Antigen processing and presentation

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Contents

Abstract

Dendritic cells are at the center of immune responses. They are defined by their ability to sense the environment, take up and process antigen, migrate to secondary lymphoid organs, where they present antigens to the adaptive immune system. In particular, they

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present lipids and proteins from pathogens, which they encountered in peripheral tissues, to T cells in order to induce a specific effector immune response. These complex antigens need to be broken down into peptides of a certain length in association with Major Histocompatibility Complex (MHC) molecules. Presentation of MHC/antigen complexes alongside costimulatory molecules and secretion of proinflammatory cytokines will induce an appropriate immune response. This interaction between dendritic cells and T cells takes place at defined locations within secondary lymphoid organs. In this review, we discuss the current knowledge and recent advances on the cellular and molecular mechanisms that underlie antigen processing and the subsequent presentation to T lymphocytes.

1. Introduction

Dendritic cells (DCs) are considered the most potent professional antigen-presenting cells (APCs) that play a central role in linking innate sensing of pathogens and antigen processing to adaptive immune responses. Although they are now considered to be at the center of immune responses, they were only discovered late in the history of immunology, first described by Steinman and Cohn (1973). They reported the presence of a cell type with long cytoplasmic projections in the spleen and other secondary lymphoid organs of mice (and later other mammals) and proposed to call them "dendritic cells" based on their morphology. However, their precise role in the immune system was unclear. Years later, Steinman published a model that set the wheels turning, and ignited active research in the field of DC biology (Steinman, 1991). He proposed that DCs pick up and process antigens from the periphery while migrating into lymphoid organs where they initiate adaptive immune responses. In parallel, the process of maturation, meaning a change in the cellular phenotype in response to the original pathogen encounter, had been reported in dermal Langerhans cells, closely related to DCs, and was associated with an increase in immunostimulatory function (Heufler et al., 1988).

DCs represent a large group of cells and their classification, ontogeny and functions have been the subject of intense debate. Initially, the presence of different lymphocytic markers on the cell surface of murine DCs, especially cluster of differentiation (CD)8α, caught the attention of scientists. It gave rise to the idea of different DC subtypes with possibly different functions and a first classification of DCs into $CD8\alpha^+$ and $CD8\alpha^-$ (Vremec et al., 1992). Later, a DC population that secreted large amounts of type I interferons in response to viral infections was described in human blood (Siegal et al., 1999)

and in mice (Asselin-Paturel et al., 2001) and was termed plasmacytoid DCs (pDCs). This population was distinct from the conventional $CD8\alpha^+$ and $CD8\alpha^-$ DCs (cDCs) (Shortman and Liu, 2002). The existence of a group of a $CD8\alpha^-$ cDCs found in the periphery led to the definition of migratory DCs (as opposed to the lymphoid-organ resident DCs). They were further subdivided according to the expression of CD103 and CD11b (Johansson-Lindbom et al., 2005) encompassing $CD103^+CD11b^+$, $CD103^-CD11b^+$ and skin Langerhans cells. These DCs are present in most mucosal tissues and migrate to the draining lymph nodes upon stimulation (Ginhoux et al., 2009).

Until recently, both cDCs and pDCs were believed to derive from a common myeloid dendritic cell precursor (CDP) (Liu et al., 2009; Naik et al., 2007) although there was evidence of some pDCs arising from a lymphoid precursor (Pelayo et al., 2005). In fact, recent work suggests that pDCs can be divided into two populations, one arising from the CDP and the other from a lymphoid precursor. While, the CDP-derived pDC subset, termed pDC-like cells, is superior in processing and presenting antigens, lymphoid-derived pDCs constitute the majority of pDCs and excel in producing type I interferons (Rodrigues et al., 2018).

The cytokine FMS-like tyrosine kinase 3 (Flt3L) drives the development of DC subsets in the bone marrow (D'Amico and Wu, 2003; McKenna et al., 2000; Waskow et al., 2008) but their differentiation depends on the expression of different transcription factors. While $CD8^+$ and $CD103^+$ DCs rely on the transcription factor Baft3 and IRF8 (Hildner et al., 2008; Schiavoni et al., 2002; Tamura et al., 2005), $CD11b^+$ DCs are controlled by IRF4 (Suzuki et al., 2004; Tamura et al., 2005).

Adding to the complexity of DC subsets at steady-state conditions, tissue inflammation has been shown to drive the differentiation of monocytes into DCs, hence termed inflammatory DCs. The first evidence came from seminal work by Sallusto and Lanzavecchia who generated DCs from human blood monocytes (Sallusto and Lanzavecchia, 1994). These DCs originate at the site of inflammation and can be later found in the draining lymph nodes in response to different pathogens such as bacteria (Serbina et al., 2003), virus (Nakano et al., 2009), fungus (Hohl et al., 2009), parasites (León et al., 2007) as well as non-infectious causes (Kool et al., 2008).

Recently, a new, ontogeny-based nomenclature for DCs and other cells of the mononuclear phagocyte system was proposed (Guilliams et al., 2014). Based on their CDP-origin and Flt3L dependency, steady-state DCs are grouped separately from macrophages, monocytes and monocyte-derived

cells. Further, DCs are divided into three groups based on the requirement of transcription factors necessary for their differentiation: Batf3-dependent DCs (cDC1), IRF4-dependent DCs (cDC2) and E2-2-dependent pDCs (pDCs). This classification reunites cells with similar developmental programs but from different locations, and proposes that their anatomical and phenotypic characteristics (e.g., lung $CD103^+$, spleen $CD8^+$) be mentioned afterward. Inflammatory monocyte-derived DCs are classified on a different branch and group with other monocyte-derived cells such as monocyte-derived M1 and M2 macrophages (Guilliams and van de Laar, 2015). However, what unites DCs in a common functional group is their capacity to sense the environment, to engulf, process and present antigens and to initiate T cell responses to direct an adequate immune effector function.

Extracellular antigens are presented by APCs following internalization, processing and loading of the resulting peptides onto Major Histocompatibility Complex (MHC) class II molecules, which are exposed on the plasma membrane to activate $CD4^+$ T cells. This process is part of the endocytic pathway of APCs and has been characterized extensively. However, recent novel evidence has fuelled to new concepts in MHC class II presentation, such as the presentation of intracellular antigen from non-canonical sources.

Traditionally, intracellular antigens, such as intracellular bacteria, parasites, viruses or tumor antigens, can be presented by all cells after cytoplasmic proteolysis. The resulting peptides are loaded onto MHC class I molecules in the endoplasmic reticulum (ER) which are then transported to the plasma membrane for presentation to $CD8^+$ T cells. However, for a cytotoxic immune response to develop, antigen-specific $CD8^+$ T lymphocytes first have to be primed by APCs.

When APCs are not directly infected by pathogens, antigens from intracellular compartments of surrounding cells are acquired from the environment and presented on MHC class I molecules by "cross-presentation," a process unique to a few cell types. This phenomenon was first reported by Bevan et al. in 1976, soon after the first report on DCs was published (Bevan, 1976a). Working in vivo, the author detected development of a specific cytotoxic response toward minor histocompatibility antigens from an allogeneic donor following adoptive transfer. They concluded that these antigens must have been transferred from the injected cells into the recipient's APCs which presented them on their MHC class I to prime cytotoxic CD8+ T cells, proposing the term "cross-priming." This process and its mechanism remained poorly understood and was object of several controversies throughout the years. Even though there are still many unanswered questions, our understanding of cross-presentation has increased over the

years thanks to the development of specific tools which enabled to study it in more detail. Today it is widely believed to have a central role in antitumor and antimicrobial immunity as well as the induction of tolerance to self. A scheme representing the different routes of antigen presentation is depicted in Fig. 1.

The induction of a cytotoxic $CD8^+$ T cell response, however, requires more than the recognition of the peptide/MHC class I complexes on the surface of a DC. Following DC activation by the antigen and the environment in a process termed "maturation," "cross-priming" APCs also deliver additional signals to T cells, both in the form of secreted cytokines and upregulation of costimulatory molecules. During DC maturation, pattern recognition receptors (PRRs) play a crucial role. DCs display a large array of PRRs that were predicted boldly by Janeway in 1989, giving rise to the theory of "nonclonal recognition of nonself patterns" (Janeway et al., 1989). These predictions were confirmed, and many families of different nonself-sensing receptors have been discovered and characterized, providing a crucial link between the innate and adaptive immune systems (Akira et al., 2006; Medzhitov, 2001). PRR families include Toll-like receptors (TLRs), Rig-like receptors (RLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), and DNA/RNA sensors. These receptors can either be cytosolic, associated with plasma or endosomal membranes, or even reside inside the cell nucleus. They are often associated with different adaptor molecules and signal through complex, multimolecular pathways. These receptors sense microbial stimuli, known as pathogen-associated molecular patterns (PAMPs), or endogenous stress signals, termed danger-associated molecular patterns (DAMPs), the latter originating from the danger theory developed by Matzinger (1994).

While it had been believed that DC maturation was a single process, evidence suggests that the maturation program strongly depends on the PAMP/DAMP stimulus and shapes the resulting immune response. Maturation induces morphological changes in DCs, as they develop cellular projections or dendrites and enhances the migratory properties of DCs (Vargas et al., 2016). More importantly, DCs maturation also results in an increased surface expression of many costimulatory molecules such as CD40, CD80, CD86, MHC class II and the secretion of different chemokines and cytokines. Additionally, while maturation initially enhances the ability of DCs to internalize antigens, this capacity is decreased as the maturation program continues (West et al., 2004). All these effects are required for DCs to trigger appropriate T cell activation and the ensuing adaptive immune response.

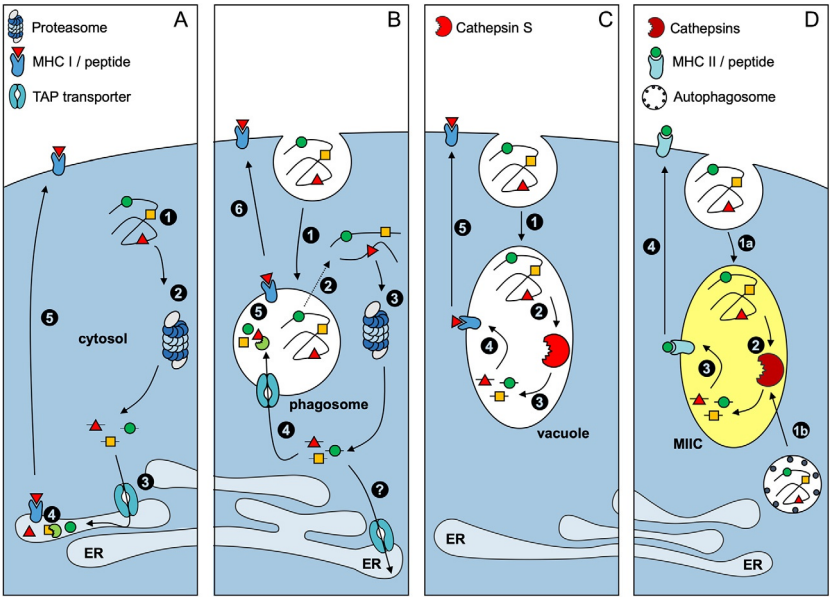

Fig. 1 Schematic representation of different pathways for antigen processing and presentation. (A) Classical MHC class I antigen presentation, also known as direct presentation. (1) Antigen synthesis in the cell cytosol (can be self or non-self); (2) cytosolic antigen degradation by the proteasome; (3) peptide translocation mediated by TAP; (4) ERAAP/ERAP1-mediated peptide trimming and peptide loading onto MHC class I molecules; and (5) MHC class I/peptide complex delivery to the cell surface. (B) Cytosolic antigen cross-presentation. (1) Internalization of antigens (macropinocytosis, endocytosis or phagocytosis) and formation of the cross-presenting organelle, GILT-promoted mild degradation of the antigen; (2) export to cytosol (dashed line, no clear evidence for a preferred mechanism); (3) further degradation by the proteasome; (4) antigens are transported back to phagosomes by TAP, or (?) transported to the ER for additional processing therein; (5) Once back to the cross-presenting organelle, antigens are trimmed by IRAP, and loaded onto MHC class I molecules; and (6) MHC class I/peptide complex transfer to the cell membrane. (C) Vacuolar antigen cross-presentation. (1) Internalization of antigens (macropinocytosis, endocytosis or phagocytosis) and formation of the cross-presenting organelle. (2) Antigen degradation by Cathepsin S and other phagosomal/endosomal proteases. (3) Potential trimming by aminopeptidases and (4) loading onto MHC class I molecules. (5) MHC class I/peptide complex transport to the plasma membrane. (D) Classical MHC class II antigen presentation. (1a) Internalization of antigens, and fusion of the early endosome/phagosome with MIIC organelle. Alternatively, (1b) autophagosomes might fuse with MIIC in order to release antigens; (2) Antigens are degraded by different cathepsins and other lysosomal proteases; (3) Resulting peptides are loaded on MHC class II molecules; and (4) Transported to the cell membrane, either by tubule formation or through the Golgi apparatus.

In the following, we describe the different antigen presentation pathways used by DCs and discuss the current knowledge about their underlying molecular mechanisms. Classical pathways include presentation of intracellular antigen on MHC class I and exogenous antigen on MHC class II molecules. As both pathways are shared with other cell types, we will give special emphasis to "cross-presentation," for which DCs are the most efficient APCs. Further, we will discuss its impact on the field of immunotherapy.

2. Classical MHC class I presentation

Classical MHC class I presentation (or direct presentation) refers to the (a) generation of antigenic peptides (self or non-self) within the cytosol by the proteasome, (b) the translocation of the resulting peptides to the ER, (c) their trimming by aminopeptidases, (d) loading of peptides onto newly synthetized MHC class I molecules to be (e) presented to $CD8^+$ T lymphocytes on the cell surface (Fig. 2). Such endogenous antigens can either be self-derived or generated following viral infections (with the associated synthesis of viral proteins). If self-peptides are produced by tumor cells, they represent a potential target to elicit antitumor responses, whereas if they are generated in normal host cells, they can lead to tolerance or autoimmunity.

MHC class I molecules are expressed in most nucleated cell types. In mice, three genes encode classical MHC class I molecules (H2-D, H2-K and H2-L) whereas in human three different genes encode for such molecules (HLA-A, HLA-B and HLA-C) (Blum et al., 2013). There are also other non-classical MHC class I genes both in human and mice, often referred to as MHC class Ib (Adams and Luoma, 2013). Several studies have documented the structure of MHC class I molecules, which are comprised of two separate proteins: a heavy chain (α), anchoring the molecule to the cell by a transmembrane domain; and a soluble protein β2-microglobulin (β2m). Although MHC class I molecules are subject to vast allelic polymorphisms, most of the protein coding sequence is strictly conserved among different class I alleles. Thus, the impressive variation in amino acid sequence is confined to a critical region in the heavy chain that interacts with peptides generated from proteolyzed antigens. This peptide-binding structure forms a groove constituted by two antiparallel α helices settled over an eight-strand β-sheet.

The peptides that bind to MHC class I molecules are generated by a two-step proteolytic mechanism: peptide generation by the cytosolic proteasome

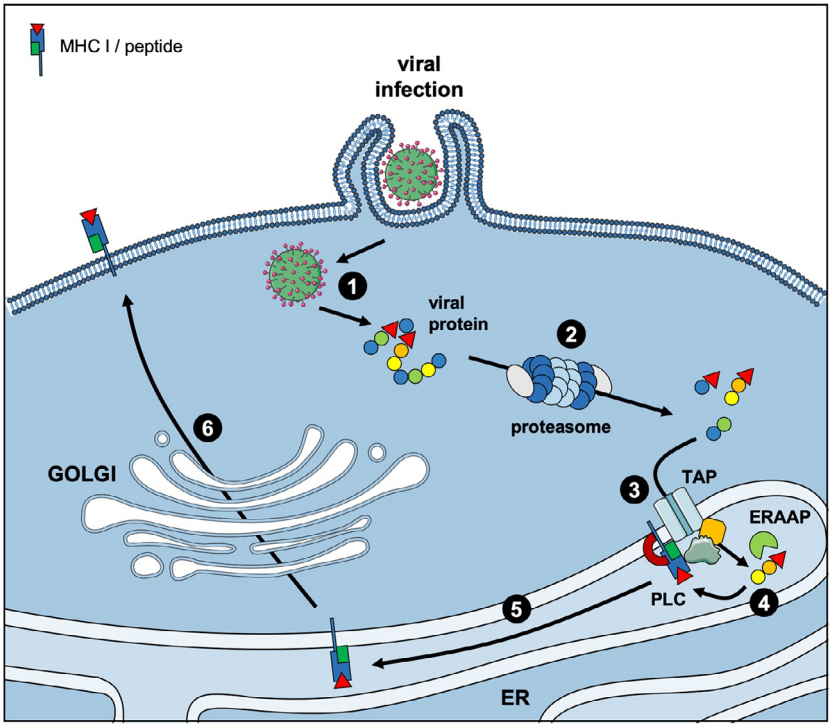

Fig. 2 Classical MHC class I antigen presentation. (1) Following viral infection, viral proteins are translated in the cytosol. Alternatively (not shown) host proteins can also be generated endogenously and follow the same route; (2) degradation of proteins—from either origin—by the proteasome; (3) TAP-dependent translocation of the resulting peptides into the ER; (4) PLC (Peptide Loading Complex, composed of TAP, calreticulin, ERp57 and tapasin) assembles and drive MHC class I folding, as well as peptide loading after ERAAP/ERAP1 (Endoplasmic Reticulum Aminopeptidase)-mediated trimming; (5) high affinity peptides loaded onto MHC class I molecules are released from the PLC; and (6) selected for transport to the cell surface.

and peptide trimming by aminopeptidases in the ER lumen (described below). Briefly, the proteasome is responsible for most of the non-lysosomal degradation of intracellular proteins (Rock and Goldberg, 1999). Its catalytic activity determines, at least in part, the antigenic peptide repertoire that will bind to MHC class I molecules and will be presented on the cell surface to T lymphocytes (Maupin-Furlow, 2012). The proteolytic activity of the proteasome is exerted by three of the seven β subunits, namely, β1, β2, and β5, which are part of the two identical inner rings of the four ringed proteasomal 20S core complex (Dick et al., 1998; Guillaume et al., 2010). Under inflammatory conditions, when cells are exposed to IFNγ,

the three catalytic subunits can be replaced by their IFNγ inducible homologs, LMP2 (β1i), MECL1 (β2i) and β5i, resulting in the formation of the immunoproteasome (IP) (Guillaume et al., 2010; Rock and Goldberg, 1999). Two additional forms of IPs exist, which contain a mixture of standard and immune catalytic subunits: either only expressing the β1i subunit or expressing both β1i and β5i subunits. Each of these proteasomes has different cleavage specificities generating a distinct set of antigenic peptides to be bound to HLA on the cell surface (Chen et al., 2001). DCs can express both proteasome and immunoproteasome at different stages of maturation (Morel et al., 2000).

Following cytosolic proteolysis, antigenic peptides are recruited to the ER and translocated to its lumen by the Transporter associated with Antigen Processing (TAP). It is constituted of two subunits: TAP1 and TAP2 which both comprise a main domain of six transmembrane α-helices (the channel) adjacent to its nucleotide-binding domain (Hinz and Tampé, 2012). Together with TAP, the proteins ERp57, tapasin and the calnexin-calreticulin system constitute the peptide-loading complex (PLC). This transient ER multimeric complex mediates translocation of proteolyzed antigens to the ER, as well as trimming and loading of peptides on MHC class I molecules. In DCs, calnexin chaperones newly synthesized MHC class I heavy chains once they reach the ER, keeping them in a partially folded state (Rajagopalan and Brenner, 1994). Subsequently, chaperoned MHC class I heavy chains assemble with β2m to form empty MHC class I molecules that will be recruited to the PLC by calreticulin (Ortmann et al., 1994). Once in the PLC, MHC class I molecules are further stabilized by tapasin and the thiol oxidoreductase ERp57 which promotes efficient peptide loading (Blees et al., 2017; Blum et al., 2013; Ortmann et al., 1997; Sadasivan et al., 1996). The exact structure and conformation of the human PLC have been recently elucidated using electron cryo-microscopy. The complex is made up of two multimeric units of calreticulin, tapasin, ERp57, and MHC class I that are centered around TAP in a symmetric orientation (Blees et al., 2017). Genetic silencing or the use of negative dominants of TAP, tapasin, ERp57 and calreticulin have shown to decrease (to different extents) surface expression of MHC class I molecules, and hence decrease MHC class I antigen presentation (Gao et al., 2002; Garbi et al., 2000, 2006; Grandea et al., 2000; Peh et al., 2000; Purcell et al., 2001; Williams et al., 2002).

Another ER luminal protein plays a critical role in peptide processing and loading, although it is not physically associated with the PLC: The

ER aminopeptidase associated with antigen processing (ERAAP in mice), or ER aminopeptidase-1 (ERAP-1 in human) (Saric et al., 2002; Serwold et al., 2002). ERAP-2 is only present in human but might also play a role in peptide processing (Saveanu et al., 2005). While peptides loaded onto MHC class I molecules are 8–11 amino acid residues long (a restriction based on the size and conformation of the peptide-binding groove of MHC class I molecules), peptides translocated by TAP can be significantly longer (Koopmann et al., 1996). These peptides will be trimmed to the correct length by ERAAP. When ERAAP was silenced in mice, peptides associated with MHC class I molecules were longer than 8–11 residues, and the resulting MHC class I heterodimers were unstable.

Depending on the affinity of the loaded antigenic peptide, MHC class I molecules will be either transported to the cell membrane, or recycled by a mechanism dependent on UDP-glucose glycoprotein transferase-1 (UGT1) that allows MHC class I/suboptimal peptide complexes to regain access to the PLC for further rounds of tapasin-mediated peptide loading (Caramelo et al., 2004; Ritter et al., 2005). High-affinity peptide/MHC class I complexes that successfully pass the aforementioned "quality controls" will be transported through the Golgi apparatus to the cell membrane to elicit antigen-specific $CD8^+$ T cell responses.

3. Classical MHC class II antigen presentation

In contrast to MHC class I molecules which are expressed in most nucleated cell types, MHC class II molecules are constitutively expressed in few immune cells, such as DCs, macrophages and B lymphocytes. Under inflammatory conditions, MHC class II molecules can also be expressed in restricted types of endothelial and epithelial cells. In humans, MHC class II molecules are encoded by three polymorphic genes (HLA-DP, HLA-DQ and HLA-DR) and in mice by two (I-A and I-E). The transcriptional control of the MHC class II locus depends on the activity of the MHC class II transactivator (CIITA), the master regulator of MHC class II molecule expression (Choi et al., 2011). In turn, the activity of CIITA is regulated by post-translational modifications and the interaction with multiple DNA-binding proteins that are mainly active in APCs.

Nevertheless, during inflammation or infection, different signaling events and chromatin modifications lead to the activation of CIITA and expression of MHC class II molecules in other types of non-immune cells (Neefjes et al., 2011). IFNγ is the main cytokine driving the synthesis of

CIITA and the induction of MHC class II expression. Other important immunological molecular stimuli, such as TLR or TGFβ signaling, can also contribute to this process. Experimental evidence shows that in mice lacking the master regulator CIITA, the expression of MHC class II molecules is drastically reduced or absent (Chang et al., 1996).

MHC class II molecules share strong similarities with MHC class I molecules with regards to their three-dimensional structure. Polymorphic amino acid residues near the peptide-binding groove and nonpolymorphic Ig-like domains that contact the $CD4^+$ and $CD8^+$ T cell molecules are conserved. However, the peptide-binding groove of MHC class II molecules is open, and hence peptides that can be bound in these molecules are longer (10–30 residues long for MHC class II vs 8–11 for MHC class I). Once they are formed, peptide/MHC class II molecules complexes are very stable and allow for sustained antigen presentation increasing the chances to encounter the matching $CD4^+$ T lymphocytes. Once $CD4^+$ T cells have become activated, they in turn trigger macrophages to eliminate pathogens that have been previously internalized, and B lymphocytes to produce pathogen-specific antibodies.

MHC class II molecules are composed of two integral membrane chains, α and β, which are synthesized and assembled in the ER. There, they associate with the invariant chain (Ii) protein that acts as a pseudopeptide and allows for the stabilization of the MHC class II heterodimer. The association with Ii also provides spatial restriction of peptide loading to a late endosomal compartment, termed MHC class II compartment (MIIC) (Cresswell, 1996; Mantegazza et al., 2013). It is still controversial whether the Ii/MHC II complexes travel to the endocytic pathway through the Golgi apparatus (Warmerdam et al., 1996) or directly via the cell surface (McCormick et al., 2005; Roche et al., 1993) (Fig. 3). Once in the MIIC, Ii is digested by resident proteases termed cathepsins, and replaced by a 24 amino acid residual fragment called CLIP (Class II-associated Invariant chain Peptide). In the same compartment, extracellular protein antigens are degraded by endo-lysosomal proteases to allow for peptide loading. For this step, MHC class II molecules require the peptide exchange factor HLA-DM (H2-M in mice) that catalyzes the removal of CLIP from the peptide-binding groove and its exchange with specific antigen-derived peptides. HLA-DM also helps select peptides with optimal affinity for MHC class II molecules (Jurewicz and Stern, 2019). Another relevant molecule in this process is HLA-DO (H2-O in mice), which tightly associates with HLA-DM in the ER and throughout the endocytic pathway, thus

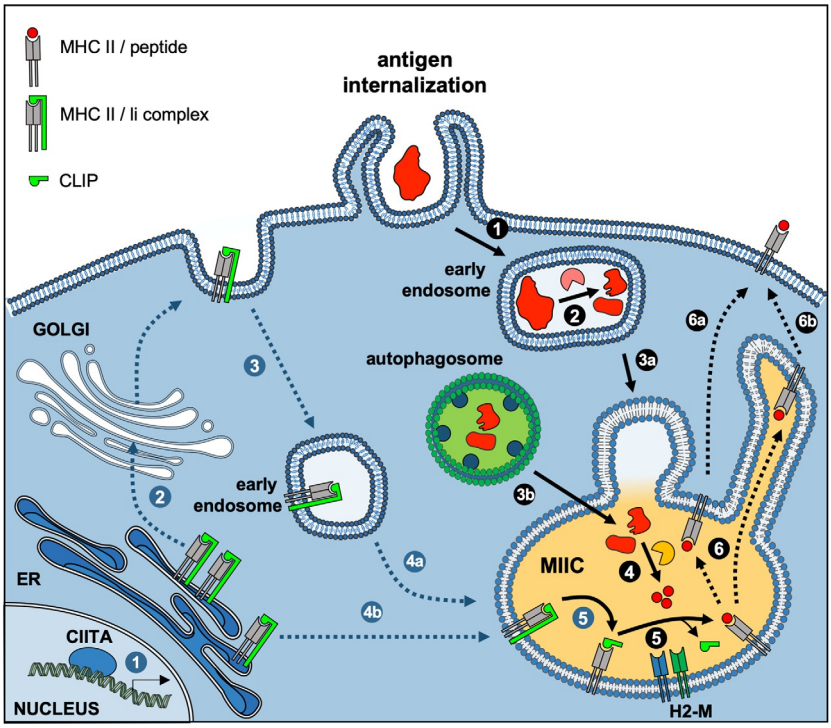

Fig. 3 Classical MHC class II antigen presentation. (A) MHC class II recruitment to the MIIC organelle (left pathway, dashed blue lines). (1) Expression of MHC class II molecules promoted by the transactivator CIITA (class II, major histocompatibility complex, transactivator). MHC class II heterodimers are stabilized in the ER through interaction with the Ii pseudopeptide; (2) MHC class II/Ii complexes are transported to the plasma membrane; (3) internalized by endocytosis and (4a) recruited to the MIIC (MHC class II compartment) organelle. Alternatively, (4b) MHC class II/Ii complexes might be transported to MIIC throughout the Golgi apparatus and the endocytic pathway. Independently of how they arrive to the MIIC, (5) Ii is proteolyzed by cathepsins and a 24 residues-long peptide named CLIP stays associated to MHC class II molecules. (B) *Antigen uptake and processing (right pathway, solid black lines)*. (1) Internalization of antigens (macropinocytosis, endocytosis or phagocytosis); (2) the antigen is partially degraded in the endosome/phagosome; (3a) membrane fusion allows delivery of endosomal cargo to the MIIC organelle. Alternatively, (3b) endogenous proteins within autophagosomes can also reach MIIC to be presented in MHC class II molecules; (4) degradation of peptides by cathepsins and other lysosomal proteases; (5) CLIP is released and replaced by the peptide, a process mediated by H2-M; and (6) MHC class II/peptide complexes are delivered to the cell surface through (6a) direct contact of the MIIC organelle or (6b) formation of tubular intermediates. Steps with unclear or conflicting evidence are showed in arrows with dashed lines.

restricting HLA-DM activity to more acidic compartments (Denzin et al., 2005). Finally, peptide/MHC class II complexes are transported to the plasma membrane and trigger $CD4^+$ T lymphocyte activation and T helper cell-mediated immune responses.

The molecular mechanisms that regulate the trafficking of peptide/MHC class II molecules complexes to the cell surface are poorly understood and a current matter of debate (Fig. 3). After antigenic peptide loading, peptide/MHC class II complexes can be delivered to the plasma membrane through direct contact of MIIC compartments with the cell surface (Pond and Watts, 1997; Wubbolts et al., 1996) or via vesiculotubular intermediates (Rocha and Neefjes, 2008; Vyas et al., 2007). It has been shown that MIIC can move bidirectionally along microtubules thanks to the activity of the motor protein dynein (inward transport) and kinesin (outward transport) (Wubbolts et al., 1999). Also, intraluminal vesicles of the multivesicular MIIC compartment can fuse with the plasma membrane for the release of peptide/MHC class II molecules complexes on nanometer sized vesicles, so-called exosomes (Kleijmeer et al., 2001; Raposo et al., 1996). In DCs, maturation stimuli induce the formation of peptide/MHC class II complexes and their transport to the cell surface (Cella et al., 1997; Pierre et al., 1997). Indeed, DC maturation also rearranges the endocytic pathway and contributes to the transition of MIIC compartments from vesicular to tubular structures (Boes et al., 2002; Chow et al., 2002), which could function as carriers of the peptide/MHC class II molecules complexes (Vyas et al., 2007). Several factors, including cholesterol, cytosolic pH, kinases and GTPases of the Rab and Arl family, have been studied as critical regulators of peptide/MHC class II complex formation and trafficking. Here, the small GTPase Rab7 controls the transition from early to late endosomes and contributes to MHC class II antigen presentation (Bertram et al., 2002; Chavrier et al., 1990). Rab7 most likely regulates the export of peptide/MHC class II complexes to the plasma membrane through the recruitment of dynein (Jordens et al., 2001). Another well characterized molecular actor is the small GTPase ADP-ribosylation factor-like protein 14 (ARL14). It localizes to MIIC in DCs and promotes the activity of the actin-based motor protein myosin 1E and thus regulates MHC class II transport to the plasma membrane (Paul et al., 2011).

As mentioned earlier, the peptide/MHC class II complexes are stable at the plasma membrane and the half-life of MHC class II molecules strongly increases after DC maturation (Cella et al., 1997; Pierre et al., 1997). Nevertheless, MHC class II molecules need to be degraded eventually, possibly

via polyubiquitylation. The E3 ubiquitin ligase MARCH1 can ubiquitylate MHC class II complexes leading to their down-regulation at the cell surface (De Gassart et al., 2008). Although it is not completely defined how MHC class II down-regulation is accomplished, some studies suggest that ubiquitylation drives the endocytosis of peptide/MHC class II complexes (Matsuki et al., 2007; Shin et al., 2006). Others support the notion that MHC class II molecules are constitutively internalized and are degraded within acidic compartments of the endocytic pathway (Walseng et al., 2010). Of note, while IL10 down-regulates the expression of MARCH1 and MHC class II molecules at the cell surface (Koppelman et al., 1997; Thibodeau et al., 2008), the costimulatory molecule CD83 inhibits the interaction between MARCH1 and MHC class II molecules, controlling their half-life in DCs (Tze et al., 2011).

MHC class II molecules present peptides derived from exogenous antigens and from self-antigens degraded within the endocytic pathway. Different cellular processes allow APCs to sample the external environment and detect potential threats for the organism. Contrary to initial believes, many studies have shown that the MHC class II peptidome is mainly composed of peptides derived from endogenous, rather than exogenous proteins (Bergseng et al., 2015; Sofron et al., 2016), and this predominance of endogenous peptides persists under inflammatory conditions (Draheim et al., 2017; Fugmann et al., 2017). In order to understand the many sources of available antigen for MHC class II peptide loading, a clear definition of the different antigen uptake mechanisms in APCs is needed.

3.1 Receptor-mediated endocytosis

This process, also called clathrin-mediated endocytosis, is highly specific and extremely efficient, as it allows for internalization of soluble material that is present in the extracellular milieu even at very low doses. It is highly evolutionary conserved and was likely present in a common eukaryotic ancestor more than a billion years ago (Wideman et al., 2014). The molecular machinery that coordinates this process is assembled at the plasma membrane by the sequential recruitment of endocytic proteins from the cytosol. Here, >50 proteins have been involved for the generation of clathrin-coated vesicles as part of the molecular machinery that drives this endocytic process. Receptor-mediated endocytosis consists of sequential and partially overlapping steps, starting with cluster-formation of endocytic coat proteins at the inner side of the plasma membrane. This first endocytic module assures

the assembly of the clathrin coat, further consisting of other components such as clathrin-adaptor proteins and scaffold proteins. Cargo recruitment concentrates foreign antigens in the coated region at the cell surface and induces membrane bending, which changes the flat plasma membrane into a clathrin-coated pit. Although receptor-mediated endocytosis is normally considered an actin-independent process, it has been described that actin polymerization cooperates with the coat and scission proteins to promote membrane shaping. This includes the activation of regulatory components, such as proteins of the Wiskott-Aldrich syndrome protein (WASp) family, and the actin filament network composed of different actin-binding proteins, such as the complex of actin-related protein 2 (ARP2). Finally, the invagination neck is constricted and cut, separating the clathrin-coated vesicle from the plasma membrane. This scission process is mostly mediated by BAR domains of different proteins that cooperate with dynamin. Complex molecular machinery composed of chaperones, kinases and phosphatases, are in charge of the disassembly of endocytic proteins leading to uncoating of the clathrin-vesicle and the release of cargo-associated material into the endosomal network (Kaksonen and Roux, 2018). Thus, exogenous antigens engulfed by APCs via receptor-mediated endocytosis arrive at early endosomes. They transit toward more acidic compartments, stopping at tubular sorting and recycling endosomes or directly passing to late endosomes/lysosomes where antigenic processing takes place.

3.2 Macropinocytosis

Macropinocytosis is a non-specific and actin-dependent endocytic process that allows for the internalization of exogenous antigens present in fluid-phase at high concentrations in the extracellular milieu. It is initiated by the formation of plasma membrane ruffles and the subsequent invagination of soluble antigens. Once macropinosomes are formed by the sealing plasma membrane ruffles, they undergo a maturation process and establishing dynamic interactions with other compartments of the endocytic network. Here, macropinosomes first fuse with early, then late endosomal compartments and finally with lysosomes to generate a tubular structure known as the macropino-lysosome, which exhibits acidic pH and a degradative environment (Canton, 2018). Mechanistic-wise, the formation of macropinosomes requires essential signaling from the cell surface that converges in an actin-driven closure process of membrane ruffles. Ras-GTPases recruit and activate PI3Ks to generate patches of phosphatidylinositol

3,4,5-trisphosphate [PtdIns(3,4,5)P3] at the plasma membrane. The negatively charged head groups of PtdIns(3,4,5)P3 induce an electrostatic interaction with the polybasic domains present in Rac1/2 and Cdc42. These Rho family GTPases coordinate actin polymerization via the activation of actin-nucleation-promoting factors, such as the SCAR/WAVE complex and WASp/N-WASp. Further maturation of macropinosomes depends on a critical switching step from Rab5 to Rab7 that facilitates the trafficking of this compartment and its fusion with late endosomes/lysosomes (Marques et al., 2017). Constitutive macropinocytosis is efficiently achieved by immature DCs (Sallusto et al., 1995) and macrophages (Yoshida et al., 2009), but is also induced in other cell types in response to growth factor, chemokine or TLR stimulation (Canton, 2018).

3.3 Phagocytosis

Phagocytosis is the process by which large foreign particulate antigens ($\geq$0.5 μm) are recognized, internalized and finally degraded by professional phagocytes. It was first observed by the Russian Élie Metchnikoff in the 1880s. Since then phagocytosis has been widely studied as the main mechanism to ingest and eliminate microbes, but also to clear apoptotic cells as part of fundamental tissue homeostasis. In brief, phagocytosis can be divided into four general steps: (i) recognition of the target particle, (ii) activation of a signaling cascade to induce particle internalization, (iii) formation of the phagosome, and (iv) maturation of the phagosome toward the phagolysosome (Rosales and Uribe-Querol, 2017). Particle recognition is achieved by the presence of a wide variety of opsonic and non-opsonic receptors on the cell surface of professional phagocytes. The former category includes Fcγ receptors, that recognize the Fc fragment of IgG-coated particles, and complement receptors, which recognize antigen coated with components of the complement cascade (C3b, C4b and iC3b). The latter category are Mannose receptors that bind mannan; Scavenger receptors and CD14 that recognize lipopolysaccharide (LPS) and lipoteichoic acid; Dectin-1 that binds LPS from some yeasts; and Apoptotic receptors, such as TIM-1 or TIM-4, which recognize phosphatidylserine from the dying cell. Foreign antigens can also be recognized by TLRs through their conserved structural motifs (PAMPs). Nevertheless, TLRs do not function as phagocytic receptors, although they often contribute to other non-opsonic receptors to stimulate particle ingestion (Freeman and Grinstein, 2014). Similar to macropinocytosis, the phagocytic uptake largely depends on actin

polymerization. After receptor-mediated antigen recognition, different signaling events trigger drastic membrane remodeling changes at the cell surface. In this way, the actin cytoskeleton leads to the formation of the phagocytic cup, which consists of pseudopods that cover the particle and allow for its internalization. The extracellular antigen is now surrounded by membrane in a new compartment termed the phagosome, that contains and isolates dangerous material from the rest of the cell. The signaling cascade initiated by the phagocytosis receptors activates the PI3K-Rac/Cdc42− SCAR/WAVE-WASp axis in order to initiate actin polymerization (Prehoda et al., 2000).

Newly formed early $Rab5^+/EEA1^+$ phagosomes progress through a maturation process and become intermediate phagosomes that are still $Rab5^+$, but no longer $EEA1^+$ compartments. Instead, these intermediate phagosomes recruit components of the endosomal sorting complexes required for transport (ESCRT) thanks to the fusion with multivesicular bodies (MVBs). Also here, the critical transition during phagosomal maturation involves the replacement of Rab5 for Rab7 through the interaction with late endosomes. Finally, $Lamp1^+/Lamp2^+$ compartments, called phago-lysosomes and fully equipped with proteolytic enzymes such as cathepsins and hydrolases contributes to antimicrobial activity through the production of reactive oxygen species (ROS) by the action of the NADPH oxidase complex. Phagosomal maturation is tightly controlled by the formation of specific *trans*-SNARE complexes that are in charge of all membrane fusion events within the cell (Fairn and Grinstein, 2012). These general steps for phagocytosis have been established under "regular" conditions and using model antigens, such as inert microparticles. The situation is certainly much more complex under inflammatory scenarios where several microbes drastically alter one or more steps of phagocytosis. Furthermore, many pathogenic microorganisms induce active invasion-mediated processes and dispose of sophisticated molecular mechanisms that drive the entry, survival and replication inside the host cell. For an in-depth revision of this issue, please refer to Uribe-Querol and Rosales (2017).

Neutrophils mainly work to eliminate pathogens as quickly and efficient as possible. To achieve this, neutrophils are characterized by a very aggressive early phagosomal response known as the oxidative burst (or respiratory burst), a fast and strong activation of NADPH oxidase that elevates the luminal pH until around 8. After 10–15 min post-engulfment, the phagosomal pH decreases abruptly thanks to attenuation of the NADPH oxidase activity and recruitment of the v-ATPase (and likely other proton channels), causing

drastic acidification of the phagosomal lumen. In macrophages, phagosomes become progressively acidic and display a high proteolytic activity to destroy the internalized microbes. Although macrophages are also able to trigger an oxidative burst, phagosomal pH is regulated differently as compared to neutrophils. While, macrophage phagosomes recruit the v-ATPase very fast and strongly, their NADPH oxidase phagosomal activity is almost insignificant. Thus, a few minutes post-internalization, phagosomal pH decreases from 7.4 to 6.5, reaching values below 6 and 5 after 20 and 60 min of phagocytosis, respectively. In contrast, DC phagosomes maintain an alkaline luminal pH and a relatively non-degradative environment for several hours post-engulfment thanks to efficient recruitment and activity of the NADPH oxidase, and a very limited acquisition of the v-ATPase toward the phagosomal membrane (Savina and Amigorena, 2007). This enables DCs to preserve important antigenic peptides for efficient antigen presentation to T lymphocytes. In DCs, these organelles display many additional, sophisticated characteristics, such as the recruitment of ER-derived components and MHC class I molecules. They may thus function as competent cross-presenting compartments adapted to promote efficient cross-priming. All these special features of DC phagosomes will be further discussed in detail later in this chapter.

3.4 Autophagy

Some non-conventional pathways allow for presentation of endogenous antigens in the context of MHC class II molecules in APCs. Presentation of endogenous proteins in MHC class II molecules was first reported 30 years ago, and to date the involvement of the proteasome, as well as the TAP transporter in antigen processing have been well documented (Dani et al., 2004; Lich et al., 2000; Malnati et al., 1992; Mukherjee et al., 2001). Although is not clear how endogenous peptides are loaded onto MHC class II molecules, or why some peptides are presented more efficiently than others, autophagy is believed to be the main intracellular pathway involved in this type of non-conventional antigen presentation. Indeed, antigenic processing via autophagy has been reported to be important in DCs for the presentation of both self and foreign antigens (Crotzer and Blum, 2009).

Autophagy is a ubiquitous process in eukaryotic cells where cytoplasmic components are processed and degraded. First, a phagophore sequesters nuclear, microsomal and cytoplasmic material destined to degradation. Then, the phagophore expands into a double-membrane limited compartment known as the autophagosome that encapsulates the invaginated material.

Autophagosomes can then fuse with endosomes to generate a new organelle termed the amphisome, which in turn fuses with lysosomes equipped with degradative enzymes, such as cathepsins and hydrolases. Additionally, the autophagosome can directly intercept lysosomes without a previous pass through the amphisomal stage. In both scenarios, the result is a strongly acidic, degradative structure referred to as the autolysosome. Finally, the engulfed material is digested within the autolysosome, and the inner membrane of this compartment disintegrates. The resulting macromolecules may be released into the cytosol for further recycling thanks to the presence of permeases in the remaining autolysosomal membrane (Klionsky, 2007).

Autophagy plays fundamental roles in diverse processes like inflammation, neurodegenerative disorders, intracellular trafficking of several pathogens and homeostasis (among others), and is directed by the evolutionary conserved autophagy-related (ATG) genes. Key molecules involved in classical autophagy are ATG13 for the initiation; ATG14 and Beclin-1 for phagophore formation; ATG7, ATG3, ATG5, ATG12 and ATG16L1 for phagophore elongation and autophagosome formation. At this point, autophagosomes are decorated with a widely used marker known as LC3-II. Lysosomal fusion and steps of autolysosome formation are regulated by LC3/GABARAP, the HOPS complex and Rab7. Membrane fusion events are commonly mediated by the SNARE proteins STX17, SNAP29 and VAMP8 (Levine and Kroemer, 2019).

3.5 Cross-dressing

The first evidence of transfer of MHC class II-peptide complexes from B to T cells was postulated >40 years ago (Cone et al., 1972). It has become now clear that such process of intercellular transfer can be used by T cells to acquire MHC molecules from donor APCs after synapse formation, a process referred to as trogocytosis (Arnold and Mannie, 1999; Hudrisier et al., 2001; Joly and Hudrisier, 2003). Later, it has been shown that membrane transfer can also occur between APCs by means of trogocytosis, exosomes or tunneling nanotubes (André et al., 2004; Davis, 2007; de Heusch et al., 2007; Herrera et al., 2004; Smyth et al., 2007). More recent studies showed that DCs can acquire fully functional MHC/peptide complexes that can elicit $CD8^+$ T cell responses both in vivo and in vitro (Dolan et al., 2006; Li et al., 2012; Wakim and Bevan, 2011). Still, the significance of this process in vivo is a matter of debate, and further experimentation is needed. For a more detailed revision, please refer to Campana et al. (2015).

4. Cross-presentation

Among the "non-classical" routes for antigen processing and presentation, antigen cross-presentation has received the most attention during the last 20 years. In 1976, a seminal study by Bevan et al. demonstrated that transplantation of allogeneic cells elicited a donor specific $CD8^+$ T cell response in mice (Bevan, 1976a,b). Previously, it was believed that MHC class I molecules only presented peptides generated within the cell, e.g., those resulting from intracellular viral infections (Zinkernagel and Doherty, 1974) and that macrophages were the most important APCs. However, following their discovery by Steinman and Cohn (1973), DCs have proven to be the most competent cells for priming cytotoxic T cell responses in vivo (Jung et al., 2002).

In particular, DCs are considered the most efficient cross-presenting cell type, although the ability to cross-present varies among different DC subsets. Early work from the labs of Bevan and Shortman demonstrated that $CD8^+$ resident DCs cross-presented both soluble as well as cell-associated ovalbumin (OVA), a widely used model antigen (den Haan et al., 2000; Pooley et al., 2001) in vivo. All splenic DC subsets were equally potent in ingesting both soluble and bead-associated OVA thus excluding that differences in antigen uptake were responsible for the differences in cross-presentation between $CD8^+$ and $CD8^-$ DCs (Schnorrer et al., 2006). However, since the route of antigen uptake impacts on their processing and presentation, more variables have to be considered when investigating differences in cross-presentation efficiency (Kamphorst et al., 2010). In line with this, $CD8^-$ DCs can cross-present antigens when these are targeted to specific receptors (den Haan and Bevan, 2002) or when they are delivered together with stimuli such as a TLR ligands (Neubert et al., 2014). Among migratory DCs, $CD103^+$ cells are considered to be the main subset cross-presenting antigens from peripheral tissues such as skin, lung and intestine (Bedoui et al., 2009; Henri et al., 2010; Houston et al., 2016; Kim and Braciale, 2009). Plasmacytoid DCs have traditionally been excluded from the list of cross-presentating DCs since absence of pDCs did not interfere with cross-presentation of peptides from influenza virus (GeurtsvanKessel et al., 2008, reviewed in Villadangos and Young, 2008). However, some studies report priming of $CD8^+$ T cell by pDCs following TLR stimulation (Mouries et al., 2008). A recent work describing two different subsets of pDCs, one of them ontogenetically closer to cDCs and with enhanced

antigen presentation properties, could explain the heterogeneity in the ability of pDCs to cross-present antigens (Rodrigues et al., 2018). Monocyte-derived DCs are very efficient cross-presenters under inflammatory conditions, however, the underlying mechanism seems to differ from that of cDCs (Cheong et al., 2010; Segura et al., 2009).

To date, two major pathways of antigen cross-presentation have been described, generally referred to as either the "vacuolar" and "cytosolic" pathway (Joffre et al., 2012). The "cytosolic" route is sensitive to proteasome inhibitors (Kovacsovics-Bankowski and Rock, 1995), suggesting that endocytosed or phagocytosed proteins have to access the cytosol, where they are degraded by the proteasome. Whether these degraded antigens are then transported back to the phagosome or the ER for further processing and MHC class I loading is still a matter of debate. However, many studies suggest that phagosomes play an important role in antigen cross-presentation (Ackerman et al., 2003, 2006; Alloatti et al., 2017; Cebrian et al., 2011; Guermonprez et al., 2003).

In contrast, cross-presentation through the "vacuolar" pathway is resistant to proteasome inhibitors but sensitive to inhibitors of lysosomal proteases, such as cathepsin S inhibitors (Bertholet et al., 2006; Shen et al., 2004). This suggests that antigen processing and loading both occur in the endocytic compartment. It is difficult to assess how both pathways contribute to antigen cross-presentation, but available evidence points toward a more important role for the cytosolic route for cross-presentation in vivo (Kovacsovics-Bankowski and Rock, 1995; Palmowski et al., 2006; Shen et al., 2004; Sigal et al., 1999).

In the following section, we will discuss how different DC subtypes preserve antigen and regulate MHC class I expression and trafficking to favor cross-presentation. We will focus our analysis on the "cytosolic" pathway, that we plan to dissect as follows: (a) antigen phagocytosis and phagosome maturation, (b) antigen translocation into the cytosol, proteasomal degradation and rerouting of peptides to phagosomes or the ER, (c) recruitment of MHC class I to the cross-presenting organelle and (d) delivery of cross-presentation machinery to the cross-presenting organelle to facilitate antigen presentation.

4.1 Endo/phagosomal antigen processing for cross-presentation

Cross-presentation is initiated once DCs internalize extracellular antigens. As mentioned above, internalization can be achieved by different uptake

mechanisms: phagocytosis, receptor-mediated endocytosis or macropinocytosis. The resulting antigen-loaded compartment will become a dynamic and highly regulated vesicle for antigen-processing and loading onto MHC class I molecules. Antigen degradation is a complex multistep process that takes place in different organelles. The nature of the antigen, the employed antigen-uptake mechanism and the type of cross-presentation pathway utilized (vacuolar vs cytosolic) govern whether peptide loading occurs in endo/phagosomes or the ER. The interaction between antigen and the intra-phago/endosomal milieu changes these organelles which then fuse sequentially first with early and then with late endosomes and lysosomes in a process termed "endosome/phagosome maturation."

One main feature of endo/phagocytic compartments in DCs is their low degradative capacity. Antigen degradation depends on the activity of proteases which increases during phagosome maturation. Initial experiments on phagosomal proteolytic activity in APCs used latex beads coupled to active site-directed probes and found that the cysteine protease Cathepsin S (CatS) was most enriched in phagosomes from APCs (Lennon-Duménil et al., 2002). The main function of CatS is the proteolytic digestion of li molecules involved in MHC class II antigen presentation to $CD4^+$ T cells (Riese et al., 1996). Interestingly, CatS was also shown to have a central role in peptide processing in the vacuolar pathway (Shen et al., 2004). The development of CatS inhibitors as immunomodulatory drugs to treat autoimmune diseases has become a promising field of investigation (Gupta et al., 2008).

The notion that DCs regulate their endocytic pathway differently compared to other phagocytes was supported by the observation that DCs exhibited a slower kinetics of active proteases acquisition compared to macrophages (Lennon-Duménil et al., 2002). The comparison of lysosomal protease expression and activity in macrophages, B lymphocytes and DCs provided further evidence for lower protein degradation capacity in DCs both in vitro and in vivo (Delamarre et al., 2005). Analysis of murine bone marrow-derived DCs (BMDCs) and DCs from secondary lymphoid organs revealed lower expression of proteases in DCs as compared to macrophages. Assays using different soluble antigens further demonstrated slower proteolysis in DCs, favoring antigen presentation and linking antigen processing to antigen presentation. These findings were further corroborated by enhanced cross-presentation of soluble viral antigens in the presence of chloroquine or ammonium chloride, two agents that prevent endosomal acidification. Here, antigen presentation in MCH class II was inhibited (Accapezzato et al., 2005; Belizaire and Unanue, 2009). Both substances increased export

to the cytosol while no effect was seen following treatment with protease inhibitors, in a model that was dependent on proteasome (Accapezzato et al., 2005). These findings led to the hypothesis that limited antigen degradation is linked to enhanced cross-presentation. Further work demonstrated that limiting the susceptibility of antigens to lysosomal proteolysis enhanced immunogenicity in vivo (Delamarre et al., 2006). However, it remained unclear how antigen degradation was regulated differently in DCs.

Endosomal and lysosomal proteases are synthesized as inactive proenzymes and become activated at different pH values that are first reached within the respective compartments. Endo/lysosomal pH is regulated by two systems: the ATP-dependent vacuolar proton pump (v-ATPase) and NADPH oxidase 2 (NOX2), that regulate the levels of H^+ and ROS, respectively, within phagosomes and endosomes (see Fig. 4 for a detailed scheme).

DCs regulate the recruitment and assembly of v-ATPase at membranes. Upon cell maturation, higher v-ATPase activity was observed, leading to accumulation of H+, lower pH, increased protein degradation and MHC class II dependent antigen presentation (Trombetta et al., 2003). At steady-state, human DCs express lower levels of v-ATPase as compared to macrophages (Mantegazza et al., 2008). More recently, the transcription factor TFEB was described as a master regulator of the lysosome biogenesis pathway (Sardiello et al., 2009; Settembre et al., 2013). TFEB regulates the transcription of many genes including cathepsins and different subunits of the v-ATPase (Sardiello et al., 2009) in a network that controls cellular processes such as autophagy (Settembre et al., 2011). Not surprisingly, DCs express lower levels of TFEB compared to macrophages adding evidence to the differential regulation of protein degradation between phagocytes (Samie and Cresswell, 2015). Remarkably, TFEB was expressed at even lower levels in splenic $CD8^+$ DC subsets specialized in cross-presentation. As expected, overexpression of TFEB in DCs lowers pH and increases protease activity resulting in decreased cross-presentation due to enhanced antigen degradation.

Nevertheless, high phago/endosomal pH in DCs suggested that mechanisms other than the reduction in v-ATPase activity was contributing to the alkalinization of these organelles. NOX2 is a multiprotein complex consisting of a transmembrane component, gp91phox and p22phox (known as cytochrome *b*558) and several cytosolic subunits. When DCs internalize antigens, cytochrome *b*558 translocates to phagosomal membranes and recruits its cytosolic components to become enzymatically active. Active

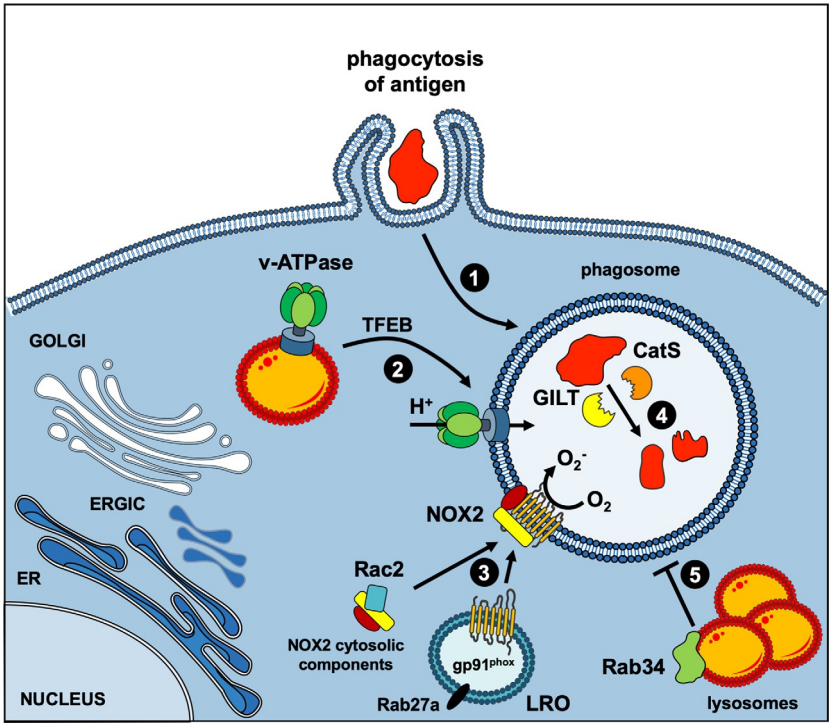

Fig. 4 Phagosomal processing of antigens during cytosolic cross-presentation. (1) Internalization of the exogenous antigen (by macropinocytosis, endocytosis or phagocytosis) and formation of the cross-presenting organelle; (2) in immature DCs, v-ATPase is incompletely assembled to phagosomes, and thereby acidification is lower than in other related cells, like macrophages. When DCs mature, v-ATPase is recruited to the cross-presenting organelle, a process mediated by TFEB. On the contrary, (3) NOX2 is assembled in phagosomes of cross-presenting DCs. NOX2 cytosolic components are recruited to the phagosome/endosome driven by Rac2, whereas gp91phox and p22phox are translocated to the cross-presenting phagosome from lysosomes-related organelles (LRO). (4) Within the phagosome, the internalized antigens are partially degraded by Cathepsin S and the GILT complex, a necessary step for further export to cytosol. (5) In a different mechanism orchestrated by TLR engagement and mediated by the small GTPase Rab34, lysosomes are prevented to fuse with the phagosome, thus maintaining the cross-presenting organelle suitable for cross-presentation.

NOX2 consumes protons for ROS production and thus slows down phagosomal acidification. In line with this, DCs from NOX2-defective mice had lower phagosomal pH were defective in cross-presentation (Savina et al., 2006). Although a clear relationship between reduced proteolysis and increased NOX2 activity was shown by many different groups in macrophages (Balce et al., 2016; Rybicka et al., 2010) and DCs

(Mantegazza et al., 2008; Rybicka et al., 2012; Savina et al., 2006), the role of phagosomal pH in this outcome remained controversial, with some papers showing pH-dependency while others involved enzyme regulation as the underlying mechanism by which NOX2 regulates proteolysis. Experimental conditions, as well as the nature of the antigens utilized, can account for these differences, as reviewed previously (Ewanchuk and Yates, 2018; Kotsias et al., 2013; Pauwels et al., 2017; Yates, 2013). The use of opsonized antigens probably overrides some of the more subtle regulatory effects present in phagosomes. Also, the regulation of the activity of different phagosomal enzymes by NOX2 activity promotes an additional qualitative control of proteolysis, and shapes the repertoire of peptides to be presented (Allan et al., 2014), as we will discuss later.

In the last decade, several molecules have been described to recruit NOX2 to the phagosomal membrane. First, it was demonstrated that the small GTPase Rab27a facilitates the fusion of vesicles containing the transmembrane elements of NOX2 with nascent phagosomes (Jancic et al., 2007). Rab27a-deficient *ashen* mice failed to cross-present antigens due to increased acidification of the phagosomal lumen and exaggerated antigen degradation. However, the question remained how NOX2 was differentially regulated in DCs compared to other phagocytes which produce ROS but do not cross-present antigens, such as neutrophils or macrophages. The discovery that one of two isoforms of the Rac GTPase was responsible for the recruitment of the cytosolic subunits of NOX2 (Savina et al., 2009) provided an answer. Rac1 promotes the assembly of NOX2 at the plasma membrane in macrophages, while Rac2 directs the cytosolic subunits toward cytosolic vesicles such as in neutrophils where the oxidative burst has been extensively characterized. A different scenario was true for DC subtypes: cross-presenting DCs depend on Rac2 to limit antigen degradation within endo/phagosomes while splenic $CD8^{-}CD11b^{+}$ DCs predominantly express Rac1. Here, NOX2 is absent at the phagosomal membrane, thus resulting in phagosomes that are less competent in cross-presentation (Savina et al., 2009). These results were recapitulated in human monocyte-derived DCs and DCs from patients suffering from chronic granulomatous disease, a genetic disorder where NOX2 is either absent or nonfunctional (Mantegazza et al., 2008). In these cells, decreased cross-presentation of tumor model antigens coincided with increased phagosomal acidification.

More recently, vesicle-associated membrane protein 8 (VAMP8), a t-SNARE protein present in endosomal recycling compartments (ERC),

was shown to participate in the recruitment of cytochrome *b*558/NOX2 to phagosomal membranes. *Leishmania donovani*, a parasite that had been shown to interfere with NOX2 assembly at phagosomal membranes and thus phagosome maturation (Lodge and Descoteaux, 2006), was found to cleave VAMP8. The resulting change in pH and phagosomal antigen degradation impaired antigen cross-presentation and thus allowed the parasite to evade immune recognition (Shio et al., 2013). In human monocytes-derived DCs NOX2 assembly was not only dependent on VAMP8, but also on SNAP23 and STX7 (Dingjan et al., 2017a).

Besides the effect of NOX2 on endo/phagolysosomal pH, other mechanisms have been proposed where ROS alter cross-presentation in a pH-independent fashion. ROS generated following enzymatic conversion of superoxides, such as hydrogen peroxide and oxygen radicals, not only modify enzymes involved in antigen degradation but also the antigen itself. Oxygen radicals can modify an essential residue in the catalytic center of serine and cysteine proteases leading to their inactivation resulting in a quantitative and qualitative change of proteolysis (Allan et al., 2014; Nagaoka et al., 2005; Rybicka et al., 2012; Suto et al., 2007). Moreover, oxidative inhibition of v-ATPase has been reported many years ago (Feng and Forgac, 1994). Several studies have found that oxidation of antigens can lead to molecular changes that influence their susceptibility to proteolytic degradation (Prokopowicz et al., 2010). In any case, the actual contribution of these pH-independent mechanisms of ROS-mediated degradation need to be investigated further and remain a matter of debate (Kotsias et al., 2013; Rybicka et al., 2012).

Surprisingly, these studies led to the discovery that NOX2-dependent ROS oxidized lipids of the endosomal membrane and that this process was dependent on VAMP8, STX7 and SNAP23 (Dingjan et al., 2016, 2017a,b). This phenomenon was shown to induce membrane instability and to promote antigen release to the cytosol, thus enhancing antigen cross-presentation. These studies offered an alternative hypothesis for antigen export to the cytosol that will be discussed below (Gros and Amigorena, 2019).

In conclusion, cross-presentation requires low antigen degradation to preserve epitopes for presentation on MHC class I molecules. In DCs, this is achieved through specialized endocytic compartments which are regulated differently as compared to other phagocytes.

However, DCs also present antigens to $CD4^+$ T lymphocytes in the context of MHC class II molecules, a process that involves increased antigen

degradation and thus seems to oppose cross-presentation. How is antigen processing balanced for cross-presentation or MHC class II presentation? Evidence points to differential delivery of antigen to different intracellular compartments based on uptake receptor. Endocytosis of the model antigen OVA through the mannose receptor (MR) was found to be delivered to early endosomes leading to cross-presentation. DCs from MR-deficient mice failed to cross-present soluble OVA, but efficiently presented antigens to $CD4^+$ T cells (Burgdorf et al., 2007). Antigen presentation on MHC class II could be inhibited by pharmacologically blocking macropinocytosis, leaving cross-presentation unaffected. Internalized antigen by macropinocytosis was shown to be targeted toward late endosomes and lysosomes. This data suggested that the internalization mechanism dictated intracellular routing of antigens. Thus, the array of endocytic receptors present on particular cells would define which presentation pathway is favored.

Based on this evidence, a model in which separate compartments are dedicated to either cross-presentation or presentation on MHC class II was proposed (Burgdorf and Kurts, 2008). Analysis of endosomes following clathrin-mediated endocytosis of different ligands revealed the presence of two distinct populations of endosomes: one being more mobile, quickly maturing into endolysosomes, and a second one with slower maturation kinetics (Lakadamyali et al., 2006). This model was further supported by data using modified liposomes to target soluble hen-egg white lysozyme (HEL) to different compartments (Belizaire and Unanue, 2009). Antigens delivered to early endosomes were cross-presented more efficiently than proteins that were macropinocytosed or targeted toward late, acidic and degradative organelles. Several studies have determined antigen routing for different receptors expressed on DCs and thus their preferential presentation on either MHC class I or class II molecules. DEC-205, DC-SIGN and scavenger receptors belong to a class of receptors that target antigens toward lysosomes, promoting CD4 T cell responses by enhancing MHC class II-dependent antigen presentation (Burgdorf and Kurts, 2008; Burgdorf et al., 2007; Chatterjee et al., 2012; Mahnke et al., 2000). Interestingly, engaging two different regions of the extracellular portion of DC-SIGN directed antigens into different compartments. This provides evidence that the nature of the antigen is important and might be exploited to manipulate the type of ensuing immune response (Tacken et al., 2011). Specific changes in the glycan composition of OVA leads to its uptake by MGL-1, a c-type lectin receptor, instead of MR. This was followed by increased cross-presentation and prolonged intracellular storage of OVA within early endosomes

(Streng-Ouwehand et al., 2016). Coadministration of the inert particulate antigen monosodium urate with soluble OVA, redirected OVA toward less degradative endosomes and favored cross-presentation (Hari et al., 2015).

It is still unclear whether there are two distinct types of compartments dedicated to presentation either on MHC class II molecules or cross-presentation of soluble antigens, or whether these compartments reflect different maturation status of a population of heterogeneous vesicles. A sequential model has been widely accepted for particulate antigens, which are internalized through phagocytosis (Amigorena and Savina, 2010; Burgdorf and Kurts, 2008). As described above, the activation of NOX2 and subsequent increase in pH upon phagocytosis slows down protein degradation. This allows peptides to be mildly processed and to proceed through the cross-presentation pathway. As phagosome maturation proceeds, elements of ER/ERGIC detach from the phagosomal membrane and subsequent fusion with acidic lysosomes renders the compartment more degradative, prone for peptide loading onto MHC class II molecules.

In line with this sequential model, TFEB activation induces phagosome acidification and enhanced degradation of particulate antigen, abrogates cross-presentation and induces MHC class II presentation in DCs exposed to LPS for 24 h. Indeed, LPS leads to TFEB translocation to the nucleus (Samie and Cresswell, 2015). Interestingly, TFEB does not affect the high capacity to cross-present antigens of immature DCs or cells exposed to LPS for short periods. This indicates that TFEB does not modulate phagosome maturation in the first hours following antigen encounter and suggests that other mechanisms regulate cross-presentation, as discussed below. TFEB activation leads to phagolysosomal fusion first at later time points, allowing for MHC class II-dependent antigen presentation to $CD4^+$ T cells.

How cross-presentation is regulated upon DC maturation has generated controversy, as studies have reported conflicting results. Studies suggest that regulation occurs at different levels and follows stimulation in a time-dependent manner. We have recently reviewed this in detail and will briefly mention the specific mechanisms regulating antigen degradation during DC maturation (Alloatti et al., 2016).

DC maturation can be divided into three phases. At early time points following activation by DAMPs or PAMPs, DCs are characterized by high levels of cross-presentation. This increase during early phases can be attributed to enhanced antigen uptake (Granucci et al., 1999; West et al., 2004),

export of antigens to the cytosol (Delamarre et al., 2003; Gil-Torregrosa et al., 2004), and changes in the intracellular trafficking of MHC class I molecules (Nair-Gupta et al., 2014). Slower recruitment of lysosomal proteases, measured shortly after internalization, possibly contributes to decreased degradation and allows for antigen export to the cytosol (Lennon-Duménil et al., 2002). It is noteworthy that in a scenario where DCs take up antigens in conjunction with a DAMP/PAMP, phagosomal autonomy was described (Hoffmann et al., 2012). This ensures that specific modulation of a given phagosome does not extend to other phagosomes. This allows for discrimination of different cargoes within the same cell and hence regulates the antigen presentation pathway for each antigen individually.

Up until 16–20 h after maturation, DCs efficiently cross-present antigens despite a decrease in internalization efficiency (Alloatti et al., 2015; Drutman and Trombetta, 2010; Vega-Ramos et al., 2014). A delay in phagolysosomal fusion and subsequent recruitment of proteases was responsible for the increase in cross-presentation (Alloatti et al., 2015). Lysosomes formed perinuclear clusters and were spatially segregated from phagosomes which in turn were moving at decreased speed compared to resting DCs. Silencing of the small GTPase Rab34 prevented lysosomal clustering, favored phagolysosome fusion and decreased cross-presentation. Comparing the proteome of phagosomes from resting and LPS-stimulated DCs, the latter contained fewer proteins of lysosomal origin, including v-ATPase subunits and cathepsins, but were enriched in proteins associated with cross-presentation, e.g., MHC class I, proteasome subunits and TAP (Pauwels et al., 2019). Additionally, maturing DCs expressed decreased levels of VAMP8, suggesting that NOX2 recruitment is not the central mechanism for decreased antigen degradation in these cells.

Fully matured DCs are possibly the most studied cells, and cross-presentation is completely shut down 20–24 h after activation. Recruitment of v-ATPase and acidification of endosomes after prolonged exposure to LPS has been known for many years (Trombetta et al., 2003). However, the underlying mechanism has only been described recently. Exposing DCs to LPS (or other TLR ligands) increases expression of TFEB and induces its nuclear translocation, leading to phagosomal acidification, proteolysis and inhibition of cross-presentation. Silencing TFEB partially restored the ability of mature DCs to cross-present by increasing the phagosomal pH (Samie and Cresswell, 2015).

4.2 Translocation of mildly degraded antigens to the cytosol, proteasomal degradation and transport to the cross-presenting organelle

While it is accepted that antigens are exported to the cytosol after internalization (Ackerman et al., 2006; Cebrian et al., 2011; Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1997; Rodriguez et al., 1999), the exact mechanism has not been fully characterized thus far. Available evidence suggests the involvement of the retrotranslocation machinery for misfolded proteins from the ER to the cytosol (ERAD machinery). The ERAD proteins Sec61 and p97 are indeed necessary for antigen export to the cytosol (Fig. 5), as well as antigen cross-presentation (Ackerman et al., 2006).

Sec61 has been proposed as an important channel for antigen export to the cytosol (Zehner et al., 2015) as siRNA-mediated silencing of Sec61 or treatment with ExoA (a bacterial Sec61-binding toxin, closing the channel) inhibits cross-presentation (Ackerman et al., 2006; Goldszmid et al., 2009; Zehner et al., 2015). Furthermore, using intrabodies against Sec61, Burgdorf et al. sequestered Sec61 in the ER, preventing its transport to the phagosome, inhibiting antigen export to the cytosol and OVA cross-presentation in BMDCs. However, a recent study inhibiting Sec61 with a specific toxin, mycolactone, did not interfere with antigen translocation to the cytosol (Grotzke et al., 2017). Of notice, mycolactone is not highly specific in Sec61 inhibition, as it might also targets different receptors (Marion et al., 2014). Hence, the proposed function of Sec61 as a peptide transporter during antigen cross-presentation awaits further clarification.

A different model for antigen export to the cytosol has been proposed by Ploegh and Rapoport et al. The authors demonstrated that Derlin-1, and not Sec61, mediates antigen transport to the cytosol (Lilley and Ploegh, 2004; Ye et al., 2004). However, genetic silencing of Derlin-1 in BMDCs (Zehner et al., 2015) and human monocyte-derived DCs (Ménager et al., 2014) had no effect on antigen export to the cytosol or antigen cross-presentation, questioning its role in cross-presentation in DCs. The protein Hdr1, an ER-resident ubiquitin ligase, has also been proposed as a candidate for antigen transport to the cytosol, as its siRNA-mediated silencing impaired both antigen translocation and cross-presentation in DCs (Zehner et al., 2015). However, MHC class II-dependent antigen presentation is changed as well, suggesting a general effect on antigen presentation, possibly associated with ER stress.

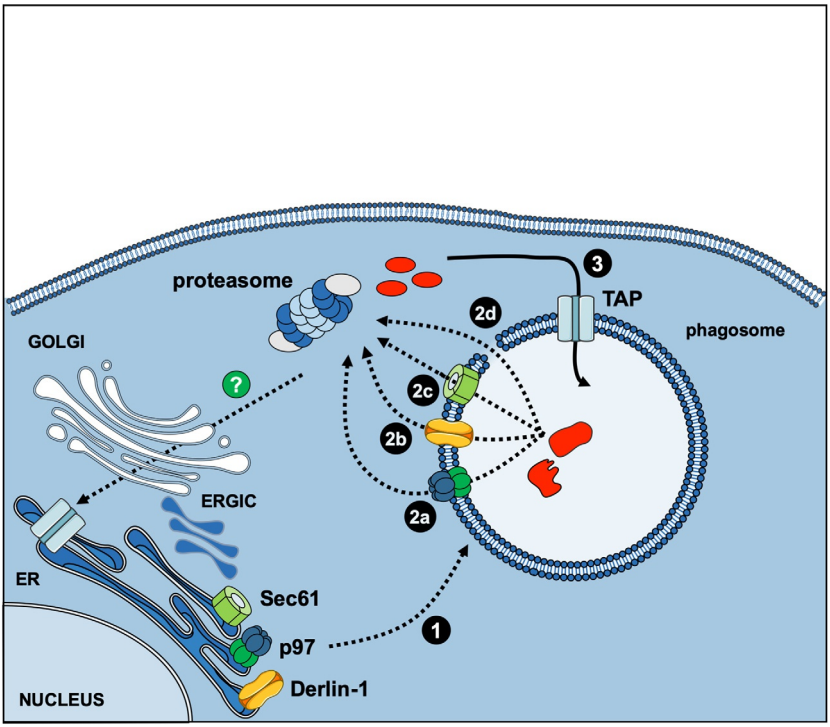

Fig. 5 Antigen translocation to the cytosol. (1) By a yet unknown mechanism, possibly mediated by Sec22b, different protein complexes from the ER reach phagosomes (Sec61, Derlin-1, p97 and others have been proposed and may contribute differentially to the process); (2) antigen can be exported to the cytosol by different means, such as (2a) through the p97 channel, (2b) the ER protein Derlin-1, (2c) the translocon complex Sec61 or (2d) by membrane disruption. (3) Cytosolic degradation of antigens exported to the cytosol, mediated by the proteasome, and transport back to the phagosomes through TAP transporter. Otherwise, (?) peptides digested by the proteasome might be translocated to the ER. Steps not fully characterized, with unclear or conflicting evidence, are showed in arrows with dashed lines.

Thus far, the most convincing evidence for the involvement of ERAD in antigen translocation comes from the ATPase p97, which forms a hexameric ring and acts as a retrotranslocation channel, likely by providing necessary energy for proteins passage (Zehner et al., 2011). Genetic silencing of p97 and overexpression of wild-type or dominant negative alleles helped establish this protein as an important player in antigen export to the cytosol and cross-presentation, at least for model antigens MelanA and OVA (Ackerman et al., 2006; Imai et al., 2005; Ménager et al., 2014; Zehner et al., 2011).

Although different mechanisms of antigen translocation to the cytosol have been proposed, all studies mentioned rely on the ERAD machinery, which is known to have a high substrate selectivity (Römisch, 2017). While there is considerable evidence for the involvement of the ERAD machinery in antigen export, the use of only few transporters for such variety of substrates seems unlikely and suggest the existence of alternative mechanisms. An alternative theory considers that the antigen might leak from the endocytic compartment as a consequence of membrane disruption. Evidence supporting this hypothesis has recently been published by Dingian et al. who demonstrated that following LPS treatment, NOX2 is recruited to endosomes by a VAMP8-dependent mechanism. The concomitant production of ROS leads to peroxidation of endosomal lipids and affects the membrane stability favoring antigen release to the cytosol and increasing antigen cross-presentation (Dingjan et al., 2016, 2017b). In addition, enrichment of sphingosine in endosomal membranes during the formation of lipid bodies, especially in cross-presenting DCs, could also facilitate membrane disruption (Bougnères et al., 2009; Ploegh, 2007). For a detailed review regarding antigen export to the cytosol, please see Gros and Amigorena (2019).

Once antigens reach the cytosol, they are either degraded by the proteasome (Fonteneau et al., 2003; Guermonprez et al., 2003; Kovacsovics-Bankowski and Rock, 1995) or the immunoproteasome (Kincaid et al., 2012; Palmowski et al., 2006) and subsequently transported to the cross-presenting organelle. Whether proteolyzed antigens are processed and loaded onto MHC class I within the ER, the phagosome or other endocytic compartments is still a matter of debate. However, there is no direct evidence of peptide loading within the ER, and most of the available data point toward phagosomal processing and loading.

Independent of the site of antigen processing and loading, peptides need to be transported into the cross-presenting organelle, likely via one (or several) transporter(s). As mentioned previously, translocation of peptides to the ER during classical MHC class I antigen presentation relies on the TAP transporter. As per cross-presentation, the vacuolar mechanism is considered TAP-independent (Bertholet et al., 2006; Shen et al., 2004), whereas the cytosolic route is mainly TAP-dependent (Burgdorf et al., 2008; Guermonprez et al., 2003; Houde et al., 2003), although a TAP-independent cytosolic cross-presentation has been reported (Lawand et al., 2016; Merzougui et al., 2011). How TAP and other relevant proteins, such as MHC class I molecules, calnexin and calreticulin reach phagosomes will be discussed in the following sections.

4.3 Intracellular trafficking of MHC class I molecules

The intracellular transport of MHC class I molecules needs to be tightly regulated by DCs in order to guarantee the efficacy of antigen loading and presentation. The different quality control steps that are required for proper MHC class I molecule folding have been extensively characterized in recent years and are reviewed elsewhere (Blees et al., 2017; Neerincx and Boyle, 2017; Springer, 2015; Thomas and Tampé, 2017). However, endocytic trafficking of MHC class I molecules and its potential impact on loading with exogenous antigens in post-ER compartments has only recently gained attention.

Besides being present at the plasma membrane and the classical secretory pathway, MHC class I molecules are also found throughout the endocytic network, either as fully conformed trimolecular complexes and as open forms. Open MHC class I conformers in the plasma membrane are present in lower numbers than their fully conformed counterparts. Both forms distributed in separate regions of the plasma membrane and can be efficiently internalized in different endocytic invaginations by a mechanism independent of clathrin and dynamin (Donaldson and Williams, 2009; Mahmutefendić et al., 2011; Zagorac et al., 2012). Following internalization, both conformers are transported to the same early endosomes, and further distributed into non-degradative membranous compartments, such as early and sorting endosomes, but also in acidic organelles, such as multivesicular bodies, late endosomes and lysosomes. However, most MHC class I molecules are concentrated in a defined perinuclear region known as the endocytic recycling compartment (ERC) (Blander, 2016; Mahmutefendić et al., 2013). Thus, MHC class I molecules can be potentially loaded with exogenous antigens within the many endocytic compartments during cross-presentation by DCs.

Moreover, MHC class I molecules are transported to the endo/lysosomal system either from the plasma membrane after their internalization or directly from the ER, chaperoned by the li, also known as CD74 (as described above). CD74 has been described to determine the intracellular fate of MHC class I in endo-lysosomes, but does not interfere with the internalization of MHC class I molecules from the cell surface (Basha et al., 2012; Sugita and Brenner, 1995). Indeed, upon depletion of CD74, MHC class I loses its localization at $Lamp1^+$ compartments and cross-presentation of exogenous antigens by DCs is hampered (Basha et al., 2012). Whether exogenous antigen-derived peptides are loaded onto MHC class I molecules within these $Lamp1^+$ compartments is still unknown. So far, experimental evidence suggests that loading of MHC class I takes place within the endocytic system, rather than in the ER, from where peptide/MHC class

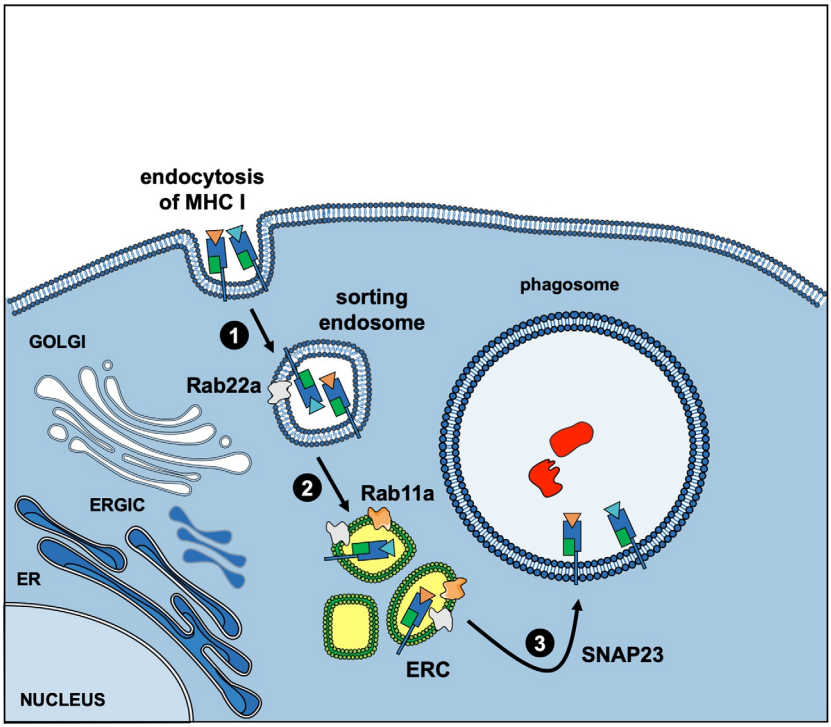

Fig. 6 MHC class I molecules transport to phagosomes. (1) plasma membrane MHC class I molecules are endocytosed and delivered to sorting endosomes; (2) MHC class I heterodimers are subsequently targeted to endocytic recycling compartments (ERC) with the participation of the small GTPases Rab22a and Rab11a; and (3) TLR engagement triggers the phosphorylation of SNAP23, allowing the recruitment of MHC class I molecules to the cross-presenting organelle.

I molecules complexes travel to the cell surface (Burgdorf et al., 2008; Cebrian et al., 2016; Nair-Gupta et al., 2014) (Fig. 6). The recruitment of relevant ER components to DC endosomes and phagosomes through a Sec22b-mediated transport further support to this concept (Cebrian et al., 2011). It is now evident that recycling of MHC class I molecules by DCs plays a pivotal role in order to achieve efficient cross-presentation (Montealegre and van Endert, 2018; van Endert, 2016).

Most information about recycling of MHC class I molecules has been obtained from nonimmune cell types, with experimental evidence in APCs being very limited. Current evidence suggests that once a cargo enters the recycling route, it can either return to the plasma membrane via the fast recycling pathway, or it can pass through the ERC in what is known as

the slow recycling pathway. While the former is the typical route utilized by, e.g., the transferrin receptor, the latter is the main recycling route for MHC class I molecules.

Following their internalization, MHC class I molecules are stored in $Rab5^+/EEA1^+$ early endosomal compartments with a pH ranging from 6.3 to 6.8 (Jovic et al., 2010). From these early endosomes, MHC class I molecules can enter into the fast recycling pathway, a process driven by the activity of the small GTPase Rab4 (Daro et al., 1996; van der Sluijs et al., 1992) or through a Rab35-dependent mechanism (Allaire et al., 2010). In contrast, the slow recycling pathway involves an intermediate transit through recycling endosomes, tubulovesicular structures exhibiting a luminal pH close to 7, which sort internalized cargo to the cell surface (Goldenring, 2015). Three small GTPases were very well described as key regulators of the MHC class I molecules slow recycling pathway; Rab11a (Ullrich et al., 1996), Rab22a (Weigert et al., 2004) and the ADP-ribosylation factor 6 (Arf6). In addition, Arf6 is important for the endocytosis of MHC class I molecules from the plasma membrane in a clathrin-independent mediated transport (Radhakrishna and Donaldson, 1997).

A pioneering study in APCs on recycling of MHC class I and endosomal peptide loading that involved cell fractionation, identified an acidic, potential MHC class I and class II loading compartment (Grommé et al., 1999). In a different experimental setting, TAP1-deficient macrophages were preincubated with peptide ligands which enhanced the stabilization of intracellular peptide/MHC class I molecules complexes and cross-presentation capacity of *E. coli*-derived OVA. This experiment suggests that peptide dissociation/exchange takes place within an endocytic compartment of controlled pH (Chefalo and Harding, 2001). A study using the DC-like murine cell line DC2.4 and performing a siRNA-based screen of 57 Rab GTPases revealed that 12 of them are potentially involved in antigen cross-presentation. The authors characterized $Rab3b/3c^+$ vesicles that colocalize with MHC class I molecules and transferrin at a perinuclear tubular structure. Moreover, $Rab3b/3c^+$ vesicles were observed at a juxtaposition with *E. coli*-containing phagosomes and colocalized with Rab27a. Although the depletion of Rab3b/3c significantly reduced the fast recycling of the transferrin receptor, a precise role for these GTPases in the slow recycling pathway of MHC class I molecules has not been addressed (Zou et al., 2009).

Two recent studies conducted in DCs shed light on the molecular basis that may govern intracellular recycling of MHC class I molecules which

might have implications for cross-presentation. The first one addresses the role of Rab11a in phagosomal recruitment of MHC class I molecules and antigen cross-presentation by DCs (Nair-Gupta et al., 2014). This small GTPase is a widely used ERC marker and controls the slow recycling pathway of MHC class I molecules in several cell types (Ullrich et al., 1996). Rab11a is localized in the *trans*-Golgi network and other dynamic post-Golgi vesicles, regulating critical intracellular trafficking steps at the interface of endocytic and exocytic pathways (Welz et al., 2014). In this novel study, Nair-Gupta et al. show that MHC class I molecules are selectively delivered to DC phagosomes following TLR signaling in a Rab11a-dependent and Sec22b-independent mechanism. The authors observed that DCs store a large intracellular reserve of MHC class I molecules in the ERC, defined as $Rab11a^{+}/VAMP3^{+}/VAMP8^{+}$ endosomes devoid of ERGIC and ER markers. Upon silencing Rab11a in DCs, phagosomal enrichment of MHC class I molecules was lost and the cross-presenting ability was strongly reduced. The authors further demonstrated that TLR signals are important to trigger phosphorylation of SNAP23 in phagosomes, thus stabilizing the SNARE complexes that mediate ERC-phagosome fusion and allowing for the delivery of MHC class I molecules to DC phagosomes (Nair-Gupta et al., 2014).

The second study focused on Rab22a, which interestingly also colocalizes at $Rab11^{+}$/MHC class I^{+} endosomal recycling compartments (Cebrian et al., 2016). In different cell types, Rab22a is distributed in early and sorting endosomes, associates with tubular recycling structures and is required for endosomal sorting and recycling of cargo (reviewed extensively in Mayorga and Cebrian, 2018). In DCs, Rab22a is found in similar subcellular compartments, but is recruited to DC endosomes and phagosomes with kinetics characteristic for early endosomal markers. After silencing Rab22a in DCs, the intracellular pool of MHC class I in the ERC was drastically reduced, and these molecules were no longer efficiently recruited to phagosomes or recycled to the plasma membrane. As a result, Rab22a KD cells failed to cross-present soluble, particulate and *Toxoplasma gondii*-associated exogenous antigens. Nevertheless, endogenous MHC class I antigen presentation was not disrupted by the silencing of Rab22a, indicating a functioning ER to Golgi transport (Cebrian et al., 2016). Interestingly, Rab22a does not play a relevant role during the delivery of ER components to DC phagosomes, but it does interfere with the normal recruitment of ER-derived proteins to DC endosomes. In line with this, endosomal maturation but not phagosomal maturation of internalized latex beads was altered following silencing of Rab22a (Croce et al., 2017).

In summary, while the existing evidence points to a major role for recycling of MHC class I molecules in cross-presenting DCs additional investigations are needed in order to better understand its role in cross-presentation. More studies might help to identify new molecular targets and develop helpful tools to further study this process.

4.4 Trimming of the antigen within the cross-presenting organelle and loading onto MHC class I molecules

In order to optimize proteasome-degraded antigenic peptides in phago/endosomes, the PLC (as described above) must be recruited to the cross-presenting organelle. Although such recruitment is subject to debate, many studies have addressed and corroborated the presence of ER-derived proteins, as well as MHC class I molecules, within phagocytic compartments. The first studies demonstrated the presence of ER-resident proteins in phagosomes of macrophages and *Dictyostelium discoideum* (Gagnon et al., 2002; Muller-Taubenberger et al., 2001). While a separate study failed to detect ER-resident proteins in phagosomes (Touret et al., 2005), many groups have reported the presence of ER proteins within phagosomes using different methods such as optical and electron microscopy. The first solid evidence of an interaction between the ER and phagosomes was published by Cresswell et al., who demonstrated that *N*-glycosylation, a specific feature of the ER, could also occur within phagosomes of human DCs (Ackerman et al., 2006), a result later corroborated in mouse DCs (Cebrian et al., 2011).

Amigorena's laboratory further demonstrated a missing link between ER and phagosomes. The SNARE protein Sec22b (resident in the ER-Golgi intermediate compartment, ERGIC) and its partner STX4 promote the fusion of vesicles containing ER cargo with phagosomes, and further recruit TAP and the PLC to the cross-presenting organelle (Cebrian et al., 2011) (Fig. 7). Genetic silencing of Sec22b impairs the recruitment of the PLC, decreases antigen export to the cytosol, accelerates phagosomal maturation and thereby inhibits cross-presentation. Of notice, classical MHC class I and II pathways are not affected by silencing of Sec22b. The importance of phagosomal maturation for cross-presentation has also been established in other articles (Alloatti et al., 2015, 2016; Samie and Cresswell, 2015). Furthermore, a mice with a DCs specific deletion of Sec22b demonstrated a defect in cross-presentation in vivo, while classical MHC class I and II presentation remained unaffected (Alloatti et al., 2017).

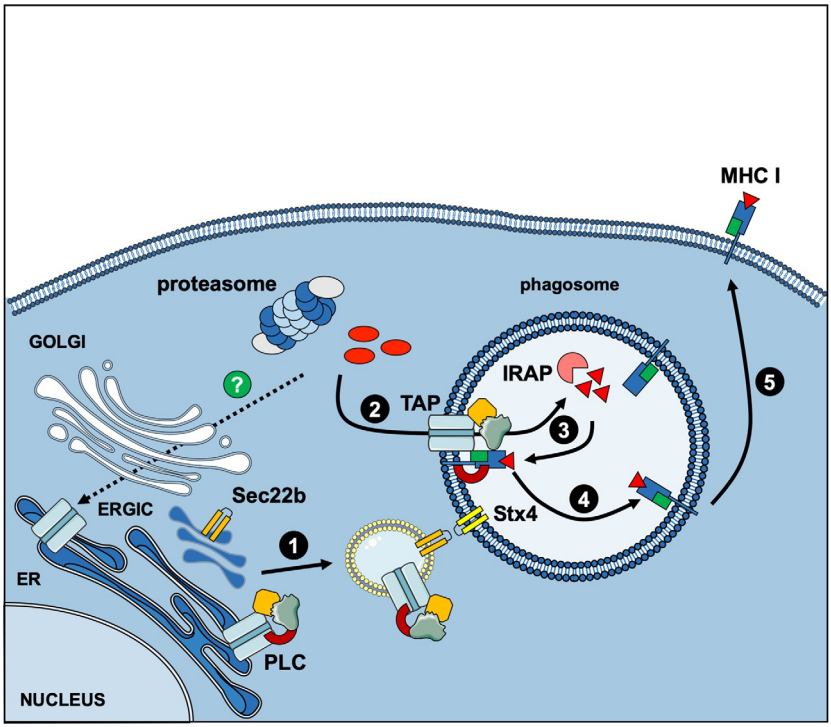

Fig. 7 Delivery of ER-resident proteins to the phagosome and peptide processing and loading. (1) the constituents of the PLC (calreticulin, ERp57, tapasin, TAP1 and 2) are recruited to the cross-presenting organelle in a mechanism mediated by the SNARE protein Sec22b, an ERGIC-resident protein. The interaction of Sec22b and its partner, Stx4 promotes membrane fusion of the vesicles containing Sec22b with the phagosome, and hence ER cargo is delivered to the organelle; (2) antigens degraded by the proteasome are translocated to the phagosomes by TAP, and (3) associated with the PLC are further trimmed by the aminopeptidase IRAP; (4) the MHC class I/peptide complex is released from the PLC and (5) transported to the cell surface where is recognized by $CD8^+$ T cells and prime specific immune responses. Steps not fully characterized, with unclear or conflicting evidence, are showed in arrows with dashed lines.

The presence of aminopeptidases in phagosomes, such as IRAP (a close relative of ERAP1) also validates the hypothesis of phagosomal loading of antigenic peptides. Silencing of ERAP1 and IRAP inhibit antigen cross-presentation (Saveanu et al., 2005, 2009; Weimershaus et al., 2012). Finally, OVA-derived peptides loaded onto MHC class I molecules have been detected in phagosomes (Ackerman et al., 2006; Basha et al., 2008; Burgdorf et al., 2008; Crespo et al., 2013; Guermonprez et al., 2003). These data strengthen the idea that the phagosome (or endosome) itself constitutes

a cross-presenting organelle and current evidence favors phagosomal loading over ER loading. Still, many features remain partially or completely unidentified such as the role of the "vacuolar" route in vivo, the mechanisms involved in antigen export to cytosol, TAP-independent "cytosolic" route, and he identification of other key participating proteins, while some of the existing data remains ambiguous (Touret et al., 2005; Wu et al., 2017). Hence more research is needed in order to understand and clarify the pathway.

5. Cross-presentation in immunity and cancer. Immunotherapy and beyond

Cross-presentation has been implicated in immune responses against multiple pathogens. However, $CD8^+$ T cell responses can be generated either by direct- or cross-presentation, which depends on the nature of the microbe. For instance, anti-vaccinia $CD8^+$ T cell responses are mainly a result of direct-presentation (Hickman et al., 2008; Xu et al., 2010), as demonstrated by dynamic intravital imaging. $CD8^+$ T cell responses to *Toxoplasma gondii*, however, are mainly generated by cross-presentation of antigens captured from their surroundings by uninfected DCs (John et al., 2009). Several other reports have suggested the relevance of antigen cross-presentation in the control of viral, bacterial or parasitic infections (Allan et al., 2003, 2006; Belz et al., 2004; Shio et al., 2013; Wilson et al., 2006). The most relevant evidence was generated by Hildner et al., who demonstrated that mice genetically silenced for Batf3, lacking the two most important cross-presenting DCs subsets (resident $CD8^+$ DCs and migratory $CD103^+$ DCs), mount weak immune responses against Sendai and West Nile viruses (Hildner et al., 2008). However, the lack of cross-presenting DC subtypes could also affect direct antigen presentation and in following studies, several groups demonstrated that the absence of such DCs subtypes promotes a marked decrease in IL-12 production, which can have significant impact on the ensuing immune response (Ashok et al., 2014; Iborra et al., 2015; Mashayekhi et al., 2011). This study addressed the relevance of cross-presentation and cross-presenting cells for the first time in vivo.

The role of antigen cross-presentation in priming antitumor immune responses is, however, less clear, but evidence highlighting the relevance of such a mechanism is accumulating. In 2003, Nowak et al. showed that the induction of apoptosis in tumor cells increased antigen cross-presentation and cross-priming of specific $CD8^+$ T cells against tumor antigens, thus

controlling tumor growth (Nowak et al., 2003). More importantly, Batf3 KO mice were found to be deficient in eliciting $CD8^+$ cytotoxic responses against syngeneic tumors (Hildner et al., 2008). Additionally, the group of Dr. Krummel identified a particular subset of DCs batf3-dependent with the capacity to infiltrate solid tumors and cross-present tumor antigens in order to prime or reprime specific $CD8^+$ T cells (Broz et al., 2014). However, other batf3-independent DC subsets have been implicated in the immune system-dependent antitumor activity of anthracyclines (Ma et al., 2013) and mediate tumor control under activating conditions in batf3-deficient mice (Tussiwand et al., 2012). In order to isolate the role of antigen cross-presentation for this finding, the group could further demonstrate that mice, deficient in the WDFY4, a protein thought to participate during the translocation of dead-cell antigen to the cross-presenting organelle, were deficient controlling tumor growth, as well as eliciting anti-viral $CD8^+$ T cell immune responses (Theisen et al., 2018). In accordance with this, mice with a DC specific deletion of Sec22b also failed to elicit $CD8^+$ T cell responses against dead cell-derived antigens and failed to control EG7-OVA tumor growth (Alloatti et al., 2017). Importantly, Sec22b-dependent antigen cross-presentation proved to be important for anti-PD-1 treatment efficacy. Melero et al. also showed that both cross-presenting DC subsets, migratory $CD103^+$ DCs and resident $CD8^+$ DCs, are required for efficient anti-PD-1 treatment and radiotherapy of tumors (Sanchez-Paulete et al., 2016). In summary, cross-presentation seems to play a critical role not only by eliciting antitumor $CD8^+$ cytotoxic responses, but also by regulating the outcome of anti-immune checkpoint therapies.

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