# Interactions of the dendritic cell

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# CHAPTER 1

## Introduction

CHAPTER 1

# THE IMMUNE SYSTEM

## The innate immune system

The immune system comprises a complex set of mechanisms and cells that work together to protect our bodies from pathogens and cancer, and to clean up after tissue damage. The immune system recognizes pathogenic bacteria, viruses and other microorganisms that have passed the physical barriers of the skin or mucosa as intruders. This is mediated by specialized receptors expressed by immune cells; the pattern recognition receptors (PRRs). PRRs can be subdivided into the Toll-like receptors, the nucleotide-binding oligomerization domain-like receptors, the C-type lectin receptors, and the RIG-1 like receptors. PRRs are devoted to recognition of Pathogen-Associated Molecular Patterns (PAMPs). These are conserved motifs present in for example the bacterial or fungal cell wall (e.g., lipopolysaccharide and chitin), lipoproteins and nucleic acids. Damaged host cells are detected in a similar fashion; damaged or dead cells expose Damage Associated Molecular Patterns (DAMPs) like intracellular proteins and DNA, which are also recognized by PRRs. PRR engagement induces a cascade of downstream signaling, eventually resulting in the secretion of inflammatory cytokines and chemokines. These secreted compounds recruit and activate neutrophils, macrophages and natural killer cells.

Activated neutrophils and macrophages combat infection by ingesting and degrading the pathogens or damaged cells. Ingestion of particles sized up to 1 µm (and solutes) is termed endocytosis, whereas uptake of larger particles is referred to as phagocytosis. Activated natural killer cells do not ingest pathogens or cells. Instead, they kill suspicious cells by secreting substances that induce apoptosis. Cells are considered suspicious if they express lower levels of certain normal 'self' proteins, or if they express proteins typically expressed by injured, infected or neoplastic cells.

This first, non-specific immune response is not dependent on previous exposure to the specific antigen and is therefore coined the innate immune response. Certain immune cell types, called antigen presenting cells (APCs), present fragments (antigens) of endo-/phagocytic cargo on their plasma membranes. The most important APCs are macrophages and dendritic cells (DCs). Although less efficient compared to these professional APCs, B cells also present antigen. The antigens are presented in the context of Major Histocompatibility Complex (MHC)-I or MHC-II molecules, and serve to activate the acquired immune system.

## The acquired immune system

The acquired immune system is embodied by B and T lymphocytes. Each naïve B or T cell expresses a different B or T cell receptor with a unique binding domain for antigen. As a result, each acquired immune response is specific for a certain antigen. To maximize the chances that a matching lymphocyte is present upon exposure to an unfamiliar antigen, millions of lymphocytes expressing different specific receptors are generated. These different receptors are not all encoded by different genes, but instead result from geometric rearrangement of gene segments of the germline DNA. In addition, during this recombination, several mechanisms induce insertion or removal of random nucleotides at the junctions of gene segments, resulting in enormous diversification of the B and T cell receptors. In later stages, B cells can edit their receptors by replacing segments of the initial rearrangement. A process further diversifying B cell receptors is the high mutation rate in B cell receptor genes (single nucleotide substitutions). This phenomenon is referred to as somatic hypermutation.



Ligands of B and T cell receptors are referred to as antigens. In contrast to T cell receptors, which only recognize processed proteins (peptides) presented on MHC by APCs, B cell receptors recognize unprocessed, intact molecules that are in their native conformation. B cell antigens therefore come in various sizes, and can be either soluble or particulate. B cell activation occurs when a repetitive antigen binds several B cell receptors on the same B cell or when in addition to B cell receptors, PRRs are also stimulated. For some antigens (T cell independent antigens), crosslinking of B cell receptors can suffice for B cell activation. However, even for T cell independent antigens, B cell receptor engagement is usually not sufficient for efficient B cell activation and proliferation. Additional stimulation is achieved through complement receptors, PRRs stimulation, and (for protein antigens) T cell help. T cell help comprises binding of the T cell receptor to a complementary antigen-presenting B cell, followed by binding of T cell costimulatory molecule CD40L to CD40 located at the plasma membrane of the B cell.

If the T cell encounters a peptide-MHC combination that is complementary to the binding domain of its T cell receptor, the high affinity of the binding induces the formation of a stable interaction. Activation of naive T cells requires co-stimulatory molecules on the APC to bind the co-stimulatory receptors on T cells. Cytokines present in the microenvironment further stimulate T cell activation. In addition, the cytokines produced by the APC govern the type of T helper cell response that is generated[1]. Certain profiles of cytokines steer the T cell towards specific effector types (e.g., T helper 1 cells that induce proliferation of activated lymphocytes or T helper 2 cells that induce differentiation of B cells into plasma cells).

In absence of co-stimulation, naive T cells can become anergic, meaning that they will not be activated in response to the antigen, also not in future encounters. Alternatively, lack of co-stimulation can trigger the T cell to go into apoptosis, or to differentiate into a regulatory T cell. Co-stimulation is not required for the activation of T cells that have encountered the specific antigen before (memory T cells).

Lymphocyte activation results in clonal expansion and differentiation into effector cells. B cells differentiate into antibody-producing plasma cells and memory cells. In the event of a repeated infection, memory B cells can quickly give rise to plasma cells secreting large amounts of antibodies.

T cells can differentiate into cytotoxic T cells, helper T cells, regulatory T cells or memory cells. Cytotoxic T cells kill infected or neoplastic cells while helper T cells assist in the activation of B and T cells. Regulatory T cells do not activate the immune system, but rather suppress the immune response, for example after an infection is cleared. The last type of T cells, memory T cells, is important during repeated infection, as these cells can mount rapid and large responses upon reinfection. Considering that lymphocyte clonal expansion takes place in response to activation, lymphocyte proliferation can be used as a readout to asses B or T cell activation.

### Tolerance

APCs present fragments of the engulfed cargo on MHC-II molecules. However, harmless self antigen can be presented too. To prevent the induction of an auto-immune response by activation of self-reactive cells, lymphocytes expressing receptors with high affinity to self-antigens either change their receptors (B cells; receptor editing) or are deleted by apoptosis (B and T cells) during maturation. The absence of costimulatory molecules on non-immune cells and tolerogenic immune cells, resulting in anergic T cells, adds another layer to immune tolerance.

Fortunately, despite these mechanisms of tolerance, malignant cells often are recognized as "foreign". Mutations leading to the production of changed, "new" proteins (neoantigens) discriminate neoplastic cells from healthy host cells. In addition, many types of cancer express proteins that are normally only expressed during embryonal development or in immune privileged organs (e.g., NY-ESO-1). Lymphocytes capable of binding these proteins are incompletely deleted during negative selection, and these proteins are therefore immunogenic.

## DENDRITIC CELLS

APCs form the bridge between the innate and the acquired immune system. Although macrophages and B cells are capable of antigen presentation, DCs are considered the most potent APCs. In contrast to macrophages, which directly send their endo-/phagosomal contents to lysosomes for degradation, DCs first transport their endo-/phagosomal contents to MHC-II rich late endosomal compartments[2]. Antigen presentation by the DC can lead to T cell activation, but DCs also help maintaining tolerance to self-antigen, by inducing T cell deletion or unresponsiveness[3].

DCs originate in the bone marrow and subsequently migrate to a tissue where they function as sentinels (Fig. 1). They continuously sample their environment, checking for PAMPs and DAMPs. Upon PRR engagement or exposure to pro-inflammatory cytokines (e.g., interleukin-1, granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-α), immature DCs undergo maturation. In addition, DCs can be potentiated by helper T cells ("DC licensing").

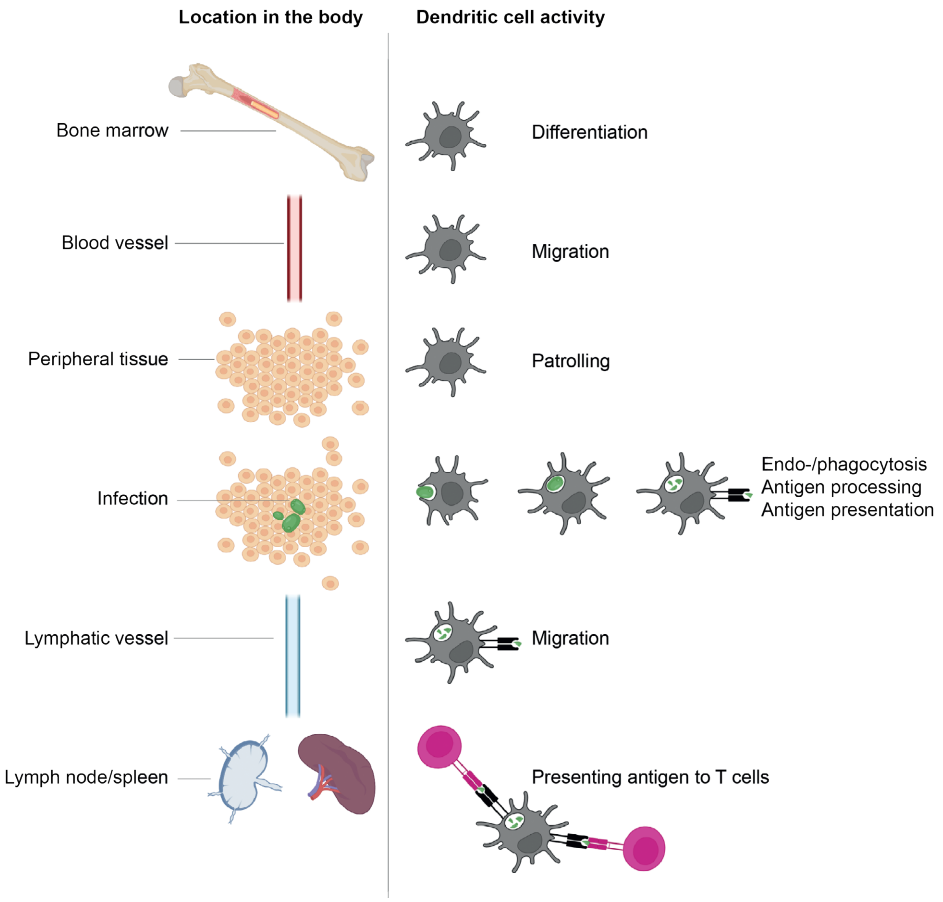


**FIG. 1: The lifecycle of the dendritic cell**
Dendritic cells originate in the bone marrow or, in the case of monocyte-derived dendritic cells, in the peripheral tissues (not depicted in this figure). They sample the tissues and circulation for possile threats, such as pathogenic microbes. Upon an infection, they engulf (endo-/phagocytose) the target particle. During the degradation of the particle, fragments of proteins (peptides) are loaded onto Major Histocompatibility Complexes, which are presented at the plasma membrane. Subsequently, the dendritic cell travels to the draining lymph node or the spleen to present these peptides (antigen) to T cells. By doing so, the dendritic cell induces either T cell activation, T cell unresponsiveness or T cell deletion. This figure is created with BioRender.com.

During maturation, the endocytic and phagocytic activity of the DC decreases while the expression of MHC and costimulatory molecules CD80 and CD86 increases[4]. DCs are named after the extremely long "arms" or dendrites they form (which should not be confused with neuronal dendrites). These dendrites elongate during maturation thus increasing the cell surface area, promoting antigen presentation. Lastly, during maturation, cell motility increases[5]. Contrary to macrophages, which reside in the tissue, mature DCs travel to the lymph nodes and the spleen upon PAMP/DAMP recognition. In these organs, the DCs interact with countless naive and memory T cells, maximizing options for antigen presentation to these cells.

## Cross-presentation

Typically, antigens obtained from the extracellular space are presented in the context of MHC-II molecules and intracellular peptides are presented on MHC-I molecules. Subsequently, antigens with an extracellular origin are presented to helper T cells (recognizing MHC-II-peptide complexes), and intracellular proteins are displayed to cytotoxic T cells (recognizing MHC-I peptide complexes). Thus, typically, antigen derived from ingested neoplastic cells are not presented to cytotoxic T cells. However, via a process termed cross-presentation, antigen with an extracellular origin can be presented on MHC-I molecules. This mechanism enables APCs to activate cytotoxic T cells expressing receptors specific for neoplastic antigen resulting in T cell-mediated killing of tumor cells. In addition, this mechanism is highly important in mounting an immune response upon vaccines. DCs are considered the best cross-presenting APCs.

## Dendritic cell subsets

DCs display a great heterogeneity. They can be categorized into three subsets based on their progenitors. These subsets are: conventional DCs (cDCs), plasmacytoid DCs (pDCs) and monocyte-derived DCs (moDCs) (Fig.2). Although most DCs are of myeloid origin, common lymphoid progenitors can also give rise to DCs, e.g., resulting in DCs highly resembling the pDC phenotype[6,7].

### Conventional DCs

Common myeloid progenitor cells can differentiate into common DC progenitors, which are the progenitors of both pDCs and pre-conventional DCs. In turn, pre-conventional DCs proliferate into cDCs. This subset is specialized at antigen-presentation and T cell priming and cDCs are therefore considered the "typical" DCs. There are two types of cDCs; cDC1s and cDC2s. CD1Cs are characterized as $CD1a^{-}CD11c^{low}$ $CLEC9A^{+}XCR1^{+}BDCA\text{-}3^{+}CD141^{+}$. CDC1s are superior at cross presentation[8,9,10] and mount cytotoxic immune responses. In

addition, they produce interleukin-12, promoting T helper 1 cell differentiation. The murine counterparts of human cD1Cs express CD8α, XCR1, CLEC9A and CD103.

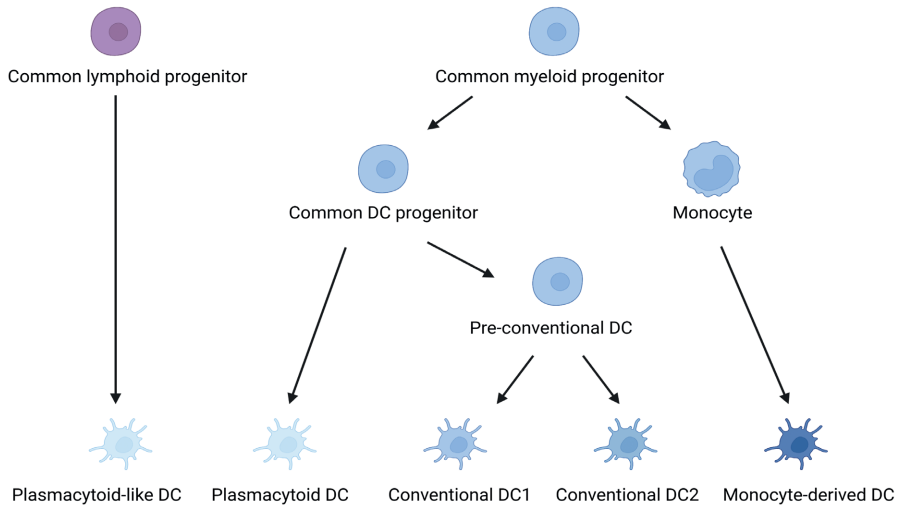


**FIG.2: Subsets of human dendritic cells.**
This Figure is created with BioRender.com.

Human CDC2s are defined as CD1a$^{+}$CD11c$^{high}$XCR1$^{-}$BDCA-1$^{+}$. CDC2s also produce interleukin-12. On top of that, CDC2 cells excel in antigen presentation on MHC-II molecules and produce interleukin-23 to promote T helper 2 and 17 cells[11]. Mouse cDCs expressing CD11b are considered the equivalent of human CDC2s.

## Plasmacytoid DCs

PDCs play an important role in the defense against viruses by producing type I interferon[12]. In addition, they stimulate T cells via interleukin-12 secretion. Resting pDCs do not activate T cells directly, because only activated pDCs present antigen. As they differentiate in the bone marrow (and not in the tissues), pDCs and cDCs are present both in the peripheral blood and the tissues.

## Monocyte-derived DCs

Next to differentiating into DC progenitors, common myeloid progenitor cells can also differentiate into monocytes. Upon inflammation, monocytes can differentiate into moDCs in the affected peripheral tissues[13]. As the differentiation into moDCs is independent of Fms like tyrosine kinase 3 ligand (FLT3L)[14] and granulocyte-macrophage colony-stimulating factor[15] (which both are required for differentiation into cDCs and

pDCs), some consider moDCs as a distinct cell type sharing some functionalities with cDCs rather than as *bona fide* DCs. Either way, these monocyte-derived cells are required for adequate T helper cell type 1 responses[16–18] and produce large amounts of the pro-inflammatory cytokine tumor necrosis factor α thereby aiding in the clearance of bacterial infections[19].

### In vitro generated monocyte-derived DCs

DCs can be generated from peripheral blood-derived monocytes *in vitro*, when cultured in the presence of granulocyte-macrophage colony-stimulating factor (promoting DC differentiation) and interleukin-4 (reducing differentiation towards CD14-expressing macrophages)[20]. As the yields of DCs directly isolated from blood are low, the generation of these monocyte-derived DCs is highly valuable for both immunotherapies and research. Although the precise physiological counterpart of the *in vitro* generated monocyte derived DC is not clear, these cells do display the main characteristics of DCs, i.e. endo/-phagocytosis, cross-presentation[21] and migration to the lymph node[22,23].

## ENDOCYTOSIS AND PHAGOCYTOSIS

During infection, pathogens are covered by specific host-derived proteins (opsonins) like antibodies, complement factors, mannose-binding lectins, fibronectin and vitronectin. This aids in the uptake of these pathogens, as phagocytes are equipped with receptors for opsonins. Phagocytes also express receptors for apoptotic corpses, most of which recognize phosphatidylserine. In addition, some PRRs recognizing PAMPS also contribute to phagocytosis. Moreover, ligation of certain of these PRRs (e.g., Dectin-1) as a single signal can suffice to trigger phagocytosis. Engagement of opsonic receptors, apoptotic corpse receptors or PRRs forms the starting point of endo-/phagocytosis. Once the target particle is recognized, a range of signaling pathways induce rearrangement of the cytoskeleton and the membrane, resulting in engulfment of the particle including the plasma membrane. Next, the endo-/phagosome matures; by fusion with early endosomes, late endosomes and eventually lysosomes. Key regulators of these fusion processes are the small GTPases Rab5 and Rab7. Rab5 recruits effector molecules like VPS34, which enables the production of phosphatidylinositol 3-phosphate (PI3P). In turn, PI3P regulates the recruitment of proteins required for fusion. Rab7 contributes to fusion by binding the homotypic fusion and vacuole protein sorting (HOPS) complex; a tethering factor central to fusion[24]. During maturation, the endo-/phagosomal lumen acidifies, activating digestive enzymes (e.g., proteases like cathepsins) leading to cargo degradation.

## LC3-associated phagocytosis

Endo-/phagocytosis results in degradation of extracellular cargo. In contrast, in autophagy intracellular constituents are degraded. Cytosolic constituents are enclosed by a double membrane thereby forming an autophagosome. Similar to in endo-/phagocytosis, fusion of the autophagosome with lysosomes leads to cargo degradation. Autophagy can be induced by certain stresses (e.g., starvation), resulting in non-selective targeting of cytoplasmic components. Under physiological conditions (in the absence of stresses), autophagy is more selective, specifically targeting damaged or superfluous organelles and protein aggregates. Autophagy can also target pathogens residing in the cytosol.



Autophagy highly relies on the actions of microtubule-associated proteins 1A/1B light chain 3B (LC3). For example, LC3 is required for elongation of the initial membrane (the autophagophore)[25]. The underlying mechanism for this is proposed to involve LC3-induced recruitment of the actin regulator JYM[26]. JYM recruitment is thought to enable actin-induced membrane remodeling, resulting in autophagophore elongation. In addition, LC3 is central to fusion of autophagosomes with lysosomes. It recruits PLEKHM1 which is required for recruitment of Rab7 and the HOPS complex[27]. LC3 is also known to recruit proteins like p62[28] and NBR1[29] that bind ubiquitinated proteins, suggesting an additional role for LC3 in cargo recruitment.

In certain phagocytic events, LC3 is recruited to the phagosomal membrane[30]. This type of phagocytosis, termed LC3-associated phagocytosis (LAP), therefore seems to be a phagocytosis/autophagy hybrid process. The pathways inducing LC3 recruitment are highly similar in canonical autophagy and LAP. For example, they both require the same autophagy related (ATG) proteins, e.g., ATG5 and ATG7[30,31].

The exact functions of LAP are currently still debated. Some studies claim that LAP accelerates phagosomal maturation[30,32–34], while others show that LAP delays[35] or does not affect phagosomal maturation[36,37].

For example, it has been shown that in GFP-LC3 expressing mouse macrophages, LC3-positive phagosomes acidified more rapidly and more extensively after engulfing dead cells compared to phagosomes incapable of LC3 recruitment due to ATG7 deletion[33]. In addition, the cells displaying LAP were shown to be more efficient at degrading phagocytosed dead cells, as measured by a more rapid decrease in fluorescence of the of the mCerulean-Spectrin-expressing apoptotic cargo. Also, cells capable of LAP produced less pro-inflammatory cytokines and more anti-inflammatory cytokines. Another study showed in a mouse macrophage cell line that phagosomes containing TLR-binding cargo (and thus capable of initiating LAP) acidify faster than phagosomes containing plain beads[34]. Phagosomes containing Listeria monocytogenes also matured faster upon LC3 recruitment in mouse macrophages. This was concluded from the finding that lysosomes pre-loaded with fluorescent latex beads colocalised more with LC3-positive phagosomes than with LC3-negative phagosomes. In addition, LAP promoted the eradication of these bacteria. In addition to Listeria monocytogenes,

LAP is also involved in the clearance of Helicobacter pylori[38] and Legionella dumoffii[39]. In addition, LAP is required for the efficient clearance of Toxoplasma gondi[40] and Aspergillus fumigatus[32,41,42] in mice.

The molecular mechanism in which LC3 recruitment would enhance phagosomal maturation remains enigmatic. Involvement of the protein FYVE and coiled–coil domain containing 1 (FYCO1), which is known to foster microtubule plus end-directed transport of autophagosomes, has been proposed[43]. In this model, LAPosomes mature by LC3-mediated recruitment of FYCO1-associated lysosomes. FYCO1 knockdown was shown to inhibit the recruitment of late endosomal marker LAMP1. Alternatively, it has been proposed that LC3 may promote phagolysosomal fusion *via* formation of a specific SNARE complex[44]. To date, it has not been investigated which SNAREs this complex would contain. It could either concern autophagosomal, phagosomal or completely different SNAREs[44]. As VAMP8 is a lysosomal SNARE, a potential LAP-specific SNARE promoting LAPosome-lysosome fusion might interact with VAMP8. It is not known whether LC3-mediated recruitment of PLEKHM1 plays a role in the effect of LC3 recruitment on phagosomal maturation.

In contrast to the accelerating effect of LAP on phagosomal maturation described above, Romao et al. show that LAP delays phagosomal maturation[35]. They show that in macrophages, LAPosomes display lower acidity compared to LC3-negative phagosomes in the same cell. As LC3-negative and LC3-positive phagosomes within the same cells were compared, these differences in acidity cannot be contributed to confounding factors such as differences in genetic makeup. In the same study, ATG5 depletion is shown to delay antigen presentation in both macrophages and DCs. However, ATG5 depletion did not affect phagosomal acidity, suggesting that phagosomal maturation is not affected. Thus, it is unlikely that the effect of ATG5 depletion on antigen presentation is caused by delayed phagosomal maturation. Possibly, the ATG5 depletion hampers antigen processing *via* a different mechanism.

LAP is also implicated in tumor immunology, as it increases tolerance to tumor cells[45]. This was shown in mice with conditional ablation of RBCN using lysozyme M (LysM/Lyz2)-Cre-lox recombination. RBCN codes for the protein Rubicon, which is required for LAP, but inhibits canonical autophagy. Inhibiting LAP by ablating RBCN reduced polarization into tolerogenic, anti-inflammatory M2 macrophages. Endosomal maturation was not affected.

Unveiling the effects of LAP is difficult as many methods targeting LAP also affect canonical autophagy, making it hard to attribute effects to either of these processes specifically.

The heterogeneity of the reported effects of LAP can be attributed to differences in experimental setup. The most important differences are the use of GFP-tagged LC3 *versus* endogenous LC3, use of different cell types (macrophages vs. dendritic cells), different species (mouse vs. human) and use of different phagocytic cargoes (interacting with different LAP-inducing receptors). It is therefore important to study different cell

types and payloads, while minimizing potential interfering factors (like a GFP-tag) and maximizing specific targeting of autophagy or LAP.

## Endo-/phagocytosis-related membrane trafficking

Once endo-/phagocytic cargo is degraded, soluble peptides and amino acids are recycled after being pumped to the cytosol, and insoluble constituents are exocytosed[46]. Alternatively, cargo receptors can be trafficked to the *trans*-Golgi network for reutilization[47]**.** Endo-/phagosomal membrane proteins can also be degraded, after being sorted into the endo-/phagosomal lumen on intraluminal vesicles. Many of these processes involving membrane trafficking require the Endosomal Sorting Complex Required for Transport (ESCRT). For example, this complex is responsible for the formation of intraluminal vesicles[48], repair of lysosomal membrane damage[49] and regulation of endocytic recycling by promoting fission of recycling tubules[50].



# THE ESCRT SYSTEM

The ESCRT system is composed of 4 subfamilies; ESCRT-0 to ESCRT-III. The different subfamilies are recruited in a consecutive manner; one subfamily recruits the next. ESCRT-0 to ESCRT-II mediate cargo sequestration[51] and membrane bending[52]. It has been shown that ESCRT-III proteins only contribute to membrane bending at concentrations much higher than physiological concentrations[53]. Rather than membrane bending, the ESCRT-III proteins mediate membrane severing. In their inactive conformation, ESCRT-III proteins are soluble monomers located in the cytoplasm. Active ESCRT-III proteins on the other hand form (hetero)polymers[54]. These polymers can form various shapes, including tubes, cones and flat spirals, which are proposed to have different roles in the process of membrane scission.

## ESCRT-III mediated membrane scission

The two models describing the mechanism of membrane scission that are most consistent with the data in the ESCRT field are the reverse dome model and the buckling model. It has been shown that negative curvature of the membrane (like in the membrane neck of a bud) catalyzes the nucleation of ESCRT-III polymers[55]. Therefore, the reverse dome model proposes that the ESCRT polymers nucleate in the neck of the bud, and subsequently grow towards the cytosolic side of the membrane neck[53,56] (Fig. 3). Next, the cone constricts by starting to grow on the side of the bud, instead of on the cytosolic side of the membrane neck. In this model, growth-mediated constriction of the ESCRT cone induces membrane scission.

In the buckling model, membrane scission is mediated by a sudden transformation from a conical conformation to a flatter spiral (Fig. 4). This conformational change pulls the membrane away from a membrane bud, thereby inducing membrane scission. As measured by atomic force microscopy, CHMP4 filaments are approximately 15 times less stiff than actin, but 5 times stiffer than DNA[57]. This has led to the suggestion that ESCRT polymers are stiff enough to form a mechanical spring capable of severing membranes. As ESCRT-III polymers have a preferred radius of curvature in which their energy is lowest[58], the inner rings of a spiral are overbent while the outer rings are underbent. It is proposed that the unfavorably overbent inner rings of the ESCRT-III cone drive the transformation into a wider and flatter spiral. The diameter of ESCRT-III tubes depends on the combination of monomers forming the heteropolymer, as different heteropolymers form tubes with different diameters[59,60]. Therefore, the transformation from a cone to a wider and flatter spiral might be the result of a change in the composition of the polymer. Extraction of the monomers possessing the highest natural curvature from the heteropolymer could result in a polymer with a lower curvature[61]. This selective extraction of monomers could be performed by the ATPase Vacuolar Protein Sorting-associated protein 4 (VPS4)[61]. Alternatively, the conformational change could be the result of VPS4-mediated release of the ESCRT-III polymer from upstream ESCRT proteins[61]. After scission, the VPS4 enables ESCRT recycling[62].

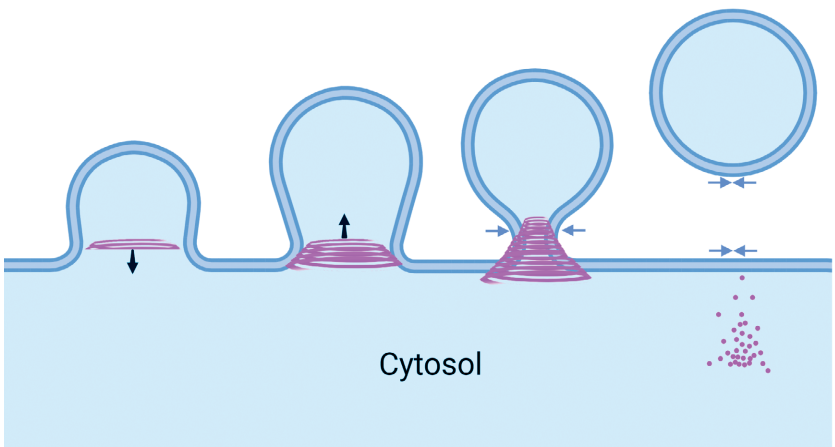


**FIG. 3: The reverse dome model of ESCRT-mediated membrane scission**
An ESCRT-III polymer (shown in magenta) nucleates at the neck of a membrane bud and grows towards the cytosol (shown in blue), thereby forming a cone which is facing the cytosol with its wide side. Next, the polymer starts growing at the narrow side of the cone, thereby narrowing this end even further. This model proposes that membrane scission is mediated by narrowing of the cone. After scission, the ESCRT proteins are located at the cytosolic side and are disassembled. This figure is created with BioRender.com.

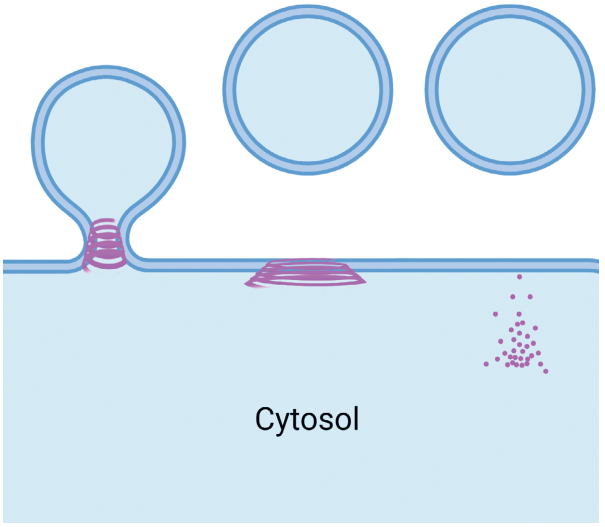


**FIG. 4: The buckling model of ESCRT-mediated membrane scission**
An ESCRT-III polymer (shown in magenta) nucleates at the neck of a membrane bud and grows towards the cytosol (shown in blue), thereby forming a cone which is facing the cytosol. Untethering of the polymer transforms the shape of the polymer into a wider, flatter spiral. This model proposes that membrane scission is mediated by the conformational change of the ESCRT-polymer, pulling the opposing sides of the membrane neck closer towards each other. After scission, the ESCRT proteins are located at the cytosolic side and are disassembled. This figure is created with BioRender.com.

ESCRT-III polymers are most commonly located at membranes budded away from the cytoplasm. However, more recently it has been shown that CHMP1B-IST1 heteropolymers can bend membranes towards the cytoplasm[59]. This is in line with the electronegative, membrane-repelling outside and electropositive inside of CHMP1B-IST1 tubes. Thus, where other ESCRT tubes seem to localize on the inside of a membrane bud, CHMP1B-IST1 polymers localize on the outside of membrane buds. However, it is not clear whether CHMP1B-IST1 polymers also mediate membrane bending *in vivo*, i.e., at physiological concentrations, or mainly contribute to membrane scission at these concentrations. Interestingly, CHMP1B and IST1 are also present at membrane sites that are bent away from the cytoplasm. To date, it is not clear how CHMP1B-IST1 polymers would contribute to membrane bending or scission at these locations. Possibly, CHMP1 and IST1 are part of heteropolymers different than CHMP1B-IST1 at these sites. However, it is not known whether tubes formed by different heteropolymers containing either CHMP1B or IST1 bind membranes on their in- or outside, so it is unclear whether these alternative heteropolymers could function at membranes curved in this direction.

# ADHESION AND MIGRATION

After ingestion of a target particle, DCs have to find their way out of the peripheral tissue towards the afferent lymphatic vessel to the lymph node. In order to migrate, the cell needs to adhere to its surroundings, so it can push and pull itself towards its direction of interest. At the same time, adhesion provides stability to tissues, so cells and extracellular matrix can together form solid entities instead of being a soup of cells. In a similar fashion, cell adhesion prevents undesired cellular displacement due to extracellular shear stress, for example in the initial phase of leukocyte extravasation.

Cell adherence is realized by adhesion receptor families including immunoglobulin (Ig)-like adhesion receptors, selectins, cadherins and integrins. Ig-like adhesion molecules, displaying extracellular domains containing Ig-like domains, bind other Ig-like adhesion receptors and integrins present on the plasma membranes of neighboring cells, thereby enabling cell-cell adhesion. The most ubiquitously expressed subtypes of Ig-like adhesion molecules intercellular adhesion molecules (ICAMs) and Nectins and Nectin-like molecules. Selectins are expressed on leukocytes and endothelial cells and bind sugar moieties present on endothelial cells and leukocytes, thereby slowing down the leukocyte, thus enabling its extravasation. Cadherins connect epithelial cells to surrounding cells in bands encircling the apex of the endothelial cells (zonula adherens).

Although cadherins and integrins are known to interact[63], adherence to the extracellular matrix is achieved mainly through integrins. Integrins are composed of two subunits; an α and a β chain. There are multiple α and β chains. The (combination of) these subunits determines ligand-affinity. For example, adhesion to collagen is mainly achieved by α1β1, α2β1, α10β1 and α11β1 (all containing the αA-domain, recognizing a certain glutamate within a specific collagenous motif), whereas fibronectin is mostly bound by α5β1, αVβ3, αVβ6, αVβ1, α8β1 and αIIbβ3 (all recognizing Arginine-Glycine-Aspartic acid active sites)[64]. Next to binding extracellular matrix proteins, certain integrins also bind ICAM, VCAM-1 and E-cadherin on the plasma membranes of other cells[64].

Integrins have different conformations. The bent, inactive state has low ligand-binding affinity, while the upright, active state allows for high affinity. Extracellular molecules can induce intracellular signaling (e.g., *via* chemokine receptors) eventually resulting in integrin activation (inside-out signaling)[65]. Ligand binding also fosters integrin clustering[66], activating Src family protein tyrosine kinases[67], thereby regulating cytoskeletal organization and cell motility (outside-in signaling)[68].

Integrins are linked to F-actin via adaptor proteins (e.g., via talin), thus enabling force transduction from to the cytoskeleton.

# SCOPE OF THIS THESIS

In this thesis we studied interactions of DCs with their surroundings. In **Chapter 2** we show that various autophagy inhibitors display different effects on LAP and cellular levels of LC3 in DCs. The V-ATPase inhibitor bafilomyin A1 and the phosphatidylinositol 3-kinase VPS34 inhibitor SAR405 decrease LC3 recruitment to phagosomes. In contrast, the lysosomotropic base chloroquine and ethyl (2-(5-nitrothiophene-2-carboxamido) thiophene-3-carbonyl) carbamate (EACC), which inhibits STX17 (syntaxin 17)-mediated fusion between autophagosomes and lysosomes, do not affect LAP. Chloroquine and EACC could therefore be used to inhibit canonical autophagy without interfering with LAP. We also show that EACC decreases the cellular levels of LC3, and our data suggest that this is due to LC3 degradation by the proteasome. In addition, we show that DCs display a very high turnover of LC3, as blocking protein synthesis decreases LC3 levels to <20% compared to control in as little as 5 hours.



In **Chapter 3** we show that dendritic cells and other adherent, migratory cells form huge spiral shaped ESCRT structures on their plasma membranes. These spirals surround integrin clusters, which are devoid of F-actin. Inhibiting F-actin increased both the numbers of ESCRT structures as well as cell adherence. Our results suggest that the ESCRT structures are part of a new type of adhesion structures, in which ESCRT proteins might take over the role of the lacking cytoskeleton by providing support to the plasma membrane.

In **Chapter 4**, we investigated the performance of a T cell proliferation assay based on incorporation of the bioorthogonally-functionalized uridine analog 5-ethynyl-2'-deoxyuridine (EdU). EdU can be coupled to a fluorophore using click chemistry and thereby enables labeling of proliferating cells. We compared this assay to an assay standard in the field; a dilution-based assay exerting the probe CellTrace. The EdU assay outperformed the CellTrace assay, as it was more sensitive, due to lower background activation in non-stimulated cells, and was less cytotoxic. In addition, it facilitated better detection of interferon gamma responses.

In summary, this thesis describes cellular processes induced by interaction of the DC with pathogens, the extracellular matrix and T cells.

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