Antigen handling and cross-presentation by dendritic cells

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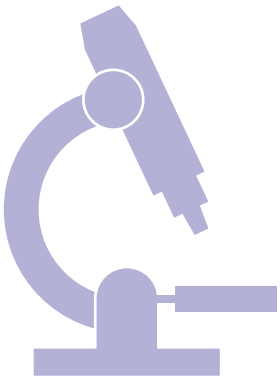


General introduction

THE IMMUNE SYSTEM

Our human body possesses a complex immune system to defend against infections with pathogens, such as bacteria, viruses and parasites but also against malignant cancer cells. The diversity of different immune mechanisms can be divided into an innate immune system and an adaptive immune system. The innate immune system is the first line of defense, and can be activated upon recognition of pathogen-associated molecular patterns (PAMPs), small molecules expressed by pathogens (1, 2). The recognition of PAMPs by the immune system relies on pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectin receptors (CLRs) (Fig. 1). Fcγ receptors (FcγRs) recognize antigens bound to antibodies. TLRs can be expressed on the cell surface (TLR1, 2, 4, 5, 6, and 11), recognizing microbial membrane components, but TLRs can also be found on intracellular vesicles (TLR3, 7, 8, and 9), where they recognize microbial nucleic acids. NLRs sense bacterial components which are directly introduced into the cytoplasm. CLRs recognize sugar structures of bacteria and fungi. Macrophages, neutrophils, and dendritic cells are sensor cells of the adaptive immune system that express PRRs and recognize PAMPs that are part of microorganisms but not of the host body's own cells. Activation of PRRs on these sensor cells can induce uptake of pathogen antigen by endocytosis or phagocytosis, resulting in killing of the pathogen, production of cytokines and chemokines to attract immune cells. In addition, inflammation and antigen presentation to other immune cells can be induced.

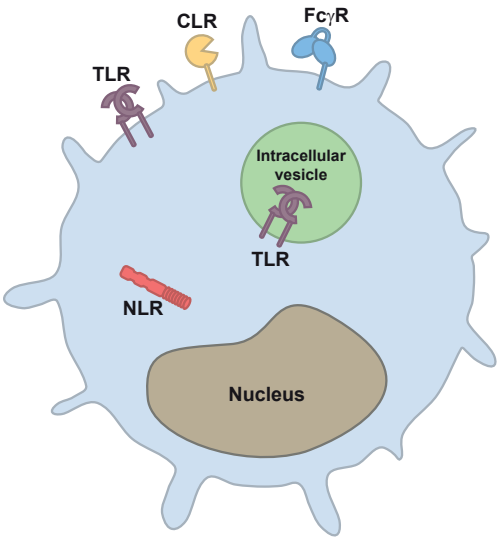

Figure 1. Dendritic cell pattern recognition receptors. Dendritic cells recognize pathogens by pattern recognition receptors, such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs). Fcγ receptors (FcγRs) recognize antigens that are bound to antibodies. TLRs can be expressed extracellular or on intracellular vesicles.

Once the pathogen overwhelms the innate defense mechanisms, the adaptive immune response comes into action. The adaptive immune system is composed of B and T lymphocytes (humoral and cellular immunity, respectively). Both B and T lymphocytes (B and T cells) express a unique repertoire of antigen receptors on each individual lymphocyte which are highly specific for a certain antigen. B cells are specialized in binding specific soluble molecules through their B-cell receptor, which facilitates the internalization of antigen via endocytosis and the process of internalized antigen, followed by the display of fragments as peptide:MHCII complexes to helper T cells (Th). When the Th have previously been activated by the same antigen, the B cells will receive signals from the Th that drive the B cells' differentiation into antibody producing cells and class switching, while others become memory B cells residing in the germinal centers. The secretion of antibodies in the blood stream can bind and mark pathogens for clearance and destruction.

T cells recognize specific antigens which are presented on professional antigen presenting cells (APCs). APCs migrate from the infection or tumor site to the lymph nodes upon antigen recognition, antigen uptake, and activation. Antigens are then processed in APCs and presented on MHC class I (MHCI) or MHC class II (MHCII) molecules on the cell surface to $CD8^+$ or $CD4^+$ lymphocytes, respectively. MHCI molecules are expressed by almost all cells, while MHCII molecules are exclusively expressed on APCs. The main function of MHCI in nucleated cells is to display intracellular proteins, derived from endogenous infections and mutations to $CD8^+$ cytotoxic T cells (CTLs). The classical MHCI antigen presentation pathway is mainly used for endogenous antigens, whereas the MHCII antigen pathway is used when exogenous antigens are encountered.

Naïve T cells circulate in the blood stream and secondary lymphoid organs (such as lymph nodes, spleen, and Peyer's patches in the small intestine) until they encounter their specific antigen, presented as a peptide:MHC complex on the surface of APCs, and get activated to proliferate and differentiate into effector T cells including $CD4^+$ Th and $CD8^+$ CTLs. $CD4^+$ T cells can differentiate into specialized effector subsets which can stimulate or regulate specific immunological functions dependent on the type of infection or pathological situation. These include T_H1 (T helper 1), T_H2, T_H17, and T_{FH} (T follicular helper) cells, which can help different immune cell types; and T_{reg} (regulatory T) cells, which inhibit or modulate the extent of immune activation. T_H1 cells help eradicating infections by microbes that are phagocytosed by macrophages. Through the release of IFN-γ, macrophages are activated and enhanced in their killing activities. T_H2 cells control extracellular parasite infections by mediating class switching of B cells to produce IgE. T_H17 cells are important in responses to extracellular bacteria and fungi by inducing neutrophilic responses to clear the pathogens. T_{FH} cells mainly provide B-cell help for high-affinity antibody production. T_{reg} cells suppress T-cell responses and prevents autoimmunity, however high levels of T_{reg} cells are often found in the tumor microenvironment and are associated with poor prognosis in many cancers. A specialized function of the adaptive immune system is the induction of memory T cells

($CD4^+$ or $CD8^+$) after an infection. This is important to enable a more rapid and effective response against pathogens that have been encountered previously. Already for almost a century we make use of the adaptive immune system by vaccinating inactive pathogens to induce long term protective immunity against aggressive infectious diseases. $CD8^+$ T cells are the main effector cells that can attack cancer and virus-infected cells. The activation and differentiation of naïve T cells by APCs is also called priming, which is often dependent on both antigen presentation and co-stimulatory signals from the APCs to the T cells. In general, $CD8^+$ T cells require more co-stimulatory activity to be activated compared to $CD4^+$ T cells. In some viral infections, DCs are sufficient to activate $CD8^+$ T cells into CTLs, however the majority of viral infections requires additional help from $CD4^+$ effector T cells. The $CD4^+$ effector T cells, triggered by MHCII presentation on APCs, upregulate CD40L which binds to CD40 on APCs. This leads to APC maturation and upregulation of various co-stimulatory molecules, including CD80, CD86, 4-1BBL, and MHC molecules. Combined with optimal specific antigen presentation on MHCI by APCs, this will license the APCs to activate $CD8^+$ CTLs (license to kill) (3). Thus APCs, especially dendritic cell subsets, play a crucial role in inducing effective CTL responses in order to eradicate tumors and infectious diseases.

ANTIGEN CROSS-PRESENTATION BY DENDRITIC CELLS

Dendritic cells (DCs) are APCs that can capture, process and present antigens to T cells. Two classical antigen presentation pathways have been described in DCs: MHCI and MHCII pathways. MHCI pathway mainly presents endogenous antigens on MHCI molecules to $CD8^+$ T cells, whereas MHCII pathway presents exogenous antigens on MHCII molecules to $CD4^+$ T cells. However, DCs have a specialized function to present exogenous antigens also on MHCI molecules, called cross-presentation, linking innate and adaptive immunity. Several studies reported the importance of DC cross-presentation for inducing T cell responses which are specific for tumor antigens and infectious diseases (4–6). In order to elicit potent $CD8^+$ T cell priming, the levels of DC maturation and DC cross-presentation efficiency are important. DCs express several uptake and sensing receptors (Fig. 1) and undergo maturation after the recognition of pathogen-derived products by PRRs (e.g. TLRs, NLRs) or antibody-antigen complexes by FcγRs. Upon DC maturation, antigen processing is increased followed by upregulation of MHC molecules and co-stimulatory molecules (such as CD40, CD80 and CD86), and induction of cytokine release, which are all important for the interaction with T cells (7). Two main intracellular pathways for antigen cross-presentation in DCs have been proposed: the vacuolar and the cytosolic pathways (Fig. 2). Antigen processing through the vacuolar pathway is proteasome independent and generally also independent of the transporter associated with antigen processing (TAP) (8). It has been suggested that antigen is degraded by proteases (e.g. cathepsin S) and that antigen

processing and loading on MHCI occur in endocytic compartments. In the cytosolic pathway, exogenous antigens are transported from endosomal vesicles into the cell cytosol, where they are degraded by the proteasome. Proteasome-generated peptides are then transported by TAP1 and TAP2 to the endoplasmic reticulum (ER) for loading on MHCI molecules (9–11) (Fig. 2). However, it has been reported that some proteasome-generated peptides may be transported back into endocytic compartments and trimmed by insulin regulated aminopeptidase (IRAP) and directly loaded on MHCI molecules (12).

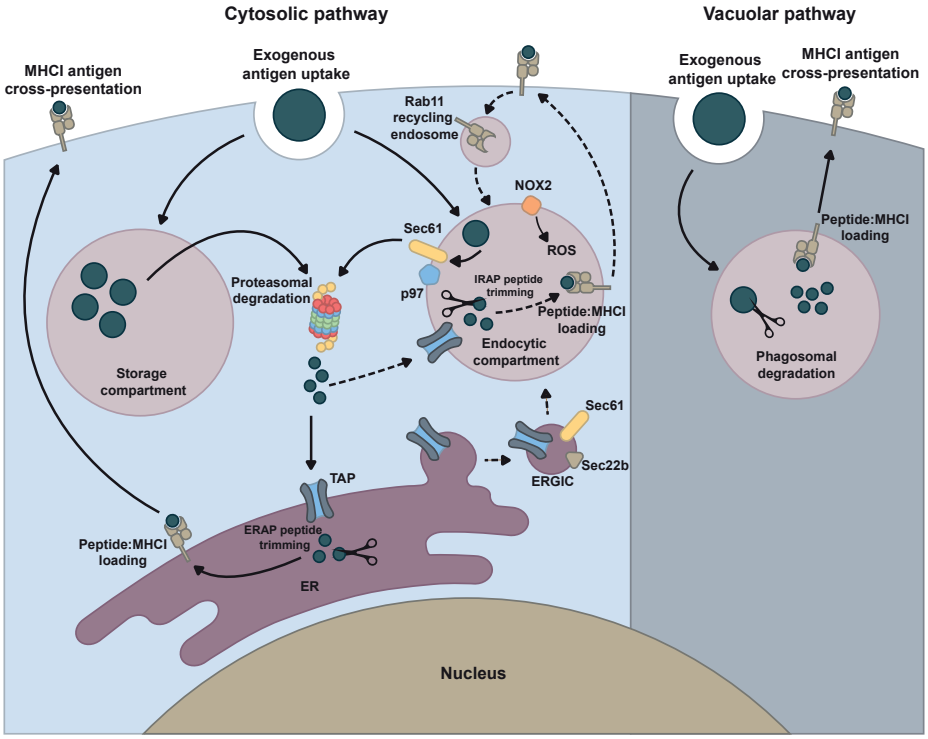

Figure 2. Antigen cross-presentation by dendritic cells. Two main intracellular pathways for antigen cross-presentation in DCs have been proposed: the cytosolic and the vacuolar pathways. In the vacuolar pathway, antigens are degraded by proteases (e.g. cathepsin S). Antigen processing and loading on MHCI occur in endocytic compartments. In the cytosolic pathway, antigens are taken up in endosomal vesicles. It has been proposed that antigens are translocated from endosomal vesicles into the cell cytosol through Sec61 or mediated through p97. Antigens are then degraded by the proteasome and transported by TAP to the endoplasmic reticulum (ER) for loading on MHCI molecules. However, some proteasome-generated peptides may be transported back into endocytic compartments for further peptide trimming and MHCI loading. MHCI molecules could be originated from the cell membrane and translocated via Rab11 recycling endosomes to endocytic compartments. There are indications that the recruitment of ER and ER-Golgi intermediate compartment (ERGIC) components to the phagosomes is mediated by the ER-resident SNARE Sec22b. Recruitment of NOX2 to endosomes and ROS production will induce alkalization and thereby preventing rapid antigen degradation in endosomes. Moreover, exogenous antigen can be taken up and conserved in storage compartments for prolonged antigen presentation. Antigen from the storage compartment is translocated to the cell cytosol where it is degraded by the proteasome and transported by TAP to the ER for MHCI loading and subsequently antigen cross-presentation on the cell surface.

How exogenous antigens are translocated from endocytic compartments into the cytosol is still not clear. It has been demonstrated using exogenous cytochrome *c*, that only cross-presenting DCs transfer cytochrome *c* to their cell cytosol, thereby triggering caspase-dependent apoptosis (13). Extensive studies in murine models identified the recruitment of ER-associated degradation (ERAD) member, Sec61, to endocytic compartments and suggested Sec61 as a possible translocator for antigen from the endosomes into the cytosol (14) (Fig. 2). By blocking Sec61 with a specific intracellular antibody they showed Sec61 was trapped in the ER, preventing its transport towards endosomes, and thereby blocking antigen translocation and cross-presentation. However, a more recent study showed severe inhibition of protein import into the ER but no inhibition of protein export from endocytic compartments when they used mycolactone, which binds Sec61α specifically (15). Although both studies showed inhibition of DC cross-presentation upon Sec61 blocking, it seems that Sec61 plays a more dominant role in inhibiting protein translocation into the ER and altering antigen cross-presentation at a different level than antigen export into the cytosol. Other evidence for the involvement of the ERAD machinery was shown by the Cresswell group, who demonstrated the requirement of p97 (also known as AAA ATPase) in protein export from phagosomes and thereby regulating cross-presentation (10) (Fig. 2). Moreover, they showed that a bead-bound synthetic peptide with an N-glycosylation site was N-glycosylated, which is a characteristic feature of the ER after DC phagocytosis. There has been an ongoing debate about the possible role of ER-resident proteins in endocytic compartments and in the membrane transport pathways. There are indications that the recruitment of ER and ER-Golgi intermediate compartment (ERGIC) components to the phagosomes is mediated by the ER-resident SNARE Sec22b (Fig. 2). The group of Amigorena showed that silencing Sec22b inhibits both the delivery of ER-resident proteins to phagosomes and the export of exogenous proteins from phagosomes to the cytosol (16). In a follow up study they showed impairment of DC cross-presentation in Sec22b-knockout DCs (17). However, conflicting results were found by another group who used similar Sec22b-knockout DCs and demonstrated that Sec22b is not necessary for cross-presentation (18). Therefore, a role for Sec22b in DC cross-presentation still needs to be determined.

Another proposed regulator of antigen cross-presentation in DCs is stromal interaction molecule 1 (STIM1), which is a calcium sensor that conveys the calcium content of the ER to store-operated channels of a cell. STIM1 can promote the contact sites between the ER and phagosomes, altering Ca^{2+} signaling and regulating phago/endosome fusion events (19). The ER membrane protein uncoordinated 93 homolog B1 (UCN93B1), which is activated by TLR triggering, interacts with STIM1 and is critically involved in antigen cross-presentation (20). Ablation of UCN93B1 impairs antigen translocation into the cytosol and antigen cross-presentation. In addition, it has been demonstrated that lipid peroxidation in DCs might play an important role in antigen translocation to the cytosol. The recruitment of NADPH-oxidase

complex (NOX2) and production of reactive oxygen species (ROS) in the endosomes can cause lipid peroxidation, resulting in leakiness of the endosomal membrane and hence, antigen access into the cytosol and enhanced antigen cross-presentation (21). Furthermore, upon NOX2 recruitment and ROS release in endosomes, mediated by Rab27a, endosomal alkalization and pH in endosomes are increased (22, 23) (Fig. 2). This will prevent rapid antigen degradation and thereby enhancing antigen cross-presentation.

We have previously published that antigen can be conserved in DCs in specialized intracellular storage compartments which facilitate prolonged antigen cross-presentation to $CD8^+$ T cells (24) (Fig. 2). These storage compartments are lysosomal-like organelles, distinct from MHCII compartments or MHCI processing/loading compartments. Surface MHCI molecules on DCs have a shorter turnover rate compared to MHCII molecules, most MHCI-peptide complexes disappear from the cell surface within 24 hours. Since the migration of DCs after antigen encountering to the T-cell zones might take up to several days, this high turnover rate of MHCI molecules is not beneficial for efficient $CD8^+$ T-cell cross-presentation (25). Also, the dose of antigen that is expressed on MHCI needs to exceed the required threshold for effective T-cell activation. Therefore, long-term antigen storage in DCs and sustained antigen display on the DC cell-surface are important to ensure T-cell cross-priming. In this thesis we investigate antigen uptake, storage, processing, and sustained cross-presentation mechanisms in DCs *in vitro* and *in vivo*.

MURINE CROSS-PRESENTING DENDRITIC CELL SUBSETS

Over the years it has become clear that DCs are organized in multiple subpopulations, each having specific functions. Only some of the DC subsets have the ability to cross present antigen efficiently. Murine DCs in secondary lymphoid organs can roughly be divided in conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (26). cDCs are further classified into $CD8\alpha^+$ DCs (cDC1) and $CD8\alpha^-$ DCs (cDC2). The development of $CD8\alpha^+$ DCs is mainly regulated by the expression of the transcription factors IRF8 and Batf3, whereas $CD8\alpha^-$ DCs is mainly regulated by IRF4 (27). Deletion of either of these genes can lead to development defects of the DC subsets. In general, $CD8\alpha^+$ DCs are considered to be more efficient at cross-presentation than $CD8\alpha^-$ DCs (28). Some explanations for the superior cross-presentation ability of $CD8\alpha^+$ DCs include higher expression of components that are associated with MHCI processing pathway, reduced antigen degradation in endosomes by ROS production, and higher efficiency in antigen transfer into the cytosol (13, 22, 29). However, studies have shown that $CD8\alpha^-$ DCs can also cross-present antigen efficiently after receptor-mediated endocytosis by CD205 or FcγRs (30, 31). The main DC subset responsible for cross-presentation in the lymph nodes, lung and skin is $CD103^+$ migratory DCs (32, 33).

Murine pDCs are generally considered as poor cross-presenting cells. Although some studies suggested their cross-presenting ability *in vitro*, *ex vivo*, or after TLR activation, their role in cross-presentation *in vivo* seems lacking during viral infections, despite the fact that they are well known for producing large amount of type I interferons (34–39). In human, the BDCA1$^+$ (CD1c$^+$) and BDCA3$^+$ (CD141$^+$) DCs in blood are proposed as the human counterparts of murine CD8α$^-$ and CD8α$^+$ DCs, respectively (40). Although in general BDCA3$^+$ are considered to be more efficient in antigen cross-presentation, it has been shown that BDCA1$^+$ DCs reached similar efficiency upon activation with TLR ligands (41). In contrast to murine pDCs, it has been reported that human pDCs can efficiently cross-present soluble and cell-associated antigens. However, a recent study identified a distinct pre-pDC subset which bears similar markers as the classical pDCs. They showed that only the pre-pDC subset was able to present antigen to CD4$^+$ T cells and that the antigen presenting ability for the classical pDCs might be a result of the "contamination" of pre-pDCs. Whether this also applies for antigen cross-presentation to CD8$^+$ T cells still needs to be elucidated. However in both murine and human, it seems that the cross-presentation ability for each subset can depend on factors including the type of antigen, antigen handling and processing, DC location, DC activation, and local inflammatory signals (42).

Recent studies identified a two-step T cell priming model in which CD4$^+$ T cells and CD8$^+$ T cells first encounter their respective antigen on different types of DCs during the first priming step (43–46). During the second priming step, lymph node resident XC-chemokine receptor 1 (XCR1)$^+$ cDC1s are recruited to receive cross-presented antigen from the DCs that carried out the first priming step. The pre-activated CD4$^+$ and CD8$^+$ T cells during the first priming step interact with the cDC1s, where CD4$^+$ T cells induce optimal signals for CD8$^+$ T cell differentiation into CTLs and memory CTLs. These findings highlight the importance of different DC subsets and their distinctive functions for the induction of efficient T cell priming. In **chapter 2** we studied the sustained cross-presentation ability of individual murine DC subsets *in vivo*.

CANCER IMMUNOTHERAPY TARGETING DENDRITIC CELLS

DCs have become the prime target for cancer vaccines since they play a critical role in inducing anti-tumor immunity responses and the formation of anti-tumor memory cells. Extensive studies on DC-based vaccination strategies have been done in order to find an optimal treatment for cancer patients. DC immunotherapy can roughly be divided in *ex vivo* activated DCs and direct *in vivo* targeting of DCs (47, 48). *Ex vivo* DCs are mainly obtained from peripheral blood mononuclear cells (PBMCs) or generated from CD34$^+$ progenitors by culturing them in the presence of cytokines such as IL-4 and GM-CSF. This very labor

intensive immunotherapy has shown some clinical successes (49). The autologous DCs cultured in Good Manufacturing Practice (GMP) setting are then loaded with tumor-derived antigens, activated with a maturation cocktail (e.g. TNF-α, IL-1β, IL-6, and PGE2), and injected back into the patients. Despite the fact that *ex vivo* generated DCs can properly initiate tumor-specific $CD8^+$ and $CD4^+$ T cell responses, there is still limited efficacy of DC-based vaccines. This is likely caused by the presence of immune escape, and immunosuppressive mechanisms in the tumor microenvironment (50). Also, determining the ideal antigen-loading is important for optimal therapy. Several methods of antigen loading of DCs have been studied, including short peptides, long peptides, tumor cell lysates, DNA or RNA coding for a specific antigen, immune complexes, and neoantigens (48, 51). Loading DCs with short peptides results in peptides that bind to a limited number of HLA class I molecules. The lack of Th induction might cause suboptimal long-lived CTL responses (3). More ideal is to load all available HLA class I- and class II-presenting molecules on DCs with tumor-derived peptides (52). *Ex vivo* loading of DCs with immune complexes, consisting specific antibodies complexed with tumor-associated protein antigen, have shown efficient MHCI and MHCII antigen presentation, potent DC activation, and efficient tumor control in mice (53).

Another DC vaccination approach is to deliver antigens to DCs directly *in vivo* by coupling the antigens to antibodies specific to DC-expressed receptors, including FcγRs and CLRs. Targeting DC CLRs, such as DEC-205, DC-SIGN, and DNGR-1, have shown efficient MHCI and MHCII immune responses (54–56). However, if these antigen-antibody conjugates are given without additional adjuvant to stimulate DC activation, this type of DC targeting can induce disease-specific tolerance (57). Therefore, additional DC activating compounds such as CD40 and TLR ligands are often required (55, 58). Another highly efficient targeting strategy of *in vivo* DCs is injecting long-peptides conjugated to TLR-ligands, sharing the peptide antigen and adjuvant in one single molecule. This resulted in enhanced antigen presentation, efficient $CD8^+$ T cell priming, and antitumor immunity in mice challenged with aggressive transplantable melanoma or lymphoma (59, 60). This might be a more promising DC targeting vaccination strategy compared to the laborious and expensive *ex vivo* loading of DCs with tumor antigens.

Currently, one of the most innovative developments in DC vaccination developments is the use of RNA sequencing to determine neoantigens derived from somatic mutations in the tumor, which are absent in non-malignant cells. Neoantigens can stimulate expansion of high-affinity $CD8^+$ T cells which are patient and tumor specific. However, the number of somatic mutations is dependent on the tumor type, which can influence the susceptibility of immunotherapy (61). Nevertheless, it becomes more clear that DCs play a vital role in the outcome of vaccines against cancer or infectious diseases.

FCΓ RECEPTORS ON DENDRITIC CELLS

Potent therapeutic vaccination against cancer relies on efficient antigen loading and activation of DCs in priming T cells. Several studies revealed that antibody-mediated targeting of protein antigen via FcγRs are highly effective in inducing T cell-mediated antitumor responses (62–64). DCs express FcγRs on their cell surface to facilitate the uptake of antibody-bound exogenous antigens. In mice, four FcγRs have been described including FcγRI (CD64), FcγRIIB (CD32B), FcγRIII (CD16), and FcγRIV (65). The activating receptors FcγRI, FcγRIII, and FcγRIV have an immunoreceptor tyrosine-based activation motif (ITAM), whereas the inhibitory receptor FcγRII has an immunoreceptor tyrosine-based inhibition motif (ITIM) (Table 1) (65). ITAM activates signaling cascades via SRC family kinases and spleen tyrosine kinase (SYK). ITIM recruits SH2 domain-containing inositol 5′-phophatase 1 (SHIP1) and counteracts activating signals by the activating receptors. Co-expression of activating and inhibitory receptors on the same cell can function as a threshold for activation, thereby defining the outcome of cellular response (66–68). In general, murine cDCs and macrophages express all activating FcγRs and the inhibitory receptor, whereas pDCs only express the inhibitory receptor FcγRII (65). FcγRs have different affinity for binding of different IgG isotypes (Table 1). Although both activating and inhibitory receptors can bind and rapidly endocytose opsonized materials or antigen-antibody immune complexes (ICs), the type of receptor that is triggered influences the degradative pathway in which antigens will be routed (69). Internalization by activating FcγRs favors the degradative route for antigen processing and presentation, whereas the inhibitory FcγR favors a retention pathway preserving the antigen for transfer to B cells. Activating receptors mainly promote antigen presentation due to their ability to activate DCs and to stimulate the MHCI cross-presentation machinery (70). We have demonstrated that FcγR-mediated uptake of model antigen OVA bound to anti-OVA IgG is at least 1000-fold more efficient in antigen cross-presentation than soluble OVA (64). Binding of ICs triggers cross-linking of the FcγRs resulting in DC maturation and internalization of the ICs toward antigen storage and presentation compartments (24, 71). DCs loaded with specific antigen-antibody ICs resulted in priming of $CD8^+$ CTLs, and tumor protection *in vivo* (64, 72). We characterized the antigen storage compartments in more detail in **chapter 4** and described the importance of these storage compartments for prolonged antigen cross-presentation *in vivo* in **chapter 2**. The role of FcγR targeting in sustained DC cross-presentation by DC subsets *in vivo* will be discussed in **chapter 3**.

Table 1. Murine Fcγ receptors

FcγRs	IgG binding affinity	Function	Motif	Expression on APCs
FcγRI	IgG2a > IgG2b > IgG3	Activation	ITAM	$CD8\alpha^+$ DCs, $CD8\alpha^-$ DCs, macrophages
FcγRIIB	IgG1 > IgG2b > IgG2a	Inhibition	ITIM	$CD8\alpha^+$ DCs, $CD8\alpha^-$ DCs, pDCs, macrophages, B cells
FcγRIII	IgG2a > IgG2b > IgG1	Activation	ITAM	$CD8\alpha^+$ DCs, $CD8\alpha^-$ DCs, macrophages
FcγRIV	IgG2a > IG2b	Activation	ITAM	$CD8\alpha^+$ DCs, $CD8\alpha^-$ DCs, macrophages

COMPLEMENT FACTOR C1Q

Complement is one of the main effector mechanisms of antibody-mediated immunity. It plays an important role in defending against bacterial infections, bridging innate and adaptive immunity, and rapid clearance of circulating ICs by binding complement coated ICs to complement receptor-1 on erythrocytes and thereby preventing IC deposition (73, 74). It has been described that patients with systemic lupus erythematosus have defects in IC clearance, resulting in tissue inflammation and damage (75). The complement system can be divided into three different pathways: the classical pathway, the alternative pathway, and the mannose binding lectin pathway (74). Each pathway is activated upon different triggering but all converge at the point of cleavage of complement protein C3 and ultimately cleavage of C5 into C5a and C5b, thereby initiating the membrane attack complex (MAC). MAC is composed of a complex of C5b, C6, C7, and C8, which binds to the cell membrane and can kill or damage the cell by inducing pores in the membrane. The classical pathway is initiated upon binding of the C1 complex (which consists of C1q, two C1r serine proteases, and two C1s serine proteases) to antibodies bound to antigen (Fig. 3). Besides binding to FcγRs, most IgG subclasses can bind to C1q, the first recognition subcomponent of the classical pathway (76). C1q is a hexameric glycoprotein assembled from 18 polypeptide chains that are formed by three types of chains (A, B, and C chain) (77). Each chain consists of a collagen-like domain (the binding site for anti-C1q auto-antibody) to which the serine proteases C1r and C1s are localized. Moreover, each chain comprises a globular head which binds to the Fc part of IgG and IgM when bound to cognate antigen (78). Importantly, it has been reported that IgG hexamerization after antigen binding leads to a more stabilized binding of C1q with high avidity (78, 79) (Fig. 3). The collagen-like regions mediate immune effector mechanisms, including complement activation through interaction with the C1r and C1s proteases (80). Upon binding of C1q to ICs, C1r and C1s are activated, resulting in activation of the classical complement pathway (81). Interestingly, C1q is mainly produced by macrophages and immature DCs (82). We and others have demonstrated that the uptake, processing of ICs, and antigen cross-presentation by DCs in the spleen were hampered in C1q-defiecient mice (83, 84). The crucial role of C1q in antibody-mediated antigen uptake and cross-presentation by APC subsets *in vivo* will be discussed in **chapter 3**.

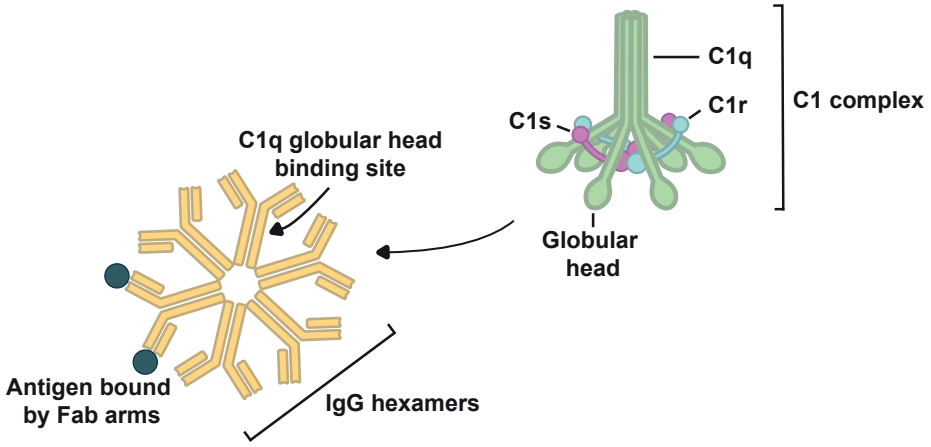

Figure 3. C1q binding to antigen:IgG hexameric complex. C1q is a hexameric glycoprotein with collagen-like domains to which two serine proteases C1r and two C1s are localized. Each chain of the hexamer comprises a globular head that binds to the Fc part of IgG when bound to cognate antigen. It is likely, based on recent data (78, 79), that IgG molecules can form hexameric structures after antigen binding which lead to a more stabilized binding of hexameric C1q multimers with high avidity.

C-TYPE LECTIN RECEPTOR MGL

CLRs have been extensively studied for the development of tumor vaccine that targets specific DC subsets. DCs express a large variety of CLRs, including DEC205, DCIR, CLEC9a/DNGR1, CLEC12, Dectin-1, Langerin, MR, DC-SIGN, and MGL (85). Most CLRs recognize glycosylated antigens through their carbohydrate recognition receptors. Directing antigens to CLRs on DCs (e.g. DEC205, MR, DC-SIGN and CLEC9a), by using antigens conjugated to CLR-specific antibodies, have shown enhanced antigen uptake and presentation (86–89). Targeting strategies using natural or artificial glycan ligands have gained interest since they are easy to develop and relatively cheap to produce. The ligands can be directly conjugated to tumor antigens or incorporated in nanoparticles, and more importantly, they mimic natural functions of the receptors, inducing "natural" signaling cascades in DCs. Glycosylated antigen specific for CLRs have shown efficient antigen uptake and presentation (90–94). However, targeting antigen to different CLRs might result in activating or suppressive downstream signaling events, resulting in different intracellular routing, DC maturation status, and antigen presentation. Therefore, it is important to bear this in mind when targeting specific CLRs for the desirable outcome.

There are two homologs of macrophage galactose-type lectin (MGL) identified in mice, MGL1 and MGL2, whereas in human only one homolog (huMGL) has been found (95, 96). MGL is exclusively expressed on DCs and macrophages and therefore considered to be a

potent target for vaccination strategies. Murine MGL1 binds to the carbohydrate structures Lewis^x (Le^x) and Lewis^a (Le^a), while murine MGL2 and huMGL have high affinities for *N*-acetylgalactosamine (GalNac) and galactose, including the O-linked Tn-antigen, TF-antigen, and core 2 (97, 98). The difference of glycan specificities between MGL1 and MGL2 provides specific targeting of each receptor. However, their targeting potency in inducing DC cross-presentation is still not fully unraveled yet. It has been shown that GalNac modifications of antigen resulted in antigen presentation to CD4$^+$ and CD8$^+$ T cells in murine BMDCs (99). Targeting dermal DCs with glycosidic Tn-based vaccines favored CD4$^+$ T cell priming *in vivo* and activation of antibody-producing B cells (100). The model protein OVA is mainly binding to the mannose receptor (MR) on DCs and has extensively been used in many DC cross-presentation studies. However, it have been shown that MR mediates cross-presentation only when high doses of OVA were used in combination with TLR-triggering (101). In **chapter 4 and 5**, we redirected OVA targeting to MGL1 on DCs by the modification of OVA with Le^x, and investigated the antigen routing, processing, and cross-presentation outcome.

AUTOPHAGY IN DENDRITIC CELLS

Autophagy is an evolutionary conserved system that degrades and recycle unnecessary or damaged cellular components by lysosomes (102). At least three different pathways for autophagy has been described: macroautophagy, microautophagy and chaperon-mediated autophagy (103) (Fig. 4). Microautophagy involves direct engulfment of small amounts of cytoplasmic material into the lysosome, whereas chaperon-mediated autophagy utilizes heat-shock-cognate protein 70 (Hsc70) and LAMP2A to translocate proteins to the lysosome. Macroautophagy degrades larger structures like damaged organelles or protein aggregates. These are taken up from the cytoplasm by a cup-shaped double membrane which fuses its ends to form double-membrane-surrounded autophagosomes and subsequently fuse with lysosomes.

Cell nutrient starvation and other stresses can initiate autophagy, which is under control of the mammalian target of rapamycin complex 1 (mTORC1) and AMP-activate protein kinase (AMPK). During starvation, mTORC1 is inactivated whereas AMPK is activated, resulting in macroautophagy activation and autophagosome elongation. The autophagosome formation is regulated by autophagy-related gene (Atg) products (104) (Fig. 5). The ULK complex (containing ULK1/2, Atg13, and FIP200) recruits the class III phosphatidylinositol 3-kinase (PI3K) VPS34 complex to the membranes. The VPS34 complex (containing Atg14, VPS34, Atg6/Beclin-1, VPS15, UVRAG, and BIF-1) generates phosphatidylinositol 3-phosphate (PI3P) which leads to the recruitment of two ubiquitin-like molecules, Atg8 (LC3) and Atg12. Atg12 is activated by Atg7 (E1-like conjugation enzyme) and Atg10 (E2-like conjugation enzyme).

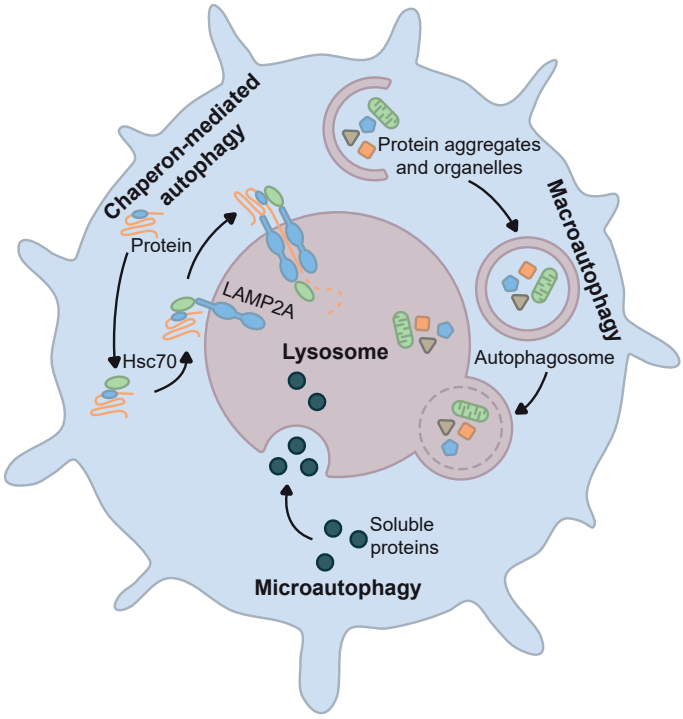

Figure 4. Autophagy pathways. Lysosomal degradation of cellular contents can involve three autophagy pathways, including macroautophagy, microautophagy, and chaperon-mediated autophagy. Protein aggregates or damaged organelles can be delivered to lysosomes via autophagosomes, a process called macroautophagy. Microautophagy involves engulfment of small amounts of cytoplasmic cargo into the lysosome. Chaperon-mediated autophagy utilizes heat-shock-cognate protein (Hsc70) and LAMP2A to translocate cytosolic proteins to the lysosome for degradation.

Activation of these conjugation systems leads to binding of Atg12 with Atg5, followed by binding of Atg16 to form the E3-like ligase of the LC3 conjugation system. LC3 is cleaved by Atg4 to LC3-I, which is then lipidated by Atg3 (E2-like conjugation enzyme) and Atg7 (E1-like conjugation enzyme) to generate phosphatidylethanolamine (PE)-conjugated LC3-II. LC3-II is incorporated into the phagophore membrane which serves as docking site of adaptor proteins. The closure of an elongated phagophore marks the formation of a mature autophagosome. LC3-II is commonly used as autophagosomal marker since it is binding covalently on newly formed autophagosomes until they fuse with lysosomes. Fusion of autophagosomes with lysosomes results in degradation of the cargo and Atg8 homologs coupled to the inner autophagosomal membrane.

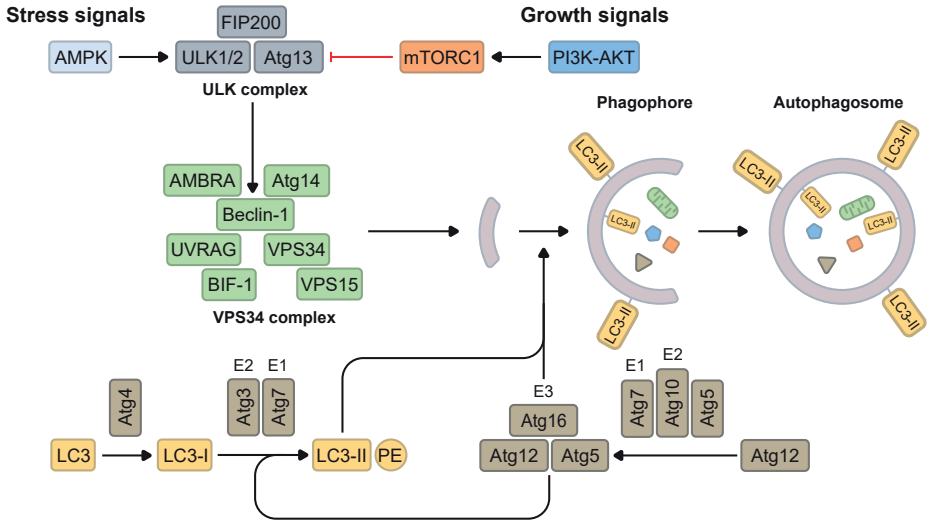

Figure 5. Formation of autophagosome. During starvation or other stress signals, mTORC1 is inactivated whereas AMPK is activated, which results in macroautophagy activation and the formation of autophagosomes. The ULK complex (ULK1/2, Atg13, FIP200) recruits the VPS34 complex (VPS34, VPS15, Beclin-1, AMBRA, Atg14, UVRAG, and BIF-1) which is required for nucleation of the phagophore membrane. The elongation and formation of autophagosomes involve the formation of two ubiquitin-like conjugation systems: Atg16 complex (Atg5, Atg12, and Atg16) and lipidation of Atg8 (LC3). For the formation of Atg16 complex, Atg12 is activated by Atg7 (E1-like conjugating enzyme) and transferred to Atg10 (E2-like conjugation enzyme) which further facilitates the formation of Atg12-Atg5, followed by binding of Atg16. The Atg16 complex is required for the lipidation of LC3. LC3 is first cleaved by Atg4 to LC3-I, which is then activated by Atg7 (E1-like conjugating enzyme) and transferred to Atg3 (E2-conjugating enzyme). The formation of Atg3-LC3-I facilitates the generation of PE-conjugated LC3-II, which is incorporated in the inner and outer membrane of autophagosomes. The closure of an elongated phagophore marks the formation of a mature autophagosome.

A role for macroautophagy has been suggested in intracellular antigen processing for MHCII presentation. Starvation-induced macroautophagy resulted in 50% increase of MHCII presentation of intracellular, cytosolic and nuclear antigens (105). DCs from mice which were Atg5 deficient showed impaired $CD4^+$ T cell priming after herpes simplex virus infection, suggesting the autophagic machinery is required for optimal phagosome-to-lysosome fusion and subsequently processing of antigen for MHCII loading (106). A role for autophagy in extracellular antigen processing for MHCII presentation was observed *in vivo* where OVA was only efficiently processed and presented to $CD4^+$ T cells in the presence of Atg5 (106). It has been suggested that LC3-associated phagocytosis (LAP) plays a role in endocytosis and degradation of extracellular material for efficient MHCII presentation (107, 108). During LAP the single phagosomal membrane recruits LC3 and LAMP phagosomes are either degraded in lysosomes, delayed in their fusion with lysosomes, or fused with compartments that contains PRRs, resulting in increased MHCII presentation of extracellular antigens (107-

110). The induction of LAP is dependent on receptor-mediated antigen uptake, and the attachment of LC3 to the phagosomes requires NOX2. However, LAP is independent of 'classical' macroautophagy proteins such as the Atg1 complex (111).

Several studies have provided evidence for enhancement of the classical MHCI antigen presentation pathway by autophagy. Reduced autophagic degradation of defective ribosomal products (DRiPs) was observed when HeLa cells were treated with the selective PI3K inhibitor 3-Methyladenine (3-MA), resulting in enhanced proteasome degradation and class I antigen presentation (112). During herpes simplex virus-1 (HSV-1) infection, it seems that macroautophagy contributes to antigen processing for efficient MHCI presentation of HSV-1 glycoprotein to $CD8^+$ T cells in a proteasome dependent manner (113). On the contrary, infection with human cytomegalovirus (HCMV) induced autophagy and increased the presentation of HCMV latency-associated protein (pUL138) in a proteasome- and TAP-independent manner that involved MHCI loading in endosomal compartments (114). Several studies have investigated the role of autophagy in DC cross-presentation with contradictory results. Some showed elevated $CD8^+$ T cell responses upon autophagy inhibition in DCs with different antigen targeting systems (115), while others showed autophagy-independent cross-presentation (106) or even lowered immune responses upon blocking autophagy (116, 117). In **chapter 6** we investigated the role of autophagy on long-term DC cross-presentation.

SCOPE OF THESIS

In this thesis we further investigate the underlying mechanisms of DC cross-presentation. Understanding and improving DC cross-presentation is key for the development of cancer vaccines to induce effective CTL responses. In **chapter 2**, we studied the sustained cross-presentation capacity of murine splenic DC subsets *in vivo* after antigen storage. The role of FcγRs and complement factor C1q in prolonged antigen cross-presentation will be described in **chapter 3**. In **chapter 4** we characterized the antigen storage compartments in DCs where antigen is preserved for long-term cross-presentation. By redirecting antigen targeting to MGL1 on DCs, we studied the antigen routing, processing, and cross-presentation outcome in DCs in **chapter 5**. In **chapter 6**, we will investigate how the autophagy machinery regulates long-term cross-presentation by DCs. Specific conjugation of fluorescent dyes to antigenic peptides to study DC uptake and routing was analyzed for cell biological properties, which will be discussed in **chapter 7**. Finally, in **chapter 8** the findings of this thesis are summarized and discussed.

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