prior to embedding in mounting medium containing 0.01% (v/v) Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 68% (v/v) glycerol in 200 mM sodium phosphate buffer at pH 7.5. The cells...
Podosomes of dendritic cells facilitate antigen sampling were imaged with an Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) with a 60x1.35 NA oil immersion objective.

**PIP₂** labeling
Human monocyte derived dendritic cells were grown on filters with 1 µm pores coated with 2.5% gelatin. Cells were disrupted by a brief ultrasound pulse as described previously (van den Bogaart et al., 2011) with some modifications. In brief, the filters with cells were washed twice with PBS at room temperature and placed on the bottom of a large glass container with PBS. The tip of the sonicator (Branson Digital Sonifier) was placed 3 cm above the filter and three pulses (10% power, 0.1 s pulse with 1 s interval) were given. Filters were then washed once with buffer containing 20 mM Hepes-KOH, 120 mM K-gluconate, 20 mM Kacetate, 2 mM ATP-Mg and 0.5 mM DTT, pH 7.4, and incubated for 30 min with 1 µm PLCδ-PH in the same buffer (van den Bogaart et al., 2011). Finally, the filters were washed twice with PBS, fixed with 4% PFA and co-stained with phalloidin–Alexa-Fluor-546.

Wiskostatin-mediated dissolution of podosomes
Dendritic cells were pre-cultured on glass or gelatin-impregnated filters for 1 h, followed by incubation for 15, 30, 60 and 120 min with 5 µm wiskostatin (40 mM stock in DMSO) and immediate fixation and immunostaining as described above.

**OVA and gp120–quantum-dot uptake assay**
For the conjugation of gp120 to quantum dots, 40 nM biotinylated gp120 was incubated with 20 nM quantum dots-655 with coupled avidin by mixing for 30 min at 4 °C. The coupling was quenched by addition of a 10-fold molar excess of free biotin (to a final concentration of 200 nM) (Cambi et al., 2007).

To test the uptake of antigen via protrusive podosome-like structures, dendritic cells were pre-cultured for 2 h on filters with 1 µm and 400 nm pore sizes that were impregnated with gelatin. Then, the filters were inverted and placed on top of plastic rings filled with culture medium such that the medium contacted the cells on the filter. The opposite (upper) side of the filter was covered with 50 µl of a suspension of 1 nM quantum dots-655 conjugated to gp120. After a 30-minute incubation, the cells were fixed with 4% PFA and actin was stained with phalloidin–Alexa-Fluor-488. Cells were imaged by confocal microscopy. To test the uptake of OVA–Alexa-Fluor-647 antigen we performed the same assay as for gp120–quantum dots, but now we placed 50 µl of a 5 µg ml⁻¹ OVA–Alexa-Fluor-647 on top of the filter.

Several control experiments were performed: (1) competitive inhibition of antigen uptake with 25 µg ml⁻¹ mannan, (2) using quantum dots conjugated to biotin instead of gp120, (3) using filters with smaller pore sizes of 400 nm, (4) using CHO cells that stably expressed DC-SIGN (Cambi et al., 2007) instead of dendritic cells, and (5) using wiskostatin and (6) Pitstop 2 block of antigen.
uptake. For the wiskostatin experiment, cells were preincubated for 30 min with 5 µm wiskostatin (40 mM stock in DMSO) and uptake experiments were performed in presence of wiskostatin. For the Pitstop 2 experiment, cells were preincubated for 15 min with 20 µM wiskostatin or the Pitstop 2 negative control (both from Abcam; 30 mM stock in DMSO) and uptake experiments were performed in presence of Pitstop 2 or negative control. All experiments were independently repeated at least three times with dendritic cells isolated from different donors and statistical significance (P values) was calculated with two-tailed Student’s t-test for paired samples.

Passive leakage of antigen through gelatin-coated filters
In order to measure passive diffusion of the quantum dots and OVA through the filters we used either uncoated or gelatin-impregnated filters with pore sizes of 400 nm and 1 µm (prepared as described above). These filters were placed on top of plastic rings filled with PBS. A 50-µl droplet of antigen suspension (0.2 nM for gp120-labeled quantum dots and 5 µg ml⁻¹ of OVA-647) was then placed on top of the filter for 30 min. The remaining volume of antigen suspension (~20 ml) was aspirated from the top of the filter and transferred to the MaxiSorp 96-well plate. The fluorescence intensity was measured with a CytoFluor II fluorescence well plate reader (PerSeptive Biosystems) (quantum dots-655, excitation 360/40, emission 645/40 nm; OVA–Alexa-Fluor-647, excitation 590/20, emission 645/20 nm filters). Each experiment was repeated at least three times.

Live-cell imaging
Cells were transfected using the Neon Transfection system (Invitrogen) as described previously (van den Dries et al., 2013a). Briefly ~200,000 cells were washed with PBS and resuspended in 100 ml resuspension buffer R (Invitrogen). These cells were subsequently mixed with 2 µg of a plasmid coding for LifeAct-GFP and electroporated (2 pulses of 40 ms, 1000 V). Transfected cells were plated in pre-warmed WillCo-dishes (WillCo Wells) containing antibiotic-free and serum-free RPMI medium (Invitrogen). After 3 h of culturing, the medium was replaced by medium supplemented with 10% (v/v) FCS and antibiotics. Prior to live cell imaging, cells were washed with PBS and imaging was performed in RPMI without Phenol Red supplemented with 10 mM HEPES at pH 7.4. Transiently transfected cells were imaged at 37 °C on a Zeiss LSM 510 microscope equipped with a PlanApochromatic 636 1.4 NA oil immersion objective.

Transmission electron microscopy
For the immunogold labeling, dendritic cells were cultured on gelatinimpregnated filters with 1 µm pore size for 1 h, washed once with 0.1 M phosphate buffer at pH 7.4 (PB) and fixed with 2% (w/v) PFA in PB for 2 h. Residual PFA was quenched with 20 mM glycine in PB for 20 min. Samples were blocked with 5% (w/v) BSA-c and 0.1% (w/v) cold water fish gelatin in PB for 20 min. The sample was incubated with the primary antibody (directed against DC-SIGN or CD206) overnight at 4 °C in 0.3% BSA-c in PB, followed by incubation with secondary rabbit-anti-mouse antibody for 30 min at 20 µg ml⁻¹. For visualization, cells were incubated with protein A labeled with 10-nm-diameter gold particles (binds rabbit IgG) in 0.3% BSA-c in PB. A final fixation step was performed
with 2% glutaraldehyde in PB for 60 min at 4 °C. Samples were treated for 60 min with 1% (w/v) OsO₄, 1% (w/v) potassium ferrocyanide in 0.1 M sodium cacodylate buffer at pH 7.4. Following dehydration with a graded ethanol series, the filters were embedded in Epon which was left to polymerize for 24 h at 60 °C. After polymerization, ~90-nm thin sections perpendicular to the surface of the filter surfaces were cut with a microtome and specimens were imaged with a JEOL 1010 transmission electron microscope.

Transwell inserts with endothelial and epithelial monolayers
Costar Transwell 6.5-mm diameter inserts containing polycarbonate membrane filters with a 5-µm pore size (catalogue number 3421; Corning Incorporated) and impregnated with gelatin were cultured with ~160,000 cells of the endothelial cell line EA.hy926 in DMEM with 4.5 g l⁻¹ glucose, 1 mM ultra-glutamine, antibiotics and 10% FBS on the outer side of the inserts. For the epithelial cell line Caco-2, ~5000 cells were cultured in DMEM with 25 mM glucose, 1% non-essential amino acids, antibiotics and 20% FBS on the outer side of the inserts and without gelatin. Confluent monolayers were formed after 10 days (for EA.hy926) and 20 days (for Caco-2) culturing time. Subsequently, ~20,000 to 100,000 dendritic cells were applied to the inner chamber of the transwell inserts and incubated for 24 h (for EA.hy926) or 5 h (for Caco-2). For the uptake experiments of Alexa-Fluor-647-labeled ovalbumin (OVA-647) through the monolayers of Caco-2 cells (supplementary material Fig. S1E), 5 µg ml⁻¹ OVA-647 in RPMI was added to the outer chamber and incubated for 1 h. Finally, the samples were fixed and immunostained for DC-SIGN and CD206 (expressed by dendritic cells).

ELISA and DO11.10 activation assay
For measuring IL-2 production, as a readout of T-cell activation, we used the T-cell hybridoma line DO11.10. DO11.10 cells were grown in IMDM with 5% FCS, 1 mM ultra-glutamine, antibiotics and 0.1% (v/v) β-mercaptoethanol. Immature mouse bone-marrow-derived dendritic cells (BMDCs) were isolated by culturing for 7 days with GM-CSF (20 ng ml⁻¹). Animal studies were approved by the Animal Ethics Committee of the Nijmegen Animal Experiments Committee. The OVA antigen (concentration used 100 µg ml⁻¹) uptake assay on filters (1 µm pores) was performed as described above for 1 h. The filters were washed three times with PBS, and DO11.10 cells in RPMI were added to the washed filters and incubated overnight. ~200,000 BMDCs and 1x10⁶ DO11.10 cells were used, but the ratio of BMDC to T-cells was in the order of 1:20–1:200 given that only a fraction of the BMDCs adhered to the filter (1000–10,000 cells). After culturing for 6 days, IL-2 production was measured by using monoclonal capture and HRP-conjugated anti-IL-2 antibodies (554424 and 554426, BD Biosciences) using standard ELISA procedures.
References

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Supplementary Figures

Fig. S1: Transmigration of dendritic cells through monolayers of endothelial and epithelial cells. (A) Confocal images of human dendritic cells cultured in inserts with gelatin impregnated polycarbonate filters with pore sizes of 5 μm. A monolayer of the endothelial cell line EA.hy926 (6 – 10 μm thick) was present on the opposite side of the filter. Dendritic cells were immunostained for DC-SIGN (green), actin was visualized by phalloidin-Alexa fluor 633 (magenta) and the nucleus by DAPI (cyan). The dotted outline indicates the position of a dendritic cell based on the DC-SIGN signal. Nuclei from endothelial cells are marked ‘E’. Nuclei from dendritic cells are marked ‘DC’. Several confocal sections underneath of the filter surface are shown (marked by the dashed yellow lines). (B) Same as A, but now for CD206 (green) instead of DC-SIGN. (C) Same as panel A, but now with a monolayer of the epithelial cell line Caco-2 (28 – 32 μm thick). (D) Schematic diagram of dendritic cell migration through the filter insert and through the endothelial or epithelial monolayer. (E) Uptake of Alexa fluor 647-labeled ovalbumin (OVA-647; magenta) through a monolayer of Caco-2 cells. Cells were immunostained for DC-SIGN (green); nuclei and actin were stained with DAPI and phalloidin-CruzFluor405 (Phal; Santa Cruz Biotechnology; both blue). Yellow arrow heads mark OVA-647 positive intracellular compartments. Scale bars, 10 μm.
Supplementary Figure 2. Dynamics of podosome-like structures. (A) Live cell confocal imaging of human dendritic cells transfected with the actin reporter LifeAct-GFP (green) and cultured on filter with 1 μm pore size impregnated with gelatin-Alexa fluor 633 (Filter; magenta). The filter was gently pressed towards the cover slip to achieve a higher imaging resolution and to allow visualization of the LifeAct-GFP distribution in rings (compare with figure 3B). Yellow arrow heads mark randomly selected actin cores. (B) Magnifications of actin rings from panel A (marked i–iii in panel A) and fluorescence intensity distributions through the cross-sections marked by the red boxes (y-averaging). Orange arrow heads mark intensity peaks at the edges of the actin rings. (C–D) Live cell imaging of dendritic cells expressing LifeAct-GFP (green) and cultured on glass (C) and filter with 1 μm pore size impregnated with gelatin-Alexa fluor 633 (magenta) (D). Shown are the fluorescence intensities over time of LifeAct-GFP at randomly selected actin-rich cores (yellow arrow heads). Original data is in Supplementary Movies 1 and 2. (E) Quantifications of the fluctuations of LifeAct-GFP fluorescence from panels C–D. The change was calculated as the difference of the maximum and the minimum fluorescence intensities ($F_{\text{max}} - F_{\text{min}}$; see panels C–D) divided over the average fluorescence intensity $F_{\text{average}}$ ($^*, P = 0.003; 2$-tailed unpaired t-test; 50 podosomes from multiple cells of 3 independent preparations). Scale bars, 10 μm.
Fig. S3: CHO cells, mouse bone marrow derived dendritic cells and T-cell activation. (A) Confocal images of CHO cells heterogeneously expressing recombinant DC-SIGN and cultured on glass or filters with 1 μm pore size and impregnated with Alexa fluor 633-labeled gelatin (Filter; grey). Actin was labeled with phalloidin-Alexa fluor 546 (Phal; magenta) and DC-SIGN was visualized by immunostaining (green). (B) Confocal images of mouse bone marrow derived dendritic cells (BMDCs) cultured on filters with 1 μm pore size and impregnated with Alexa fluor 633-labeled gelatin (Filter; grey). Actin was labeled with phalloidin-Alexa fluor 546 (magenta) and vinculin, MMP-14 and CD206 were visualized by immunostaining (AB; green). The yellow lines indicate the positions of the orthogonal views. The yellow arrows heads indicate randomly chosen actin cores. The red arrow head indicates the approximate filter surfaces. (C) IL-2 production by mouse hybridoma DO11.10 T-cells induced by BMDCs that were loaded with OVA-antigen through filters and measured after 6 days of T-cell activation. Due to (i) the great sensitivity of T-cells, (ii) the limited amount of dendritic cells we could culture on the filters (~10^3 cells) and (iii) the inability to completely wash OVA from the gelatin coated filters (due to non-specific binding), we cannot exclude the possibility that antigen was directly taken up from the medium by the BMDCs during the prolonged T-cell incubation step or that antigen leaked out of the BMDCs or was regurgitated to be presented to the T-cells in trans rather than in cis. Experiments were independently repeated at least 3 times. Data from a representative experiment is shown and error bars indicate the spread of data for at least 2 technical repeats. Scale bars, A: 10 μm; B: 2 μm.
Reaching for far-flung antigen: How solid-core podosomes of dendritic cells transform into protrusive structures
Abstract
We recently identified a novel role for podosomes in antigen sampling. Podosomes are dynamic cellular structures that consist of point-like concentrations of actin surrounded by integrins and adaptor proteins such as vinculin and talin. Podosomes establish cellular contact with the extracellular matrix (ECM) and facilitate cell migration via ECM degradation. In our recent paper, we studied podosomes of human dendritic cells (DCs), major antigen presenting cells (APCs) that take-up, process, and present foreign antigen to naive T-cells. We employed gelatin-impregnated porous polycarbonate filters to demonstrate that the mechanosensitive podosomes of DCs selectively localize to regions of low-physical resistance such as the filter pores. After degradation of the gelatin, podosomes increasingly protrude into the lumen of these pores. These protrusive podosome-derived structures contain several endocytic and early endosomal markers such as clathrin, Rab5, and VAMP3, and, surprisingly, also contain C-type lectins, a type of pathogen recognition receptors (PRRs). Finally, we performed functional uptake experiments to demonstrate that these PRRs facilitate uptake of antigen from the opposite side of the filter. Our data provide mechanistic insight in how dendritic cells sample for antigen across epithelial barriers for instance from the lumen of the lung and gut.

SHORT COMMUNICATION
Dendritic cells (DCs) are antigen-presenting cells (APCs) ubiquitously present in all parts of the human body and constantly sample for antigens via PRRs on their surface (1-3). DCs constitute the link between the innate and adaptive immune systems, because they are the only cells that can induce a primary immune response in naive T-lymphocytes (4). In order to perform this function, DCs have to migrate between their sites of origin (bone marrow), sites of sampling activity and lymph nodes where T-cells are activated by the antigens presented by major histocompatibility complex (MHC) molecules on the surface of the DCs. DCs are thus a very motile cell type, that travel inside the body not only passively within the blood stream, but can also “crawl” between cells thereby reaching almost any part of the body within a relatively short time. For this crawling, DCs need to adhere to the ECM and this adhesion is facilitated by podosomes (5-9).

Podosomes are cellular structures that consist of dense actin cores surrounded by adaptor proteins such as vinculin, talin, and paxillin that connect the actin cytoskeleton to the membrane, regulatory proteins WASP, and Arp2/3 as well as integrins that allow cellular adhesion to the ECM (10-13). Podosomes are also points of local degradation of ECM, which is achieved by concentrated release of proteases such as MMP14 (also known as MT1-MMP) (7, 9, 14-16). Podosomes are well-known to facilitate cell migration through endothelium, epithelium, and connective tissues (8, 17, 18). A more recently discovered fact is that podosomes are mechanosensitive and can sense the local stiffness of the substrate (19, 20).

In an elegant study, Gawden-Bone and coworkers demonstrated that when grown on porous polycarbonate filters, podosomes of dendritic cells can evolve into protrusive structures (16).
How solid-core podosomes of dendritic cells transform into protrusive structures

Fig. 1: Membrane trafficking at protrusive podosomes-derived structures. Human monocyte-derived DCs transiently expressing MMP14, clathrin, Rab5, Rab8, and VAMP3 tagged with fluorescent proteins. Confocal imaging of cells co-transfected with the Factin reporter either LifeAct-RFP or LifeAct-GFP (red) and plasmids carrying the genes of interest (either GFP or mCherry-tagged; green) cultured on polycarbonate filters with 1 μm pore size and impregnated with gelatin labeled with Alexa fluor 633 (Filter; blue) at least for 1 h prior to imaging. (A) MMP14-mCherry and LifeAct-GFP. (B) Clathrin-GFP and LifeAct-RFP. (C) Rab5-GFP and LifeAct-RFP. (D) Rab8-GFP and LifeAct-RFP. (E) VAMP3-GFP and LifeAct-RFP. Transfections and imaging were performed as described (24). Scale bars represent 5 μm.
Although these protrusive structures morphologically resemble invadopodia of cancer cells, they are more dynamic with shorter lifetimes and lower protrusion depths and, in contrast to invadopodia, they still depend on the protein WASP for their formation. Very similar to invadopodia, these protrusive podosome-derived structures turned out to contain stretches of tubulin which likely mediate trafficking of metalloproteinases to the protrusive tips for degradation of ECM. Indeed, in our recent paper we showed the presence of the metalloproteinase MMP14 at the tips of protrusive podosome-like structures of human monocytederived DCs by immunofluorescence. We recently confirmed this finding by overexpression of MMP14 tagged with the fluorescent protein mCherry (Fig. 1A). Here, we co-expressed the F-actin binding reporter protein LifeAct tagged to GFP to visualize the protrusion of the actin cores into the filter pores.

![Fig. 2: C-type lectins locate to protrusive podosomes-derived structures. (A) Confocal images of human dendritic cells cultured on polycarbonate membrane filters with pore sizes of 1 μm. Actin was stained with phalloidin-Alexa-Fluor-546 (Phalloidin, red), DC-SIGN was labeled by specific primary antibody and secondary antibody conjugated to Alexa-Fluor-488 (DC-SIGN, green). Yellow arrow heads indicate the positioning of protrusion and filter pore. (B–D) Transmission electron microscopy of protrusive podosome-like structures. (B) Electron micrographs of human dendritic cells cultured on polycarbonate filter with 1 μm pore size, impregnated with gelatin and immunogold labeled for CD206. (C) An outline of part of the cell with the protrusion shown in the micrograph in panel (B) (yellow dashed box). (D) Magnification of the protrusion indicated by the yellow box in panel (B). Yellow arrowheads mark positions of clusters of gold-beads which indicate the localization of CD206. Scale bar represents 5 μm (A) and 1 μm (B–D).

Not only is MMP14 released at the tips of protrusive podosome-like structures, but endocytosis also occurs at these spots and Gawden-Bone et al. already demonstrated uptake of degraded gelatin. In agreement with this notion that when podosomes become invasive, membrane trafficking is increased at these sites, we showed the presence of several endocytic and endosomal markers at these protrusive structures. We first established the presence of clathrin at protrusive podosome-like structures by immunofluorescence and recently confirmed this by overexpression of GFP-tagged clathrin (Fig. 1B). Several overexpressed GFP-tagged versions of other markers for endocytic activity also located to these protrusive podosome-evolved structures: Rab5, Rab8, and VAMP3 (Fig. 1C–E) (24). Localization was specific for these proteins, as free GFP and GFP-tagged VAMP7 did not or only little protrude into the filter pores. Rab5
and Rab8 are members of the Rab family of small GTPases, and are well-known regulators of intracellular membrane trafficking of early and recycling endosomes (27-29). VAMP3 is a soluble NSF attachment protein receptor (SNARE) that catalyzes membrane fusion of early endosomes (30,31) and is also involved in the extension of pseudopods in phagocytosis (32). Although Rab5, Rab8, and VAMP3 clearly localized in vesicles in the cytosol, our resolution was insufficient to discern a vesicular localization of these proteins in protrusive podosome-derived structures. Nevertheless, our results show that protrusions are sites of membrane trafficking and corroborate with the study of Gawden-Bone et al (16).

Since Gawden-Bone et al. (16) and we (24) demonstrated that endocytosis occurs at protrusive podosome-like structures and since the main function of DCs is antigen presentation, it seemed logical to suggest that these protrusive structures might be involved in endocytosis of antigens. Indeed, protrusive podosome-evolved structures contain various PRRs that can recognize foreign antigens and our immunofluorescence data showed the localization of several members from the C-type lectin family: DC-SIGN (CD209; Fig. 2A), DCIR (CLEC4A), dectin-1 (CLEC7A), and the mannose receptor (CD206) (24). Further investigation by transmission electron microscopy and immunogold labeling confirmed that DCSIGN and CD206 were present on the protrusive structures (Fig. 2B–D for CD206).

We then established that the PRRs residing at the protrusive podosome-like structures indeed were capable to sample for foreign antigen (24). We designed a functional assay where DCs were cultured on gelatin-impregnated filters and where fluorescently labeled mock antigen specific for either DC-SIGN or CD206 was present at the opposite side of the filter (24). With this assay, the ability of dendritic cells to endocytose antigens exclusively via protrusive podosome-like structures could be tested. Control experiments were performed: 1) without mock antigen, 2) with filters containing too narrow pores for podosome protrusion (< 1 μm), 3) competitive blockage of PRRs with mannose, 4) with the endocytic inhibitor Pitstop II, and 5) with the WASP inhibitor wiskostatin. These functional uptake experiments demonstrated the exclusive role of protrusive podosome-evolved structures for uptake of antigen through the filter pores (24). We were also able to show that antigen which was taken up via protrusive podosome-like structures could be
actively processed in the cell and loaded onto MHC class II to finally be presented to T-cells (24).

Our study directly demonstrates a novel role for podosomes in antigen sampling (Fig. 3). Podosomes sense for spots of low physical resistance of the substrate where they exert mechanical forces and actively degrade the ECM. This process results in remodeling of the ECM and podosomes become increasingly invasive and start protruding in the extracellular environment (16, 19, 20, 24, 33). At some point, when the pores in the extracellular environment reach beyond a threshold size of about 3 μm, these protrusive podosome-derived structures facilitate migration of dendritic cells through ECM and across endo- and epithelial barriers (16, 17, 24, 34, 35). It is increasingly well established that podosomes thereby help DCs to rapidly migrate for instance to sites of infection in the body (36, 37). In our study, we showed that protrusive podosome-derived structures are sampling stations that actively probe for antigen which would otherwise not be accessible because of its deeper localization within the substrate or across epithelial/endothelial membranes (24). In these cases, PRRs locating to the protrusive tips of podosome-derived structures can trigger receptor-mediated uptake and this propels subsequent antigen degradation, MHC class II presentation and finally T-cell activation (24). Our findings provide a mechanistic understanding for the well-known capability of DCs to probe for antigens across epithelial barriers, for instance in the lumen of the gut, lungs and small intestine (34, 35, 38-44). Thereby, our data constitute a novel way of how DCs sample for antigen in the human body and this aids our understanding of the immune system.

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General discussion & future perspectives
Dendritic cell subsets
Dendritic cells (DCs) and macrophages contribute to innate immunity via uptake and killing of cancerous cells, apoptotic cells and pathogens. Adaptive immunity is fulfilled when DCs and macrophages communicate information about encountered antigens to other cells, mainly T cells. Both DCs and macrophages can degrade ingested material to 18-20 amino acid long peptides and present them in the context of MHC type II to CD4+ “helper” T-cells.

Why do we still use monocyte derived DCs (moDCs) in our experiments and what is the physiological relevance of these cells? Monocytes are abundant in blood and can be easily cultured from the blood of human donors in comparison to blood-circulating DC subsets, plasmacytoid pDCs (which are morphologically close to plasma cells) and myeloid mDCs. When placed in a setting consisting of pro-inflammatory GM-CSF with homeostatic IL-4, monocytes differentiate into moDCs. Phenotypically, moDCs have a larger cell size than pDCs and cDCs and can ingest many particles of 3-5 μm in size. This feature of moDCs makes them a more useful model system for phagocytosis research. Moreover, moDCs can sustain electroporation for transfection with DNA expression constructs or siRNA for knock down, in contrast to primary DC subsets [1-3]. MoDCs can be easily derived from the blood of patients carrying mutations affecting phagosomal physiology and be directly used for studies in vitro, as was shown in our lab for DCs derived from patients with chronic granulomatous disease (CGD) [4]. Unlike macrophages, moDCs are strong antigen presenters and can be used both in MHC-class II and MHC-class I (cross)-presentation studies [2, 4]. In the past moDCs were used in anti-cancer therapy and still present an interest in translational research [5]. MoDCs can be linked to intestinal DCs for their ability to form protrusive structures through endo/epithelial barriers, meaning that our findings on protrusive podosome sampling for antigens [2, 6] can be directly linked to relevant situations present in the gut [7, 8].

Historically DCs, macrophages and monocytes were grouped together as mononuclear phagocytes due to eliciting comparable immune responses, similarity in produced cytokines, ability of migrating to sites of infection and strong phagocytic specialization [9, 10]. However, this classification no longer holds and it is increasingly clear that primary DC subsets are professional antigen-presenting cells that orchestrate immune responses [11] via activating naïve T-cells [12, 13], constituting only 1% of peripheral white blood cells in humans and animals [14]. These primary subsets are less heterogeneous, elicit better ability to migrate to lymph nodes and exhibit better T-cell priming functions than monocyte-derived cells [15]. Through lineage-tracing analysis it is well-established that macrophages and blood monocytes are different and maintained independently from DCs [15]. Naturally occurring DCs consist of pDCs and conventional cDC1 and cDC1 subsets [15, 16]. Before entering the blood stream these subsets originate in bone marrow from haematopoietic stem cell (HSC)-derived precursors (pre-DC). Interestingly, precursors of pDCs separate quite early from pre-DC committed to cDC1 and 2 phenotypes. The origin and differentiation of these subset is a matter of continuous research and debate [17], but it is clear that mononuclear phagocytes can be divided into naturally occurring DCs derived from
common pre-DCs of HSC origin, embryonic-derived and self-renewing macrophages and (HSC)-
derived common monocyte progenitors (cMoPs) cells, giving rise to moDCs [15]. Nevertheless, DC
nomenclature remains to be a dynamic, debated and still rapidly changing subject.

There is a general lack of consensus in the literature on defining particular cell types as DCs.
Monocyte-derived cells of the intestine are defined as DCs by some researches based on a CD11c+
signature [18, 19] and presence of transepithelial dendrites [7, 8], while others have reasons to
classify these cells as macrophages [20]. Mouse mononuclear phagocytes undergo phenotypical
changes in inflammatory environments making them different from steady state and some
researchers define them as tumour necrosis factor (TNF)- and inducible nitric oxide synthase
(iNOS)-producing DCs (TIP-DCs) [21-23]. This highlights the remarkable quality of monocytes as
transient accessory cells capable of changing their steady state phenotype and acquire an array
of different functions in changing micro-environments [7, 24, 25]. It is certain from transcriptomic
analysis that human and mouse monocytes are precursors for dermal CD14+ DCs and intestinal
mouse CD103−CD172a+ DCs [26, 27], as well as inflammatory DCs hallmarked by CD1c, CD1a and
CD14 [28, 29].

Another difficulty is the comparison between murine and human subsets of mononuclear
phagocytes. Mouse monocyte-derived cells exhibit similarities to cDCs by expressing CD11c
and MHC class II, presenting antigens for activation of naïve T-cells [30-32]. At the same time,
mouse monocyte-derived cells have strong similarities to macrophages which is highlighted by
expression of F4/80, MER kinase and CD64 [23, 24, 30, 33], making them efficient phagocytes.
Mouse monocytes are highly plastic and making a clear distinction between mouse monocytes
and macrophages or mouse monocytes and cDCs is a challenging task [15]. Thorough
transcriptomic analysis it has been shown that non-classical CD14lowCD16+ human monocytes
are comparable to the LY6Clow non-classical mouse monocytes, while CD14+CD16 are more
comparable to LY6C+ classical mouse monocytes [34, 35]. In this work we also use mouse-derived
DCs generated from mouse bone marrow applying the murine cytokine GM-CSF. As reported for
mouse bone-marrow-derived mononucleocytes, the GM-CSF and IL-4 stimulation method leads
to high purity of mouse DCs based on CD11c, CD40, CD80, CD86, and MHC-II expression markers
[14]. However, more recent studies show that mouse bone-marrow cells treated with GM-CSF will
result in production of heterogeneous population of CD11c+MHCII+ macrophages and DCs [36].
Comparing mouse and human DCs might be complicated by the finding that some functional
specializations of DCs between species may not be necessarily conserved, as shown by IL12p70
secretion and cross-presentation by cDC1s [37, 38].

Thus, our understanding of DC subsets and the development of a nomenclature is still work
in progress [15, 17]. In our work we focused on studying phagocytosis in moDCs, mainly for
experimental reasons (higher cell counts and ease of handling). Given the role of monocytes
as precursor of moDCs and M1 and M2 macrophages, it would be of interest to compare the
molecular machinery driving phagocytosis in moDCs with monocytes as well as pro-inflammatory M1 and anti-inflammatory M2 macrophages. Human monocytes, unlike mouse monocytes, also exhibit strong phagocytic properties [39]. We have not investigated what signaling mechanisms are activated in these cell types in response to pathogenic and non-pathogenic stimuli triggering phagocytosis, and how this relates to phagosomal maturation properties. The complete picture of why monocytes reserve the ability to differentiate into different types of phagocytes and what cues control that process remains to be investigated.

Phagosomal shape
In our work we identified novel roles of SWAP70 in phagosomal uptake and the PIKfyve kinase in phagosomal maturation, but several questions concerning their roles remain. In all of our studies we used spherical zymosan particles or spherical latex beads as model cargo for phagocytosis. This approach is commonly used due to uniformity and easy accessibility of the material, as well as for availability of standard protocols and quantification methods [1, 40]. However, not all pathogens are round and they may adopt different shapes and forms. Interestingly, not only the pathogen’s ligands and opsonization can drive phagocytic efficiency, but also the shape and roughness of the particle is a key defining point in antigen uptake as shown by theoretical simulations [41-43] and experimental data [44-52]. Fungi developed a protective mechanism impeding uptake by switching from short and round shape to elongated hyphae (reviewed in [53-56]) which is further described in recent studies [57]. Interestingly, theoretical and experimental studies reveal that smaller sized particles can be engulfed without involvement of F-actin [41], suggesting SWAP70 might be dispensable for this process. It would be of value to investigate how the results from our work could be translated into a situation where the pathogen would be capable to change its form within the phagosome. Proteins (integrins, ERM, BAR, etc.) and lipids (phosphoinositides, PtdEtn, PA, DAG, etc.) involved in cytoskeletal anchoring, are sensitive to the curvatures of the particle shape [58-60]. It has been reported that after uptake, phagocytes can rotate particles to adjust to its shape [61, 62] and a number of protocols and probes were created for imaging of such rotation [63]. Particle shape can trigger different immune activation programs in phagocytes such as the type of cytokines produced [51, 64] or activation of the inflammasome pathway [65]. It would be of interest to investigate how phagocytes would employ SWAP70 and PIKfyve to adjust to different shapes or dynamic alterations in the shapes of the ingested pathogens and how loss of these proteins may affect antigen degradation, presentation and cytokine production.

Routes of F-actin polymerization
The specific mechanisms of phagosomal F-actin polymerization depend on the type of pathogen, phagocytic cargo shape and size, ligands, receptors involved, presence of co-stimulatory or co-inhibitory signals and the type of phagocyte [45, 48, 66-74]. These aspects add a significant level of complexity to our understanding of phagocytosis.

In our studies we confirmed that SWAP70 localization to phagosomes was not affected by the
type of ligand on the phagosomal surface [1, 3]. In both DCs and macrophages, SWAP70 was excluded from other F-actin rich compartments such as podosomes and the immunological synapse [1, 3]. It was also reported that SWAP70 can be found on macropinosomes [75]. It would be of value to visualize how SWAP70 is recruited to the phagocytic cup. Experimental setups involving frustrated phagocytosis on coated glass surfaces [76] and our newly developed Förster resonance energy transfer (FRET) employing fluorescence lifetime imaging microscopy (FLIM) imaging protocols [77, 78] could help to answer these questions, as it will allow to resolve with a far higher temporal resolution the recruitment of SWAP70 in relation to different gradients of phosphoinositides at the phagocytic cup. This would also help to determine in what order SWAP70 is recruited to the phagocytic cup in comparison to other F-actin adaptors or GEFs for GTPases. With these approaches, we could investigate if SWAP70 knock-down would cause some changes in recruitment stoichiometries of other F-actin stabilizing proteins such as Rho and Cdc42 GTPases, vinculin, talin, paxilin and integrins to compensate for SWAP70 loss. Since topical localization of these proteins is sometimes difficult to observe by conventional microscopy at phagocytic cups formed by phagocytosis of regular size particles of 3-5 μm, it would be much easier to define such localization patterns on the flat phagocytic cup formed during frustrated phagocytosis. This approach also makes it easier to employ super resolution microscopy for resolving protein clustering or F-actin bundling.

How do individual F-actin filaments anchor themselves on the surface of the phagosome? F-actin anchoring differs depending on the intracellular niche such as lamellopodia, pseudopodia and phagosomes [66, 79-81]. From personal communication (Grinstein Lab) we know that solid core podosome-like structures were observed in phagocytic cups in frustrated phagocytosis. Since SWAP70 was practically absent from the classical podosomes formed on the ventral side of moDCs, it would be interesting to test how SWAP70 is positioned in relation to podosomes observed in phagocytic cups. We believe that powerful imaging approaches such as super-resolution microscopy imaging will be able to answer this question, as it would allow to resolve these structures with sufficient spatial resolution to discern individual F-actin filaments.

SWAP70 can directly bind to PI(3,4,5)P$_3$ and PI(3,4)P$_2$ at the phagocytic cup as was shown by us [1]. It is also well-described that phagocytic cup closure cannot be accomplished in the absence of these phosphoinositides, due to their role in Ca$^{2+}$ signaling and F-actin polymerization [82]. Mass spectrometry analysis of phosphoinositide-binding proteins revealed quite a limited number of binders for PI(3,4)P$_2$ [83]. Interestingly, SWAP70 harbors an EF-hand domain which might sense Ca$^{2+}$ [84, 85] and we showed in our studies that SWAP70 mutants lacking this domain are less efficiently recruited to the phagosome [1]. Since the role of calcium signaling in phagocytosis is profound [86], it will be interesting to further decipher the input of the EF-hand domain in SWAP70 functions at the phagosome.

Another point of interest is the concentric rings of SWAP70 formed on the surface of the
phagosome. Mammalian septins couple GTP or GDP, interact with F-actin and can self-organize in higher order structures such as filaments and rings [87-89]. In RAW264.7 and J774 mouse macrophage cell lines, septins SEPT2 and SEPT11 are co-localized with sub-membranous actin-rich structures during the early stages of FcγR-mediated phagocytosis. SEPT2 and SEPT11 knock-down may inhibit phagocytosis. Since similar structures are described for septins, it would be interesting to investigate a possible connection of these septin structures with SWAP70.

**Phagosomal pH**

According to the canonical model of phagocytosis, early endo/phagosome with neutral pH gradually convert into acidic late endo/lysosomes by exchanging PI(3)P to PI(3,5)P 2 and increased activity of the v-ATPase [66, 90]. A recent study using elongated bacteria as phagocytic model showed that direct manipulation of pH can control acquisition of phagocytic markers [91]. This suggests an inside-out sensing, where cytosolic proteins are capable of sensing luminal pH in tubular phagocytic cups (tPCs). The suggested mechanism for such sensing could be pH-dependent conformational changes of the transmembrane region of the protein, or proton leakage (also suggested by our lab [4]) from the lumen of the phagosome into the cytosol. The latter mechanism might generate a potential signaling-response coming from pH-sensitive proteins in the cytosol of alter Ca 2+ signaling since some cation channels release Ca 2+ in response to local pH changes [92]. In addition, the v-ATPase itself can sense pH [93] and can transmit signals to mTOR with can sense and regulate the luminal concentration of amino acids in lysosomes [94].

**Cathepsin expression levels and activity**

Cathepsins are a class of proteases with remarkable roles in antigen degradation and presentation in endo-/lysosomes. Cathepsins selectively recognize specific substrates for degradation. Cathepsin S is expressed in professional antigen-presenting cells (APCs) and is crucial for the processing of the invariant chain in bone-marrow derived antigen-presenting cells thereby controlling MHC-II presentation [95]. The different cathepsin activation states at different stages of endo-/phagosomal maturation are still discussed and the reported pH optima for different cathepsins may differ depending on the literature source [40, 95, 96]. Cathepsins can also be active in non-endosomal settings, for example extracellularly for degrading extracellular matrix.

The mechanism of cathepsin trafficking to the phagosome is still under discussion. Cathepsin H, D, S, L and B are commonly referred to as lysosomal [90, 96, 97], but several pieces of evidence suggest they may already be present at phagosomes earlier during maturation. In FcR-mediated phagocytosis, the treatment of cells with PIKfyve antagonists (MF4, YM201636, apilimod) inhibited recruitment of LAMP1+ and lysosomal aspartic cathepsin D [90]. In contrast, the overexpression of a kinase-defective dominant-negative form PIKfyve K1831E did not affect lysosomal targeting of newly synthesized pro-cathepsin D [97]. In another study, inhibition of PIKfyve or manipulation with PIKfyve downstream targets (such as TFEB; master transcriptional regulator of lysosomal biogenesis) caused an increase in the levels of inactive pro-cathepsins A and D in B-cell lymphoma.
cell lines [98]. A similar increase of the inactive version of pro-cathepsin D expression by a factor of 20 and of the active version of cathepsin B by factor of ~3 was observed in PIKfyve<sup>Ab</sup> Pf4-Cre platelets from mice [99]. Similar results on accumulation of an immature form of cathepsin D were obtained in MNT-1 melanoma cells upon apilimod treatment [100]. Accumulation of the immature form of cathepsin D was observed in podocytes upon overexpression of a dominant negative mutant of PIKfyve-dependent Vac14 (L156R) [101]. In contrast, apilimod treatment of the prostate cancer epithelial cell line PC-3 had no effect on cathepsin D activity [102]. In our work we focused on cysteine cathepsins (S, B) and not aspartic cathepsins, such as cathepsin D. We did not detect significant differences in total protein levels of cathepsin X, B and S while the effect of PIKfyve inhibition had the strongest effect on cathepsin S activity. It is of note that PIKfyve inhibition with YM201636 caused an enlargement of lysosomal compartments, but these remained acidic and retained cathepsin B activity, as shown with a membrane permeable substrate for cathepsin B [103].

The activity-based probe we used in our work is also designed to detect cathepsin L [104]. It is reported that cathepsin L is only active in steady state DCs and becomes inactive in matured DCs [96]. It is remarkable that activity of cathepsin L in our moDC lysates is almost undetectable, likely because down regulation of cathepsin L expression is a common feature for moDCs. It is believed that cathepsin S and L have similar functions at li-processing leading to MHC-II-driven activation of CD4<sup>+</sup>, but cathepsin L is mainly expressed in thymus, for example by cortical thymic epithelial cells generating cues for CD4<sup>+</sup>-T cell selection [105].

Literature data linking PIKfyve perturbation to cathepsin B activity shows that inhibition of PIKfyve results in an upregulation of cathepsin B expression in platelets [99] or retention of function in enlarged lysosomes in HeLa cells [103]. This partially correlates with our observations were we do not detect a strong effect of our PIKfyve inhibitors on cathepsin B activity in moDCs. We also could not detect any significant differences in total cathepsin expression differences, possibly due to a limited length of our standard experiments for cathepsin activity which we measured for 3 hours in cell lysates after addition of inhibitors.

**NOX2 activity and cathepsin activity**

Phagosomal maturation is largely shaped by a redox microenvironment in the phagosomal lumen created by the activity of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) complex. Unlike macrophages, DCs adopt the strategy of slowing down phagosomal maturation to prevent the engulfed cells or pathogens from too fast degradation [106, 107]. This is particularly important because DCs have to migrate to lymph nodes and communicate the information about phagocytosed material to helper and effector T-cells. This migration may take days and reduction of antigen degradation speed allows for sustained surface presentation of the ingested antigen within MHC-I and II [4, 40, 108-114]. NOX2 is recruited early to the phagosomes already at the stages before phagocytic cup sealing and the fully functional complex resides on
the phagosome carrying early markers for hours after uptake [115, 116]. Self-produced ROS can partially damage NOX2 and it needs to be replenished from the lysosomal LAMP1+ compartment for sustained NOX2 activity [40, 117]. NOX2 complex produces the free radical superoxide (O$_2$•$^-$), that can dismutate to hydrogen peroxide (H$_2$O$_2$) leading to the generation of hydroxyl radicals (-OH) in the presence of transition metals [4, 111]. Sustained presence of NOX2 at the phagosome may facilitate reactive oxygen species (ROS)-mediated pathogen killing [118-120]. At the same time NOX2 can inhibit proteases such as cathepsins through either a direct effect of ROS on cysteines located in their active sites (reported in this thesis and in [112, 113]) or through a higher pH due to alkalinization of the phagosomal lumen [40, 108, 110].

Lysosomal cysteine cathepsins (such as B, S, L and Z) constitute a major basis for proteolytic activity within phagosomes [95, 121]. Our data show that pharmacological inhibition of PIKfyve caused an increased ROS production and a remarkable decrease of cathepsin S activity, which is in line with reported ROS effects on cathepsin activity [121]. Additionally, it would be of value to demonstrate how the cathepsin content changes in the phagosome lumen over time after uptake. As reported, cathepsin S has a higher pH optimum than cathepsins B and L [40], suggesting that cathepsin S might already be active in early phagosomes before reaching complete maturation. We observed the strongest effects of PIKfyve inhibition on cathepsin S activity, compared to other cathepsins, allowing us to conclude that inhibitors acting on PIKfyve caused not only a maturation defect and prolonged NOX2 presence, accompanied by an increase in ROS, but also might already affect the activity of proteases present in early phagosomes. In our studies we primarily used zymosan, which is a potent activator of NOX2 activity [4, 117]. Since it is known that FcR-stimulation and LPS also stimulate ROS production by NOX2 [114, 122, 123], it seems likely that ligands for those receptors elicit a similar NOX2-mediated reduction of cathepsins S activity. As NOX2 activity can inhibit phagosomal cysteine cathepsins, but not aspartic cathepsins [112, 114, 124], we do not expect aspartic cathepsins to be affected by NOX2 activity, but their trafficking to phagosomes might be disrupted upon PIKfyve inhibition.

NOX2 activity does not only affect proteolysis of ingested antigens by lysosomal cysteine cathepsins, but also ROS produced by NOX2 can result in disulfide reduction of protein antigens as was recently reviewed [121]. It is of note that only linear peptide/protein stretches are accessible to proteases for cleavage, hence all inter- and intra-disulfide bonds have to be reduced before antigen processing [125], as has been also demonstrated for MHC-II with model antigens [126, 127]. GILT (γ-interferon-inducible lysosomal thiol reductase) activity can be inhibited by ROS and this may cause different antigen processing patterns leading to altered repertoires of peptides for presentation, because some of the proteins cannot be unfolded and are therefore not accessible to proteinases for degradation [121].

MHCII loading depends from its dissociation from the li-chaperon, which is degraded by cathepsin S. MHCII-li complexes reside in endo-lysosomal compartments and on the cell surface where MHC-
Il can be internalized into the phagosomal lumen. As our Western blot data shows that cathepsin S and Il degradation were affected upon PIKfyve inhibition, it would be of value to resolve the compartment of Il degradation and determine whether MHCII-Ii and cathepsin S co-localize to early phagosomes with confocal or super-resolution microscopy. A current culprit for solving this issue is the lack of suitable antibodies or expression constructs.

SWAP70 was shown to be able to directly binding RhoA and through that interaction, SWAP70 can affect MHCII trafficking to the surface [128]. Although we studied MHCII presentation in the context of PIKfyve, we did not address this role of SWAP70. This could be studied using our novel biorthogonal labeling of MHCII-presented peptides.

**PIKfyve and downstream effectors**

The mechanism underlying PIKfyve control of phagosomal maturation is not completely understood. Taken into account the redundancy of many PIP-kinases [129-131], the non-redundant role of PIKfyve in this process is remarkable but the precise trafficking role of its product (PI(3,5)P₂) is not understood. PI(3,5)P₂ may be involved in vesicular trafficking along tubules towards their “-” end. Evidence in support for this comes from our live imaging experiments with PIKfyve fused to GFP which is present on zymosan-containing phagosomes but also on tubular structures that move from the cell periphery towards the nucleus (unpublished data). It would be interesting to study if phagosomal PIKfyve causes a TRPML1-dependent calcium release from phagosomes, thereby activating dynein adaptor ALG2 as it is described for endolysosomal transport towards “-” ends in the direction of the microtubule-organizing center (MTOC) [132, 133]. However, of course, we cannot exclude that phagosomes as very large structures rely on other mediators of directional vesicle transport.

Other potential PIKfyve effectors are D-repeat proteins interacting with phosphoinositides (WIPI), WIPI1 and WIPI2 can bind specifically to PI(3,5)P₂ but the function of this binding remains to be understood [134]. WIPI2 has been recently reported in linking of a Rab11a⁺ recycling endosomal compartment to PI(3)P₂-enriched membranes, creating a platform for autophagy LC3-conjugation [135]. Rab11a⁺ is also known to recruit MHC-I to the phagosomes from the endosomal recycling compartment (ERC) [136], but the relationship between these roles is unclear. MHCII is assembled in the ER and eventually resides in late endosomal compartment from where it can be recruited to the maturing endo-/phagosomes [137]. Since PIKfyve facilitates fusion with lysosomes as we extensively discussed above, it would be of particular interest to study the role of PIKfyve in both MHC-class I and II trafficking to the phagosome.

The role of PIKfyve in autophagy is well-established, disruption of PIKfyve leads to accumulation of autophagic proteins such as NBR1, p62, LC3, WIPI2, which is sometimes explained by an inactivity of proteases, dysregulation of fusion with lysosomes, dysfunctional formation of multivesicular bodies (MVBs), or problems the with ESCRT machinery [102, 138-140]. It would be
interesting to investigate how this involvement of PIKfyve in autophagy might be linked to MHC-II antigen loading, since it has been shown that phagosomal LC3-II facilitates presentation of fungal antigens within MHC-II [141]. A possible mechanism underlying this process is LC3-II dependent recruitment of FYCO1, an effector of Rab which is necessary for fusion with lysosomes [142-144]. Contrary to this, TLR2-ligand cargo phagosomes require LC3-II to prevent them from fusion with lysosomes and stabilize NOX2 production of ROS for prolonged MHC-II presentation [145]. Interestingly, TLR4-ligands such as LPS-coated beads stimulated lysosomal marker recruitment and cargo degradation [146]. It would be interesting to compare the rates of PIKfyve recruitment and PI(3,5)P$_2$ abundance with phagosomes carrying different stimuli.

**Mechanism of phagosomal escape by pathogens**

In our study we used very simple model particles for phagocytosis, that do not take into account the possible alternative mechanisms that pathogens can employ for avoiding classical route phagosomal maturation. Even though host cells developed profound mechanisms of defense, many parasites still can escape phagocytosis. Bacterial avoidance strategies are reviewed extensively, showing that bacterial shape [147, 148], filamentation [149-152] or virulence factors can be utilized by bacteria to avoid phagocytosis [153] or to perturb phagosomal maturation [154, 155]. Fungal filamentous structure can also impede phagocytosis [91, 156, 157]. It would be interesting to study the characteristics of SWAP70 and PIKfyve following uptake or infection with pathogens employing such unconventional avoidance strategies.

**Phagocytosis and cancer**

How does our work contribute to the understanding of immune defense mechanisms in cancer clearance? We found that a SWAP70 deficiency may lead to a reduced phagocytosis and pinocytosis. At the same time, SWAP70 is described as an oncogen promoting cancer spreading in prostate cancer (CaP) cell lines [158] and ovarian cancers [159], promoting invasiveness of human malignant glioma cells [160] and formation of larger tumors formed by SWAP70 transformed cells in nude mice [161]. Since the role of SWAP70 in cancer spreading is emerging, it would be interesting to study how SWAP70 deficiency in animal models affects the clearance of tumors.

In our studies we employed drugs (apilimod) that were used in clinical trials targeting Crohn’s disease [162, 163], rheumatoid arthritis [164], psoriasis [165, 166]. Clinical development of these drugs was stopped due to unforeseen side effects [162-166]. We show that apilimod can lead to inhibition of crucial mechanisms of antigen degradation and presentation in DCs, leading to suppression of immune responses. This is a potential explanation for the observed toxicity of apilimod in the clinical trials.

**Podosomal protrusions and medical conditions**

Shortly after protrusive podosomes were described by Gawden-Bone et al. [167], we discovered a role of these structures in antigen sampling by DCs and a possible role in diapedesis. How this
can be linked to anti-cancer therapy? Interestingly, cell spreading cancers use invadopodia, which in some ways are similar to protrusive podosomes because both are F-actin based, dynamic protrusions capable of degrading extracellular matrix and share similar molecular signaling mechanisms for maintaining their structure [168]. Our results suggest that therapies targeting invadopodia in cancer spreading [169] may potentially affect the immune cell ability to sample antigens and migrate to sites of infections.

Conclusion and future perspectives
The summary models outlined in Fig. 1 and 2 showed that DCs can resort to different mechanisms of antigen uptake (phagocytosis and endocytic uptake via podosome protrusions are the focus of this thesis). The mode of uptake depends on the type of antigen, shape and size, as well as the niche where antigen sampling is performed. Our findings explain how F-actin is anchored and stabilized at large phagosomes via the adaptor protein SWAP70 which facilitates particle uptake. This contributes a new role for SWAP70’s in immunity [128] and cancer development [158-161]. Our findings further provide a new mechanism for antigen processing and degradation, as we show that blockage of PIKfyve activity results in endo-/phagosomal defects of maturation leading to prolonged presence of NOX2 on the phagosome. New aspects of the role of NOX2 in antigen processing and presentation are still being uncovered [4, 108-113, 117, 121, 124, 145] and these explain the antigen presentation defects observable in patients suffering from chronic granulomatous disease (CGD) [4]. Although knock-out of PIKfyve in mice is embryonically lethal [170], PIKfyve mutations in humans result in François-Neetens fleck corneal dystrophy (CFD) which manifests in white flecks scattered in all layers of the stroma of the eye [171].

Thus, our data showed the involvement of PIKfyve in immunity by controlling endo-/phagosomal maturation, antigen processing and MHC-II loading and presentation. This important function of PIKfyve might explain the reason why clinical trials targeting PIKfyve to prevent cancer viability and spreading [162-166] have had a negative effect on immune functions of DCs and possibly other phagocytes. Our data clearly shows that phagocytes rely on PIKfyve to assure pathogen clearance and for triggering of adaptive immunity via MHCII-dependent antigen presentation to T-cells. Overall, our findings add to our understanding of antigen uptake, degradation and presentation mechanisms by DCs, that can be utilized in clinical target design for combating autoimmune-diseases, cancers or infections.
Figure 1 A. Pathogen uptake and phagocytic cup formation. (1) Larger antigen particles (>0.5 μm) are internalized via phagocytosis, which is initiated by receptor clustering and PI(4,5)P₂ accumulation at the site of particle-cell contact. (2) PI(3,4,5)P₃ is dephosphorylated by SHIP1 and 2 producing PI(3,4)P₂. Surface expressed MHCII-li might be internalized, facing the phagosomal lumen. (3) The initial phagocytic cup is decorated by SWAP70 utilizing its pleckstrin homology (PH-)domain for binding to PI(3,4)P₂. (4) Via its Dbl-homology domain, SWAP70 participates in recruitment and stabilization of an active (GTP-bound) version of Rac1. (5) Rac1 triggers downstream events resulting in F-actin polymerization at the phagocytic cup. (6) This, in turn, triggers further recruitment of SWAP70 binding more F-actin via its F-actin binding domain. (7) F-actin is anchored at the phagosome and completes phagocytic cup closure and particle uptake. (8) After closure, PI(3,4)P₂ is converted to PI(3)P triggering recruitment of early endo/phagosomal markers such as Rab5 and EEA1. (9) NOX2 completes its assembly on emerging phagosomes and initiates ROS production in the phagosomal lumen. Early phagosomes might already contain cathepsin S becoming active at alkalinized pH and initiating proteolytic degradation of the li-chaperon, thereby releasing the antigen-loading site of MHCII. (10) Approximately 10 min after phagocytic cup closure, the PIKfyve

Fig. 1: Model of all findings described in this thesis showing different routes of antigen uptake via phagocytosis. (A) Pathogen uptake by phagocytic cup formation. (1) Larger antigen particles (>0.5 μm) are internalized via phagocytosis, which is initiated by receptor clustering and PI(4,5)P₂ accumulation at the site of particle-cell contact. (2) PI(3,4,5)P₃ is dephosphorylated by SHIP1 and 2 producing PI(3,4)P₂. Surface expressed MHCII-li might be internalized, facing the phagosomal lumen. (3) The initial phagocytic cup is decorated by SWAP70 utilizing its pleckstrin homology (PH-)domain for binding to PI(3,4)P₂. (4) Via its Dbl-homology domain, SWAP70 participates in recruitment and stabilization of an active (GTP-bound) version of Rac1. (5) Rac1 triggers downstream events resulting in F-actin polymerization at the phagocytic cup. (6) This, in turn, triggers further recruitment of SWAP70 binding more F-actin via its F-actin binding domain. (7) F-actin is anchored at the phagosome and completes phagocytic cup closure and particle uptake. (8) After closure, PI(3,4)P₂ is converted to PI(3)P triggering recruitment of early endo/phagosomal markers such as Rab5 and EEA1. (9) NOX2 completes its assembly on emerging phagosomes and initiates ROS production in the phagosomal lumen. Early phagosomes might already contain cathepsin S becoming active at alkalinized pH and initiating proteolytic degradation of the li-chaperon, thereby releasing the antigen-loading site of MHCII. (10) Approximately 10 min after phagocytic cup closure, the PIKfyve.
kinase converts PI(3)P into PI(3,5)P. (11) PI(3,5)P initiates TRPML-1 activation and Ca\(^{2+}\) release, initiating ALG2 dynein adaptor recruitment which might execute transport towards the minus-end of microtubules towards the MTOC and perinuclear region. (12) PI(3,5)P-containing phagosomes fuse with endolysosomal compartments hallmarked by LAMP1 and the V-ATPase, triggering phagosomal lumen acidification to low pH. (13) Endolysosomal cathepsins such as B and D may become active at this point facilitating further antigen degradation for loading to MHCII. Additional MHCII might be recruited to phagosomes from endolysosomal compartments. Cathepsin S has a wide range of pH optima therefore it will likely continue degrading the li-chaperon. (14) Antigen peptides of 18-20 amino-acids in length are loaded onto MHCII with the help of HLA-DM in endolysosomal compartments and will be trafficked to the surface of the cell to initiate CD4\(^{+}\) T-cell responses. (15) Novel assay for measuring MHC-II presentation: non-natural amino acid L-C-propargylglycine of MHCII-loaded antigen can be bioorthogonally labeled with CalFluor dyes to quantitatively access levels of antigen presentation.

< Fig. 2: Model of all findings described in this thesis showing different routes of antigen uptake via protrusive podosomes. Protrusive podosomes for antigen sampling. (1) Podosomes can protrude and dissolve their solid F-actin cores. (2) Tubulin microfilaments penetrate into the lumen of protrusive podosomes and facilitate kinesin-dependent delivery of MT1-MMP proteinase for further matrix degradation at the protrusive tip. (3) Pattern recognition receptors are also delivered at the tip of protrusive podosomes along microtubules, likely via kinesins. (4) Sample antigens are internalized through epithelial barriers via such protrusive structures.
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Het immuunsysteem

Schadelijke pathogenen (d.w.z. organismen of virussen die ziekte kunnen veroorzaken) en kankercellen kunnen beschermende afweermechanismen van ons lichaam tegenkomen – het zogenaamde immuunsysteem. Het proces van het herkennen en elimineren van pathogenen of kankercellen wordt een immuunreactie genoemd. Aanvankelijk worden pathogenen of kankercellen aangevallen en zonder onderscheid opgeslokt door bepaalde cellen van ons immuunsysteem. Deze eerste verdedigingslijn wordt “innate” immuunsysteem genoemd. De tweede verdedigingslinie wordt “adaptieve” immuunsysteem genoemd. Adaptieve immuniteit heeft enige tijd nodig om zich te ontwikkelen, maar resulteert in een zeer specifieke reactie tegen bepaalde soorten pathogenen, en een “geheugen” om sneller te treden bij terugkerende infecties.

Dendritische cellen (DC) spelen een belangrijke rol in het adaptieve immuunsysteem. Ze kunnen actief pathogenen van de infectieplaats (zoals een wond) of kwaadaardige cellen (bij kanker) overspoelen. Daarna kunnen DCs ongewenste pathogenen of cellen verteren tot kleinere fragmenten en later een deel van deze resterende fragmenten van slechte indringers terugsturen op het celoppervlak van de DC. Blootstelling van fragmenten van ziekteverwekkers aan het oppervlak van DCs wordt “antigeenpresentatie” genoemd. Fragmenten van afgebroken pathogenen worden gepresenteerd binnen eiwitcomplex genaamd MHC1 of MHC2 (major histocompatibility complex).


Fagocytose / Endocytose

Simpel gezegd, opname van deeltjes of moleculen kleiner dan 0.5 µm (0.0005 mm) kan endocytose worden genoemd, en deeltjes groter dan 0.5 µm worden meestal door de cellen opgegeten door het proces genaamd fagocytose. Cellen kunnen ook vloeistoffen “drinken” via een mechanisme genaamd micropinocytose. Alle cellen worden beschermd door membranen die bestaan uit lipiden. Al het materiaal dat van buiten komt moet de celmembranenbarrière passeren. Elke opname / drinkgedrag door cellen wordt gedaan door membraanstructuren die vouwen kunnen vormen (een zogenaamde fagocytische “cup”) en pathogene deeltjes kunnen vangen in een lipide-envelop. Deze envelop blijft op het endo / phagosomale oppervlak, zelfs nadat deeltjes zich in de cel bevinden en de “spijsvertering” van de opgenome deeltjes vindt binnen in deze envelop plaats. De lipide samenstelling van de membraanomwikkeling bepaalt de efficiëntie van fagocytose of endocytose. Een interessant type lipiden zijn de zogenaamde
fosfatidyl inositolfosfaten (phosphoinositide, PI). Deze PIs worden in een lipide-membraan ingebracht door een vet staart, maar de suikerhoofden van deze lipiden steken uit. Deze suikerkop (inositolering) kan op meerdere plaatsen worden gefosforyleerd, wat resulteert in 7 verschillende soorten PIs. Wat interessanter is, is dat één soort fosfoinositide in een andere soort kan worden omgezet, dit gebeurt na endocytose, pinocytose en fagocytose en dit kan optreden op het oppervlak van de lipide-envelop van het pinosome, fagosoom of endosoom in de cel. Vanwege de unieke structuren van verschillende PIs kunnen ze specifieke eiwitten die vrij in de cel ronddrijven binden. Als één type PI op één plek (bijv. op het fagosoom) in overvloed aanwezig is, kunnen al deze eiwitten in grote aantallen binden en een bepaalde plek op bijvoorbeeld het fagosome binden die een verandering van fagomiale eigenschappen veroorzaakt.

Alle stadia van de vorming van endo- / fagosomen kunnen worden gevolgd: van vroege stappen tot late stappen. In de vroege stadia van endo- / fagosomale opname moet het celmembraan zich uitstrekken tot “armachtige” verlengingen om een pathogeen te “omhelzen”. Interacties tussen eiwitten van het cytoskelet en PI-lipiden stimuleert de vorming van deze uitbreidingen. Vervolgens wordt de membraanenvelop gevormd en wordt fagosomen in een binnenruimte van de cellen opgenomen. Nu is het cytoskelet niet langer nodig en beginnen andere eiwitten met het verteren in het vroege fagosoom.

Andere soorten PIs zullen later eiwitten rekruteren die het vroege fagosoom omzetten naar een laat fagosoom. Dit late fagosoom zal uiteindelijk fuseren met lysosomen. Lysosomen zitten vol met componenten die klaar staan om een pathogeen of kankercel die gevangen zitten in de fagomiale membraanomhulling te “verteren” en “vernietigen”.

Nieuwe biologische inzichten in de uitwisseling tussen organellen door superresolutiemicroscopie

Processen van endo- / fagosomale conversies berusten op fusie van de membraanvesicles en organelen in de cellen. Fagosomen en andere organelen zijn erg klein, dus deze conversies zijn op submicroscopische schaal en daarom moeten zeer krachtige methodes zoals microscopie met superresolutie worden gebruikt. In Hoofdstuk 2 bespreek ik de snel ontwikkelende superresolutie benaderingen (zoals STED, PALM, STORM) die worden gebruikt voor het bestuderen van membraanfusion. Ik bespreek de gepubliceerde studies die zich richten op cytoskelet re-arrangementen en dynamica in endocytose en fagocytose. Ik onderzocht ook de interacties van DCs met T- en B-cellen doormiddel van immunologische synapsvorming en probeerde het belang van superresolutie in dergelijk onderzoek te demonstreren. Ik heb ook sleutelgebieden in het subcellulaire organelverkeer geïdentificeerd waar superresolutiemicroscopie vereist is om belangrijke vragen aan te pakken.

PIs in vroege stadia van fagocytose

In Hoofdstuk 3 bestudeerde ik de rol van een zeldzaam soort PI genaamd PI(3,4)P$_2$ in
antigeenopname via fagocytose door DCs. In eerste instantie heb ik gekeken naar gebpubliceerde screens om nieuwe eiwitten die binden aan PI(3,4)P₂ te identificeren. Van de slechts een paar voorspelde interactoren, heb ik besloten om me te concentreren op het eiwit SWAP70. Ik bevestigde met verschillende soorten microscopie dat SWAP70 een sterk binding heeft aan PI(3,4)P₂ in fagocytische cups tijdens vroege stadia van fagocytose. Ik kon SWAP70 niet vinden in andere cytoskeletische structuren zoals podosomen, wat eerdere waarnemingen bevestigt. Superresolutie microscopie onthulde dat SWAP70 langs parallelle bogen of ringen van het celskelet uit F-actine “kabels” uittijgt. SWAP70 is ook uitgelijnd met kabels van Rac1 GTPase op de fagocytische cup. Ik heb ook SWAP70 gemuteerd en SWAP70 uit de cellen verwijderd met behulp van zogenaamde siRNA knock-down. Ik gebruikte specifieke medicijnen om de productie van PI(3,4)P₂ in humane immuuncellen te remmen. Alles werd gedaan om te bewijzen hoe belangrijk SWAP70 is in Rac1-activering, F-actinevorming en fagocytose. Dus, ik heb een nieuw PI(3,4)P₂ bindend eiwit SWAP70 identificeerd dat in grote hoeveelheden op vroege fagosomen te vinden is. SWAP70 bindt tegelijkertijd aan PI(3,4)P₂, F-actine en Rac1, hetgeen noodzakelijk is voor een succesvolle antigeenopname.

In Hoofdstuk 4 heb ik mijn onderzoek over SWAP70 uitgebreid en zijn rol getest niet alleen in menselijke monocytaire DCs, maar ook in andere DC-subsets zoals pDCs en mDCs. Ik bevestig in dit hoofdstuk de belangrijke rol van SWAP70 bij fagocytose in beenmergafgeleide DCs (BMDCs) van de muis gegenereerd met de groeifactoren FLT3 en GM-CSF en in de muizenmacrofaagcellijn RAW264.7. Ik presenteer aanvullende gegevens die de rol van SWAP70 in het controleren van F-actine in het fagosoom aantonen, maar niet op de immunologische synaps waarbij DCs in contact komen met T-cellen. Ik bevestigde verder de rol van SWAP70 door zijn functie te remmen met het molecuul sanguinarine. De bevindingen van dit hoofdstuk ondersteunen onze bevindingen met SWAP70 knock-down en mutatie-analyse in Hoofdstuk 3. Bovendien heb ik waargenomen dat actieve SWAP70 niet alleen F-actinevorming ondersteunt, maar ook activering het de F-actine verstorende eiwit coffiline blokkeert.

PIs in latere stadia van fagocytose
In Hoofdstuk 5 heb ik gekeken naar de fagosoomovergang van het vroege naar late stadium en heb ik de rol van de fosfoinositide kinase PIKfyve in dit proces besproken. PIKfyve is het eiwit in de cel dat het PI type PI(3,5)P₃ kan produceren. Dit is weer een onderbestudeerd type PI dat cruciaal is voor membraanfusie van fagosomen met lysosomen. Deze fusie wordt gekenmerkt door lage pH en verhoogde protease-activiteit van cathepsines, dat zijn eiwitten die andere eiwitten kunnen “verteren” of “vernietigen”. We toonden aan dat remming van PIKfyve met specifieke geneesmiddelen (apilimod of YM201636) een stop veroorzaakte van fagosomal rijping en leidde tot de verlengde aanwezigheid en verhoogde activiteit van een ander eiwitcomplex - NOX2. NOX2 produceert reactieve zuurstofradicalen (ROS), wat resulteert in verminderde activiteit van cysteinecathepsines. Ik heb onderzocht hoe blokkering van PIKfyve en lagere niveaus van cathepsine-activiteit afbraak en presentatie van antigeen in MHC II beïnvloeden. Daarnaast heb
ik samen met collega’s een alternatieve bioorthogonale MHCII-presentatietest ontwikkeld om
presentatie van het griepvirus op MHCII op het celoppervlak te traceren. Mijn experimenten
onthullen een belangrijke rol van PIKfyve bij antigeenafbraak in fago / endo-lysosomen, wat
leidde tot antigeenpresentatie binnen MCH II die adaptieve immunititeit teweegbrengt.

Nieuwe routes van antigeenopname door uitstekende structuren
In Hoofdstuk 6 identificeerde ik een nieuwe rol van podosomen bij de opname van pathogenen.
Podosomen zijn structuren die uit de cel steken en in verschillende celtypen kunnen worden
gevonden. Podosomen kunnen celmigratie begeleiden door de extracellulaire matrix en door
endo / epiteel barrières (zoals huid, bloedvaten, ingewanden, enz.). Mijn studie is geïnspireerd door
waarnemingen dat de dichte en ronde kernen van podosomen kunnen worden omgezet in lange
en uitstekende structuren wanneer ze zachte plekken op substraten tegenkomen. Dit verhoogde
de mogelijkheid van een rol van podosomen bij het vangen van pathogenen. Inderdaad, in mijn
experimenten onthulde ik dat DCs met podosomen, als een soort van armen, kunnen doordringen
in de dichte lagen gevormd door andere cellen (die darm of vaten nabootsen) om pathogenen aan
de andere kant van de laag te vangen. Dit betekent dat DCs deze lange “armen” kunnen gebruiken
om pathogenen te vangen zonder helemaal door de laag te hoeven migreren.

In een korte studie gepresenteerd in hoofdstuk 7, bespreek ik verder de lange en doordringende
aard van podosomen. Ik heb de aanwezigheid van verschillende endocytische en exocytische
eiwitten aangetoond op uitstekende podosomen.

Ten slotte bespreek ik in hoofdstuk 8 onze resultaten en relateren onze observaties aan de
mamenteel beschikbare gegevens in de literatuur. Ik bespreek mogelijke nieuwe richtingen in
fosfoinositide-onderzoek met een focus op superresolutie microscopie en nieuwe bioorthogonale
labelingstechnieken.

De bevindingen in dit proefschrift zijn samengevat in een overzichtsfiguur die ons in staat stelt
de unieke en complexe mechanismen van antigeenopname en antigeenverwerking door
antigeenpresenterende cellen te traceren vóór presentatie aan T-cellen. Deze fundamentele
ontdekkingen leiden tot een beter begrip van welke routes van fosfoinositidemetabolisme cruciaal
zijn voor endo / fagocytische functies van DCs. Daarnaast bieden de bevindingen een mechanisme
verklaring voor de bevindingen van verschillende klinische onderzoeken dat remmers van
fosfoinositide-gerelateerde eiwitten zoals apilimod of YM201636 een algemeen toxisch effect
hebben.
Acknowledgments

Hurray! We have made it and now it is time for me to express my deepest gratitude to all the wonderful people I have met, was inspired by, learned from, and, most importantly, could simply rely on. All the people I will be thanking here made these 6 years of my life in the Netherlands an almost extra-terrestrial experience. Moving to NL from a country separated just by a three-hour plane flight actually felt like crossing outer space on the intergalactic ship. It never seemed possible given the resources I had, but, eventually, the universe conspired and I am where I am now!

Geert, you are like a beam of bright light in our lifes (sorry for optical analogies). Even the darkest days were brightened by your presence, support, attention and simply companionship. You started at Tumour Immunology as a tenure tracker who rejected the option of having private office space and willing to place your desktop on a pipetting bench just not to separate yourself from your group. Well, thanks to you I learned what optimism, enthusiasm and belief in humanity can do! Just in less than 5 years you transitioned from a tenure tracker in Nijmegen to a person with a private office and responsibility of running the whole department in Groningen. Though willing to stay transparent and approachable you picked an office with a glass door and, thankfully, I am working as a post-doc at your department keeping an office next door.

Carl, the first real encounter I had with you during my final master presentation. I was a bit nervous knowing that I was the first ever student supervised by Geert, who just started in your department as a tenure tracker in 2013. Of course, now I know that you do not often come to the master internship presentations unless something interests you. I think you wanted to see how your tenure tracker Geert was doing and what kind of supervision he could offer. Well, I think the presentation went well and we were all able to publish the result in JCS! I always liked your kind and attentive attitude, your comments on my presentations were always valuable. I thank you for picking me, Jorieke, Edyta and Till for organizing a one-day symposium “Hacking the immune system: Exploiting computational immunology to understand the immune system”. It was an honor to be trusted with such a responsibility and I definitely gained a valuable experience from it!

Martin ter Beest, I do not really know what my scientific life would be like if I have not met you in my master internship and you would not have given me a profound training in experimental work. Your protocols are always the best, your work ethic is the example to all of us! You are literally a guru in every subject and your opinion is one of the most valued. In my PhD life I relied on you when it came to cloning and you were always so kind to offer your help. Once again, I would like to thank you for always being there for us (and I am sure many would sign under these words)!

Natalia, my dear friend and colleague, thank you for always being by side, helping with BMDC
experiments, lending mCLING, critical analysis and broad scientific knowledge. I thank you for our cozy dinners and tea-times, it is always interesting listening to you! Traveling to Leipzig and spending time at your wedding party with the rest of our research group was so much fun!

Laurent, you are one of the friendliest people I have ever met! You and Rosalie make such a lovely couple and I loved our BBQ time together! Your presentations were always so clear and your word choice is always so sophisticated. You succeeded in writing scientific texts and I know that your paper drafts were always of superior quality.

Peter L., you strike me as a confident and extremely knowledgeable person. Your entire personality leaves no doubt that you are just few steps away from being a group leader. The level of your expertise in complex wet lab techniques and bioinformatics is jaw-dropping. I hope we can stay in touch, and I should mention, that even if you don't notice it yourself, you inspire other people. Speaking of which; I was so amazed by your programming skills, that I got inspired to take classes in Python on Udemy.

Elke, thank you for being so optimistic, for giving an example of positive life attitude and showcasing a talent for networking! You are in the beginning of your PhD, but already got a travel grant from EMBO and your network is spanning from the USA to Sweden. I think you are destined for success and great scientific future!

Sjors, you came to our group as a master student and now you are a PhD, but it always felt like you are a Post-doc! You deliver ideas with confidence and everything you say has a significant weight. Thank you for optimizing click-chemistry protocols, with it our iScience paper gained a lot! Frans, I want to thank you for being always so jovial and always adding a bright spark to the day! Your quirky remarks and sense of humor make you stand out. Alas, it is always easy for me to be in your company, because we can always practice the art of caustic remarks and sarcasm! I am proud of you acquiring Veni, you are not only a great scientist but also a great father! I am glad you also moved to Groningen for work!

Ilse, you deserve special thanks for advice and help! Your protocols were always so meticulously written. You helped us with the entire rebuttal section leading to publication in Cell Reports. You were the first PhD student to graduate, and you laid the ground for the next to come. Your talent for productivity is legendary. And even recently, when I had some organizational questions about my PhD defense you provided me with very helpful information!

Danielle, I want to thank you for letting me be part of your JoVE video publication. It was quite some experience to read a script while being filmed on a professional camera. I will never forget our happy times at pub quizzes or having a deep conversation on a plane from Sardinia to Eindhoven. You left an imprint on our lives!
Dear new members of membrane trafficking family at RUG: Femmy, Pieter G. and Harry, it is so nice to meet you all and be able to work with you every day! Femmy you are so willing to learn and be good at what you are doing! Keep up the good work! Pieter G., you are so helpful with organizing the lab space and making timely orders! Harry, you seem to be so knowledgeable and nice, you represent all the best qualities of a true Englishman!

I also want to thank some of the current and ex-TIL member as well. Florian W., thank you for your protocols and scientific advice. Jurien T., I remember your presentation to potential interns when we were still MMD students; how enthusiastic you were! Dear Jolanda, thank you for letting me tutor MM1CF, it was a great teaching practice! Mika and Yusuf I want to thank you both for consulting me on how to run OT-II experiments and sharing your precious OVA-coupled PLGA particles with me. Eric, your computer help desk assistance was always timely and invaluable! You also organized so many fun events helping our big TIL family grow closer to each other. Martijn V., your critical remarks and amazing cathepsin-activity sensing probe (BMV109) were strategically crucial in our iScience paper! I would like to thank all the great people in our lab which were always so eager to help. Jorieke, Fleur Schaper, Anne van Duffelen, Olga I. and Natalia R. thank you for sharing BMDCs or FLT3 cells, without you my experiments with mouse cells would not be possible! Florian W., Duffelen, S., Jeanette P. thank you all for sharing adherent monocytes. Tom, Tjitske and Glenn thank you for FACS trainings and timely consultations! Alessandra Cambi, Ben Joosten, Koen van den Dries and Svenja Mennens, thank you all for your important comments and contributions in our scientific discussions and process. It was sad to see you all leave our department, but understandable. Alessandra, thank you for helping us with shaping our podosome paper into a final product! Ben, thank you for your expertise in microscopy. Koen, your expertise in cell biology, cloning and microscopy is impressive, your expert opinion is very valuable! Svenja, thanks for all good times I had in your company! Ilja Smit-deWitte, thank you for all that help and assistance you have been providing me with! You were always so nice and so ready to help. Thanks to your organization and diligence, so many issues such as visas, work contacts, vacation planning and PhD graduation process were so rapidly resolved! Inge R., thank you for your beautiful EM images, that added a lot to our podosome paper! Inge W., thank you for all that fun time on the ice-skating court! Bas and Jasper, thank you for letting me share a house with you in the city center of Nijmegen! Stephanie, thank you for your companionship, I hope you are enjoying England! Georgina, thank you for explaining me the Marie Curie process, your advices are very valuable! Dear Jorieke, Edyta and Till, what a challenging and fun experience it was to be all involved in organization of a symposium with the guests from abroad! I learned something valuable from all of you! Jorieke, how diligent and professional you always are! Edyta, you were always so organized and ready to be of help! I am your biggest Instagram fan! Your talent for baking is unbelievable, so is your talent for showcasing your baking in the form of beautiful pictures. Till, thank you for lending your bike! You saved our winter vacation in 2015. Dear mouse group led by Annemiek van Spriel, thank you all for your wise and valuable input during our DLMs and TIMs. Vera Dunlock and Sjoerd van Deventer I always appreciated your deep thinking and
through approach, your comments were always so valuable (and thank you, Vera, for explaining the stamp experiment)!

Of course, my research would not be possible without the students Baranca Buijsers, Thomas van Ee and Melissa A.C. van Aart who chose to do their internships under my supervision. Baranca, you were my first student and you were working on your Bachelor internship, though it was a short internship you managed to do a lot! You never feared extra hours of work and your performance was beyond expectations! Thomas, you were the next bachelor student I supervised, but somehow, I never felt like we were bound only by a work place. Above all, I see you more as my friend not a colleague. Thank you for showing me around your home town – Apeldoorn – and giving me a broad overview the city’s rich history. Somehow, we ended up seeing a monkey town – Apenheul – and it was a good reminder to us all that we are not the only primates on Earth. Melissa, my dearest master student, thank you very much for all your dedication and supreme lab skills making a huge contribution to our publication in iScience!

I would like to thank our international collaborators for their invaluable help bringing our publications to the next level. Alf Honigmann and Riccardo Maraspini, your STED data revealed peculiarities of SWAP70 and F-actin on the phagosomal surface, and I believe our publication in Cell Reports got even stronger thanks to your data. Carolyn R. Bertozzi and Samantha G.L. Keyser thank you both for generating and providing us CalFluor, without it we would not be able to develop a novel protocol for detecting an antigen presentation in MHC-II.

Here I would like to thank all the wonderful people I have met during my internship at the Research group Molecular biology of ciliopathies at Radboud UMC. I want to thank Ronald Roepman for providing a space and opportunity for completing my internship at his lab and I want to thank two of my daily supervisors Machteld Oud and Heleen Arts. You both were so nice to me and I learned a lot from you both! Machteld taught me the art of planning and showed me how to accomplish many tasks done in a short period of time. Heleen, thank you for always being so calm and patient and always having a great vision for future experiments. I also would like to thank the people in the lab Kaman Wu-Gerretsen, Ideke Lamers, Min (Nguyen Duy Thanh Minh) and Brooke Lynn LaTour for creating such fun working environment!

My arrival to the Netherlands and acquiring a master degree here would not be possible without an Eranet Mundus grant. I would like to thank Marijke Delemarre (an Eranet program coordinator in the Netherlands) for giving me life advices helping me out with administrative issues. I would like to thank Roland Brock, Helma Pluk and Loes Vaessen for their efforts to organize and run Master’s programme in Molecular Mechanisms of Disease (MMD). You created one of the most exciting and competitive master degree program in the Netherlands, offering great training and making so many students from all over the world willing to be admitted!
I would like to express appreciation to my mentor and teacher Wiljan Hendriks for your willingness to listen, always giving wise advises and finding time for meetings in your busy schedule. You are not only a person with great positive and at the same time calming energy, but you also a gifted teacher. Luckily for me I also had a chance to attend your classes and it is clear to me that there is a good reason why so many times you have been nominated and won the “best lecturer of the year” price!

John Brumell, thank you for inviting me over to SickKids research center in Toronto where I was able to present my research to your group. I am looking forward to future collaborations. Johannes, you moved to Toronto from Sweden and know everything about EMBO fellowship. Thank you for giving me a clear perspective on how things work in Canada.

Of course, this journey would not have been possible without my close friends and family. Mark Gorris, I have met you on the first day of our MMD master program and it led to many years of close friendship and so many good memories. We went through two years of our master program together, we both chose TIL for our internships, and later we came back to TIL for our PhDs. I value you for your confidence, clear vision, sense of humor and sharp mind. I thank you for countless moments of fun, joy and true companionship! Stefanie, my friend, I have not been in contact with you for a while, but I know that you are doing great and you found your true passion in life. Thank you for all the wonderful memorable moments we lived through while trying to get the best out of our master degree education. Olga Illina and Pasha Grystenko, we have met only recently, but I feel like we have known each other forever. You both can relate to me in some aspects where nobody else would. You were standing by my side and helped me to go through the final years of my PhD. You gave me desire to explore more about the world and acquire new skills I would not have been able to gain on my own. Thanks to you I was taking Latin dancing classes, learning Spanish and professing myself in water color painting. Together we traveled to Romania, Venice and the South of France. We survived a car accident on an icy snowy mountain road in Romania, missed boats connecting us between islands in Venice, dealt with the car burglaries in Germany and France. Your nonchalant attitude and outer cosmic energy helped us to levitate over these simple inconveniences of this material world! Ivan and Nataliya Zhovannik you both are even more recent friends of mine, but I value our friendship greatly. You revealed a world of Data Science for me. Your stories and experience is so different from the rest of the people I know! Berenice Rösler, my friend! Thank you for being there for me on that sunny island of Sardinia in May of 2016. I will never forget our morning gymnastic on a pier before we plunged into cold water of Mediterranean Sea. I am glad, that after returning from that summer school on immunology we stayed in contact. You are a great German girl, who speaks English with Californian accent. You care for your horses and wonderful dog. You excel yourself in martial arts and you seemed to have an endless fountain of energy! I simply love your personality and hope to be your friend forever!

My dear parents Olga and Valery, we had our disagreements, but it seems like it is all resolved by
now. My dear grandmas Asya and Vera – without your love and support I would not have been able to have a truly happy childhood. My dear brothers - Elisey and Matthew and sisters – Melita and Milana, you are making your older brother so proud. Elisey you are a school principal and are an example of hard work for me. Matthew, your knowledge about the world is amazing. You never stop reading and I hope you will become a computer scientist in future as you want it to be. My dear sisters, you are only 12 and 8, but you have been working so hard day after day on your figure skating career since both of you were just 4. My dear family, I love you all very much and you never cease to amaze me!

I would like to thank my amazing American friends Randy Blair, Bob Schiel, Jimmy, Rya, Joey you all helped me to accept myself in many ways. You showed me an example of true happiness, love, care for your partners. I spent so many cozy unforgettable moments with you all! You shared a wealth of knowledge with me about life. Randy, your spiritual and philosophical wisdom is boundless. Bob, your care for others is the example to us all. Jimmy and b, you both are wonderful couple who helped us out a lot. Joey, you are dear friend a forever young nonchalant lawyer.

My loving husband, Joshua VanWormer, you have been always there for me all these years and I could not be more thankful for that. I have known you for 12 years now and we have been though a lot together. 6 years ago, we sealed our bond in Maryland, a year later the Supreme Court of the USA recognized our marriage nationwide. One year after that, we acquired our Victorian folk house in Toledo, OH working many summers and winters turning it into a cozy house with antiques. Now we continue our life in the Netherlands as we are learning the challenging art of being landlords. Over the course of all these years we have traveled all over Europe and the Middle east. I should be thankful for your American upbringing, you drove a car through Appalachian Mountains of the USA, enormous traffic jams of Toronto, merciless speeding cameras in Germany, calming countryside of Poland, mountains of Sicily and Tenerife, narrow roads of Macedonia, crazy traffic in Bulgaria, beautiful “cote d’azur” roads of France and getting accustomed to driving on the left side of highways in Cyprus. You are the only one I can always ask for help with technical issues, since you know how to fix almost anything. Your enormous patience and dedication are a driving force leading us forward. “Thank you for being a friend” (as they sing in the Golden Girls theme song). I love you very much!

Sincerely,

Maksim Baranov (Максим Баранов)
Curriculum Vitae

Maksim (Maxim) Valeryevich Baranov was born on 17th of November 1987 in Balashikha, Moscow region. He graduated from high school in 2005 (Tver Lyceum, Tver city) and was admitted to a 5-year Specialist degree program (equivalent to a Bachelor’s degree) at Tver State University, Tver, Russia. In his 5th year of Specialists degree program, Maksim studied in Pushchino, Moscow region, working on his graduation thesis dedicated to “Prediction of folding nuclei in tRNA molecules” at the Institute of Protein Research (Russian Academy of Sciences) in the group of Dr Oxana V. Galzitskaya.

In 2010, he obtained his Specialist’s degree in Biology (specialization Biochemistry and Biotechnology) with distinction “Cum Laude” and moved to Moscow where he was admitted to a Master Program in Bioengineering at a top-ranking Lomonosov Moscow State University. Within two years of his master degree education he worked on a “Heterogeneous expression of polyphosphatase and exopolyphosphatase in the yeast” in the group of Dr. Michael A. Eldarov at the Centre for “Bioengineering” (Russian Academy of Sciences) in Moscow and also completed a 3-month summer internship dedicated to “Creation of an analysis pipeline for in-house Galaxy Server” in the group of Dr. Thomas Manke at the Max Planck Institute of Immunobiology and Epigenetics (MPI-IE), Freiburg, Germany.

Upon completion of his master degree in 2012 in Russia, Maksim applied for Eranet Mundus grant and was awarded a full tuition coverage for a competitive master degree in Molecular Mechanisms of Disease (MMD) at the Radboud University, Nijmegen, the Netherlands. In his first year, he performed a 7-month internship in the group of Prof. Dr. Geert van den Bogaart at the Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences (RIMLS), Nijmegen, Netherlands. In the course of his internship he investigated how “Podosomes of dendritic cells facilitate antigen sampling”.

In the second year of MMD Maksim was involved in a 7-month internship under the supervision of Dr. Machteld Oud and Dr. Heleen Arts at the Department of Human Genetics, Radboud University Medical Center (UMC), Nijmegen, Netherlands. There Maksim worked on a project entitled “Studying the disrupting effects of Sensenbrenner-associated missense mutations in WDR35 using a proteomics approach”.

In 2014 Maksim competed his MMD master’s degree with distinction “Bene Meritum” and was admitted to perform a PhD project in the group of membrane trafficking led by Prof. Dr. Geert van den Bogaart, at the Department of Tumor Immunology, Radboud Institute for Molecular Life Sciences (RIMLS), Nijmegen, Netherlands. Under supervision of Prof. Dr. Geert van den Bogaart and Prof. Dr. Carl Figdor, Maksim worked on a topic described in this thesis. In the course of his PhD, Maksim was awarded multiple travel grants from different institutions which he applied for attending international conferences such as Keystone in Ireland and Gordon Research in the USA among others. Maxim also presented his work at research institutes abroad including The Hospital for Sick Children, Toronto, Canada.

Maksim is currently involved in a post-doc research project under the supervision of Prof. Dr. Geert van den Bogaart at the Department of Molecular Immunology and Microbiology, Groningen Biomolecular Sciences & Biotechnology Institute (GBB), Groningen, The Netherlands.
APPENDIX

Published research articles


Grants & Awards

2018  Travel grant, Radboud Internationalisation Grant, Radboud University Nijmegen
       (15th International Symposium on Dendritic Cells in Aachen, Germany)
2017  Travel grant, The Society for Leukocyte Biology (SLB)
       (Gordon Research Symposium on Phagocytes, New Hampshire, USA)
2017  Travel grant, Radboud Internationalisation Grant, Radboud University Nijmegen
       (Gordon Research Conference on Phagocytes, New Hampshire, USA)
2017  Travel grant, European Network of Immunology Institutes (ENII)
       (ENII Summer School on Advanced Immunology in Sardinia, Italy)
2017  Travel grant, Radboud Internationalisation Grant, Radboud University Nijmegen
       (Key Stone Conference on Cell Death and Inflammation, Dublin, Ireland)
2017  Travel grant, Erasmus+ Staff Mobility Grant, European Union
       (ENII Summer School on Advanced Immunology in Sardinia, Italy)
2015  Study Prize, Radboud University Nijmegen
       (Best master thesis: “Podosomes of dendritic cells facilitate antigen sampling.”)
2012-14  Eranet-Mundus, Euro-Russian Academic Network-Mundus
         (Full tuition coverage for obtaining a Master degree in the Netherlands)
2011  IMPRS Scholarship, Max Planck Institute of Immunobiology and Epigenetics (MPI-IE)
       (Living expenses coverage during 3 months summer internship)
2009  DAAD Scholarship, German Academic Exchange Service (DAAD)
       (Intermediate (B2) German Language course at the University of Mainz, Germany)
APPENDIX

PhD portfolio

<table>
<thead>
<tr>
<th>Name PhD candidate: M. V. Baranov</th>
<th>PhD period: 01-10-2014 – 30-09-2018</th>
</tr>
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<tbody>
<tr>
<td>Department: Tumor Immunology</td>
<td>Promotor(s): Prof. Dr. Carl Figdor</td>
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<tr>
<td>Graduate School: Radboud Institute for Molecular Life Sciences</td>
<td>Co-promotor(s): Prof. Dr. Geert van den Bogaart</td>
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<tr>
<th>TRAINING ACTIVITIES</th>
<th>Year(s)</th>
<th>ECTS</th>
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</table>
a) Courses & Workshops                   |         |      |
| - Introduction day Radboudumc           | 2015    | 0.5  |
| Courses:                                |         |      |
| - Graduate School specific introductory course | 2015    | 2    |
| - The Art of presenting science         | 2015    | 1.5  |
| - Scientific Integrity course           | 2015    | 1    |
| - Perfecting your Academic Writing Skills | 2015    | 2    |
| - MIC course Imaging                    | 2016    | 1    |
| - Achieving your Goals                  | 2017    | 1.5  |
| Workshops:                              |         |      |
| - BD Horizon Tour – FACS workshop       | 2016    | 0.4  |
| - Online workshop Adobe Illustrator and InDesign on Udemy | 2018    | 1.25 |

b) Seminars & lectures                   |         |      |
| - Radboud Research Rounds               | 2015-2016 | 0.3  |

c) Symposia & congresses (*oral; # poster) |         |      |
| - Radboud New Frontiers                 | 2014-2016 | 3    |
| - NVVI Winter school, Noordwijkershout, Netherlands | 2016 | 0.75 |
| - NVVI symposium, Lunteren, Netherlands | 2015    | 1    |
| - PhD retreat *                         | 2014-2018 | 4.75 |
| - CHAINS conference, Veldhoven, Netherlands * | 2016 | 1.5  |
| - ENII-EFIS/EII International Summer School on Advanced Immunology, Sardinia, Italy * | 2017 | 2.75 |
| - International conference Keystone, Dublin, Ireland – Cell death and Inflammation * | 2017 | 2.25 |
| - International conference Gordon Research, Waterville Valley, NH, USA – Phagocytes * | 2017 | 2.75 |
| - DC 2018, Aachen, Germany – 15th International Symposium on dendritic cells * | 2018 | 1.5  |

d) Other                                 |         |      |
| - Co-organizing 1 day conference: "Hacking the Immune System", Nijmegen, Netherlands | 2017 | 2.0  |
| - Journal Clubs at Tumor Immunology Department | 2015-2018 | 3    |
| - Tumor Immunology Meeting presentations | 2015-2018 | 5    |
| - MACHT meeting presentation            | 2015    | 1    |

| TEACHING ACTIVITIES                     |         |      |
e) Lecturing                             |         |      |
| - Oral presentation at the scientific seminar at the Department of Cell Biology, Sick Kids hospital, Toronto, Canada | 2017 | 0.5  |
| - Teaching MM1CF to 1st year MMD students |         |      |
| - Teaching MM1MC to 2nd year MMD students | 2015 | 0.6  |
|                                          | 2016 | 0.6  |
f) Supervision of internships / other    |         |      |
| - Supervision of a Bachelor internship project (2x) | 2016 | 2.3  |
| - Supervision of a Master internship project (1x) | 2017 | 2.2  |
| - Supervision of a Master literature thesis writing | 2017 | 0.25 |

TOTAL 49.15