Antigen handling and cross-presentation by dendritic cells

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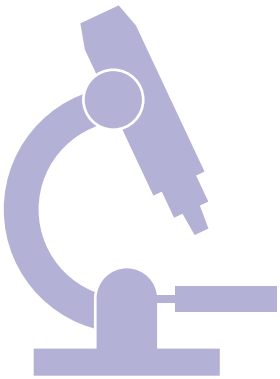


Summary and general discussion

In the current thesis, we provide novel insights in antigen uptake, storage, processing, and sustained cross-presentation mechanisms in dendritic cells (DCs) *in vitro* and *in vivo* (Fig. 1). We have studied antigen handling functions by dendritic cells in three different antigen delivery routes: antibody targeting involving Fcγ receptors (FcγRs) and complement factor C1q, C-type lectin receptor (CLR) targeting, and toll-like receptor (TLR) ligand targeting systems. Our data highlights that antigen storage in specialized compartments in DCs, despite the chosen uptake route, is beneficial for prolonged antigen cross-presentation by DCs and sustained T cell activation. Further *in vivo* studies in different antigen presenting cell (APC) subsets confirmed the presence of antigen storage compartments by isolating APC subsets after *in vivo* antigen uptake. Besides, we revealed a dominant role of C1q in antigen-antibody immune complex (IC) uptake and cross-presentation *in vivo* in contrast to the crucial role of FcγRs *in vitro*. Furthermore, we demonstrated that autophagosomes have a negative impact on the storage of antigen in those specialized compartments and thereby affecting DC cross-presentation efficiency. With the current studies, we unraveled some mechanics of antigen processing in DCs which contribute to future vaccine designs against diseases such as cancer. A general summary and more detailed discussion of remaining questions will be provided below. Finally, future applications and directions for DC-based immunotherapy will be discussed.

ANTIGEN STORAGE IN DENDRITIC CELLS

Prolonged cross-presentation by dendritic cells

What happens with antigen after its uptake by DCs? This is one of the questions that has gained lots of interest by researchers since the discovery of DCs and their key mediator function in bridging innate and adaptive immune responses. The distinction between MHCI and MHCII antigen presentation pathways in expressing intracellular and extracellular antigens, respectively, is well known now. But the mechanisms of presenting exogenous antigen, derived from e.g. cancer cells, on MHCI to cytotoxic T cells require in depth unraveling of DC uptake, processing and cross-presentation machinery. This basic knowledge is of central importance to develop efficient cancer vaccines for the activation of immune responses to specific tumor antigens. One observation from our group was that antigen can be conserved in DCs for several days in specialized intracellular storage compartments which facilitate prolonged antigen cross-presentation to $CD8^+$ T cells (**chapter 4** and (1)) (Fig. 1). This storage compartment functions as a depot for the continuous supply of MHCI ligands. One can appreciate its importance since the trafficking of DCs from peripheral organs to lymphatic sites can take up to several days (2). In addition, due to the rapid turnover rate of MHCI-peptide complexes on the cell surface, newly synthesized peptide

loading could be beneficial to achieve long-lasting and potent antigen presentation capacities of DCs (3). Further characterization of this storage compartment with the use of different antigen targeting system (including FcγRs and C-type lectin receptor MGL1) revealed that, despite different antigen targeting routes, the antigens ended up in the same endosomal compartments (LAMP1$^+$) in DCs (**chapter 4**) and induced sustained antigen cross-presentation by DCs (**chapter 4 and 5**). Importantly, the storage compartments are distinct from early endosomal (EEA1$^+$/ Rab5$^+$), MHCI or MHCII loading compartments. Our results indicate that these specialized storage compartments play a central role in DC cross-presentation where antigens, taken up via different surface receptors, are congregated in the same endosomal organelle for further processing.

One of the initial suggested concepts of endosomal routing of captured antigens is that antigens first enter early endosomes (Rab5$^+$), then late endosomes (Rab7$^+$), and end up in lysosomes (LAMP1$^+$). The pH in these endosomes drops accordingly to the maturation state of the endosomes, which is in favor of antigen degradation. Despite the fact that an overall pH of 4 was reached by DCs after 24 hours of IC uptake, antigen degradation was limited (**chapter 6** and (1)). How antigen is protected from degradation in the storage compartments is still not fully understood. We have shown in **chapter 4** that the activity of cathepsin S was lacking in the compartments where antigen was stored, which could explain why antigen was degraded at a slower rate. Besides, there is a possibility that antigens in ICs are protected by the bound antibodies. It has also been suggested that the intracellular neonatal FcR (FcRn) facilitates the transport of IgG-bound antigens, but not monomeric IgG, to endosomes where it protects the degradation of antigen and mediates efficient antigen delivery to the cytosol (4, 5). However, preliminary studies with FcRn deficient mice in our group suggested no clear involvement of this receptor in antigen cross-presentation of immune-complexed OVA (unpublished data).

An interesting finding we observed in **chapter 4** was the presence of cathepsin X in the storage compartments. It has been described that cathepsin X expression is restricted to immune cells, such as monocytes, macrophages, and dendritic cells (6). It can regulate the proliferation, maturation, migration, and adhesion of immune cells. Inhibition of cathepsin X resulted in lower expression of co-stimulatory molecules, hampered cytokine production, diminished DC migration, and decreased stimulation of CD4$^+$ T cells (7). During DC maturation, cathepsin X translocates to the membrane, where it activates Mac-1 integrin receptor, resulting in cell adhesion and development of podosomes (8). After DC maturation, cathepsin X redistributes from the membrane to the perinuclear region, resulting in de-adhesion of DCs. In **chapter 4,** we observed similar redistribution of cathepsin X towards the perinuclear region and co-localization with antigen in storage after FcγR targeting. Cathepsin X is a fairly new discovered cathepsin, and its role in DC cross-presentation is not known yet. However, the presence of this particular cathepsin in the storage compartments could

imply a role in antigen processing for cross-presentation. Interestingly, preliminary data from our group observed a higher presence of cathepsin X in CD8α⁺ DCs compared to CD8α⁻ DCs, pDCs, and macrophages (unpublished data). Further studies with cathepsin X deficient DCs are needed to elucidate whether there is a direct role of cathepsin X in antigen cross-presentation.

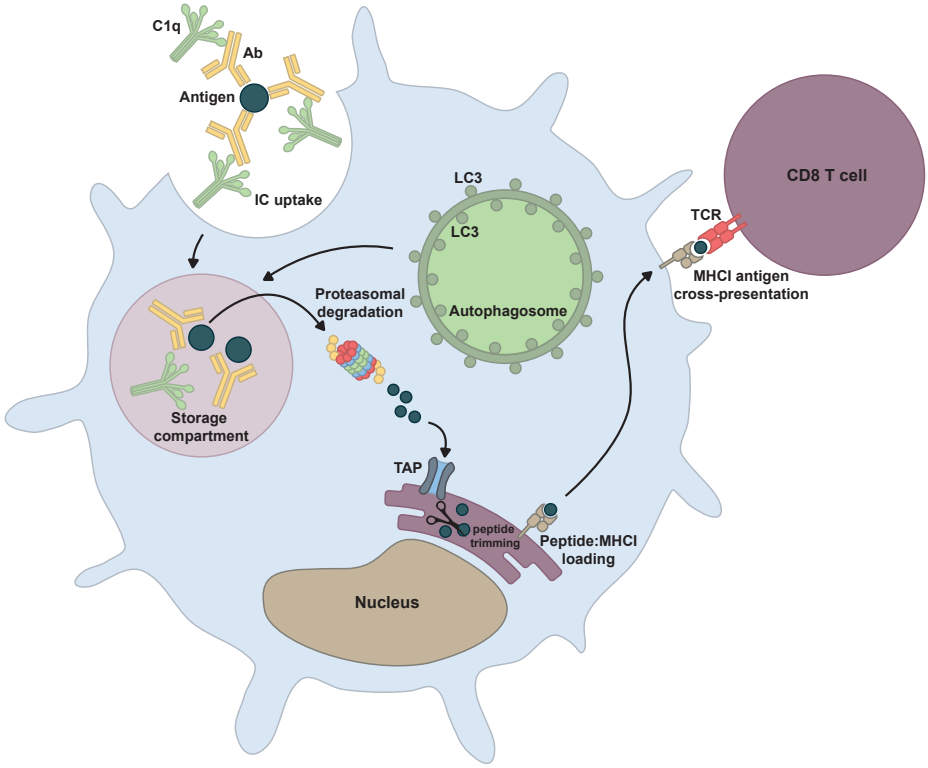

Figure 1. Overview of antigen cross-presentation mechanisms by DCs studied in the current thesis. Soluble protein antigen binds to antibodies in circulation to form antigen–antibody immune complexes (IC). C1q facilitates the uptake of these complexes by DCs via an as yet undefined uptake route *in vivo*. After uptake, antigens are 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 to CD8⁺ T cell through the T cell receptor (TCR). Moreover, autopaghosomes (positive for LC3) can degrade antigen storage compartments and thereby affecting the sustained cross-presentation capacity of DCs.

Differential cross-presentation ability by dendritic cell subsets *in vivo*

Since we have observed the existence of antigen storage compartments in DCs cultured *in vitro* and their contribution to prolonged antigen cross-presentation, the next step was

to confirm their existence and role in the *in vivo* setting. In order to mimic the natural formation of antigen-antibody ICs, we injected mice sequentially with anti-OVA IgG and OVA to form OVA ICs *in vivo*, which previously have been shown by our group to induce efficient antigen cross-presentation to $CD8^+$ T cells (9). We now show in **chapter 2** for the first time that different APC subsets, including $CD8\alpha^+$ DCs (also called cDC1), $CD8\alpha^-$ DCs (also called cDC2), pDCs, and macrophages, have the ability to store antigens for several days *in vivo*. This corresponds with long-lasting *in vivo* antigen presentation to $CD8^+$ and $CD4^+$ T cells up to a week after antigen injection. These data confirm our earlier *in vitro* work and emphasize the importance of antigen storage also in DCs *in vivo*. Interestingly, a clear distinction between antigen presentation ability was found between $CD8\alpha^-$ DCs and $CD8\alpha^+$ DCs, where the later subset was superior in antigen cross-presentation. Other studies have shown similar distinction in antigen presentation capacities by $CD8\alpha^+$ and $CD8\alpha^-$ DCs (10–13). It is still unclear why specific APC subsets are more potent in cross-presenting antigen. It has been suggested that cross-presentation by $CD8\alpha^-$ DCs depends on activating Fcγ receptors (FcγRs) (14) or an additional stimuli such as TLR ligands (15). MHCI antigen presentation by $CD8\alpha^-$ DCs is hampered in γ-chain-deficient mice, but not in $CD8\alpha^+$ DCs, indicating that the activation of $CD8\alpha^+$ DCs is not required for efficient cross-presentation. However, we observed in **chapter 3** that complement factor C1q, rather than FcγRs, plays a major role in antibody-mediated antigen uptake from blood circulation and presentation *in vivo*, which will be discussed later in this chapter.

Other explanations for the superior cross-presentation ability of $CD8\alpha^+$ DCs include lower degradation of antigen in endosomes by ROS production (16) or lower levels of lysosomal proteases (17). DCs have the ability to recruit NOX2 to the endosomes which mediates the generation of ROS that capture protons to build hydrogen peroxide. This results in active alkalization and impaired pH-dependent activation of lysosomal proteases. However, preliminary data from our group with NOX2 deficient mice did not show differences in antigen cross-presentation of IC (unpublished data). It has been demonstrated that DCs express lower levels of lysosomal proteases compared to other immune cells (17). Expression of cathepsins L, S, D, and B in phagosomes in DCs is also more reduced compared to the levels in macrophages. This results in slower phagolysosomal antigen degradation and prolonged antigen presence in DCs. Others showed more efficient transfer of exogenous antigens into the cytosol (18), and higher expression of components that are associated with MHCI processing pathway (12) in $CD8\alpha^+$ DCs, which can also contribute to their potent cross-presentation capacity.

Despite the fact that we observed efficient antigen uptake by splenic pDCs and macrophages *in vivo*, strikingly both subsets showed no detectable antigen presentation to T cells *ex vivo* (**chapter 2**). pDCs are well known for their ability in producing large amounts of type I interferons. Some studies had shown their potential role in cross-presentation *in*

vitro or *ex vivo* (19–21), however their cross-presentation capacity *in vivo* seemed lacking which is in line with our observations (22, 23). It is also important to take into account that pDCs only express the inhibitory FcγRII, suggesting a role in controlling tolerance in steady state. Although, it has been demonstrated that pDCs are capable in inducing effective cross-presentation upon TLR stimulation (20). A more recent study showed that the activation of pDCs by TLR ligands induced the production of mitochondrial ROS and thereby increased the cross-presentation capacity by pDCs (24). Macrophages mostly function as a first line defense against pathogens by their rapid degradation ability and are less well known for their role in antigen cross-presentation. Although it has been suggested that macrophages have a more acidic endosomal environment compared to DCs (17, 25), in our current system we could still detect similar amounts of antigen stored in splenic macrophages compared to DCs several days after antigen and antibody injection without detectable MHCI or MHCII presentation.

Antigen transfer between APC subsets: A role for antigen storage compartments?
Although only specific APC subsets can cross-present antigen, that does not mean that other APC subsets are not relevant for efficient T cell induction. There are studies suggesting that macrophages and pDCs act in concert with DCs to promote cross-priming to T cells (reviewed in (26)). Upon infection, pDCs migrate to lymphoid organ areas which are rich in $CD169^+$ macrophages, and produce large amounts of IFN-I (27). It has been shown that IFN-I is critical for the antigen cross-presentation by DCs and that pDCs can promote the generation and survival of antigen-specific $CD8^+$ T cells upon infection (28–31). Moreover, several studies suggested antigen transfer from $CD169^+$ macrophages to DCs for CTL induction (32, 33). A more recent study discovered that CD169, a sialic acid binding lectin involved in cell-cell contact, preferentially binds to sialic acid containing ligands on $CD8\alpha^+$ DCs and thereby facilitated antigen transfer to DCs (34). In addition, also carry-over of antigen from one DC type to another is currently accepted as a feasible model of cross presentation (35–37) especially when different DC subsets seem to have different roles in a two-step T cell priming model (38–41). In this two-step priming model, naïve $CD4^+$ and $CD8^+$ T cells are activated by different DC populations. The activated $CD8^+$ T cells recruit lymph node-resident $XCR1^+$ DCs which receive cross-presented antigen from the DCs that carried out the first priming step (42, 43). The $XCR1^+$ DCs interact with both activated $CD4^+$ and $CD8^+$ T cells and thereby inducing optimal signals for $CD8^+$ T cell differentiation into cytotoxic T lymphocytes (CTLs) and memory CTLs. We showed with multiple antigen-targeting systems that antigen could be conserved in DCs for sustained cross-presentation. This does not only increase the significance of antigen storage by different cell types, but we speculate that prolonged antigen storage and presentation would be beneficial for antigen transfer between subsets and multi-step T cell priming mechanisms.

FATE OF ANTIGEN IN DENDRITIC CELLS

Antigen routing in dendritic cells

One remarkable observation we had was that timing seemed to correlate with different antigen processing phases in DCs. Despite the fact that antigen targeted to either FcγR or MGL1 ended up in the same storage compartment, antigen targeted to MGL1 induced DC cross-presentation which was TAP independent (**chapter 5**). This seemed to be in contradiction with our previous work showing TAP and proteasome dependent antigen cross-presentation after FcγRs targeting on DCs (1). However, an important note is that relatively early cross-presentation was measured in the MGL1 setting, only 4 hours after antigen pulse, whereas antigen cross-presentation from the storage compartments mediated by FcγRs targeting was measured after 2 hours antigen pulse and 48 hours chase. It could be possible that there is a distinction between early and late antigen processing and cross-presentation in DCs. One can speculate that during the early stage after antigen uptake, antigen is processed and loaded on MHCI directly in early endosomes, which is TAP and proteasome independent. In this case, MHCI molecules could be derived from Rab11 positive endosomal recycling compartments (44). Similar antigen routing had been shown before for antigens targeted to the mannose receptor and DC-SIGN, where antigens were mainly residing and presented from early endosomal compartments (45, 46). However, when antigen stays longer in DCs and resides in LAMP1 positive compartments, which lack direct processing and loading machineries, it requires antigen translocation from the storage compartments to the cytosol for proteasomal degradation and subsequently transportation via TAP to MHCI loading sites. Indeed, the lack of TAP-1 and PA28 in the storage compartments suggests that antigen is rather translocated from the endosomes back into the cell cytosol for further processing and loading of MHCI in the ER for antigen cross-presentation (**chapter 4**). We had previously demonstrated almost complete inhibition of MHCI cross-presentation by DCs from the storage organelles upon inhibition of proteasomal activity or using TAP deficient DCs (1). Although it is not excluded that peptides, after proteasome degradation, are transported back into endocytic compartments and trimmed by IRAP and loaded on MHCI (47).

The ability of antigens to induce DC maturation signaling pathways might also contribute to the maturation and reorganization of endosomes and further trafficking of antigens from early endosomes to late endosomes. We showed in **chapter 5** that the modification of OVA with the glycan-structure Lewis^{X} (Le^{X}) re-directs OVA to the C-type lectin receptor MGL1, skewing naïve CD4^{+} T cell differentiation towards IFNγ-producing Th1 cells. MR targeting often requires high amounts of soluble OVA and additional activation signals from e.g. TLR ligands. Le^{X} modification did not only reduce the required OVA amount by 100-fold, but also obviated the additional TLR activation signal. More importantly, OVA-Le^{X}was routed to

Rab11$^+$ LAMP1$^+$ compartments where it was stored for sustained antigen cross-presentation, whereas soluble OVA was routed towards EEA1$^+$ Rab11$^+$ compartments. Targeting different regions of the same receptor might also influence the routing and processing of antigen by DCs, as shown by a study targeting the carbohydrate-recognition domain (CRD) and the neck region of DC-SIGN (46). Antigens coupled to antibodies specific for CRD were delivered to lysosomal compartments, resulting in rapid antigen degradation and poor cross-presentation, whereas antigens coupled to antibodies specific for the neck region were directed to early endosomal compartments and induced effective cross-presentation.

Different categories of endosomes with distinct functions had been proposed before where endosomes were described as "dynamic" or "static" (48) (reviewed in (49)). The "dynamic" endosomes were suggested to mature rapidly towards late endosomes and provide antigens for MHCII loading, whereas "static" endosomes (Rab14 positive) were matured more slowly towards late endosomes and favor cross-presentation due to lower proteolytic activity and antigen degradation. However, since these "static" endosomes possess MHCI loading components, such as MHCI and IRAP (47), it is unlikely that the endosomal storage compartments that we described here are the same. A distinct marker expressed on storage compartments is LAMP1. Although LAMP1 is a classical lysosomal marker, it is unlikely that the storage compartment is a classical lysosome due to the lack of active proteases such as cathepsin S (**chapter 4**) and slow antigen degradation rate. However, endosomal trafficking and maturation pathways are dynamic and complex, it would be interesting to further investigate the expression of other endo-lysosomal or subcellular markers on the storage compartments, for instance by proteomic analysis of isolated organelles.

In addition, new approaches for tracking antigen in DCs will be available. Currently, most of the fluorophores are chemically conjugated to the molecule of interest which may influence the experimental outcome. In **chapter 7** we investigated new possibilities in coupling different fluorophores to TLR-ligand conjugated peptides. However, interpreting data by using fluorescently labeled compounds could sometimes be challenging due to their bulky and hydrophobic structures compared to the relatively small peptides. One of the new possibilities to overcome these problems is the use of click chemistry (reviewed in (50)). By using biorthogonal peptides and ligation of a complementary fluorophore to the biorthogonal amino acid side chain at the end of the experiment, surface labeling of MHCI loaded minimal epitopes was quantified on APCs (51). Potentially this technique can be used for accurate tracking of the compound of interest, even in vivo, bypassing solubility problems or characteristic changes caused by the type of fluorophore. However, the click chemistry approach is still technically difficult in cells and further optimization is needed for future use. When optimized, this could further unravel the routing and processing of antigens in DCs in subcellular detail.

Autophagy and antigen degradation

Since the fate of antigen from the DC storage compartment is still unclear, we investigated the possible role of autophagy in antigen storage and cross-presentation. In **chapter 6** we revealed that DCs treated with common autophagy inhibitors or gained from Atg5$^{-/-}$ mice showed prolonged antigen storage and significantly enhanced antigen cross-presentation to CD8^{+} T cells. Thus, autophagosomes degrade antigen storage compartments and consequently less antigens will be available for cross-presentation (Fig. 1). This was rather unexpected since it was reported that autophagy inhibition can negatively influence MHCI cross-presentation (52–54). We could confirm that blocking autophagy at an early stage inhibited MHCI cross-presentation, but the opposite enhancing effect was found on long term antigen cross-presentation. The discrepancies between the beneficial or detrimental role of autophagy in DC cross-presentation were reflected in studies providing evidence for both. Some groups showed elevated CD8^{+} T cell responses upon autophagy inhibition in DCs (55), while others showed that MHCI cross-presentation by DCs was still intact in the absence of Atg5 (56). Tumor antigens conjugated to nanoparticles delivered to autophagosomes were efficiently cross-presented to CD8^{+} T cells resulting in potent antitumor responses (54). Interestingly, it had been shown that XCR1 positive DCs had the highest steady-state levels of macroautophagy, indicating that autophagy is highly active in specialized cross-presenting DCs (57). The reasons for these discrepancies are unclear, although it seems that the outcome depends on the type of antigen, cell subset and time point of measuring antigen presentation, as we already pointed out in the section above. Nevertheless, our data provide new insight in the role of autophagy in antigen degradation and thereby affecting cross-presentation to T cells. It had been suggested that Atg5 or Atg7 deficient DCs had decreased endocytosis and degradation of MHCI molecules resulting in elevated surface expression of MHCI (55). This could subsequently induce antigen cross-presentation to CD8^{+} T cells. However, in our study conditions in **chapter 6**, we did not find significant differences in MHCI surface expression levels on Atg5 deficient DCs upon maturation. We propose that enhanced cross-presentation is rather caused by increased peptide production.

In addition, we showed in **chapter 6** that LC3 positive autophagosomes were in close proximity with the antigen storage compartments. However, it cannot be ruled out that the LC3 positive compartments are vesicles mediated by LC3-associated phagocytosis (LAP). Some receptors that can stimulate LAP include FcγRs, TLRs, CLR Dectin-1, and the phosphatidylserine binding receptor TIM4 (58–61). Therefore, additional staining for p62, which is present on autophagosomes, could distinguish LAP from autophagosomes. However, it has been shown that LAP formation is dependent on the recruitment of NOX2 to the membranes and the generation of ROS (59, 62, 63). Preliminary functional studies with NOX2-deficient mice showed no effect on sustained antigen cross-presentation by DCs (unpublished data), therefore it is unlikely that LAP is playing a crucial role in our setting.

How autophagosomes are affecting antigen degradation in the storage compartment still needs to be elucidated. It seems that it is not controlled by lysosomal activity within the compartments since we could not detect significant differences in the pH of antigen containing compartments between wildtype and Atg5 deficient DCs. Moreover, we already showed in **chapter 4** that the activity of endosomal proteases, such as cathepsin S, was lacking in the antigen storage compartments. One possible explanation is that autophagosomes prevent translocation of antigen from the storage compartment to the cytosol for further processing and loading on MHCI molecules. It had been reported that during autophagy, the edges of the isolation membrane of autophagosomes were sealed to prevent leakage of hydrolases which could cause cellular damage and apoptosis (64, 65). Another possibility is that, under normal conditions, antigens are slowly leaked from the storage compartments into the cytosol for further processing. It had been shown that autophagosomes can degrade leaky endosomes (66), which makes it plausible that autophagosomes degrade antigen storage compartments and thereby hampering antigen cross-presentation.

THE ROLE OF FCΓRS AND C1Q IN ANTIBODY-MEDIATED ANTIGEN TARGETING TO DCS

The importance of antigen targeted to FcγRs in T cell-mediated anti-tumor responses had been documented well by our group and others (1, 67–69). However, to our surprise, FcγRs seem to play a limited role in the uptake of ICs by DCs *in vivo* (**chapter 3**). We discovered that the uptake of *in vivo*- formed OVA ICs, by injecting mice sequentially with anti-OVA IgG and OVA, was not hampered in DCs from mice lacking FcγRI/II/III/IV (FcγR quadruple$^{-/-}$). More interestingly, our results indicate a dominant role of complement factor C1q in controlling antigen targeting and handling by DCs *in vivo* (Fig. 1). Mice lacking C1q (C1qa$^{-/-}$) showed no antigen uptake in APCs and severely reduced antigen presentation to T cells.

Complement plays a main role as effector mechanism of antibody-mediated immunity and one of its function is to dispose immune complexes from circulation. C1q was initially discovered as part of the C1 initiation component of the classical complement pathway upon binding to antigen-bound IgM or IgG (70). However it has been shown that C1q regulates a variety of cellular processes independent of complement activation, such as enhancement of phagocytosis of apoptotic cells, decrease in pro-inflammatory cytokine release and induction of anti-inflammatory mediators in macrophages and DCs, skewing the adaptive immune system towards a more regulatory state (71).

Although many potential C1q receptors has been described, it is still unclear which C1q receptor is expressed on DCs to mediate IC uptake. C1q receptors that are known

to be expressed on DCs, such as RAGE and DC-SIGN, mainly function for apoptotic cell phagocytosis and DC differentiation, respectively (71). Both the collagen-like region and the globular head are suggested as binding sites for C1q receptors. Importantly, it has been suggested that monomeric IgG binding to one C1q head is of low affinity and results in poor complement activation. IgG molecules form hexamers after binding to antigens and bind to C1q with high affinity promoting efficient complement activation (72). Further work by the same group, using different Ab mutants which promote or inhibit hexamerization in solution, showed that IgG hexamerization was a prerequisite to C1q binding and C1 activation (73). More recently, it has been shown that the C1q arms were condensed upon hexameric antibody binding, resulting in the rearrangement of the $C1r_2$-$C1S_2$ proteases and tilting the C1q's cone-shaped stalk. C1r could therefore activate C1s within single, strained C1 complexes, or between neighboring C1 complexes (74). These data suggest flexible movements of C1q which can modulate the positions of the six globular domains and cross-activities with neighboring C1 complexes. Additional studies on the interaction between C1q and ICs, and the identification of the C1q-IC uptake receptor on DCs are needed to further fine-tune C1q-mediated IC uptake and immune responses by DCs.

It is still puzzling why FcγRs have a less essential role in IC uptake *in vivo*, while *in vitro* we had demonstrated that the uptake of OVA IC by FcR γ-chain$^{-/-}$ BMDCs was hampered (1). In **chapter 3** we also showed hampered IC uptake and cross-presentation by FcγR quadruple$^{-/-}$ BMDCs *in vitro*. Although FcγRs are known to enable DC activation to augment antigen cross-presentation *in vitro*, mainly through signaling of ITAM, we did not detect an increase in DC maturation after co-injection of antigen and antibody *in vivo* (data not shown). These results indicate that FcγRs are the more dominant uptake and activation receptors under *in vitro* conditions and that there is a distinct contribution of FcγRs in antibody-mediated antigen uptake by DCs *in vitro* and *in vivo*. The elimination of circulating antigen-antibody ICs is generally assumed to be mediated by Kupffer cells and endothelial cells expressing FcγRs in the liver. It is presumable that FcγRs *in vivo* are more important in IC clearance from the circulation by FcγR expressing liver cells than in IC uptake by DCs in the spleen. Indeed, serum from FcγR quadruple$^{-/-}$ mice showed slower clearance of antigen from circulation, prolonged presence of antigen in circulation, and slightly higher antigen uptake by APCs *in vivo* (**chapter 3**). It has been suggested that FcRn mediates cross-presentation of ICs by CD8α$^-$ DCs and that FcRn and FcγRs work in cooperation (5). Since we showed hampered antigen cross-presentation by CD8α$^-$ DCs in FcγR quadruple$^{-/-}$ mice, it is not excluded that FcγRs or FcRn play a role in the activation and signaling pathway for cross-presentation in this particular subset.

Another possible explanation for the distinct role of FcγRs *in vivo* could be the fact that DCs *in vivo* are strategically positioned for efficient uptake of antigen to initiate adaptive immunity. Studies on the anatomy of mouse spleen showed high organization

of immune cells in different zones within the spleen. $CD8\alpha^+$ DCs that express higher levels of DEC205 are more restricted to periarterial lymphoid sheaths in the spleen, whereas DCIR2 expressing $CD8\alpha^-$ DCs are restricted to the bridging region of the marginal zone (75). Langerin/$CD207^+CD103^+$ $CD8\alpha^+$ DCs are mainly localized in the marginal zone, but upon phagocytosis of apoptotic cells they migrate into T cells zones for cross-presentation (76). Since DC populations are distributed differently in the spleen, and considering the fact that we only found a small percentage of antigen-positive cells within each APC subset, it is possible that DCs expressing a receptor for C1q-mediated IC uptake are positioned more favorable for better access to circulating ICs compared to DCs that lack the C1q receptor. Although higher C1q receptor expression levels in DC populations cannot be ruled out. Further anatomic studies on the spleen are required to determine the position of FcγR- and C1q receptor-expressing DC subsets.

CONCLUSION AND FUTURE PROSPECTIVE

Dendritic cells have an increasing role as foundation for effective cancer immunotherapy. As central regulators of the adaptive immune responses, DCs are crucial for antigen recognition, transport to draining lymph nodes and cross-presentation to T cells. However, the precise mechanisms of antigen cross-presentation by DCs are still not fully unraveled. Nevertheless, DCs became an attractive target for vaccination against diseases, such as cancer, for which cellular immunity is important. Different strategies were explored for the development of DC vaccines, including *ex vivo* generated DCs and *in vivo* DC targeting. The majority of *ex vivo* generated DCs are monocyte-derived DCs (moDCs) differentiated from purified blood monocytes in the presence of cytokines and subsequently loaded with tumor-derived antigens. Although moDCs have the advantage in terms of practicality, since monocytes are easy to isolate in high amounts and efficiently differentiated into moDCs, it is important to keep in mind that *ex vivo* generated moDCs could be functionally different from natural DCs. Some studies showed that moDCs differ in their lysosomal pH and are limited in their ability to migrate *in vivo* with most moDCs residing at the injection site (77–79). Another approach for DC based anti-cancer therapy is using *In vivo* DC targeting. By coupling tumor antigens to specific monoclonal antibodies (mAbs), DC receptors can be targeted *in vivo*. Many studies have been investigating C-type lectin receptors, such as DEC-205, fused with tumor antigen, however most of the time an additional adjuvant is needed to activate DCs in order to overcome tolerance (80–83).

We have published before that targeting FcγRs on DCs with IC can efficiently activate DCs and prime T cells leading to prophylactic and therapeutic tumor control *in vivo* (84). In **chapter 2**, we discovered that DC subsets *in vivo* have the capacity to store antigen

for several days in specialized antigen storage compartments, which corresponds with sustained antigen cross-presentation to $CD8^+$ T cells. Prolonged antigen cross-presentation is crucial since it takes time for DCs to mature and travel to lymph nodes to encounter T cells. It is important for the development of next generation DC vaccines to consider DC targeting which can enhance antigen storage in DCs and induce efficient DC maturation. Since a natural infection usually includes multiple antigenic ligands, it is plausible to further enhance DC targeting and activation by combining different patter-recognition receptor (PRR) ligands. Our group has shown before that TLR-ligand- peptide conjugates also lead to the formation of intracellular antigen depot and induce $CD8^+$ and $CD4^+$ T-cell priming capacity leading to efficient induction of antitumor immunity in mice (1, 85). In **chapter 5**, we showed that targeting C-type lectin receptor MGL can also lead to prolonged antigen storage and antigen cross-presentation by DCs. Several studies have suggested cross talk between multiple PRRs, such as TLR and NLR (86–88). Moreover, triggering TLRs, NLRs, and CLRs can all activate downstream NFκB activation, indicating the signaling pathways of different PRRs contain overlapping functions. Therefore, combining different PRR ligands might induce a synergistic effect on DC activation and antigen cross-presentation.

In **chapter 3**, we showed that *in vivo* DC targeting is much more complex and not all *in vitro* DC studies can be directly translated to the *in vivo* situation. Therefore, *In vivo* targeting of DCs by using antibodies can be a difficult approach since antibodies can be taken up by other cell types expressing similar receptors and filtered out of the system before reaching the targeted DCs. Understanding the *in vivo* uptake mechanisms of DCs might improve the design of modified antibodies which can be specifically targeted to DCs. Other promising targeting approaches are using nanoparticles or liposomes to deliver antigens specifically to DCs. One of the FDA approved nanoparticles is PLGA-based, a biodegradable slow-release polymer that effectively encapsulates drugs and antigens (89, 90). An advantage of these antigen carriers is the flexibility in property modulation, e.g., size, charge, and composition, which can influence the outcome of the vaccine (91).

Targeting the right APC subsets is also a crucial aspect for an effective vaccine. As discussed in **chapter 2**, all APC subsets have the capacity to take up and store antigen for several days, but only the $CD8\alpha^+$ DCs showed superior antigen cross-presentation ability to $CD8^+$ T cells, while the $CD8\alpha^-$ DCs were better antigen presenters to $CD4^+$ T cells. Although $CD8\alpha^+$ DCs are effective inducers of $CD8^+$ T cell priming and cytotoxic killing of tumor cells, the role of $CD4^+$ T cells cannot be neglected. It was already demonstrated by our group and others that the Th response is essential for the induction of antitumor immunity, which is mainly mediated by the upregulation of CD40L on $CD4^+$ T cells triggered by MHCII presentation by DCs (92–94). CD40L will engage CD40 on DCs to cause maturation of the DCs and efficient induction of $CD8^+$ killer T cells. Therefore, it is important for a vaccine to deliver

antigens both to the $CD8\alpha^+$ and the $CD8\alpha^-$ DCs. Designing vaccines that contain both $CD8^+$ as $CD4^+$ epitopes of the target antigens have already shown effective antitumor immunity (85).

Beside specific targeting and activating DCs, choosing a highly tumor specific antigen that is delivered to the DCs is also an important aspect for designing an effective antitumor therapy which evades tolerance induction. Many sources of antigen have been used in DC vaccines, including e.g. short peptides, synthetic long peptides, tumor cell lysates, and DNA/RNA transduction with viral vectors (95–99). Although these approaches show promising results in combination with maturation signals, it remains unclear what the optimal method for antigen loading is. A different approach to improve DC vaccines might be the use of neoantigens, which are generated by somatic mutations in the tumor. Vaccination with neoantigen-loaded DCs have shown to promote neoantigen-specific T cell responses (100). However, neoantigens requires labor-intensive sequencing of the tumor of patients, and the frequency of neoantigens is strongly dependent of the tumor type (101). One of the most recent and innovative developments in DC vaccine is the use of RNA encoding tumor antigen derived epitopes combined with immunostimulatory motifs which were delivered by nano-sized lipoplexes to DCs (102, 103).

Future directions for specific immunotherapy include combining DC targeted vaccination with the now widely clinically applied immune checkpoint blockade antibodies like PD-1 and CTLA-4 to block the interactions between APCs and T cells and thereby releasing the inhibitory signals for tumor-specific T cells. Moreover, the combination of immunotherapy with other therapies such as chemotherapy or tumor ablation techniques has been shown to be beneficial for improved tumor eradication. Since the discovery of DCs more than a century ago, these specialized cells have established a crucial role, either directly or indirectly in immunotherapeutic strategies of cancer. More studies on the mechanisms of DC cross-presentation are required to use these cells, gifted by nature, to their full potential.

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