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## The Endocytic Activity of Dendritic Cells

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In this issue, Sallusto et al. (1) report that dendritic cells can vigorously internalize solutes by macropinocytosis, delivering these to MHC class II-rich vesicles (MIIVs). In the most familiar form of endocytosis, clathrin-coated pits invaginate to form small (<0.1  $\mu\text{m}$  in diameter) coated vesicles (Fig. 1). Ligands adsorb to receptors in the coated pit and are subsequently delivered to lysosomes, where the ligands are degraded down to the level of amino acids. During macropinocytosis, which occurs at sites of membrane ruffling, larger droplets of fluid enter the cell, and an actin-based cytoskeleton rather than clathrin seems to be required (Fig. 1). Sallusto et al. (1) describe solutes that rapidly enter dendritic cells within macropinosomes, both in the fluid phase or attached to the membrane. In both cases, the solutes are delivered to MIIVs, where they presumably can be converted to peptides and recycled to the cell surface as MHC II-peptide complexes. This set of events would explain a previous report from these same authors on the efficiency with which dendritic cells present protein antigens (2). In that study, the presentation by dendritic cells of antigens, apparently from the fluid phase, was as effective as presentation by B cell lines that adsorb the same antigen via surface Ig receptors.

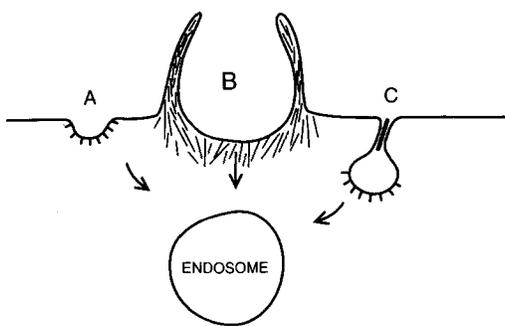
These new observations represent a striking turn of events. In most previous studies of dendritic cells, little or no uptake

could be detected. Here we comment on this apparent discrepancy, and we consider the capacity of macropinocytosis to enhance the presentation of fluid phase or nonbinding antigens. Our emphasis is on the uptake and cycling of endocytic vesicles rather than on the subsequent steps in presentation.

**Historical Aspects.** The lack of endocytic activity in dendritic cells was one of the cardinal traits that initially allowed their identification as a distinct lineage. At that time, the early 1970s, the antibody response by mouse spleen cells was the prototype immune response to antigens in culture. Mishell and Dutton had pioneered this system (3). Then it was found that the antibody response required the cooperation of two populations. These were distinguished on the basis of differences in buoyant density or in the capacity to adhere to glass, plastic, or Sephadex G10. The high density nonadherent cells were primarily lymphocytes, while the low density adherent cells were considered to be macrophages that had to interact with antigen.

When the adherent cells were examined for the traits that were available to identify macrophages, both macrophage and nonmacrophage populations were apparent (4, 5). The macrophages had numerous lysosomes (by electron microscopy and by staining for lysosomal acid phosphatase) and actively internalized particles and smaller tracers (microorganisms, antibody-coated particles, soluble horseradish peroxidase [HRP], and colloidal carbon). The other stellate population, named "dendritic cells," had distinct features that included a paucity of lysosomes and an inability to accumulate a variety of particulate and fluid phase offerings. The distinctions were apparent *in vivo* as well. If colloidal carbon or HRP was administered intravenously to mice, the macrophages isolated from the spleen were heavily labeled (5). Little or no labeling was evident in the dendritic cells isolated from the same mice. If colloidal thorium was given and spleen sections were examined by electron microscopy, the macrophages showed labeling of numerous lysosomes, while the dendritic cells had few lysosomes and were unlabeled (6). Multivesicular vacuoles were evident in all these early descriptions of dendritic cells (4, 5, 7, 8), and recent evidence indicates that the vacuoles are MIIVs, i.e., rich in MHC II products (9, 10).

A lack of endocytic activity was reported in all of the initial descriptions of dendritic cells. Klinkert et al. (11) isolated these cells from several tissues of the rat and noted their inability to accumulate colloidal carbon. In studies of dendritic cells from afferent lymph of rabbits (12) and rats (13), Kelly et al. described a lack of uptake of colloidal carbon and im-



**Figure 1.** Possible pathways for the uptake of soluble proteins into dendritic cells. (A) Coated pit pathway. Soluble proteins enter in small coated vesicles, either in the fluid phase or more efficiently if adsorbed to receptors in the pit. (B) Macropinosome pathway. Soluble proteins and small particles enter in the fluid phase or adsorbed to the membrane of large vesicles that form at "ruffles" that are subtended by actin. (C) Birbeck granule pathway. This can occur in Langerhans cells, the dendritic cells of stratified squamous epithelia. The Birbeck granule, which may fuse features of pathways A and B, has a distinct "tennis racket" appearance consisting of a handle that is formed by juxtaposed membranes that can connect to the cell surface, as well as a large electron-lucent vesicle that can be coated.

mune complexes (12), while Pugh et al. observed a lack of uptake of ferritin-, latex-, and antibody-coated red cells (13). Wolff examined uptake of HRP into guinea pig epidermal Langerhans cells and found some uptake, but it was lower than the adjacent weakly endocytic keratinocytes (14). Schuler and Steinman, in their identification of epidermal Langerhans cells as dendritic cells, described a lack of uptake of HRP and particulates in vitro (8). Van Voorhis et al. could not detect uptake of HRP or latex and zymosan particles by dendritic cells from human blood (15). Hart et al. came to similar conclusions on a lack of latex uptake by dendritic cells from human tonsil (16). Eikelenboom (17) and Dijkstra (18) reported limited staining for lysosomal acid phosphatase and little uptake of colloidal carbon for dendritic cells in the T cell regions of spleen in situ.

After methods were developed to separate dendritic cells from macrophages, several laboratories found that dendritic cells exhibited two features that had not been encountered previously (reviewed in reference 19). These were very high levels of MHC II products and unusual stimulatory capacity for several T cell-dependent responses. In situ, dendritic cells were the principal cell in spleen and lymph that could capture antigen in a form that was presented to MHC II-restricted T cells. Dendritic cells could also prime MHC II-restricted T cells in mice in the absence of any adjuvant other than the APCs themselves.

The paucity of lysosomes and weak endocytic activity of dendritic cells, coupled with strong APC function, soon became enigmatic to many. Several developments indicated that endocytosis followed by digestion to form peptides was the principal pathway in which antigens came to be presented on MHC II molecules. What are some of the ways that one might reconcile these two contrasting sets of data, i.e., the need for endocytosis to process internalized antigens for binding to MHC II and the potent APC function of weakly endocytic dendritic cells? We comment on five possibilities (Table 1).

**Table 1.** *Some Attempts to Reconcile the Weak Endocytic and Strong Antigen-presenting Activities of Dendritic Cells*

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Processing takes place extracellularly.
Presentation, unlike clearance and scavenging, requires only small amounts of uptake.
Dendritic cells are like B cells in that they use adsorptive mechanisms to target small amounts of antigen for presentation rather than scavenging.
Substantial rates of uptake are followed by recycling, not delivery to lysosomes.
Endocytosis is regulated to defined periods in the life history of dendritic cells or after specific stimuli.

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*Could Processing Take Place "Extracellularly"?* Perhaps dendritic cells do not require endocytosis, and they either degrade antigens at the cell surface or they acquire peptides from other cells (20, 21). There still is no direct evidence for extracellular processing by dendritic cells, and efforts to detect transfer of peptides from other cells have failed. In the latter situation, the response of MHC-restricted T cells was monitored with a mixture of APCs, e.g., dendritic cells of the appropriate MHC that had not been pulsed with antigen, together with macrophages of the inappropriate MHC that had been pulsed with antigen. No regurgitation of immunogenic MHC II-binding peptides from macrophages to dendritic cells was detected (22, 23).

*Might Antigen Presentation by Dendritic Cells, in Contrast to Scavenging by Macrophages, only Require Low Levels of Endocytosis?* The criteria that were used to detect endocytosis in dendritic cells were those used to monitor the bulk scavenging function of macrophages, particularly the accumulation of a broad spectrum of substrates in digestive lysosomes. Scavenging is a high capacity form of endocytosis that is used to clear substrates, such as damaged cells and immune complexes, and it typically leads to complete digestion. When a fluid phase solute is applied to macrophages at 1 mg/ml (a dose that is often used in studies of antigen presentation), the cells on average accumulate  $10^5$  soluble protein molecules per hour, and these are digested down to the level of amino acids (24). Adsorptive uptake via Fc receptors increases the clearance and scavenging properties of macrophages by roughly 1,000-fold (25). In contrast, antigen presentation requires very small numbers of MHC-associated peptides. MHC II-restricted T<sub>H</sub> hybrids can respond to APCs that present a few hundred specific MHC-peptide complexes (26, 27). For naive T cells that are stimulated with dendritic cells, only a few hundred complexes of MHC II and superantigen are needed (28). APCs are unlikely to have higher levels of a particular MHC peptide since most APCs express <100,000 copies of a given MHC II locus (29) and the array of different peptides that are presented is >1,000 (30).

Therefore, low amounts of MHC-associated ligands are presented, so low levels of solute uptake may suffice. Inaba et al. made this suggestion when they observed relatively low levels of rhodamine-OVA uptake by dendritic cells, which nevertheless were capable of priming mice to rhodamine-associated antigens in situ (31). However, the efficacy with which internalized antigens are processed into MHC-binding peptides remains unknown. APCs conceivably have to take in  $10^5$ – $10^6$  molecules to successfully present 10–100 peptides.

*Are Dendritic Cells Similar to B Cells, Using Adsorptive Endocytosis to Target Antigens for Presentation Rather Than Scavenging?* It is difficult to observe bulk uptake of solutes or particles in primary B cells, which like dendritic cells, are potent APCs with few lysosomes. Yet antigens that bind to surface Ig receptors are known to be internalized and processed onto MHC II molecules (32–34). Might dendritic cells also have receptors that mediate adsorptive uptake, in an analogous fashion to surface Ig, and deliver these as peptides to

MHC products, rather than end-stage lysosomes? Dendritic cells can present small amounts of self immunoglobulins (35) and immune complexes (2) by Fc $\gamma$  receptors. The Birbeck granules that are found in epidermal Langerhans cells (Fig. 1) can be coated and can mediate adsorptive uptake (36–38). Sallusto et al. (1) report that dendritic cells can take in both HRP and FITC-dextran by adsorption to mannosyl-fucosyl C-type lectin receptors (1). The NLDC-145 antigen on dendritic cells has recently been cloned and shown to have 10 C-type lectin domains and to mediate adsorptive uptake in coated pits and antigen presentation (39).

*Might Dendritic Cells Have High Rates of Endocytosis but Recycle Contents to the Cell Surface Rather Than Deliver Them to Lysosomes?* A general feature of endocytosis is the rapid recycling of much of the internalized membrane and contents to the cell surface (reviewed in reference 40). When dendritic cells internalize solutes, might only small amounts be retained as peptides and the majority recycled back out of the cell? Levine and Chain described a substantial traffic of endocytic vesicles through endosomes, but not lysosomes, in dendritic cells. They used fluorescent solutes and cytofluorography to monitor endocytic activity. They concluded that after internalization, much of the solute is discharged by recycling (41). Rapid ( $t_{1/2}$  of 5 min) and slow ( $t_{1/2}$  of 100 min) recycling compartments were observed, corresponding to 80 and 20% of the internalized solute. These results differ from those of Sallusto et al. (1), who show that dendritic cells accumulate significant amounts of fluorescent solutes for long periods. Their cell populations had more abundant intracellular MIIVs, relative to the cells studied by Levine and Chain. Perhaps, for purposes of presentation, MIIVs trap internalized solutes for longer periods.

*Is Endocytosis Regulated and/or Confined to Stages in the Life History of Dendritic Cells?* Dendritic cells may endocytose readily detectable amounts of bulk substrates (solutes and particles), but this may be limited to discrete stages of their life history or to responses to specific stimuli. Romani et al. (42) and Streilein and Grammer (43) described the fact that upon isolation from skin, dendritic cells efficiently present protein antigens. Within 12 h, this capacity is lost. Then the cells develop strong binding and stimulatory activity for T cells, upregulating a number of accessory molecules such as ICAM-1, B7-1 and B7-2, LFA-3, and CD40. Splenic dendritic cells also capture antigens in vivo (22) and upon isolation from spleen (31), but they lose this capacity after overnight culture. Dendritic cells in effect seem to be able to segregate in time two broad functions of accessory cells. Antigens are captured at one point in their life history, e.g., as sentinels in a tissue like the skin, and then the captured antigens are presented at a later phase of their life history when the cells are rich in adhesion and costimulatory molecules (42).

What specific steps in antigen presentation might be regulated? Biosynthesis of MHC products is actually not constitutive in MHC-rich dendritic cells. In vivo (9) and after isolation (44, 45), epidermal dendritic cells actively synthesize MHC II and invariant chain. Within 12 h, at least  $10^6$  (8) cycloheximide-sensitive (46), I-A molecules are expressed on

the cell surface. Then biosynthesis diminishes dramatically. Sallusto et al. indicate that regulation also can lie at the uptake step, the formation of macropinosomes. This also was suggested by Stössel et al. (47), who noted large numbers of acidic endosomes in freshly isolated Langerhans cells, but not in cultured cells. Regulation at the endocytosis level is also evident with particulates. Proliferating progenitors to dendritic cells in the bone marrow can phagocytose some latex Bacille Calmette-Guérin particles and BCG microorganisms, whereas the progeny are weak or inactive (48). Freshly isolated epidermal dendritic cells can phagocytose certain particles such as yeast and leishmania, whereas cultured cells are inactive (49).

The regulation that is considered in the paper of Sallusto et al. (1) may be mediated by cytokines. In their experiments, cells from human blood were cultured for long periods in GM-CSF and IL-4 (2). Sallusto et al. (1) reason that exposure to cytokines may freeze the state in which dendritic cells can capture antigen in bulk, a state that is otherwise short-lived in the case of cultured skin and spleen dendritic cells. Interestingly, the antigen capture mode can be reversed by several ligands: TNF- $\alpha$ , LPS, IL-1, and CD40 ligand. A loss of intracellular MIIVs occurs simultaneously with an increase in cell surface MHC II.

*Macropinocytosis as a Mechanism of Solute Capture.* The proposal that cytokines increase macropinocytosis in dendritic cells is consistent with earlier findings that growth factors induce macropinocytosis in other cells (50–52). The pinocytic response to growth factors is large and quick (minutes), but it is followed by a lower but sustained level of pinocytosis as long as the stimulus remains (53). Sallusto et al. (1) observed active macropinocytosis even after the removal of GM-CSF and IL-4, suggesting that the dendritic cells had differentiated. An alternative explanation is that sufficient cytokine remains in the system, even within recycling vacuoles. Fibroblasts transformed with oncogenic ras have high constitutive rates of pinocytosis (54). Salmonella enter epithelial cells and macrophages (55–57) in macropinocytic vesicles that are induced immediately and at a local site upon addition of the organism.

Macropinosomes form from surface ruffles that fold back against the cell or against each other to enclose a vesicle up to 5  $\mu$ m in diameter (58). Ruffling uses the actin cytoskeleton, and macropinocytosis can be selectively inhibited by cytochalasins (51). Dendritic cells exhibit many cell surface extensions or veils. While it has always been assumed that these projections are used to survey and contact T cells, they could act as ruffles for making macropinosomes as well.

Macropinocytosis may provide an efficient sampling mechanism for fluid phase or nonbinding antigens. Since macropinosomes have lower surface/volume ratios than small vesicles, more extracellular fluid is sampled per unit of membrane internalized. Sallusto et al. (1) calculated that individual dendritic cells internalized  $\sim 2,400$  fl/h, the equivalent of the cell volume. If this sampling were carried out by coated vesicles, whose internal volume is  $\sim 0.001$  fl, each dendritic cell would have to internalize  $>40,000$  vesicles per minute. Estimates

of the rate of coated vesicle formation in cells are 40 times lower (59). Spherical macropinosomes that are 1.6  $\mu\text{m}$  in diameter carry  $\sim 2$  fl. An uptake rate of 20 such vesicles each minute (vs 40,000 coated vesicles) would explain the observed rate of solute uptake.

Inside cells, macropinosomes may interact with other organelles such as endosomes, lysosomes (60), or MHC II-rich vesicles. Yet, at some point, the large volume of internalized fluid must be disposed. This could occur in either of two ways. If the macropinosomes themselves recycle to the plasma membrane, most of the internalized membrane and fluid would reflux. Solute would not accumulate, consistent with the rapid recycling observations of Levine and Chain (41), but small amounts of peptide would have to be captured via MHC products. Alternatively and more likely we feel, macropinosomes would shrink inside the cell, with membrane recycling to the cell surface via smaller vesicles or shrunken multivesicular bodies, and water leaving by diffusion across membranes (61). In this case, internalized solutes might remain trapped inside the shrinking macropinosome for longer periods. This model would better fit the findings of Sallusto et al. (1) in two respects: (a) significant amounts of solute would accumulate in the cells; and (b) one would have a greater opportunity to carry out all the critical steps required for presentation, i.e., limited proteolysis of the antigen, fusion with vesicles carrying newly synthesized MHC II and invariant chain pro-

teins, proteolysis of the invariant chain, and peptide binding to MHC II products.

Evidence for both kinds of macropinosome disposal, i.e., direct recycling vs diffusion of internalized fluid, have been obtained. In cultured fibroblasts and epithelial cells, macropinosomes interact little with other endocytic compartments and eventually return to the cell surface (62). In macrophages, macropinosomes fuse readily, including with lysosomes, and most of the internalized solute remains in the cell (60). Solute retention clearly takes place in the GM-CSF + IL-4-stimulated dendritic cell, and at the same time, fully formed MHC II-peptide complexes cycle to the cell surface for presentation.

**Conclusion.** We comment on the enigma that dendritic cells are potent APCs but do not readily accumulate solutes and particles in lysosomes. Mechanisms are now emerging whereby dendritic cells do endocytose and link uptake to antigen presentation. One such mechanism, as shown by Sallusto et al. (1), is bulk uptake by macropinocytosis after stimulation by the combination of GM-CSF and IL-4. This is followed by the delivery of solutes to vacuoles that are rich in MHC II products (MIIVs). Unlike macrophages, dendritic cells lack a well-developed scavenging pathway for complete digestion of substrates to amino acids in lysosomes. Instead, endocytosis appears to be regulated and relegated to present peptides via MIIVs.

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