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REVIEW

Lysosome-Related Organelles: a View from Immunity and Pigmentation

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ABSTRACT. **Lysosomes are ubiquitous organelles that carry out essential household functions. Certain cell types, however, contain lysosome-related organelles with specialized functions. Their specialized functions are usually reflected by specific morphological and compositional features. A number of diseases that develop due to genetic mutations, pathogen exposure or cell transformation are characterized by dysfunctional lysosomes and/or lysosome-related organelles. In this review we highlight adaptations and malfunction of the endosomal/lysosomal system in normal and pathological situations with special focus on MHC class II compartments in antigen presenting cells and melanosomes in pigment cells.**

Key words: MHC class II compartments/exosomes/melanosomes/lysosomes/multivesicular bodies

Lysosome-related organelles (LRO) comprise a heterogeneous group of cell-type specific organelles that have features in common with lysosomes and other late endocytic organelles. Most LRO have been defined in cells of the hematopoietic lineage (Table I), and include dense granules and α-granules in platelets, major histocompatibility complex (MHC) class II compartments in antigen presenting cells (APCs) and cytotoxic granules of T lymphocytes and natural killer cells (NK). LRO are, however, also found in non-hematopoietic cells, including lamellar bodies in type 2 lung epithelial cells and melanosomes in melanocytes of the skin and retinal pigment epithelium (RPE) (for review see Marks and Seabra, 2001). LRO share compositional and physiological characteristics with conventional lysosomes, such as the presence of resident lysosomal proteins and a low lumenal pH. They often also have ultrastructural characteristics of lysosomes, including an electron-dense content composed of protein deposits and/or intraluminal membranes. In contrast to the classical lysosome, for which the primary function is to hydrolyse exoplasmic macromolecules, LRO have additional and/or separate unique functions peculiar to their cell type. Among these functions for most LRO, often referred to as secretory lysosomes (Blott and Griffiths, 2002), is the secretion of their contents into the extracellular milieu.

Depending on the cell type LRO may either compose the entire pool of lysosomes or co-exist in the host cell together with *bona fide* lysosomes. The co-existence of some LRO and lysosomes reflects a distinct adaptation of the host cell for the biogenesis and/or secretion of LRO. The distinction between LRO that coincide or co-exist with conventional lysosomes is reflected by the existence of a number of pathological situations in which the structure and function of lysosomes and LRO are either similarly or unequally affected. In recent years, the identification of proteins that affect the biogenesis of lysosomes and LRO, encoded by genes that are defective in certain disease states, has accelerated research in this field. Lysosome and LRO dysfunction is an elemental component of a group of genetic diseases in mice and man, including Chediak-Higashi syndrome (CHS) (Ward *et al.*, 2000), Hermansky-Pudlak syndrome (HPS) (Huizing *et al.*, 2000), and Griscelli syndrome (GS) (Sanal *et al.*, 2002). The genes implicated

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Abbreviations: AP, Adaptor protein; APC, Antigen presenting cell; BSA, Bovine serum albumin; CHS, Chediak-Higashi syndrome; DC, Dendritic cell; EBV, Ebstein Barr virus; ER, Endoplasmic reticulum; FDC, Follicular dendritic cells; GFP, green fluorescent protein; GS, Griscelli syndrome; HLA, Human leukocyt antigen; HPS, Hermansky-Pudlak syndrome; IEM, Immuno electron microscopy; Ii, Invariant chain; LRO, Lysosome related organelles; MHC, Major histocompatibility complex; MIIC, MHC class II-enriched compartment; MVB, Multivesicular bodies; PAG, Protein A-Gold; RPE, Retinal pigment epithelium; TGN, Trans golgi network.

Table I. LYSOSOME-RELATED ORGANELLES

Organelle	Tissue distribution	Physiologic function
Melanosomes	Melanocytes, retinal pigment epithelial cells	Melanin formation, storage, and transfer
Platelet dense granules	Platelets, megakaryocytes	Release of ATP, ADP, serotonin and calcium for blood clotting
Lamellar bodies	Lung epithelial type II cells	Storage and secretion of surfactant for lung function
Lytic granules	Cytotoxic T lymphocytes, NK cells	Destruction of virally-infected or cancerous target cells
MIIC (Major histocompatibility complex class II compartments)	Antigen presenting cells (dendritic cells, B lymphocytes, macrophages, others)	Processing and presentation of antigens to $CD4^+$ T lymphocytes for immune regulation
Basophilic granules	Basophils, mast cells	Triggered release of histamines, other inflammatory stimuli
Azurophilic granules	Neutrophils, eosinophils	Release of microbicidal and inflammatory agents
Osteoclast granules	Osteoclasts	Bone resorption and remodeling
Weibel-Palade bodies	Endothelial cells	Maturation and regulated release of von Willebrand factor into blood
Platelet α granules	Platelets, megakaryocytes	Fibrinogen and von Willebrand factor release for platelet adhesion and blood clotting

in these syndromes all appear to encode proteins that play key roles in organelle biogenesis or organelle motility. The biogenesis or function of lysosomes and/or LRO may also be affected in acquired diseases, such as cell transformation. Finally, pathogens, such as certain viruses and parasites, may enter the cell via lysosomes or take advantage of the biogenetic and secretory capacities of LRO and lysosomes for their replication and propagation. In this review we focus primarily on two distinct LRO model systems, MHC class II compartments in APCs and melanosomes in pigmented cells (Fig. 1).

MHC class II compartments are modified late endosomes and lysosomes

Acquired immune responses are initiated and controlled by the presentation to T lymphocytes of antigen-derived peptides at the cell surface of target cells or APCs by MHC molecules (Germain, 1994). MHC class I molecules are ubiquitously expressed, whereas MHC class II molecules are expressed predominantly by specialized APCs, including macrophages, B lymphocytes, dendritic cells (DCs) and thymic epithelial cells; MHC class II molecules can also be expressed upon cytokine stimulation of other cell types, including mast cells, melanocytes and epithelial cells. With the aid of associated invariant chain (Ii), newly synthesized MHC class II molecules are routed from the endoplasmic reticulum (ER) via the Golgi to compartments in the endocytic tract. Here the Ii is degraded, ultimately allowing binding of peptides to MHC class II. Such peptides may arise from the proteolysis of self-proteins, present within the endocytic pathway, or of exogenous proteins, which can be internalized by receptor-mediated endocytosis, fluid phase pinocytosis or phagocytosis (Watts, 1997; Wolf and Ploegh, 1995). After binding peptide, MHC class II is transported to the plasma membrane for presentation to $CD4^+$ T cells. Our understanding of how and where MHC class II meets antigen-derived peptides has increased significantly during the last few years through the combined use of high-resolution immunoelectron microscopy (IEM), subcellular fractionation and biochemical methods (Kleijmeer *et al.*, 1996; Pierre and Mellman, 1998). These studies have in turn taught us a great deal about the conventional endocytic system.

The endocytic system is composed of a series of sequential endosomal compartments and lysosomes. Endocytosed antigens and newly synthesized MHC class II meet each other at a number of stages within the endocytic tract ranging from early endosomes to lysosomes. Likewise, both the proteolytic processing of MHC class II-associated Ii and endocytosed proteins and the peptide loading of MHC class II are initiated in earlier endosome compartments but may

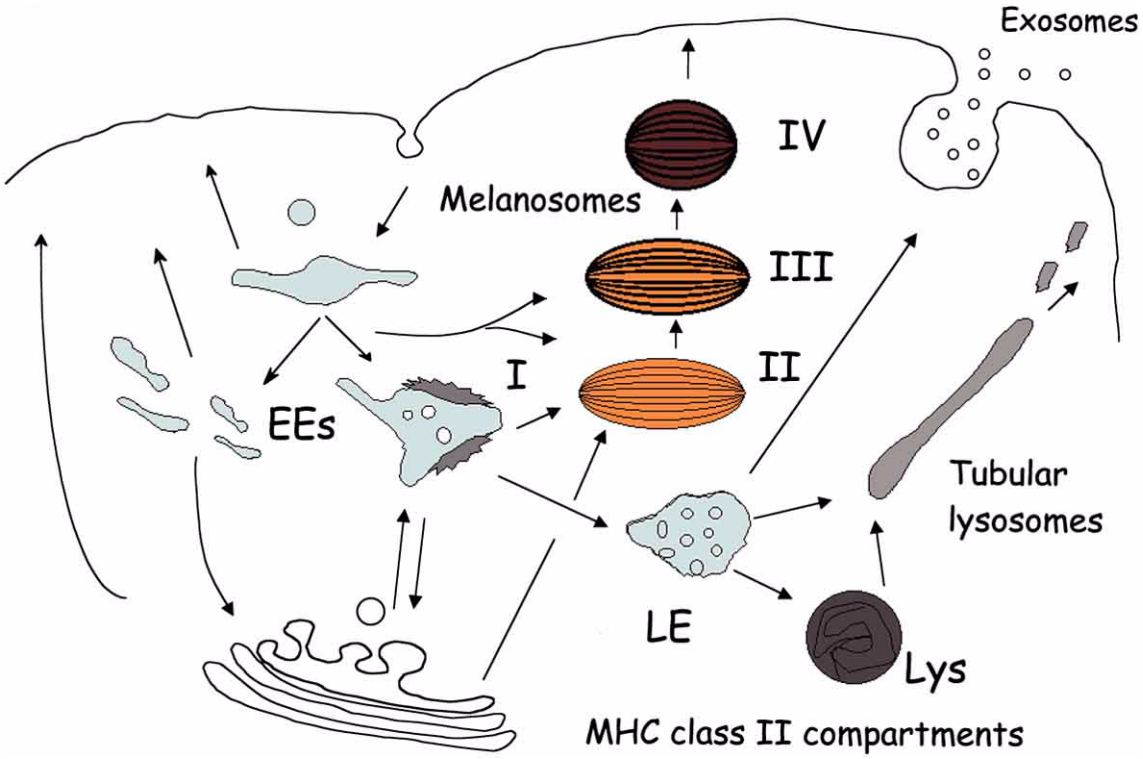

Fig. 1. Simplified schematic representation of the intracellular compartments of melanocytes and APCs and their relationship with the organelles of the endocytic pathway. Stage I premelanosomes correspond to maturing multivesicular endosomes characterized by abundant planar bilayered clathrin coats. Specific sorting and processing events drive the formation of striated stage II pre-melanosomes (Pmel-17 plays a key role in this step). Melanin synthesis starts in stage III melanosomes with melanin deposits apparent on internal striations. Stage IV melanosomes are mature, fully pigmented, and ready to be transferred to keratinocytes. During melanosome maturation, newly formed stage II pre-melanosomes receive melanosomal components (including melanogenic enzymes such as ,Tyrosinase and Tyrp1) from the biosynthetic and endocytic pathways. Despite their derivation from and interrelationship with endosomes, melanosomes in highly pigmented cells are distinguishable from lysosomes. In antigen presenting cells MHC class II compartments correspond to late multivesicular endosomes and to multilaminar lysosomes. Bilayered coats are less abundant in APCs. Multivesicular MIICs can fuse with the cell surface, resulting in exosome release. Both subtypes of MIICs are able to tubulate upon membrane reorganisation in response to antigen stimulation and T cell contact. These tubular lysosomes fuse with the cell surface, exposing MHC class II-peptide complexes at the plasma membrane. As detailed in the text, each transport step is regulated by different molecular machineries.

continue in late endosomes and lysosomes from where they are finally delivered to the cell surface (see below). MHC class II expressing cells thus exploit the ubiquitous endocytic system to load MHC class II with foreign antigens (reviewed in Kleijmeer and Raposo, 2001).

Initial ultrastructural studies on human B lymphocytes revealed the presence of MHC class II molecules in late endosomal and lysosomal compartments (Peters *et al.*, 1991a). These compartments were collectively designated MHC class II-enriched compartments (MIICs) (Peters *et al.*, 1991a), and characterized by internal, typical concentrically arranged, multilaminar membranes (Fig. 2B), relatively low internal pH, and the presence of lysosomal hydrolases (β-hexosaminidase and cathepsin D) and lysosomal membrane proteins (Lamps). Since this initial characterization, numerous ultrastructural and immunocytochemical studies on mouse B cells, human Epstein Barr Virus (EBV)-transformed B cell lines, mouse macrophages, and human and mouse DCs, revealed that MIICs are a more heterogeneous population of structures than originally thought. At least three types of MIICs could be morphologically defined, multilaminar, multivesicular and intermediate types (Kleijmeer *et al.*, 1997; Peters *et al.*, 1995; Raposo *et al.*, 1996). Multivesicular MIICs that contain internal vesicles are reminiscent of a class of late endosomes referred to as multivesicular bodies (MVBs) (Stahl and Barbieri, 2002) (Fig. 2A). Late endosomes are formed by maturation of early endosomes, a process that involves the formation of vesicles within the lumen of endosomes by invagination of the limiting membrane (Stoorvogel *et al.*, 1991). Multilaminar MIICs are reminiscent of conventional lysosomes in other cell types. Intermediate MIICs may result as a consequence of the fusion of multivesicular and multilaminar MIICs, consistent with the notion that transfer from late endosomes to lysosomes may occur through direct fusion of these compartments as suggested by *in vitro* fusion experi-

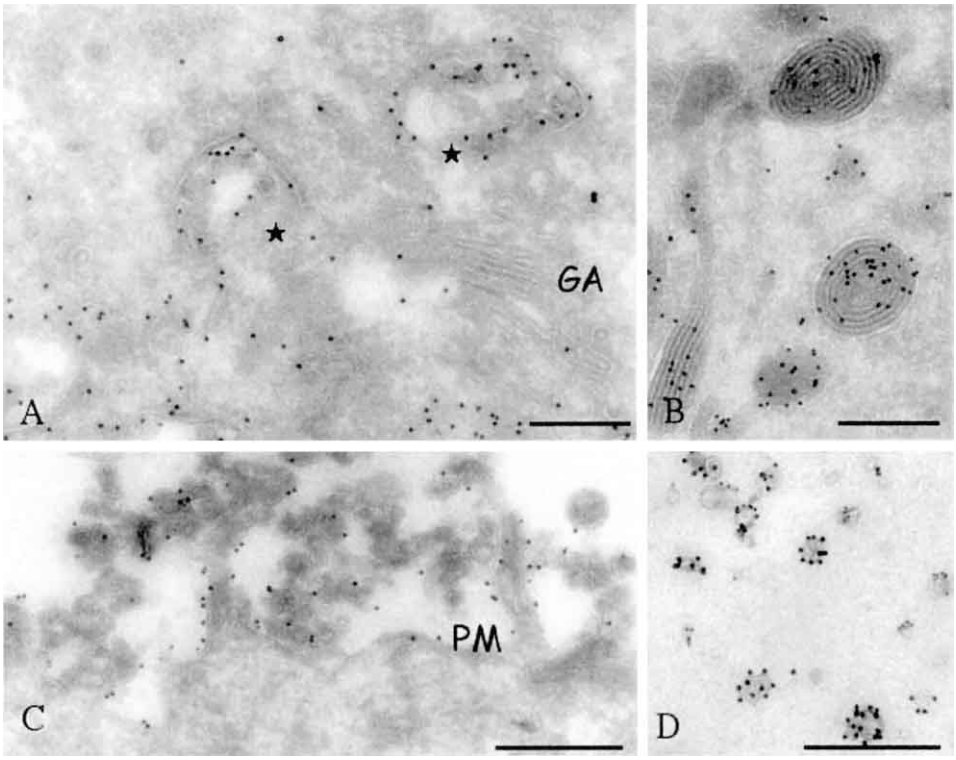

Fig. 2. MHC class II compartments and exosome secretion. A: Ultrathin cryosections of human B cells immunogold labeled for MHC class II. Multivesicular MIICs are indicated by stars. GA, Golgi Apparatus. B: Ultrathin cryosections of immature human dendritic cells immunogold labeled for MHC class II. Abundant labeling for MHC class II in multilaminar MIICs. C: Exocytic fusion of multivesicular MIICs in human B cells. Ultrathin cryosections were immunogold labeled for MHC class II (PAG 15) and ICAM-1 (PAG 10). Exosomes carrying MHC class II and ICAM-1 are secreted into the extracellular space. D: Exosomes purified from the cell culture supernatants of dendritic cells. Exosomes were immunogold labeled for CD63 (PAG 15) and MHC class II (PAG10). Bars: 200 nm.

ments (Luzio *et al.*, 2001). MIICs contain ubiquitous lysosomal components as well as specific proteins such as Ii, HLA-DM, and in some APCs, HLA-DO (Neefjes, 1999). The relative concentrations of these markers, however, differ significantly between MIIC subtypes. In addition to their morphological appearance and protein composition, MIICs — like endosomes — can be classified based on their accessibility to endocytic tracers. Multivesicular MIICs are accessed by endocytic tracers (e.g. BSA coupled to gold) 20 min after uptake, whereas 1 to 2 hrs are required to reach multilaminar MIICs (Kleijmeer *et al.*, 1996). Thus, consistent with their morphological characterization, multilaminar MIICs are positioned later in the endocytic tract than multivesicular MIICs. Interestingly, the amount of intact Ii, but not MHC class II or HLA-DM, decreases while progressing through the endocytic tract (Kleijmeer *et al.*, 1997). These studies, together with the demonstration of MHC class II-peptide complexes in MIICs and the competence of purified MIICs to present antigen (Morkowski *et al.*, 1997; Turley *et al.*, 2000; West *et al.*, 1994), clearly indicate that MHC class II peptide loading occurs predominantly in late endosomes and lysosomes.

MIICs represent a heterogeneous set of secretory lysosomes

The interaction of APCs with T cells is dependent on their ability to correctly expose MHC class II-peptide complexes on the cell surface. MHC class II peptide complexes thus need to be transferred from late endosomes and/or lysosomes to the plasma membrane.

Interestingly, in many cell types the majority of intracellular MHC class II is localized to the internal membranes of multivesicular MIICs (Kleijmeer *et al.*, 2001; Raposo *et al.*, 1996). These internal vesicles have three distinct potential fates. Firstly, as discussed above, fusion of the limiting membrane of multivesicular MIICs with lysosomes (or multilaminar MIICs) results in targeting to these compartments. Secondly, as demonstrated for immature DC, multivesicular MIICs can serve as a compartment for long term storage of MHC class II until they are bound to peptide and delivered to the cell surface upon receipt of a maturation stimulus. Finally, the limiting membrane of multivesicular MIICs may fuse with the plasma membrane, resulting in the release of their internal vesicles (reviewed in Stoorvogel *et al.*, 2002) (Fig. 2C, 2D). Our ultrastructural studies (Raposo *et*

al., 1996; Zitvogel *et al.*, 1998), together with *in vivo* time lapse video of green fluorescent protein (GFP)-tagged MHC class II constructs (Wubbolts *et al.*, 1997), captured evidence of just such a process for multivesicular MIICs in B cells, immature dendritic cells and melanoma cells. This fusion allows transfer of MHC class II from the limiting membrane of MIICs to the plasma membrane (Wubbolts *et al.*, 1997). However, given that the majority of MHC class II in multivesicular MIICs is associated with the internal vesicles rather than the limiting membrane, this process mainly results in the secretion of MHC class II in association with vesicles (Raposo *et al.*, 1996). Analogous to similar vesicles first reported to be secreted by reticulocytes (Harding *et al.*, 1984; Johnstone, 1992), these class II-positive vesicles were called exosomes. In B cells, ~10% of all newly synthesized MHC class II is secreted in association with exosomes after 24 hrs; the majority of MHC class II, however, is transferred to the plasma membrane within 3–4 hours of synthesis (Raposo *et al.*, 1996). Direct fusion of MIICs with the plasma membrane thus cannot account for the transfer of the majority of MHC class II to the plasma membrane. The idea that other transport routes also operate to transfer MHC class II-peptide complexes to the cell surface is consistent with the sensitivity of this process to brefeldin A, a drug known to interfere with the ARF-dependent assembly of several classes of coated transport vesicles (Pond and Watts, 1997); this implicates a vesicle-mediated process, rather than ARF-independent direct fusion of MIICs with the plasma membrane. Among coat proteins known to be involved in endosomal sorting processes, only clathrin has been observed on MIICs, but clathrin coats do not seem to recruit MHC class II for transport to the plasma membrane (Ramm *et al.*, 2000).

A clue for a major transport pathway of class II-peptide complexes to the plasma membrane in DCs comes from recent ultrastructural and time lapse video microscopy studies in which the incredible plasticity and dynamics of the late endosomal/lysosomal system is illustrated (Boes *et al.*, 2002; Chow *et al.*, 2002; Kleijmeer *et al.*, 2001). Immature DC in peripheral tissues act as sentinels for the immune system, constantly sampling extracellular material through macropinocytosis. Curiously, the material internalised during this time is not efficiently degraded in endosomes and lysosomes, and peptides derived therefrom fail to be efficiently loaded onto MHC class II molecules. The MHC class II in these cells is largely associated with the intralumenal vesicles of multivesicular MIICs (in murine DCs) (Kleijmeer *et al.*, 2001) and in multilaminar MIICs (in human DCs) (Barois *et al.*, 2002). After stimulation with pathogens and/or cytokines, DC mature and migrate to secondary lymphoid organs to present acquired antigens to specific T lymphocytes. During this process, internalisation slows, and internalised material that had been previously stored within the DCs is now degraded to peptides (Thery and Amigorena, 2001). The peptides are now efficiently loaded onto MHC class II, providing a rich source of intracellular peptide-loaded MHC class II molecules on the intralumenal vesicles of multivesicular MIICs. However, rather than being secreted from the cell in the form of exosomes, these vesicles now fuse with the MVB limiting membrane. As a consequence, MIICs develop long tubular extensions, and the DCs lose their ability to secrete exosomes (Kleijmeer *et al.*, 2001). Tubular lysosomal structures, carrying MHC class II molecules may then have the ability to fuse directly with the cell surface (Boes *et al.*, 2002; Chow *et al.*, 2002) in a process reminiscent of the fusion of conventional lysosomes for plasma membrane repair in response to mechanical rupture (Reddy *et al.*, 2001). Alternatively, transfer may be mediated by 100 nm transport vesicles that derive from the tips of these tubules (Kleijmeer *et al.*, 2001). Ultrastructural studies on human DC indicate that multilaminar MIICs, which are positioned later in the endocytic pathway than multivesicular MIICs, also have the ability to form tubules (Barois *et al.*, 2002). Stimulated fusion of tubulating MIICs or vesicles that derive therefrom with the plasma membrane results not only in surface exposure of MHC class II-peptide complexes but also in a rapid increase of the amount of plasma membrane required for the formation of the many dendrites that are characteristic of mature DCs (Mellman and Steinman, 2001). Similar MIIC re-organisations may also occur in B lymphocytes upon stimulation through the B cell receptor (Lankar, Raposo, Bonnerot, unpublished data), and likely contributes to the facilitated presentation of antigen for which the B cell receptor is specific (Lanzavecchia, 1985).

Characteristics and putative functions of exosomes

In contrast to maturing DC, the internal vesicles of MVBs in immature DC, B cells, cytotoxic T cells, mast cells, platelets and many other cell types do not fuse with the MVB limiting membrane but rather are released from the cell as exosomes upon fusion of the MVB with the plasma membrane (Peters *et al.*, 1991b; Raposo *et al.*, 1996; Zitvogel *et al.*, 1998). Exosomes are present in blood (unpublished data) and can be isolated from cell culture supernatants. Depending on their origin, their diameters range from 30–90 nm (Fig. 2D). Their protein composition is unique and distinct from that of other organelles, such as, early endosomes and the plasma membrane, as determined by immunoelectron microscopy, Western blotting and mass spectrometry (Escola *et al.*, 1998; Raposo *et al.*, 1996; Thery *et al.*, 1999; Thery *et al.*, 2001). Among their constituents are both ubiquitous and cell type-specific proteins, and the content of ubiquitious proteins can vary from cell to cell. For example, exosomes from B lymphocytes, dendritic cells, mast cells and intestinal epithelial cells are enriched in MHC class II and MHC class I molecules, whereas cytotoxic T cell derived exosomes contain primarily perforin and

granzymes (Peters *et al.*, 1991b). Some of the common proteins are cytosolic chaperone proteins (e.g. Hsc70, and Hsc 90), subunits of trimeric G proteins, components of the MVB formation machinery (e.g. Tsg 101), cytoskeletal proteins (e.g. actin, tubulin, moesin), and tetraspanins (e.g. CD9, CD63, CD81, CD82) (reviewed in Stoorvogel *et al.*, 2002; Thery *et al.*, 2002). Consistent with their origin, the protein composition of exosomes is similar to that of the internal vesicles of MVBs and distinct from that of the MVB limiting membrane. For example, in B lymphocytes and dendritic cells, MHC II, MHC I and tetraspanins are largely confined to the internal vesicles, whereas Lamp-1 and HLA-DM reside primarily at the limiting membrane and are largely excluded from exosomes (Escola *et al.*, 1998; Hammond *et al.*, 1998; Kleijmeer *et al.*, 2001). The lipid content of exosomes and the internal vesicles of MVBs is also unique. For example, they are enriched in cholesterol and sphingomyelin (Mobius *et al.*, 2002; Wubbolts *et al.*, 2003), two lipids that have been found to be critical in the maintenance of membrane rafts (van Meer, 2002). Indeed, the fact that exosomes and internal vesicles of MVBs are enriched in tetraspanins and glycosylphosphatyidylinositol-anchored proteins, two classes of proteins that are known to partition into raft-like microdomains (Claas *et al.*, 2001; Rabesandratana *et al.*, 1998), suggest that membrane rafts may play an important role in sorting of proteins into the internal vesicles at MVBs.

The enrichment of peptide-loaded MHC class II and MHC class I, co-stimulatory molecules, and cell adhesion molecules within exosomes, suggested that they may have immuno-modulatory functions. Indeed B cell derived exosomes were demonstrated to stimulate $CD4^+$ T cells *in vitro* (Raposo *et al.*, 1996). Furthermore, isolated DC-derived exosomes that were loaded with tumor derived peptides *in vitro* were shown to induce anti-tumor responses in mice (Zitvogel *et al.*, 1998). *In vivo*, their physiological relevance is far from being understood, but exosomes may play a critical role in the function of follicular DC (FDC). FDC are accessory cells of the immune system essential for affinity maturation and immunoglobulin isotype switching of B cell clones within germinal centers (Tew *et al.*, 1997). FDC do not express MHC class II themselves nor secrete exosomes, but abundantly display MHC class II on exosomes at their cell surface (Denzer *et al.*, 2000). While antigen-immunoglobulin complexes retained on the surface of FDC are pivotal for selection of high-affinity B lymphocytes, FDC docked B cell exosomes might select and recruit specific T helper cells. In this way, FDC might sieve and facilitate the interaction between matching B and T cells, ultimately resulting in B cell differentiation (Tew *et al.*, 1997). Thus exosomes may represent a refined and regulated way of intercellular communication (Stoorvogel *et al.*, 2002; Thery *et al.*, 2002). This type of communication is not limited to the immune system, but may function in numerous cell and tissue developmental processes. An example is pattern formation in *Drosophila* embryos, which is critically dependent on the graded distribution of morphogens in the developing tissue. Small membranous particles that resemble exosomes, called argosomes, were recently proposed to facilitate the dispersion of the membrane associated morphogen, Wingless, over large distances through the imaginal disc epithelium (Christian, 2002; Greco *et al.*, 2001). Other membrane associated morphogens, such as Hedgehog and Decapentaplegic, may be spread in a similar manner (Entchev and Gonzalez-Gaitan, 2002). With the discovery of exosome-like vesicles in Drosophila it is plausible that their functions have been conserved during evolution. The emerging theme is that exosomes may play important functions in developmental biology.

Melanosomes coexist with lysosomes

MHC class II compartments appear to correspond to endosomes and lysosomes, modified only by the expression of cell-type specific proteins such as MHC class II and Ii. Other LRO, however, appear to be entities distinct from lysosomes within their host cells, and pose novel problems regarding organelle biogenesis and integration with the endocytic pathway. Recent studies suggest that melanosomes are representative of this class of LRO.

Melanosomes are membrane enclosed compartments in epidermal and ocular melanocytes and pigment epithelia of the eye that synthesize and store the pigment melanin. In the skin, they are also secretory organelles, facilitating transfer of melanin from melanocytes to keratinocytes, and thereby generating the characteristic pigmentation of skin and hair (King *et al.*, 1995). In retinal pigment epithelial cells, melanosomes rather appear to function in focusing light and in the detoxification of phagocytosed photoreceptor outer membranes (Marmorstein *et al.*, 1998). In contrast to MIICs, which have specialized as well as conventional functions, melanosomes have unique morphological features and coexist with conventional lysosomes in the same cell. Melanosomes nevertheless share a number of properties associated with conventional lysosomes, such as a low luminal pH (Bhatnagar *et al.*, 1993) and the presence of lysosomal hydrolases and membrane proteins (reviewed in Orlow, 1995). The biogenesis of melanosomes is reflected by defined sequential stages, each with characteristic morphological features (Seiji *et al.*, 1963) (Fig. 3). Stage I premelanosomes are electron-lucent, membrane-enclosed structures containing limited but variable amounts of internal membranes similar to MVBs in other cell types, including multivesicular MIICs in MHC class II expressing cells (Piper and Luzio, 2001). The unique features of melanosomes become more evident in stage II premelanosomes. These are ellipsoid structures with a striated appearance, generated by non-membranous fibers in their lumen. Melanins are deposited on these fibers as they are synthesized, resulting in blackened and thickened striations in stage III

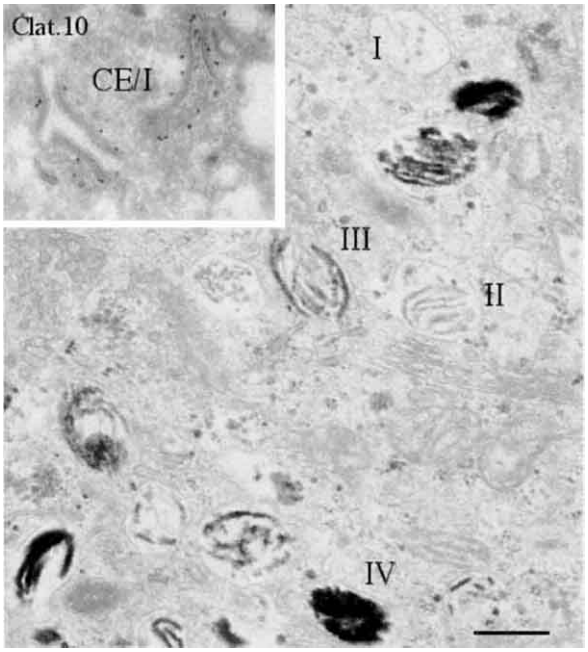

Fig. 3. Distinct melanosomal stages. Detail of an ultrathin section of epon-embedded MNT1 cells. Examples of the different melanosomal stages are indicated by numbers. Inset: Detail of an ultrathincryosection of MNT1 cells immunogold labeled for clathrin. Anti-clathrin antibodies are visualized with PAG 10. Note the stage I pre-melanosome with few internal vesicles and the characteristic planar clathrin-positive coats on its cytosolic side. Bar: 200 nm.

melanosomes. Eventually, the entire luminal space is filled with melanin in stage IV melanosomes. The striations not only function in the sequestration and concentration of melanins, but possibly also to prevent toxification of cells by melanin intermediates as well as to prevent diffusion of melanin after secretion during transfer to keratinocytes. Stage I and II premelanosomes are enriched in Pmel17/gp100, a pigment cell-specific structural protein that associates with the fibrous striations (Raposo *et al.*, 2001). Expression of Pmel17/gp100 in non-pigment cells by transfection is sufficient for the formation of striated endocytic organelles, suggesting that this protein is a major component of the striations (Berson *et al.*, 2001). Pmel17/gp100 is not detected in large amounts in stage III and IV melanosomes, most likely due to encapsulation by melanin and masking of epitopes rather than to its actual absence (Donatien and Orlow, 1995) (and our unpublished observations). By contrast, Stage III and IV melanosomes are enriched in other pigment cell-specific proteins, such as tyrosinase and tyrosinase related protein 1 and 2 (Tyrp1 and 2), enzymes involved in melanin synthesis (Raposo *et al.*, 2001). Although lysosomal proteins are present in these organelles, they are more enriched in cells that make black melanins in separate structures that resemble late endosomes and lysosomes. Why melanosomes and conventional lysosomes develop as separate entities in the same cell is not clear. One possibility is that such an organization would ensure transfer of pigment, but not massive amounts of lysosomal hydrolases, to neighboring keratinocytes.

Adaptations of the biosynthetic and endosomal system for the biogenesis of MHC class II compartments and melanosomes

Newly synthesized MHC-II/Ii complexes accumulate at trans Golgi network (TGN) exit sites that are different from those enriched in other late endosomal cargo, such as mannose 6-phosphate receptors (Glickman *et al.*, 1996; Peters *et al.*, 1995; Peters *et al.*, 1991a). They pass through early endosomal structures and are targeted to late endosomal multivesicular MIICs by virtue of their association with Ii (Brachet *et al.*, 1999; Pond and Watts, 1999). The class II-like molecule HLA-DM, required for the exchange of Ii-derived CLIP fragments for other peptides within the peptide binding groove of MHC-II molecules, seems to be targeted to MIICs by a distinct clathrin-dependent pathway, perhaps bypassing the early endosomal system (Liu *et al.*, 1998; Marks *et al.*, 1995). An additional route to MIICs has been recently shown for the CD1b molecule which may be transported from TGN or endosomes via a pathway distinct from class II and DM and dependent on the adaptor protein AP-3 (Briken *et al.*, 2002; Sugita *et al.*, 2002). Taken together, these data suggest that multiple conventional transport routes are utilized to access late endosomal MIICs in APCs. In these cells, however, the final destination for all of the pathways is the same.

The use of multiple distinct sorting pathways is also a feature of the formation of melanosomes. Residents of premelanosomes, such as Pmel17/gp100 and MART-1, seem to be targeted to these compartments indirectly via vacuolar regions of early endosomes (Berson *et al.*, 2001; De Maziere *et al.*, 2002; Raposo *et al.*, 2001). Markers of mature melanosomes, such as tyrosinase and Tyrp1, appear to be targeted directly from the TGN and/or from tubular early endosomes to preformed stage II melanosomes (Raposo *et al.*, 2001) (and GR, unpublished data). In melanocytes synthesizing black pigments, melanosomal proteins do not accumulate substantially in conventional late endosomes and lysosomes within the same cell. The molecular mechanisms behind this specialisation is unknown. However, it has been observed that Pmel17, tyrosinase and Tyrp1 do accumulate in late endosomes and lysosomes when expressed by transfection in non-pigmented cell types (Berson *et al.*, 2001; Calvo *et al.*, 1999; Vijayasaradhi *et al.*, 1995; Simmen *et al.*, 1999). Thus, melanocytes have developed unique mechanisms within the endocytic pathway that allow melanosomal components to be sorted from conventional lysosomal components, such as Lamps and cathepsins.

Conserved protein complexes seem to function in controlling multiple sorting steps to lysosomes. Among those,

the heterotetrameric adaptor complexes AP-3 and AP-1 are involved in selective protein recruitment at the TGN and in endosomes (Robinson and Bonifacino, 2001). AP-3 binds to a cytoplasmic targeting determinant of tyrosinase (Blagoveshchenskaya *et al.*, 1999; Honing *et al.*, 1998) and appears to regulate sorting of tyrosinase from endosomes and/or the TGN. Indeed melanocytes from HPS2 patients, which lack functional AP-3, accumulate tyrosinase in endosomal structures (Huizing *et al.*, 2001b). Our unpublished studies on the subcellular localization of AP-3 and on the phenotype of mouse AP-3-deficient melanocytes suggest that AP-3 may facilitate transfer of tyrosinase from early endosomes to pre-formed premelanosomes. AP-3 may also regulate the localization of a melanosomal protein from quail, QNR-71 (Le Borgne *et al.*, 2001). On the other hand, another resident of mature melanosomes, Tyrp1, does not appear to be mislocalized in AP-3 deficient melanocytes (Huizing *et al.*, 2001b; GR, unpublished observations) and localizes instead extensively to AP-1 coated membranes (Raposo and Marks, 2002; Raposo *et al.*, 2001). The PDZ domain-containing protein, GIPC (Liu *et al.*, 2001) and a pigment cell-specific rab protein, Rab38 (Loftus *et al.*, 2002) may participate in sorting and/or delivery of Tyrp1 to melanosomes. Transport of neither MHC-II/Ii nor HLA-DM is affected by AP-3 deficiency (Caplan *et al.*, 2000; Sevilla *et al.*, 2001), and there is conflicting evidence regarding a role for AP-1 in MHC-II/Ii transport (Brachet *et al.*, 1999; Salamero *et al.*, 1996). By contrast, emerging evidence demonstrates a requirement for AP-3 in the transport of a related antigen presenting molecule, CD1b, to MIICs (Briken *et al.*, 2002; Sugita *et al.*, 2002). Thus, APCs and melanocytes utilize multiple conserved coat proteins at the TGN and/or early endosomes to regulate trafficking to their specialized compartments.

The multivesicular body: a common sorting station in the biosynthetic and endocytic pathway of APCs and melanocytes

The early endosomal system corresponds to a heterogeneous set of sorting and recycling domains, which, at the ultrastructural level, appear as a network of tubules and irregularly shaped vacuoles surrounded by buds and transport vesicles. They are distributed throughout the cell although the tubular structures are often enriched in the pericentriolar region close to the Golgi apparatus (Geuze *et al.*, 1984; Tooze and Hollinshead, 1991). Endocytosed molecules that are not destined to recycle travel down the endocytic pathway ultimately to lysosomes. MVBs constitute a spatial and temporal intermediate between early endosomes and lysosomes (Piper and Luzio, 2001; Stahl and Barbieri, 2002). Their internal vesicles originate from inward invaginations of the endosome limiting membrane, enwrapping cytosolic material in the process (van Deurs *et al.*, 1993). During this process selected membrane proteins are sequestered in the internal vesicles, whereas others remain at the limiting membrane. Sorting of some proteins to the internal vesicles may occur by virtue of their direct or indirect association with lipid microdomains (see above), whereas other proteins contain specific sorting signals for incorporation into newly forming internal vesicles. Sorting into internal vesicles may serve either to target proteins for degradation upon fusion of MVBs with lysosomes, such as occurs for ligand-stimulated growth factor receptors, or for sequestration of specific functions, such as occurs for MHC class II (Piper and Luzio, 2001).

Similar to the sequestration of MHC class II in internal vesicles of MVBs, the premelanosomal proteins (Pmel17 and MART-1) are sequestered in internal vesicles of MVBs within melanocytes prior to their incorporation within stage II premelanosomes. By contrast, Tyrp1 and Tyrosinase are sorted distinctly to the maturing melanosome and are not incorporated into the internal vesicles (see above). This segregation allows for the enzymes involved in melanin synthesis to be transported to melanosomes after the formation of fibrous striations within pre-melanosomes in a Pmel17-dependent process (Berson *et al.*, 2001; Raposo and Marks, 2002). Sorting of premelanosomal proteins into the internal vesicles may be facilitated by the abundant flat clathrin-containing coats on the cytosolic side of newly forming MVBs in melanocytes (Raposo and Marks, 2002; Raposo *et al.*, 2001). Pre-melanosomal membrane proteins appear to be segregated into coat-enriched domains at the MVB limiting membrane. Similar clathrin coats have been observed, although much less abundant, in other cells (Raiborg *et al.*, 2002; Sachse *et al.*, 2002) and have been proposed to concentrate cargo destined to be incorporated into the MVB internal vesicles; in addition to clathrin, these coats also contain the multifunctional clathrin adaptor Hrs and the tSNARE syntaxin 13 (De Maziere *et al.*, 2002; Sachse *et al.*, 2002). Hrs is an essential component for MVB formation and protein sorting in this compartment (Bilodeau *et al.*, 2002; Bishop and Woodman, 2001; Lloyd *et al.*, 2002; Raiborg *et al.*, 2002), and syntaxin 13 is thought to be involved in endosomal fusion events (Pelham, 2001). The excessive abundance of clathrin coats on maturing melanosomes suggests that this ubiquitous sorting system is used to target pre-melanosomal proteins into the intralumenal vesicles and then to stage II premelanosomes. When expressed in non-melanocytic cells Pmel17 accumulated in typical multivesicular late endosomes, demonstrating that intrinsic properties of this protein are sufficient to drive its transfer into MVBs of non-melanocytic cells (Berson *et al.*, 2001). Similarly, expression of MHC class II in embryonal kidney cells resulted in the formation of MHC class II compartments (Calafat *et al.*, 1994). These observation support the idea that certain unique morphological features may arise from cargo rather than the sorting mechanisms *per se*, and/or that conventional sorting mechanisms can be perverted to provide for unique sorting events.

Dysfunctional MHC class II compartments and melanosomes in genetic diseases, cancer and infection

During the last two decades many pathological conditions have been identified as lysosomal storage diseases (Winchester *et al.*, 2000). These include genetic deficiencies in specific lysosomal enzymes, in modifications required for the transport of these enzymes to lysosomes, and in proteins required for the biogenesis of lysosomes and/or lysosome-related organelles. For the latter class at least three heritable human diseases and their corresponding mouse models have been identified: Griscelli Syndrome, Chediak-Higashi Syndrome and Hermansky-Pudlak Syndrome. GS and its corresponding mouse models are disorders of organelle motility, and result from mutations in genes encoding an unconventional myosin (Myosin Va/*dilute*), a Rab protein (Rab27a/*ashen*) or — in mice — a rab effector protein (melanophilin or *leaden*) (reviewed in Hammer and Wu, 2002; Seabra *et al.*, 2002). These three proteins coordinate transfer of melanosomes within epidermal melanocytes from microtubules to the cortical actin cytoskeleton for their ultimate transfer to keratinocytes. Melanocytes from mutant mice or patients abnormally accumulate stage IV melanosomes in the perinuclear area. Rab27a-deficient *ashen* mice also are deficient in cytotoxic T cell activity due to a failure in appropriate polarization of their cytotoxic granules (Haddad *et al.*, 2001; Stinchcombe *et al.*, 2001), suggesting a general role for Rab27a in regulated secretion from lysosome-related organelles. Such defects are not observed in *dilute* mice, suggesting that Rab27a may act on distinct effectors in different cell types. Indeed, Rab27a participates in the localization of melanosomes to the subapical membrane in RPE by association with a distinct effector, MyRIP (El-Amraoui *et al.*, 2002) and a distinct myosin, myosin VIIa; myosin VIIa is deficient in a heritable form of blindness and deafness known as Usher syndrome (Weil *et al.*, 1995). An analysis of MHC-II processing in mice or humans with GS or related syndromes has not yet been reported.

In contrast to GS, CHS and HPS are diseases of organelle biogenesis. Lysosomes and LRO in patients or mouse models of these diseases have altered morphologies and/or contents as a consequence of failed transport to or from these organelles. CHS (and the corresponding *beige* mouse) is due to mutation of a single gene which encodes a large protein known as Lyst or CHS1p. All lysosomal compartments in CHS patients and *beige* mice are dramatically enlarged. *CHS* mutants are also immunocompromised with known defects in the secretion of cytotoxic granules (Baetz *et al.*, 1995; Stinchcombe *et al.*, 2000) and MIIC formation (Faigle *et al.*, 1998; Lem *et al.*, 1999). B lymphoblastoid cells from CHS patients show defects in transfer of MHC class II to MVBs, perhaps reflecting a role for Lyst/CHS1p in intraorganellar protein sorting (Faigle *et al.*, 1998). Lyst contains two domains that are thought to associate with lipids, including a perilipin domain and a pleckstrin homology domain that is associated with the BEACH domain (Jogl *et al.*, 2002); the involvement of perilipin domains in binding cholesterol-enriched membranes (Servetnick *et al.*, 1995) and of pleckstrin homology domains in binding phosphoinositides (Lemmon and Ferguson, 2000) suggests a potential role of Lyst in interfacing with lipids at the MVB. Analysis of the specific protein transport defects in melanocytes from these mutants may shed light on the biogenetic steps controlled by CHS1p.

HPS is a complex disorder characterized by pigmentation and bleeding defects, lung fibrosis and ceroid accumulation in lysosomes. In contrast to CHS, where both conventional lysosomes and LRO are affected, in HPS major defects are observed in specific LRO including melanosomes and platelet dense granules (Huizing *et al.*, 2000; Spritz and Oh, 1999). HPS can result from mutations within any of at least four genes, and a similar disorder in mice results from defects within any of up to 16 genes. Some of these genes, including HPS1/*pale ear*, HPS3/*cocoa*, and HPS4/*light ear* encode proteins with no known conserved structural domain or function. Others encode proteins known to regulate protein traffic, including subunits of the AP-3 complex (defective in *mocha* and *pearl* mice and HPS2 patients), an enzyme that regulates Rab protein localization (rab geranyl geranyl transferase α subunit, defective in *gunmetal* mice), and a protein of 25 kD (defective in *pallid* mice) that is found within a complex with other HPS-related proteins (defective in *muted* and likely in *reduced pigment* and *cappuccino* mice) and that potentially interacts with the tSNARE, syntaxin 13 (Falcon-Perez and Dell'Angelica, 2002; Falcon-Perez *et al.*, 2002). The disease phenotype varies with the deficient gene, but most forms of HPS are characterized by the absence of platelet dense granules, morphological defects in melanosomes, and eventual fibrosis of the lung (Spritz and Oh, 1999). Some HPS patients are immunocompromised as well, but the bases for these defects have not been characterized. The observed dependence of CD1b transport in APCs on AP-3 (Briken *et al.*, 2002) opens the possibility that presentation of microbial antigens via the this MHC-like molecule is affected in HPS2 patients (Sugita *et al.*, 2002). The definition of specific transport steps that are affected in HPS cells will undoubtedly provide further insights into the molecular mechanisms underlying the biogenesis of MIICs, melanosomes and other LRO.

Dysfunctions of LRO may also result from cell transformation. During transformation to melanoma cells, some melanocytes lose their ability to synthesize pigment while others harbor disorganized melanosomal structures (Hearing, 1999; Sakai *et al.*, 1997). These changes are partially due to variations in the expression of melanosomal resident proteins perhaps a reflection of selective pressure from the immune system but may also be due to alterations in the

biogenetic machinery. For example, changes in the oxidative metabolism of melanoma cells may affect the pH or oxidative state of endocytic compartments and thereby alter formation of vesicles, fusion events, or intralumenal functions. Whether these changes are merely a consequence of transformation or a causative factor remains to be tested.

Another critical feature of transformation is the role of LRO in anti-tumor immunity. The main melanosomal components, Tyrosinase, Tyrp1, Tyrp2, Pmel17/gp 100, and MART-1 all have been identified as major tumor-associated antigens in melanoma, capable of eliciting both $CD4^+$ and $CD8^+$ T cell responses and, in some cases, specific antibody production (Kawakami *et al.*, 1998; Kawakami *et al.*, 2000; Overwijk and Restifo, 2000; Sakai *et al.*, 1997). Indeed, melanosomal proteins are the most prevalent of known human tumor-associated antigens for which $CD4^+$ T cell responses have been demonstrated in cancer patients. The prevalence of these LRO-associated proteins among MHC class II-restricted antigens is not likely to be coincidental. Two potential mechanisms may account for their enhanced presentation relative to other tumor-associated antigens by MHC class II. First, given that melanosomes and MIICs share molecular and structural characteristics, it is conceivable that MHC class II and melanosomal proteins expressed by melanoma cells meet intracellularly in a common LRO. MHC class II expression can be induced by γ-interferon in cutaneous melanocytes, some melanoma cells constitutively express MHC class II as a consequence of up-regulation during the process of malignant transformation (Brady *et al.*, 2000; Cerundolo, 1999; Zarour *et al.*, 2000) and melanoma cells can stimulate tyrosinase-specific $CD4^+$ T cell clones (Topalian *et al.*, 1994). Furthermore, melanosomal proteins and MHC class II can in principal be targeted to a common LRO, since expression in a B cell line of a chimeric protein consisting of ovalbumin fused to the melanosome targeting signal of Tyrp1 resulted in efficient presentation of an ovalbumin peptide by MHC class II (Wang 1999); this finding may not be surprising given the localization of melanosomal proteins to conventional lysosomes in non-pigment cells (Wang *et al.*, 1999) and the lysosome-like nature of MIICs. On the other hand, interferon-γ-treatment of melanoma cells results in a potent down-regulation of the expression of melanosomal proteins (Le Poole *et al.*, 2002 and our unpublished studies), and there appears to be an inverse correlation between the expression of MHC class II and melanosome proteins (unpublished data). Moreover, since interferon-γ treatment induces the expression of MHC class II on untransformed melanocytes, it is unlikely that melanocytes or their derivative melanoma cells, as "unprofessional" APCs, would be capable of eliciting a $CD4^+$ T cell immune response; otherwise, the immune system would constantly attack melanocytes.

The second potential mechanism for association of melanosomal proteins with MHC class II molecules relies on the ability of LRO to fuse directly with the plasma membrane. Fusion of multivesicular melanosome intermediates would result in the release of exosomes bearing melanosomal proteins (Wolfers *et al.*, 2001). Uptake of these exosomes by dendritic cells could then result in the processing and presentation of the associated proteins. In support of this idea, some melanosomal antigens, including MART-1 and Tyrp1, are enriched in exosomes secreted by melanoma cells (Wolfers *et al.*, 2001). Interestingly, such exosomes have been found in malignant effusions *in vivo* in patients bearing melanoma and could induce production of melanoma-specific T cells when pulsed with dendritic cells (Andre *et al.*, 2002). Perhaps it is only when local DCs are stimulated by infection or cytokine activity that the antigens from melanoma-derived exosomes are processed and presented to the immune system. The existence of a such a natural source of tumor rejection antigens opens new avenues for anti-tumor immunisation.

Defects in the biogenesis and secretory capacities of LRO can be induced not only by genetic diseases or cell transformation, but also upon hijacking of lysosomal compartments by pathogens. There are several examples in which pathogens invade cells through the endocytic pathway, and then alter the morphology and function of endosomes, lysosomes, or LRO for their own advantage. One example is, the parasite *Trypanosoma cruzi*, which exploits the process of lysosome exocytosis (Andrews, 2002; Rodriguez *et al.*, 1999). Lysosome exocytosis normally functions as a housekeeping process in the local repair of physically ruptured plasma membrane. Upon binding to the plasma membrane, *T. cruzi* triggers the host cell to direct its lysosomes to the site of contact followed by exocytic fusion. The parasite then utilizes the local surplus of membrane to enter the host cell. In addition, pathogens may profit from the secretory capacity of MVBs, lysosomes, or LRO to escape into the extracellular milieu, and infect neighbouring cells. Our recent studies on the replication of HIV-1 in human macrophages highlight such an expropriation of the lysosomal compartment. In these cells HIV assembles and accumulates in multivesicular MIICs, sharing features with the internal vesicles of MVBs (Raposo *et al.*, 2002) (see Fig. 4). This study correlates well with others showing that the subcellular machinery necessary for HIV-1 budding is similar — if not identical — to that used in all cells for the biogenesis of the internal vesicles of MVBs (Garrus *et al.*, 2001). The accumulation of virus in macrophage MIICs may have two major consequences. First, the virus acquires membrane proteins and lipids normally targeted to the internal vesicles of MVBs, such as MHC class II, adhesion molecules, sphingomyelin and cholesterol; indeed, HIV-1 is known to associate with lipid rafts, and high cholesterol levels may be critical for the stability of the virus particles (Suomalainen, 2002). The second consequence of HIV replication in MIICs (or other LROs) is the release of fully assembled viruses into the extracellular environment upon fusion of MIICs with the cell surface (Raposo *et al.*, 2002).

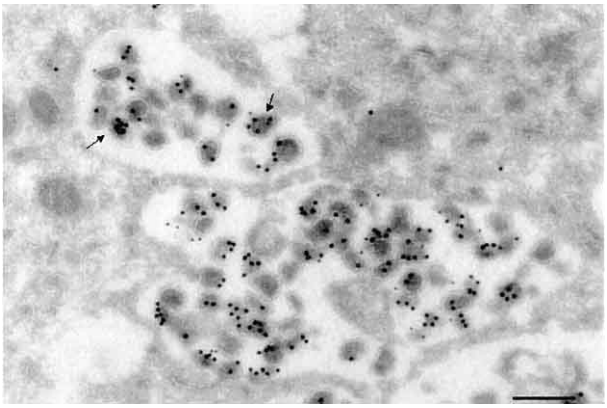

Fig. 4. In human macrophages HIV-1 accumulates in MHC class II compartments. Ultrathincryosections of human macrophages were double immunogold labeled for HIV-1 p24 (PAG 15) and MHC class II (PAG 10). Numerous p24 positive virus particles accumulate in membrane enclosed compartments that are also labeled for MHC class II. Numerous virus particles contain MHC class II. Bar: 200 nm.

It is tempting to speculate that other pathogens may also exploit the MVB machinery and LRO secretion in order to propagate.

Conclusions

The formation of LRO, as exemplified here by MIICs and melanosomes, seems to require multiple protein sorting pathways. Although different types of LRO are often depicted within one single family it is becoming evident that despite common determinants and shared protein sorting machineries, the cellular mechanisms involved in their biogenesis and/or secretion may be adapted differently. Conventional lysosomes in non-specialized cells can under certain situations, such as infection, inflammation and cell surface injury, fuse with the plasma membrane and secrete their contents. This broadens the previous view that only certain specific LRO behave as secretory lysosomes. Further unraveling of protein sorting events that lead to formation of other LRO will likely also provide valuable information on the molecular mechanisms required for the biogenesis of conventional lysosomes. More specifically it will help to elucidate how specialized cells exploit and expand on ubiquitous organelles. Finally, understanding these pathways is valuable if not indispensable for the development of effective treatments of LRO related diseases.

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