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Old lysosomes, new tricks: MHC II dynamics in DCs

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MHC II-restricted antigen presentation typically results in recognition of exogenous foreign peptides by T lymphocytes and the subsequent generation of a cellular immune response. It is well known that the intracellular events involved in the assembly of peptide–MHC II complexes occur in the endocytic pathway. However, a point not often considered by immunologists is that the nature of these events suggests contradictions between the fundamental characteristics of the endocytic pathway as defined in non-specialized cells and those features necessary to facilitate efficient MHC II antigen processing in antigen-presenting cells. We believe that one reason why dendritic cells are so efficient at T-cell stimulation is their ability to reconcile these contradictions by regulating the activities and dynamics of their otherwise 'normal' complement of endocytic organelles.

Generating an immune response requires T-cell recognition of antigen in the form of short peptides bound to MHC molecules. Cells overcome ostensibly impossible barriers to form peptide–MHC complexes (pMHC) and deliver them to the plasma membrane. This situation was first illustrated in MHC I-restricted antigen presentation. Here, antigenic peptides formed in the cytosol must traverse the compartment barrier of the endoplasmic reticulum (ER) membrane to bind MHC I molecules. Seemingly in violation of a basic principle of cell biology, careful analysis led to the identification of specific ATP-dependent translocators (TAP1/2) that enable this 'disallowed' event.

For MHC II molecules, the encounter between antigen and MHC occurs in the endocytic pathway, without obvious compartment barriers separating peptide and MHC. However, consideration of the organization and function of the endocytic pathway [1] (Box 1) creates some logical difficulties for rationalizing where productive pMHC II formation occurs [2]. Lysosomes, even in antigen presenting cells (APCs), such as macrophages, rapidly degrade proteins completely, making it difficult to understand how they accumulate immunogenic peptides, 12–25 amino acids in length. Because they are inefficient sites of membrane recycling, they might not allow easy escape, even if pMHC II complexes did form. However, endosomes in most cell types (at least early endosomes) are protease poor. Individual receptors generally reside within them for only a few minutes, making it difficult to see how pMHC II can form, given the low affinity of MHC II molecules for their cognate peptides.

These considerations helped promulgate the idea that professional APCs might exhibit specializations (e.g. modified organelles, atypical patterns of membrane traffic, highly specialized proteases or chaperones), which provide favored sites for antigen processing within the endocytic pathway. Indeed, many APCs express chaperones, such as HLA-DM and HLA-DO, to assist in peptide loading and that also contain proteases, such as cathepsin S, which selectively assist in invariant chain processing and some antigen catabolism [3]. Despite extensive analysis, however, the endocytic pathway organelles themselves appeared fundamentally similar to those in most cell types. Here, we give a historical perspective of studies on the cell biology of MHC II antigen presentation and make the case that, although conventional, the endocytic pathway in professional APCs is specialized. The specializations are most apparent in recent investigations of dendritic cells (DCs), which show developmental regulation of proteolytic activity and transport out of lysosomes.

Intracellular localization and function of MHC II

Classical studies of APCs, mostly transformed B-cell lines, melanoma cells and macrophages, used immuno-electron microscopy, which demonstrated that MHC II and MHC I molecules exhibit distinct intracellular locations. MHC II is largely in endocytic organelles with features of late endosomes or lysosomes [4–6]. Subcellular fractionation and light microscopy studies revealed similar results in B-cell lines, although some (e.g. A20 murine B lymphoma cells) exhibit MHC II predominantly in endosomal compartments [7,8]. These possibly non-physiologically relevant cell types were used because they are easily accessible, highly abundant *in vitro* and express elevated levels of MHC II.

The MHC II-containing compartments in these studies were given names to differentiate them from other endocytic organelles. The term MIIC (for 'MHC II compartment') [4] was used to designate structures similar to late endocytic organelles, whereas CIIV (class II vesicles) [7] denoted earlier, endosomal structures. MIIC and CIIV were initially presumed to be specialized compartments dedicated to various aspects of MHC II function, perhaps adapted to solve problems associated

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Box 1. The classical pathway of endocytosis: a one-way ticket to Dante's 9th circle

In perhaps the earliest cell biological studies of the immune response nearly 100 years ago, Ilya Mechnikov showed that phagocytes engulf foreign particles and, when exposed to an acidic milieu, they are degraded in an energy-dependent fashion. 50 years later, Christian deDuve and colleagues defined the intracellular compartment in which such events occur as the lysosome. Lysosomes were thus first seen as digestive organelles containing high concentrations of proteases and other acidic hydrolases, which exhibit a requirement for low pH for optimal activity. Acid pH in lysosomes (and other endocytic organelles) was eventually found to be due to a distinctive F_1–F_0-type proton ATPase (vacuolar ATPase or V-ATPase).

Early studies of endocytosis demonstrated that lysosomes (Figure I) comprise the predominant site for accumulation and degradation of internalized macromolecules [42], like Dante's deepest level of the inferno, the 9th circle, a place from which substances, and souls, are generally unrecoverable. This is perhaps clearest in the case of macrophages, which have long been known to convert internalized proteins into amino acids and dipeptides with exquisite efficiency [43].

At the same time, it became clear that receptors and a variety of other plasma membrane components, despite being continuously internalized, are not degraded but rapidly recycled back to the cell surface for re-use [44]. Recycling components generally avoid lysosomes and instead reach only a 'pre-lysosomal' sorting compartment, termed an endosome [45].

Several populations of endosomes can be distinguished [1] (Figure I). Early endosomes (sometimes referred to as sorting endosomes), generally found in the peripheral cytoplasm, host the dissociation of most incoming receptor–ligand complexes, as well as the sorting of receptors from components (including discharged ligands) in the early endosome lumen. Although most receptors recycle rapidly (<5 min) from early endosomes, ~25% accumulate in a perinuclear endosomal compartment, termed a recycling endosome, before returning to the surface [46]. The dissociated ligands are transferred from early to late endosomes, a lysosome-like compartment, which often contains abundant intravesicular inclusions. These 'multivesicular bodies' consist of membrane derived from invaginations of the early and late endosomal limiting membrane, typically containing that subset of membrane proteins, such as signaling receptors marked for 'downregulation', which are destined for lysosomal degradation [47]. In the case of dendritic cells, these internal vesicles also accumulate abundant MHC II [23,48].

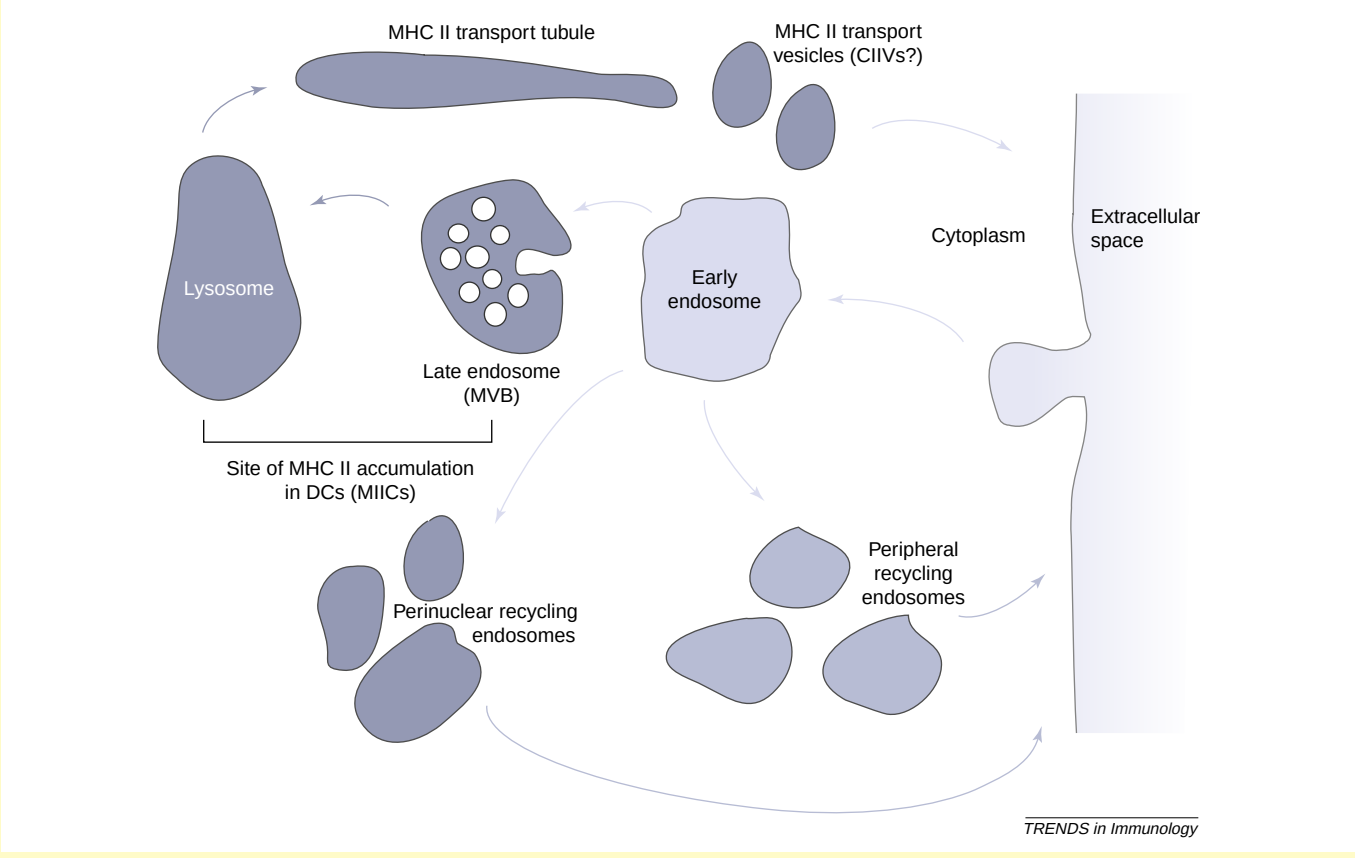

Figure I. Representation of the endocytic pathway. Internalized molecules first enter the early endosome, then transit through the late endosome and finally to lysosomes. Alternatively, sorting from the early endosome, in which internalized molecules are re-routed to either perinuclear or peripheral recycling endosomes, might occur. From these compartments, molecules are returned to the cell surface. Compartments involved in MHC II transport are also indicated. Lighter shading reflects earlier compartments whereas darker shading indicates later compartments. Abbreviations: CIIVs, MHC II vesicles; DCs, dendritic cells; MIICs, MHC II compartments.

with antigen processing in conventional endosomes or lysosomes. Through a precise comparison of conventional endocytic markers and MHC II distribution, however, MIICs were shown to be standard late endocytic or lysosomal compartments, which contained MHC II [9,10]. CIIVs are physically distinct by cell fractionation, and do not contain classical endosomal markers, such as the transferrin receptor; functional evidence indicates a role in a late step in transport of newly synthesized MHC II (possibly pMHC II complexes) to the surface [7,11]. It is unclear from the B-cell work, however, if they have a specialized antigen-processing function.

Physiological relevance for the localization studies was sought by assaying for peptide loaded MHC II, initially by

the indirect criteria of SDS (sodium dodecyl sulfate) stability [8,11,12]. Moreover, MHC II localization was studied relative to the distribution of other molecules required for MHC II function (invariant chain, HLA-DM/H2-M) and to features, such as disulfide bond reduction, required for the processing of some antigens [3,5,13–15]. Indeed, SDS stable complexes are detectable in almost any compartment, however, the fate and functional significance of the complexes (i.e. surface transport versus degradation) remains unclear, except in the case of tetanus toxoid, where an individual immunogenic peptide could be associated with SDS stability [12].

The proposition that peptide loading occurs in endosomes reconciled the problems that lysosomal loading posed [2]. Endosomes contain some proteases but generally comprise a less degradative compartment that might provide for both processing and complex formation [1]. Moreover, this compartment enables efficient recycling, and for some B-cell lines, is a major site of MHC II localization [7]. Problems with this suggestion remain, however, because passage through endosomes results in too short a residence time for the required events and conditions are possibly too mild for the protein hydrolysis necessary for generating appropriate length peptides. An analysis of the antigen-presentation capabilities of B cells and macrophages following a low temperature (20 °C) block, which inhibited endosome to lysosome maturation but not initial endocytosis, suggested that T-cell stimulation was inefficient for some antigens unless lysosomal exposure occurred [16].

Yet, if lysosomes act as an antigen-processing site, how do immunogenic peptides survive in an environment that evolved to completely degrade incoming proteins? How do pMHC II complexes escape a compartment associated, in most cells, with slow, if any, membrane recycling?

Regulated transport of MHCII in DCs

DCs are uniquely suited for antigen processing and presentation because many aspects of their cell biology seem optimized for this purpose [17]. B cells also exhibit exquisite efficiency at presentation under physiological conditions but only for their cognate antigen. DCs, the most professional of all APCs, must be able to take up, process and present a seemingly limitless array of foreign and self-proteins and at exceedingly low concentrations [18]. DCs also capture antigens in peripheral tissues, conveying them to lymphoid organs where productive interactions with naïve T cells occur. Although there are many different DC subtypes *in vivo*, the ability to generate relatively homogeneous populations *in vitro* has provided a venue for study that appears to be more biologically significant than previous work with B-cell lines, lymphoblasts, lymphomas and macrophages (macrophages being inefficient presenting cells relative to both DCs and B cells).

Another intriguing and experimentally useful feature of DCs is that their antigen-presentation capacity is tightly controlled according to their maturation status [17]. Maturation is a designation that broadly distinguishes between two functionally and phenotypically distinct states, which are termed immature and mature [19,20]. Immature DCs acquire antigen through active endocytosis but express relatively low levels of MHC I, MHC II and co-stimulatory molecules on their plasma membrane – characteristics that result in a reduced capacity for T-cell stimulation. By contrast, mature DCs reduce their endocytic capacity in favor of high surface levels of MHC and co-stimulatory molecules, making them capable of activating even naïve T lymphocytes. This regulation of cellular processes by DCs make them ideal for studying the cell biological basis of antigen presentation, providing a conditional switch that can be used to correlate the acquisition of a new functional property with some alteration in underlying cell biology. In particular, DC-regulated peptide loading and transport of MHC II have revealed specializations of the endocytic pathway that solves many of the problems for antigen processing implicit in the properties of 'classical' endocytic organelles.

DC studies have shown that MHC II accumulates in lysosomes of immature cells, co-localizing with resident lysosomal membrane proteins, HLA-DM/H-2M and hydrolytic enzymes [20]. These compartments accumulate exogenous antigen internalized either as soluble protein or phagocytic particles. Yet, at least in bone marrow-derived murine DCs, immunogenic pMHC II complexes from these antigens form and accumulate inefficiently, as measured using antibodies to pMHC II complexes or by T-cell presentation assays [21,22]. Some SDS-stable MHC II could be detected; however, the functional significance or fate of these molecules is unclear [20]. In human monocyte-derived DCs, however, pMHC II containing a tetanus toxoid-derived peptide was detected in immature cells but these complexes were rapidly turned over, producing the same net effect [19], that is, inefficient production or accumulation of immunogenic complexes in immature DCs. Also noteworthy is that, in immature DCs, MHC II accumulates on the internal vesicles of multivesicular compartments [or multivesicular bodies (MVBs)] of the late endocytic pathway [23] (Box 2). The internal vesicles of MVBs are occasionally released into the extracellular space, thereby generating exosomes [24,25]. The MVBs are dynamic, at least in melanoma cells expressing GFP-tagged MHC II, in which they have been seen to move in a microtubule-dependent fashion and possibly fuse with the plasma membrane [26,27].

The situation changes markedly after immature DCs are induced to mature by a variety of microbial products (Toll-like receptor ligands), cytokines [e.g. tumor necrosis factor-α (TNF-α)] or T-cell ligands (e.g. CD40 ligand). Immunogenic pMHC II complexes form more efficiently and/or accumulate [19–21], and indeed, first appear within the same lysosomal compartments that had previously been unable to accumulate pMHC II efficiently [28]. Interestingly, these complexes can form using antigen internalized at least 72 hours before receiving a maturation stimulus, suggesting at least partially intact retention of antigen in immature DCs. [29]. Of further note was that, at least in fixed cells sampled at various times following induction of maturation, the pMHC II complexes could be found sequentially in peripheral, non-lysosomal compartments, reminiscent of CIIV compartments seen previously in B cells [7], and are finally found

Box 2. Multivesicular bodies and exosomes

The MHC II that accumulates in the late endocytic pathway in immature dendritic cells (DCs) often resides in multivesicular compartments [or multivesicular bodies (MVBs)] (Figure I). Within MVBs, MHC II is concentrated more highly on the internal vesicles than on the limiting membrane [23]. Recent electron tomography studies in B lymphocytes and a DC cell line indicated that the internal vesicles of MVBs are not contiguous with the limiting membrane [49]. Given that, in most examples studied thus far, membrane proteins sequestered on these internal structures are marked for degradation, the question arises as to whether MHC II molecules in MVBs are similarly degraded, somehow rescued or undergo some combination of these two options, which might not be mutually exclusive for the total population of molecules. One possibility, which is thus far without direct evidence, is that, on maturation induction, these internal vesicles fuse back with the late endosome or lysosome limiting membrane. Such an event would, in principle, enable these MHC II molecules to be included in lysosome-derived tubules for retrograde transport, given that the tubules are likely to form from the limiting membrane. If such an unprecedented type of fusion is shown to occur for MHC II transport, this might represent yet another lysosomal specialization in DCs. Alternatively, the internal vesicles might simply be degraded, such as occurs during signaling receptor downregulation, and there is enough MHC II on the limiting membrane to account for that which accumulates on the cell surface. Yet another possibility is that the nature of the internal MVB inclusions in DCs is somehow different from those in other cells. Indeed, a crucial unknown is the mechanism by which MHC II molecules are sequestered on these internal vesicles in the first place. Signaling receptors subject to downregulation are so internalized following mono-ubiquitination and recognition by ESCRT (endosomal sorting complex required for transport) complex members [47], however, similar information is not yet available for MHC II molecules.

Another possible fate of the internal vesicles that accumulate MHC II is release into the extracellular space, also known as exosome release [24,25]. Any MHC II that was on the limiting membrane of the MVB is subsequently presented on the cell surface [25]. This is not, however, the main process by which MHC II is transported to the plasma membrane. In fact, exosomes are more commonly released from immature DCs, which have less MHC II present on the cell surface, than from mature DCs [50]. Exosomes could function in immune modulation because they contain several stimulatory molecules (e.g. MHC I, co-stimulatory molecules) in addition to MHC II.

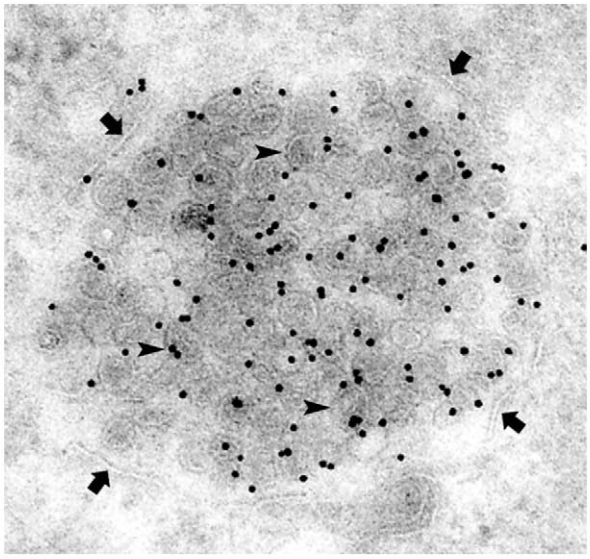

Figure I. Representative electron micrograph of a multivesicular compartment from an immature $CD34^+$ stem cell-derived human dendritic cell (DC) labeled with an anti-MHC II antibody. Wide arrows highlight limiting membrane and arrowheads point out some internal vesicles containing MHC II. Micrograph courtesy of Jeoung-Sook Shin, Marc Pypaert and Lindsay Murrell.

at the plasma membrane, while becoming progressively depleted from lysosomes [28]. DC lysosomes can therefore host the regulated assembly of immunogenic pMHC II complexes and facilitate their vectorial escape. Neither of these events would have been predicted on the basis of conventional views of the lysosomal compartment.

The possible escape function is intriguing because, as mentioned earlier, lysosomes have long been considered highly inefficient sites of membrane recycling to the plasma membrane. In recent years, some membrane proteins have been identified that are transported between the surface and late endocytic compartments; some of these (e.g. the lectin DEC-205 and the tetraspanin CD63) are also expressed by DCs [30,31]. However, these examples traffic constitutively and, moreover, MHC II molecules have never been so characterized. Consequently, several groups have investigated this pathway. Kleijmeer and colleagues used immuno-electron microscopy to show that lipopolysaccharide (LPS) treatment of a murine DC cell line induced marked tubule formation [23] of MHC II-containing compartments that also included some resident lysosomal membrane proteins. These studies were extended to murine bone marrow-derived DCs in live cell imaging experiments using cells expressing green fluorescent protein (GFP)-tagged MHC II molecules. Stimulation with LPS (or T cells) elicited the formation of lysosomal tubules [32,33], which moved in a retrograde manner to fuse directly with the plasma membrane, as demonstrated by total internal reflection fluorescence microscopy [33] (Figure 1). For T-cell-dependent tubule formation, transport was suggested to be directional, supported by data showing that a small majority of the tubules were directed towards a T cell. It seems likely that DCs will polarize, at least partially, to sites of T-cell interactions, however, it remains unknown as to whether these particular tubules actually fuse. Moreover, by the time most DCs engage T cells, they would already have matured [34] and thus translocated their MHC II to surface. Mature DCs also typically interact with multiple T cells simultaneously.

In any event, the DC tubes seen in MHC II transport appear to be different from macrophage tubular lysosomes because the latter have not been shown to transport material or fuse with the plasma membrane [35]. Although their role in post-loading MHC II transport suggests that the tubules might correspond to CIIVs, such a relationship remains unclear, although a CIIV population has been isolated from DCs [20]. The results with the tubules, although striking, do not exclude the possibility that MHC II transport can also occur by vesicles as demonstrated by studies using melanoma cells [27]. The main difference between the two observations is that tubules are formed only in DCs after stimulation, whereas the melanoma vesicle transport was

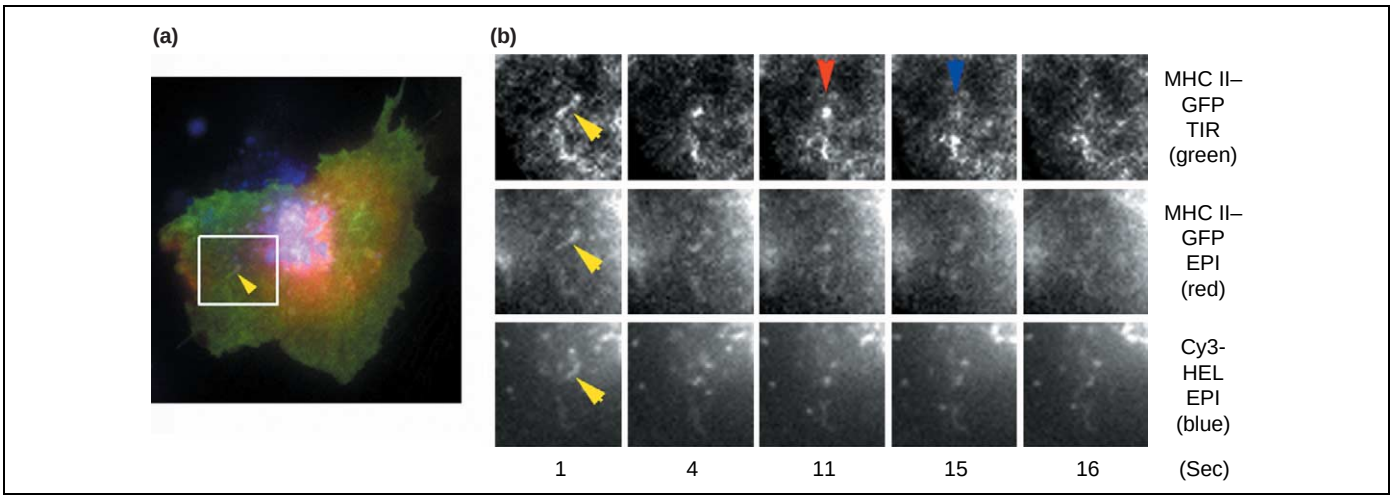

Figure 1. MHC II–GFP (green fluorescent protein)-expressing bone marrow-derived dendritic cells (DCs) fed fluorescently labeled hen egg lysozyme (Cy3-HEL) and stimulated with bacterial products generate endocytically derived tubules, which are targeted to, and fuse directly with, the plasma membrane. **(a)** Three color merged image highlighting tubule (white box with yellow arrowhead) visible by total internal reflection (TIR, green) and conventional epifluorescence (EPI, red) of MHC II–GFP and epifluorescence of Cy3-HEL (blue). **(b)** Color separated sequence lasting 16 seconds shows prototypical fusion profile, in which TIR signal peaks at 11 seconds (red arrowhead), followed by dissipation at 15 seconds (blue arrowhead) and disappearance at 16 seconds. MHC II epifluorescence signal also disappears at 16 seconds, thus confirming the fusion event. The Cy3-HEL signal at 15–16 seconds is still slightly apparent, suggesting partial fusion of the content marker only. Reproduced with permission from *Nature* (418, 988–994) (Ref. [33]). Copyright (2002) Macmillan Magazines Ltd.

constitutive and might be more closely related to the export of MVBs during exosome release.

Thus, lysosomes in DCs seem to be specialized. Rather than merely degradative dead-ends, they can reversibly accumulate proteins, form new protein complexes (pMHC II) and allow transport of the complexes to the cell surface, all in a highly regulated fashion.

The rule that lysosomes represent an end stage organelle, from which there is little if any escape, although fundamentally true, has additional exceptions. Secretary lysosomes implicated in plasma membrane wound healing or trypanosome entry and in cytotoxic lymphocyte killing [36,37] represent a similarly 'disallowed' transport event. In contrast to the secretary lysosome pathway, however, retrograde MHC II transport in DCs does not appear to cause significant extracellular release of lysosomal contents. In maturing DCs, previously internalized dextran is not released on MHC II surface arrival [29] nor has the lysosomal enzyme β-hexosamidase been detected in the medium of cells transporting MHC II to the plasma membrane [23]. Although some constitutive release of lysosomal contents occurs in most cell types, presumably including DCs, regulated retrograde transport of MHC II does not greatly increase this amount.

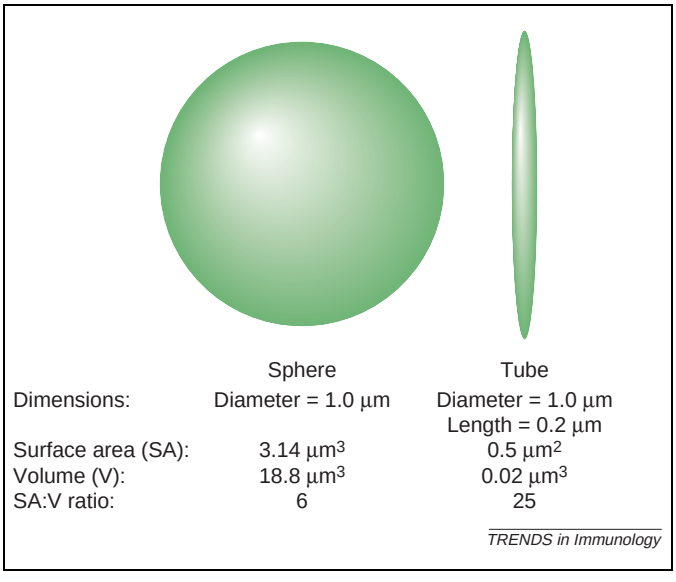

Figure 2. Example calculations comparing volume and surface area for a sphere and tube with physiologically appropriate dimensions. The surface area to volume ratio for the tube is more than four times that of the sphere. Thus, transport mediated by an elongated tube is able to deliver more membrane-bound components and less content to the cell surface than a spherical vesicle following fusion.

The observation that lysosomal membrane components, but not lysosomal contents, reach the cell surface in compartments of a tubular morphology brings to mind the type of Euclidean sorting that also occurs for tubular endosomes and recycling compartments. Geometrically, a tube-like shape results in a high surface-to-volume ratio, providing the desired effect in the case of MHC II transport from a lysosomal compartment, with the result being surface arrival of membrane-bound MHC II molecules but keeping loss of lysosomal contents at a minimum (Figure 2). It will be both interesting and important to determine the precise numbers that describe these events.

DC lysosomes regulate proteolytic capacity: a kinder, gentler inferno

In addition to allowing for escape of pMHC II complexes, DC lysosomes are also surprising in that they allow the complexes to form in the first place, in a fashion highly regulated in most DC types. Most striking is the fact that antigen can be sequestered by immature DCs for up to 3 days before being mobilized as a donor of immunogenic peptides [21]. This observation suggests, indirectly, that DCs might regulate lysosomal proteolysis in a developmentally linked fashion. At first, hints of a diminished degradative capacity in lysosomes came from studies on specific proteases. In particular, a natural cathepsin S inhibitor, known as cystatin C, was found more highly

expressed in immature DCs than in mature DCs. As a result, invariant chain and other protein substrates for cathepsin S were not completely cleaved before stimulation [38]. However, apart from cathepsins L and B, cystatin C is a relatively poor inhibitor of almost all other lysosomal proteases and might not be invoked to single-handedly control hydrolytic capacity.

A more attractive explanation comes from evidence showing that, relative to mature DCs, immature DCs have reduced lysosomal acidification resulting in diminished proteolysis of internalized antigens. Moreover, the higher pH correlated with the presence of more pro-forms (inactive) of proteases in lysosomes of immature but not mature DCs. An attenuation of lysosomal acidification can further be explained by differences in the assembly of the proton pump (V-ATPase) on lysosomal membranes. The V-ATPase consists of two subcomplexes, V_1 (consisting solely of peripheral membrane proteins, including the catalytic subunit of the pump) and V_0 (consisting of membrane proteins that form the actual proton pore) [39]. In immature DCs, a significant fraction of V_1 is free in the cytosol, whereas in mature DCs, the pump is almost entirely in its assembled, functional state [29]. Thus, greater lysosomal acidification and rate of proteolysis would be expected in mature than in immature DC lysosomes. It will now be important to establish signaling pathways leading to V-ATPase assembly because they might not necessarily be identical to those leading to other hallmarks of DC maturation.

Summary

Studying the mechanisms of antigen presentation by DCs has revealed several unexpected and important hints into understanding how DCs activate T cells so efficiently and potently. This work has also provided some novel insights into previously unappreciated aspects of fundamental cell biology of the function and dynamics of late endocytic compartments.

The ability of DCs to developmentally regulate their capacity for lysosomal proteolysis is not unique to this cell type but does represent a specialization that contributes to the ability of the cell to regulate the production of pMHC II complexes. Because the induction of DC maturation also causes DCs to migrate from peripheral tissues to lymph nodes carrying previously encountered antigen with them, this particular specialization might also regulate where in the body pMHC II production occurs. Delaying lysosomal activation until the cells have begun migrating might delay pMHC II production until maturing DCs have started to reach secondary lymphoid tissues. *In vivo*, cells in the periphery generally exhibit an immature phenotype [20,40]. Interestingly, and as expected, DCs in lymph nodes display a range of maturation phenotypes, although the functional consequences of these various cell populations are not yet fully understood.

Similarly, the pathway of retrograde transport of pMHC II from lysosomes might not unique to DCs. What is unique, however, is the ability of DCs to couple pMHC II formation and retrograde transport to the receipt of maturation signals. Much remains to be learned about the events leading to tubule formation and fusion at the plasma membrane. Apart from the SNARE ([SNAP (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein)]-associated molecule synaptotagmin VII [41], no other potential fusion molecules have yet been associated with a lysosome to plasma membrane fusion pathway.

These examples, although dramatic, probably represent just the first few surprises held for us by DCs. Moreover, it might emerge that different subpopulations of DCs accomplish these various tasks using different strategies, and as these differences are characterized, we will be in a better position to assess the function and regulation of each subpopulation. These are important considerations, not only for fundamental immunology and cell biology, but also for understanding how best to intervene for the purposes of immunotherapy.

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