

Development of the adaptive immune response necessitates uptake of an antigen inside antigen-presenting cells (APC), such as dendritic cells, B cells and macrophages and liberation of antigenic peptides for binding and presentation by major histocompatibility complex class II (MHC-II) molecules to CD4 T cells. The classical MHC-II antigen presentation pathway uses newly-synthesized MHC-II molecules co-chaperoned by Ii chain and H2-DM molecules to present antigenic peptides generated in late endosomal/phagosomal compartments. Antigenic peptides can also bind MHC-II molecules recycled from the cell-surface in early endosomes/phagosomes in a recycling MHC-II antigen presentation pathway, which is less dependent on invari-

## Dichotomy of MHC Class II Antigen Presentation of M Protein from *Streptococcus pyogenes* into Classical and Recycling Pathways in Macrophages

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ant chain and H2-DM. In this review, we summarised evidence that presentation of T cell epitopes from a single bacterial antigen (surface fibrillar M protein from *Streptococcus pyogenes*) engages either classical or recycling MHC-II pathways.

### ● Introduction

The majority of peptides recognized by CD4 T cells are presented by the classical MHC-II presentation pathway operating in both professional and non-professional antigen-presenting cells (APC) (1). In this pathway, exogenous antigens are processed in late endosomal/lysosomal compartments at low pH, and peptide epitopes generated are loaded on newly-synthesised MHC-II molecules under the control of HLA-DM (H2-DM in mice) and HLA-DO (H2-DO in mice) chaperones. Furthermore, presentation by the classical pathway depends on the intracellular transport of newly-synthesised MHC-II molecules from the endoplasmic reticulum (ER) via the trans-Golgi network to MHC-II containing compartments (MIIC), which has been shown to be controlled by another molecular chaperone Ii-chain (2-5).

Alternatively, there is evidence to suggest that antigenic peptides generated in

early endosomes at pH above 6.5 can bind MHC-II molecules recycling from the surface of APC independently of Ii-chain, DM and DO chaperone function (6-8). Several

factors can facilitate peptide loading in the recycling MHC-II antigen presentation pathway, such as localization of peptide epitopes within flexible regions of protein antigens and expression of particular MHC-II alleles or alternative splice variants of Ii-chain in APC (5,9,10). Here, we have summarised our data on the mechanisms of antigen uptake, intracellular transport and proteolytic processing of two T cell epitopes from the surface M protein of *Streptococcus pyogenes* for presentation by MHC-II molecules in macrophages.

### ● Experimental system

We have developed an experimental system to study the mechanisms of antigen presentation of two immunodominant T cell epitopes (Ed-restricted M517-31 and Ad-restricted M5308-319) from the surface fibrillar M5 protein of *S. pyogenes* (11-14). The system uses mouse bone-marrow-derived macrophages and/or macrophage-like cell line J774A.1 treated or untreated with metabolic inhibitors and pulse-chased with viable *S. pyogenes* for different periods of time. Antigen-dependent IL-2 production of T cell hybridomas specific for M517-31 and M5308-319 T cell epitopes is measured as the proliferative response of the IL-2-dependent cytotoxic T cell line-2 (CTLL-2).

We showed that presentation of M517-31 was characterised by fast kinetics

(15 min), whereas presentation of M5308-319 was much slower (60 min). Moreover, M517-31 presentation was independent of acidification of endosomal/phagosomal compartments (suggesting the involvement of early endosomes/phagosomes in antigen processing), as well as of protein synthesis (suggesting independence of newly-synthesised MHC-II molecules), whereas presentation of M5308-319 was dependent on these mechanisms. These data suggested that presentation M517-31 and M5308-319 engage recycling and classical MHC-II pathways, respectively (15-18). We used this experimental system to address whether antigen presentation via classical and recycling MHC-II pathways depend on different routes of antigen uptake and intracellular transport, as well as proteolytic processing enzymes.

### ● Antigen uptake

Antigen-presenting cells use several mechanisms of antigen uptake, including phagocytosis, macropinocytosis and receptor-mediated endocytosis. Phagocytosis is referred to as an F-actin microfilament-dependent mechanism of internalisation of large particles (>0.25 µm) and bacteria into cells, which is induced by ligation of specific surface receptors (2,19,20). Membrane ruffling induced by growth factors, phorbol ester or other stimuli results in macropinocytosis, which is also dependent on F-actin microfilament recruitment but is receptor-independent. Macropinocytosis is employed by cells for uptake of fluids and particulate material including bacteria *E. coli* and *Salmonella* species into large endocytic vesicles 0.15-5.0 µm in size (21,22). Both phagocytosis and macropinocytosis are independent on clathrin-coated pits and vesicles. In contrast, receptor-mediated endocytosis is described as a clathrin-dependent, F-actin-independent uptake mechanism of soluble antigens and smaller bacteria, such as *Ehrlichia risticii* (23) and some *Chlamydia* species (24). All three

mechanisms of uptake are employed for intracellular antigen processing (20,25,26), although clathrin-dependent and clathrin-independent mechanisms of uptake have been shown to transport antigen largely to distinct endosomal compartments (27), suggesting that different uptake mechanisms could be employed for processing via classical and recycling MHC-II pathways.

We investigated whether the engagement of a particular uptake mechanism results in preferential antigen presentation via the classical and/or recycling MHC-II pathway (16). Electron microscopy studies showed that upon brief pulse with *S. pyogenes*, bacteria were attached to the APC cell surface and then internalised into tight and spacious vacuoles, probably corresponding to phagosomes and macropinosomes, respectively (Figure 1 A, B). Upon inhibition of phagocytosis and macropinocytosis with cytochalasin D (blocks F-actin recruitment (28)) no internalization of bacteria was observed by electron microscopy, and no presentation of M517-31 and M5308-319 was detected at the functional level. Similarly, blockade of M517-31 and M5308-319 presentation was observed in

the presence of wortmannin (Figure 1 C), which blocks phosphoinositide 3-kinase (PI3-kinase)-dependent completion of vesicle formation in macropinocytosis and phagocytosis (29). Interestingly, about 10 times higher concentration of wortmannin was necessary to block M517-31 presentation, compared to M5308-319, suggesting that classical and recycling MHC-II pathways have differential requirements for the PI3-kinase activity. We have also shown that protein kinase C (PKC, inhibited by staurosporine) activity is essential for presentation of both M5-specific T cell epitopes from bacteria and synthetic peptides (Figure 1 D). PKC activity is known to control actin polymerization and recruitment of cytoskeletal proteins to the binding sites of particles at the cell surface, and hence particle internalisation (30-32). Collectively, our data suggest that microfilaments, as well as PKC and PI3-kinase activity are essential for bacterial uptake and downstream presentation by both classical and recycling MHC-II pathways (Figure 1).

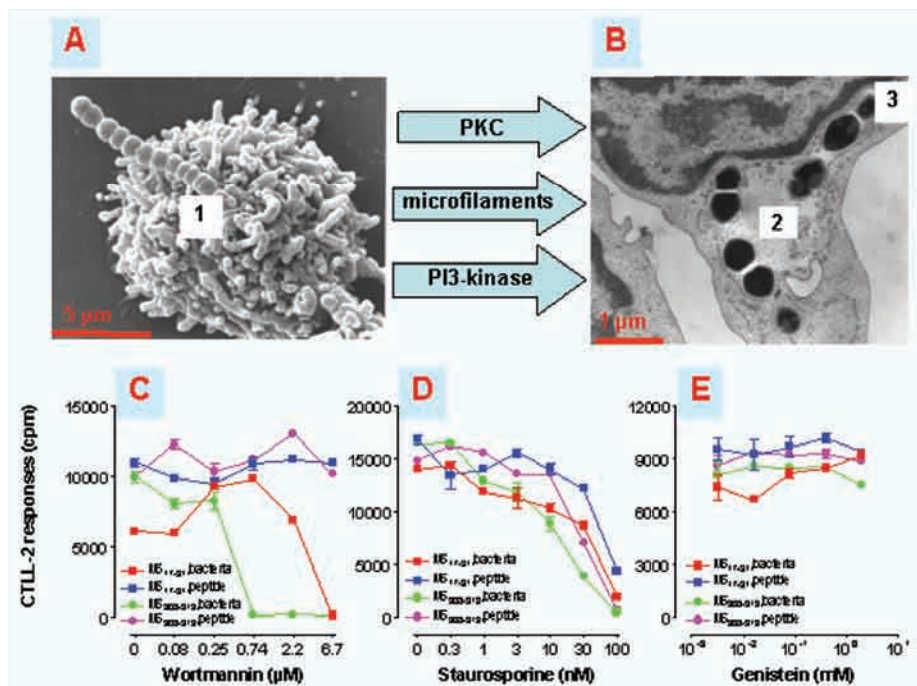
Importantly, uptake of bacteria into spacious, but not tight, compartments

was eliminated in macrophages pre-treated with amiloride (inhibits membrane ruffling and macropinocytosis (33)), leading to selective blockade of M5308-319 presentation (16). These data suggest that uptake of *S. pyogenes* by phagocytosis results in M517-31 presentation via the recycling MHC-II pathway, while macropinocytosis targeted bacteria for selective M5308-319 presentation via the classical pathway (Figure 2). This conclusion is consistent with previous reports that macropinosomes deliver antigen to the multivesicular and multilamellar compartments reminiscent of MIIC (2,26), possibly due to their preferential fusion with tubular lysosomes (34).

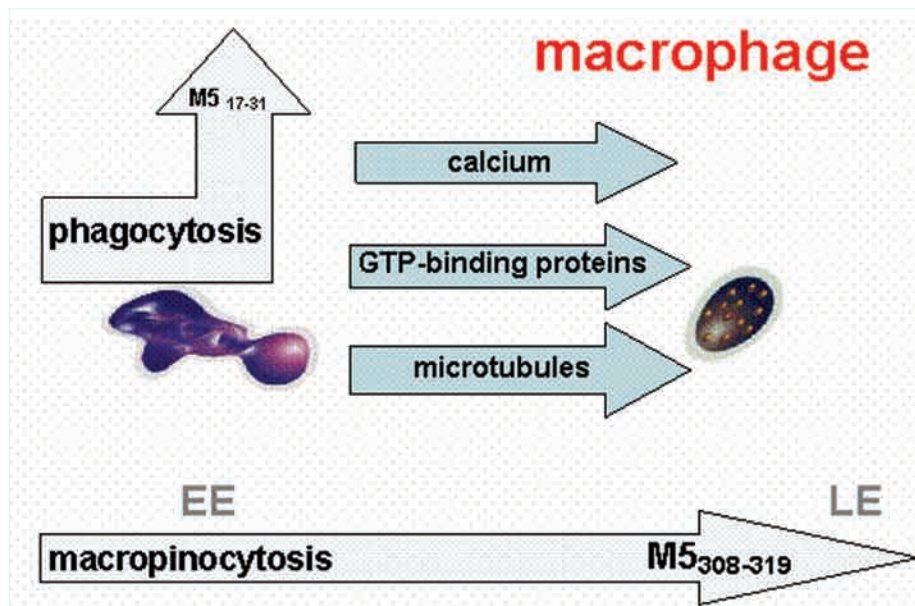
### ● Intracellular transport

Upon internalisation, antigen is transported along the endosomal pathway for antigen-processing and loading on MHC-II molecules. Membrane transport in the endosomal pathway has been shown to be dependent on several molecular mechanisms (35), including intact microtubule cytoskeleton (blocked by vinblastine (36,37)) and protein tyrosine kinase activity (PTK, inhibited by tyrphostin A25 (38)). Endosomal transport and endosome-phagosome fusion are also known to be controlled by GTP-binding proteins (blocked by aluminium fluoride (AlF<sub>4</sub><sup>-</sup>) ions (39)) and mobilization of intracellular calcium (blocked by thapsigargin (40,41)) (Figure 2). In the course of antigen processing peptide epitopes are generated and loaded on newly-synthesized MHC-II molecules, which are transported from ER via the trans-Golgi network to MIIC (blocked by Brefeldin A (42)).

We addressed the role of endosomal/phagosomal transport, as well as transport of newly-synthesized MHC-II molecules between endoplasmic reticulum and trans-Golgi for presentation of streptococcal M protein-specific T cell epitopes from bacteria (15,17). Presentation of M5308-319, but not M517-31, was found to be dependent on intact microtubules, GTP-binding proteins and intracellular calcium, suggesting that these molecular mechanisms control antigen presentation via the classical, but not recycling, MHC-II pathway. In addition, presentation of M5308-319, but not M517-31, depended on intact ER-Golgi transport, suggesting that newly-synthesised MHC-II molecules were engaged in M5308-319, but not M517-31, presenta-



**Figure 1: Internalization of *S. pyogenes* for MHC-II presentation.** Human monocytes were pulsed with viable *S. pyogenes* for 10 min and the attachment of bacteria was analyzed by scanning (A) (magnification  $\times 7,550$ ; bar 5  $\mu\text{m}$ ) or transmission electron microscopy (B) (magnification  $\times 11,500$ ; bar 1  $\mu\text{m}$ ). Bacteria are seen attached to the cell surface (1), internalized into spacious (2) or tight (3) vacuoles. Signalling requirements for presentation of two M5-specific T cell epitopes from *S. pyogenes* were studied in J774A.1 macrophages treated with wortmannin (C), staurosporine (D) or genistein (E). Macrophages were pulsed with bacteria or peptides, and presentation of M517-31 and M5308-319 was measured as IL-2 response of specific T cell hybridomas in CTLL-2 bioassay. Internalization of *S. pyogenes* and presentation of both M5-specific T cell epitopes from viable bacteria were found to be dependent on microfilaments, as well as the activity of protein kinase C (PKC) and phosphoinositide 3-kinase (PI3-kinase), as indicated by arrows.



**Figure 2: Model of intracellular transport of *S. pyogenes* for MHC-II presentation.** Internalization of *S. pyogenes* by phagocytosis results in presentation of M517-31, while macropinocytosis leads to M5308-319 presentation. Transport between early (EE) and late (LE) endosomes is controlled by at least three mechanisms, such as microtubules, GTP-binding proteins and mobilization of intracellular calcium, which are crucial for presentation of M5308-319, but not M517-31.

tion. Furthermore, presentation of both epitopes was independent of PTK activity (blocked by genistein (43)) necessary for transport between late endosomes and lysosomes (Figure 1 E). These data suggest that bacterial antigen is transported to late endosomes/phagosomes, but not lysosomes, for M5308-319 presentation via the classical MHC-II pathway. In contrast, bacteria are processed in early endosomes/phagosomes for M517-31 presentation via the recycling MHC-II pathway (Figure 2). In our recent study, we used subcellular fractionation of bone-marrow macrophages pulsed with viable *S. pyogenes* to show fast appearance of M517-31/Ed complexes and slow accumulation of M5308-319/Ad complexes in light fractions corresponding to plasma membrane/early endosomes (44). This data suggest a role for endosomes in presentation of antigens from phagocytosed bacteria.

### ● Proteolytic processing

Antigens taken up by APC are processed during endosomal transport principally by pre-processing enzymes, such as asparagine endopeptidase (AEP) and g-interferon-inducible lysosomal thiol reductase (GILT) (45,46), and then by cysteine- and aspartic proteinases (47,48). We have studied the participation of all four known families of intracellular proteinases (49), aspartic-, serine-, cysteine-, and metallo-proteinases, in bacterial anti-

gen processing (18). To this end, we used family-specific metabolic inhibitors pepstatin A, phenylmethylsulfonyl fluoride (PMSF), (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methyl-butane (E-64d) and 1,10-phenanthroline, respectively. The data showed that processing of bacteria for M5308-319 presentation was dependent on aspartic-, cysteine- and serine proteinases, while M517-31 presentation was dependent only on serine proteinase activity. The involvement of intracellular serine proteinases in bacterial antigen processing has not been described previously. We envisage that serine proteinases with neutral pH optimum (49,50) may be strategically positioned in early endosomal/phagosomal compartments and therefore can participate in processing of recycling T cell epitopes and pre-processing of the classical epitopes prior to further processing by lysosomal enzymes.

### ● Concluding remarks

Our studies show evidence for the segregation of bacterial antigen processing and MHC-II presentation into classical and recycling pathways. In the classical pathway, *S. pyogenes* is preferentially taken up by macropinocytosis and then transported via a microtubule- and GTP-binding protein-dependent mechanism to late endosomes/phagosomes for pH-dependent processing by serine-, aspartic- and cysteine-proteinases. The peptide epitopes containing M5308-319 core

sequence are generated and bound on newly-synthesised MHC-II for presentation to specific CD4 T cells. The recycling pathway involves bacterial uptake via phagocytosis, followed by microtubule-, and GTP-binding protein-independent transport to early endosomes/phagosomes for pH-independent processing by serine proteinases, liberation of M517-31 and binding of recycling MHC-II molecules. Further studies are necessary for establishing functional consequences of the described antigen processing dichotomy for immunity to infections, cancer, tolerance and autoimmunity (10).

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