DISCUSSION

Chapter 4. Discussion

The unique process of endocytosis and nature of endocytic vesicles have traditionally attracted scientific interest that dates back to the earliest reports of Ellas Metchnikoff in 1882 (212). Since then, an ever-growing pool of scientific literature on endosome research corroborates unabated appreciation for the critical role of endocytosis and endocytic vesicles in cell biology. The capacity of endocytic process is indeed impressive; the latter allows direct uptake of small molecules to entire microorganisms into host cell(s), without compromising cellular integrity or energetics (142). Consequently, the compartmentalized function of endocytic vesicles permit a unique access of an otherwise tightly segregated intracellular milieu to the macromolecular contents of extracellular environment.

Arguably, nowhere is the significance of mammalian endocytosis more pronounced than in macrophages (2, 3). Adult macrophages vigorously internalize small antigenic particles by pinocytosis and larger bacteria, viruses and other microbial cells by phagocytosis. Besides containing systemic spread of potential pathogens and toxins, the endocytic milieu neutralizes endocytosed agents by oxidative and/or nonoxidative mechanisms (3, 42). Subsequently, a complex array of enzymes along the endocytic pathway perform controlled degradation of the endocytosed material; a process that is ultimately completed in the lysosomes. Recent reports have highlighted the critical role of specific macrophage endosomes in extra-lysosomal antigen processing and major histocompatibility complex (MHC) mediated presentation - both of which are critical in mammalian cell mediated immunity (84, 85). Macrophage endocytosis is also linked to specific diseases, where microbes exploit the endocytic process for selective entry and proliferation within the host (57). Together, mammalian macrophage endosomes offer an ideal system for characterizing the critical role of endosomes in disease and health. The latter fact offered the rationale for selecting the macrophage system for studying endosome characteristics described in this report.

In the mammalian system, two major anatomic site-specific pools of mature macrophages exist: the alveolar and peritoneal. Macrophages from each pool exhibit number of characteristic differences. The macrophages from yet other anatomic sites, like, pleural macrophages also exhibit site-specific differences (55). Alveolar macrophages (AM) and peritoneal macrophages (PM) respond differently to oxygen, reflective of the high and low oxygen tension in the alveolar and peritoneal sites, respectively, that is based on differences in their key enzymes of oxidative phosphorylative and glycolytic pathways (194). Differences exist in their activation, spontaneous spreading behavior (14) and chemokine production/induction patterns when activated (10). Within the gamut of endocytosis (including uptake, processing and presentation), possible differences between individual pools have not been analyzed fully. However, several reports have helped underline such possibilities. In a seminal paper, Todt *et al.* reported significant differences between the two pools in phagocytosis of apoptotic cells (PMs exhibiting higher phagocytic efficiency) and attributed this to difference in expression of specific class(es) of protein kinase C (PKC) in these two pools (215). In context of endosomal (enzymatic) processing, differences are reported in cathepsin activities, specifically Cathepsin D (149, 220); AMs and PMs also exhibit marked differences in their efficiency of antigen processing and presentation of agents like *Listeria* (228). Together, these facts underline the importance of choosing site-specific macrophage pools for studying specific functions of endocytosis.

The importance of the peritoneal membrane in host immune functions and general homeostatsis is unambiguous (26). First, the semipermeable peritoneal membrane covering multiple vital organs acts as a highly selective barrier against possible infectious threat. Second, an inducible fibrin network within the peritoneal cavity is capable of trapping particulate matter. Third, specialized immune cells like polymorphonuclear lymphocytes and PMs perform phagocytic elimination of most pathogens. These latter cells are also associated with diaphragmatic lymphatic absorption and antigen presentation that induce humoral immune functions whenever required (26).

In terms of numbers, resident PMs are the single largest immune effector cells in the peritoneal cavity and originate from unique sets of locally proliferating progenitor cells, which offer site-specific characteristics (49). Mature PMs produce copious amount of cytokines, activate the complement cascade and induce migration of granulocytes from the intravesicular space into the peritoneal cavity (35). Functionally, PMs act as the first line of defense in the local peritoneal niche (26, 204).

Although, considerable information is available on macrophage endocytosis in general, few studies have focused specifically on peritoneal macrophages, till date. Reported studies of macrophage endosomes have largely been based on macrophage cell lines as the system for study. Cell line systems offer many advantages over primary macrophage cultures: ease of handling, an unlimited self-replicating source without significant quantitative limitations in sampling and relatively high degree of homogeneity within the cells (34). However, these systems suffer from limitations like they do not reflect the inherent heterogeneity of native macrophage cells, and more importantly, the diversity of phenotypes between macrophages isolated from different anatomical sites as described earlier (34, 144, 178, 204). Keeping this in view and the fact that very few representative cell lines of peritoneal macrophages exist (36), the present program focused on primary cultures of rat peritoneal macrophages as the system for study.

Following the selection of an appropriate system for study, the focus shifted to isolation of intact endosomes from such cells. A critical aspect in the study of subcellular organelles is standardizing effective techniques for cell disruption (without damage to target organelles) and subsequent high-yield purification of the same. In case of endosomes, this is all the more challenging due to (a) inherent heterogeneity in the morphology/types of vesicles along the endocytic pathway as well as similarities with lysosomes that make selective isolations difficult and (b) a highly active endosomal enzymatic milieu that exerts risk of degradation of endocytic components during purification. In past, three basic techniques have been frequently used for purification of endocytic vesicles: density gradient centrifugation (88, 137),

FFE (free flow electrophoresis) and magnetic affinity chromatography (13, 15, 52, 87, 150, 151). Electrophoretic isolation technique like FFE depends on the unique charge/mass ratios of endocytic vesicles that leads to specific (electrophoretic) migration pattern(s) and consequent purification of such vesicles within a 'free-flow' cell (87). Although there are comparatively few reports, FFE has been successfully used either independently (87, 126), or in combination with other techniques (61, 121) for endosome purification. Using a different principle, density gradient centrifugation relies on the unique weight/size ratio of endocytic vesicles, which generate distinct bands in density gradients (196). Owing to its ease of operation, this technique has found acceptance in endosome purification protocols, including purification of mammalian endosomes (88, 196, 214). However, one of the downsides of this technique is its 'non-specific' nature, a fact that carries the risk of contaminating subcellular organelles like mitochondria in the purified preparations (214). Besides, the close similarities of endosomes of different classes and between endosomes and lysosomes - often results in large overlaps of endosomes and lysosomes during purification thereby making purification of specific endosomal classes from a mixed population, untenable (214). In contrast with FFE or centrifugation, magnetic purification exploits the unique biomagnetic properties of iron-dextran laden endocytic vesicles from the cells that are fed with the ferromagnetic probe prior to their isolation. Since electromagnetic purification targets only iron-dextran containing vesicles, the system offers a high degree of specificity towards specific (endocytic) vesicles that actively participate in endocytosis and can therefore result in purer preparations. In past, magnetic purification has been successfully used to purify endosomes from diverse systems including *Dictyostelium discoideum*, (150, 151, 174) *Entamoeba histolytica* (130) and *Xenopus* oocytes (13, 171). However, there are very few reports for the use of this technique in mammalian system; interestingly, majority of these have used cell lines for their study and not primary cultures (15, 160).

Based on this background, the present project attached major emphasis on standardizing endosome purification protocols specific to primary cultured cells of peritoneal macrophages. An array of biochemical, immunological or morphological

assays with defined objectives were strategically used to independently assess individual steps of the purification in order to obtain highest quality endosomal preparations and examine if it were possible to purify endosomes representing discrete stages of the endocytic pathway.

At the onset, these independent investigations revealed several interesting observations. The first of these was the determination of sonication as the best disruption method for macrophage cells from primary cultures; related techniques like, forced-passage through syringe reported by Padh *et al.* did not offer satisfactory results. The magnetic chromatography system was developed according to the prodecure by Rodriguez Paris *et al.* and Padh *et al.* using a stainless steel wire (150 mg) matrix compacted to a length of 1 cm, in a magnetic field of strength 0.8 Tesla. However, a modification was introduced by using circulating cold water jacketed glass column that resulted in dramatic improvement of viable endosome yield (data not shown). Since a major determinant of purification is the quality of the iron-dextran probe, systematic analysis was conducted to ensure low cytotoxicity, efficient internalization and subsequent retention of iron-dextran fed endocytic organelles in the magnetic matrix. Given that endocytic dose response of iron-dextran varies considerably between different cell systems (52), the present studies independently standardized the dose/time of endocytic probes that resulted in efficient purification without being cytotoxic to the peritoneal macrophage cells. In past, iron-dextran concentration as high as 37 mg/ml was used by Diettrich *et al.* for fibroblast lysosome purification (52), whereas Glombitza *et al.* reported 24 h pulse periods for optimal probe internalization in human epidermal keratinocytes (73). In contrast, concentration of 2 mg/ml iron-dextran with incubation period of 45 min closely agrees with that reported in *Dictyostelium discoideum* (150, 151, 174) or U937 macrophage cell line (160). The comparatively lower concentration and exposure time of iron-dextran containing medium for *Dictyostelium discoideum* and macrophages may be due to higher pinocytic activity in these systems. In a related study Becich *et al.* used 0.2 mg/ml of iron-dextran covalently conjugated to Gal-BSA or asialofetuin to isolate endolysosomes from Hep G2 cultures by exploiting the

asialofetuin receptors on the cells and recorded 8 - 9% recovery of the PNS lysosomes followed by 9 - fold enrichment (15). In contrast, the present studies obtained approximately 50% of the PNS endocytic vesicles with 3 - 5 fold purity as judged by pinocytic ligand FITC-dextran. Whereas this data indicates a high degree of efficiency of magnetic purification, several other groups have also reported similar observations. Working with U937 macrophages, Perrin-Cocon *et al.* reported 50% recovery (endocytic retention) of endocytic probe (160), whereas in case of fibroblasts Diettrich *et al.* obtained lysosomes with 25 - 29% yield with 6 - 40 fold purity (52). In the *Dictyostelium discoideum* system, Rodriguez-Paris *et al.* obtained 82% fluorescent yield with 60 fold purity (174); while Padh *et al.* reported preparations with 60 - 70% yield and 20 - 25 fold purity with <1% contamination from other organelles (151). Although the reason for the relative differences between purification data from individual reports is not clear, the data from the present analysis agrees with published observations and indicates high degree of purification. The uptake rate was fairly reproducible between different sets of primary cultures and did not show significant differences between the two probes, fed individually or together. Interestingly, uptake kinetics revealed a biphasic nature: following a relatively slower kinetic in the first 10 - 15 min, the uptake rate increased after 15 min and remained overall constant upto 40 min.

Following the determination of the time required for uptake saturation, the next set of experiments determined the effect of physical conditions like temperature on the iron-dextran and FITC-dextran uptake. Quite a few reports have indicated temperature as a critical determinant of endocytosis - a feature that seems to be conserved across species (59, 227). Whereas the reason(s) for temperature sensitivity of the endocytic process is not completely understood, many reports have indicated that discrete functions associated with the endocytic pathway respond differently to temperature (227). For example, in *Trypanosoma brucei* endocytosed material is not transported from the cell periphery (flagellar pockets) to discrete endosomes in the cytoplasm proper below 4°C; above 12°C the endocytosed material is transported into recycling endosomes but does not seem to fuse with late endosomes (25). These defects are quickly reversed at 37°C (25). In yet other reports, temperature sensitive

mutants in the well studied *Dictyostelium discoideum* showed inhibition of specific endocytic functions in response to restrictive temperatures. Whereas early endocytic events were severely affected, other membrane-trafficking events such as phagocytosis, secretion of lysosomal enzymes and contractile vacuole function were normal (11). Related observations in mammalian macrophages also report that temperature dependent uptake of ligands is remarkably different from secretion and endocytic processing leading to degradation of ligands (118, 227, 231).

In the present program, studies were limited to investigating the effect of temperature on total uptake capacity within a 45 min period determined to encompass the whole endocytic pathway. The dramatic decrease in fluid phase uptake at low temperatures in the present observation is reminiscent with reports in *Trypanosoma* system as well as that reported in mammalian macrophages (25, 118, 227); the present studies extend the information further in defining the upper extreme above 40°C that inflicts near total loss of uptake function. Between these two extremes, the kinetics of uptake was multiphasic; there were two inflection points: one at temperatures exceeding 15°C and another above 30°C. Interestingly, working with pig platelets, Wolkers *et al.*, also reported two temperature sensitive inflections (231). Using FTIR studies the same group determined a major membrane phase transition in these cells at approximately 12°C and two smaller transitions at 26°C and 37°C, which indicated that temperature dependence of fluid phase endocytosis coincides with membrane properties (231). Whereas such investigations were beyond the scope of present program, the current analysis allowed a valuable insight on the effect of temperature on endocytosis in peritoneal macrophages.

Following establishment of conditions for endosome isolation from primary cultures of macrophages, appropriate strategies were designed to acquire adequate number of peritoneal macrophages for detailed characterization of endosomes. A typical problem in studying peritoneal macrophages is the paucity of macrophage numbers naturally obtained in native peritoneal lavages. This often necessitates use of large number of animals for harvesting adequate cell numbers, leading to logistical and ethical problems. Such problems have been circumvented largely, by use of phlogogenic agents like thioglycolate - which, following administration into the

peritoneal cavity - leads to massive elicitation of local macrophage numbers (115). Following establishment of this protocol in the murine system (69), thioglycolate induced peritonitis has been accepted as a standard procedure for providing large number of macrophages for studies and has offered a background to assess effects of pro-inflammatory or anti-inflammatory mediators (115).

Therefore, keeping with well-established approaches, primary cultures of thioglycolate elicited macrophages offered the basic system for studies in the present program (set I). Given the potent role of macrophages in inflammation and immunity, a parallel set (using thioglycolate elicited background) stimulated by a potent inflammatory stimulus, formed the second system (set II). Further, in order to explore possible differences induced by thioglycolate elicitation, a third set (set III), consisting of resident (non-elicited) macrophages was studied.

Magnetic affinity chromatography adapted for the program, allowed purification of high quality endocytic vesicles from all three systems. Biochemical analysis performed after each set of analysis confirmed high enrichment of endosome specific markers and did not detect significant contaminating enzymes/factors known to be associated with other subcellular organelles. Electron microscopic analysis of the purified endosome preparations agreed with these findings and showed vesicular structures were free from contaminating organelles – otherwise observed in the PNS fractions (data not shown) while fluorescent microscopic analysis confirmed high enrichment of intact endosomes in the magnetically purified fraction. The integrity of the isolated endocytic vesicles was analyzed by their ability to undergo ATP-dependent acidification. ATP hydrolysis by V-H^+-ATPase (vacuolar ATPase) present in the endosomal membrane generates an acidic pH within the membrane bound endosomal milieu (39, 152, 155, 174, 206). This unique function has therefore found use as an indicator for intact, biochemically functional endosomes (160, 174). The most frequently used assay in context is quantitating fluorescence of endocytosed FITC. The fluorescence is high under neutral pH and is quenched as the internal milieu is acidified by V-H^+-ATPase in presence of extraneously added ATP (152, 160). In the present study, pH of the isolated vesicles dropped with subsequent

maturation. Further addition of 1 mM ATP quickly resulted in decrease in the intravesicular FITC-dextran fluorescence intensity, as expected from ATP driven intravesicular acidification. The dissipation of the proton gradient with nigericin indicated, intravesicular acidification was due to ATPase activity and the integrity of the vesicles was intact after purification.

Following acidification of endosomes in the purified preparations, efforts were directed at studying endosome biogenesis in rat peritoneal macrophages. The knowledge of the consistent association of unique Rab proteins with endosomes has seen their well-accepted use as markers to identify specific classes of endosomes (162, 187, 213, 223). For example, Rab 5 regulates the internalization of molecules via clathrin coated vesicles and their subsequent delivery to early endosomes (32, 195). It regulates the fusion of plasma membrane-derived clathrin coated vesicles with early endosomes and homotypic fusion between early endosomes (32). Thus, Rab5 functions as a regulatory factor in the early endocytic pathway and acts as a marker for early endosomes (32, 39, 214). In contrast Rab 7, a 22 kDa GTPase, was determined to be a regulator of late endocytic membrane transport leading from early to late endosomes and is prominently associated with late endosomes but not the lysosomes (31, 224). Hence, Rab 7 is widely regarded as a marker for late endosomes (214, 224). Therefore, in the present study these two markers were used to identify the different endocytic vesicles.

In the present findings Rab 5 immunoreactivity was highlighted in early pulsed (and chased) cells as a discrete ~23 kDa band that agreed with expected size of Rab 5 in published reports (214). Rab 5 was detected strongly in the 5'/0' and 15'/0' endosomes but not in longer pulsed and chased cells (15'/30' and 5'/60'). In contrast, Rab 7 was insignificant in the short pulsed cells and was detected only after 30 min chase. The above feature was highly reproducible in independent batches of thioglycolate stimulated cells. Purified preparations that stained for Rab 5 (short pulsed/chased) did not stain for Rab 7 (long pulsed/chased) and *vice versa;* only exception was 15'/0' pulsed cells which were positive for both the Rabs thereby

indicating an intermediate population. Whereas such sharp demarcation of Rab 5 and Rab 7 expression patterns perhaps was not unexpected, these results, together with specific enrichment of these markers in the purified fractions (RF) indicated a surprisingly high degree of purity of the endosomal preparations. Subsequent electron microscopic (EM) studies lend further credence to the Western blot based protein analysis, where dramatic differences were observed between endocytic vesicles in the short pulsed/chased and long pulsed/chased cells. The preparations subjected to shorter pulse/chase appeared as smaller 0.4 - 0.8 μm structures characterized by lighter appearance with electrolucent centers. The high incidence of such vesicles in this group largely negates the likelihood of errors (electrolucent vesicles without significant endocytosed iron) in observation(s) due to the plane of section. Similar arguments also hold true for vesicles isolated with extended chase periods. Increase in chase time allowed detection of relatively larger structures; many of these vesicles contained internalized membranes. With longer pulse/chase the size of the vesicles were significantly larger, spherical or globular and noticeably more electron-dense. A few of these vesicles were seen fusing with one another. Earlier Tjelle *et al.* working with endosomes from mouse peritoneal cell line J774 and Perrin-Cocon *et al.* working with human macrophage cell line U937 observed similar profiles in early and late pulsed cells (160, 214). Interestingly, Western blot analysis of such endosomal preparations stained positive for both Rab 5 and Rab 7 (with relatively higher signals for Rab 5 in early endosomes and vice versa). Such discrepancy was explained by possibilities of overlapping endosomal populations during purification, or presence of both Rab 5 and Rab 7 in early and late endosomes in the respective macrophages. In that context, the present findings negate simultaneous presence of Rab 5 and Rab 7 GTPases in early and late endosomes of rat peritoneal macrophages. Another interesting observation in this report was the association of Rab 7 signals even in long pulse-chased 15'/60' cells; sets which showed significant number of lysosome like vesicles.

Lysosomes are the terminal and major digestive compartments of the endocytic pathway. They are responsible for the degradation of materials internalized

by endocytosis and autophagocytosis. The highly acidic intralysosomal environment is maintained by lysosomal membranes. The latter sequesters the acid hydrolases and helps in interaction and fusion of lysosomes with other membrane organelles. One of the primary components of lysosomal membranes is LAMPs. These are membrane glycoproteins located in the intralumenal domains of mouse, rat, chicken and human lysosomes; hence ubiquitously regarded as late endosomal/lysosomal markers (223). Both LAMP-1 and Rab 7 signals were detected in 15'/30' and 15'/60' pulsed/chased chased cells. This observation indicated the presence of late endosome like vesicles in the above cells. Earlier studies by Griffith *et al.* lead to similar conclusions (75). Interestingly, biochemical, immunological and electron microscopic data on the 15'/60' set, offer consistent evidence for the presence of both late endosomes and lysosome in the purified population. This finding supports and extends earlier observations of 'tubular lysosomes' in peritoneal macrophages and J774 cell lines by Racoonsin *et al.* and Harding *et al.* (84, 167). Furthermore, absence of sequential transport of endocytosed ligands from late endosomes to lysosomes with longer chase period rules out a vectorial one-way transport in peritoneal macrophages, thereby arguing for the existence of a retrograde fusion step between lysosomes and late endosomes. Earlier studies by Griffith *et al.* showed that when cells were fed with colloidal gold and chased in gold free medium for days together the markers were invariably distributed in late endosomes and lysosomes (77, 78). Also supporting a retrograde transport between the lysosomes and late endosomes was the finding by Jahraus *et al.* This group showed that sucrose that had accumulated in lysosomes could be hydrolyzed by invertase, conjugated to latex beads present in late endosomes/phagolysosomes (96). Hence, such retrograde fusion might help the lysosomes to inject mature hydrolases into late endosomes whenever necessary (96).

Contrary to conventional belief that endocytic, enzymatic, processing is a strictly lysosomal feature, many recent reports have highlighted discrete proteolytic processing machinery in the endosomes (21, 53, 214). Keeping this in view, significant efforts were directed at studying the role of key endosomal proteases in peritoneal macrophages. Initial assays with purified preparations at different pH

ranges showed higher proteolytic activity in the acidic pH (pH 4) and lower, but detectable activity at neutral pH and no detectable activity in alkaline pH range (data not shown). The activity was not inhibited by serine or metalloprotease inhibitors (PMSF and EDTA respectively), but significantly inhibited by pepstatin, a reversible aspartic protease inhibitor; and E-64, an irreversible cysteine protease inhibitor. The activity was also considerably affected by leupeptin – an inhibitor of both serine (trypsin) proteases and cysteine proteases. However, the fact that the activity was not inhibited by PMSF (an inhibitor for serine protease) indicated that the target of leupeptin might well be the cysteine proteases in this case. Together, these results strongly indicated that acid active aspartic and cysteine proteases as well as neutral cysteine proteases were active in the purified endosomes from rat peritoneal macrophages.

In past, analyses of endosomal proteolysis have highlighted cathepsins as the most significant endosomal/lysosomal protease(s) (129). 'Cathepsins' are often used as a generalized term for most mammalian cysteine proteases or aspartate proteases (129) that are active in acidic pH, although few cathepsins like Cathepsin B are also reportedly active at neutral pH (21). Most cathepsins are synthesized as proforms between 40 - 50 kDa which are subsequently processed to active forms between 20 - 35 kDa (129). In the present report the potent proteolytic activity at low pH and the size of the proteolytic bands observed in zymography analysis, encouraged the investigation towards cathepsins in the isolated preparations. Since both Rab-specific Western blots and EM analysis indicated feasibility of highly pure stage-specific endosomal preparations, an interesting question was - whether stage-specific cathepsins are associated with these discrete endosomal pools to contribute to their unique proteolytic activity? Consequently, three most important endosomal cathepsins - D, B and H were chosen for their stage-specific association in related mammalian endosomes.

Cathepsin D is understood to be one of the most significant endosomal protease (53, 149, 197). In a seminal paper, Diment *et al.* reported degradation of the mannose- BSA ligand is evident as early as 6 min, after endosomal uptake (54). The

degradation was efficiently conducted in cell free system containing purified endosomes, in the absence of lysosomes - confirming that complete endosomal enzymatic degradation can occur specifically in pre-lysosomal compartments of endosomes. Further, the investigations showed the proteolysis to be fundamentally acid protease mediated since degradation could be induced by the addition of ATP and consequent intravesicular proteolysis was inhibited by pepstatin - a reversible inhibitor of aspartic (acid) proteases (54). Later, the group purified and characterized this acid protease as Cathepsin D. The latter was shown to exist as a 45 kDa proform and a processed 29 kDa mature form. Subsequently, the authors determined Cathepsin D as 'the most significant acid protease' in the endosomal milieu (54). Other researchers have identified this enzyme in multivesicular endosomes in macrophages, hepatocytes and B-lymphoblastoid cells (8, 80, 149). Two possible mechanisms explain the localization of Cathepsin D in endosomes. The cation-independent mannose 6-phosphate receptor (CI-MPR) dependent form transits from trans-Golgi network to lysosomes via endosomes while the (CI-MPR) independent membrane bound endosomal pool is synthesized as an inactive precursor which is further cleaved to yield the membrane associated active form and the soluble lysosomal form (53). In the present investigation, Cathepsin D immunoreactivity was fairly constant in all purified samples, indicating a stable association of this protease along the endocytic pathway in rat peritoneal macrophages. The data conforms well to previous immunofluorescence and flowcytometric evidence that active Cathepsin D was selectively retained within the early EEA1 (early endosomal associated antigen 1) positive vesicles in BHK (baby hamster kidney) cells (165) and rat hepatic endosomes (8) as well as Rab7 positive late endosomes (132). Consistent with the previous studies in rat iris pigment epithelial cells (205) and hepatocytes (6), detection of both the pro (43 kDa) and mature (28 kDa) forms of this enzyme in endosomal preparations in the current study suggests that significant processing of Cathepsin D occurs in the endosomes. Moreover, greater signal intensity of the active form with an increase in chase period shows higher activity of Cathepsin D in mature endosomes.

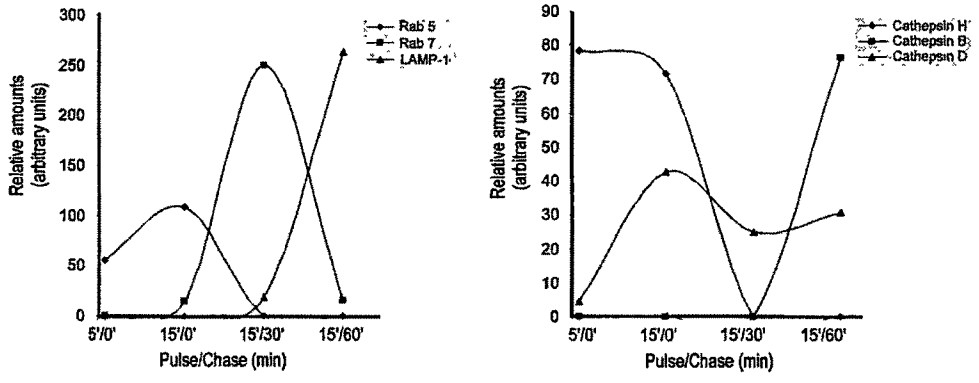
Likewise, Cathepsin B is regarded as the most significant cysteine endo-lysosomal protease. The above fact called for an analysis of Cathepsin B in context with endosome biogenesis. Cathepsin B is one of the few endosomal proteases that are active in both acidic and neutral pH ranges (21). This is significant in case of early endosomes, which are characterized by near neutral pH (221). Blum *et al.* have identified both forms of this enzyme in mouse macrophages at pH 6.2 using a fluorescent substrate ben-zyloxycarbonyl - arginyl - arginyl – amidomethylcoumarin (21). However, studies by Authier *et al.* and Lautwein *et al.* have provided evidence for early endosomal localization of Cathepsin B in hepatocytes and human B lymphoblastoid cells thereby arguing for its role in initial proteolytic attack (8, 113). In contrast with the previous results, the present analysis revealed that in rat peritoneal macrophages, Cathespin B was predominantly associated with late endosome/lysosomes and not with early endosomes. The immunoreactive 30 kDa band for Cathepsin B was prominently detected in late endosomal/lysosomal vesicles along with acid phosphatase, Rab 7 and LAMP-1. Earlier, Claus *et al.* also observed high levels of Cathepsin B in late endosomes in J774 macrophages (40). Incidentally, several authors have reported that most significant expression of this enzyme is in lyososomes (40, 63), a fact that might explain the discernable signal for Cathepsin B in the PNS fraction of shorter pulsed cells.

In contrast to the expression pattern of Cathepsin B, analysis for Cathepsin H was deemed interesting, because the latter enzyme is reported to be a major cysteine protease in early endosomes of macrophages by Claus *et al.* (40). Whereas, the above mentioned group could not detect the protease form by immunoblotting, the present investigation successfully determined a strongly immunoreactive 27 kDa band that agreed with the earlier observations by Haraguchi *et al.* on rat Cathepsin H (83). This band was detected selectively in short pulsed endosomes and not long chased cells. Hence, the present data extends the findings of Claus *et al.* and demonstrates the unique localization of this protease in early endosomes of mouse and rat macrophages. Available information from kidney and spleen tissues also specifies Cathepsin H to be an early endosomal enzyme with an optimum pH of about 6.5

(110); an exception is the results by Lautwein *et al.* in human B lymphoblastoid cells where Cathepsin H is defined as a late endosomal/lysosomal hydrolase (113).

Thus, the Western blot analysis for endosomal proteins successfully demonstrated (a) the unique efficiency of the electromagnetic chromatography technique towards high quality purification of discrete classes of endosomes and (b) the class-specific association of key cathepsins in these endosomes.

Taken together the present results indicate that the early endosomal compartments of rat peritoneal macrophages are rich in Rab 5 and the cysteine protease Cathepsin H. Subsequently, the ligand travels through intermediate endosomal vesicles rich in Rab 5, Rab 7 and Cathepsin H. Finally it enters the late endosomal/lysosomal compartments that are enriched in Rab 7, LAMP-1 and Cathepsin B. The aspartyl protease Cathepsin D is active throughout the endocytic pathway. It acts synergistically or in combination with cysteine proteases to process the internalized ligands. The maturity profiles of the endosomal markers and proteases are given below.



After successful isolation and temporal profiling of endosomal maturity, in the next phase of analysis we studied the total lipids of three distinct classes of endosomes. To separate both polar and non-polar lipids of the membrane samples by TLC analysis two solvent systems were used; the first solvent system resolved both phospholipids and neutral lipids while the second system specifically separated the neutral species. This method of separation has been used earlier by Rodriguez-Paris *et al.* and Ruiz *et al.* and reportedly offers high resolution of major phospholipids and

neutral lipids (174, 177). In the present studies, the current analysis successfully determined several characteristics; few were confirmatory and few were unique – between the lipid profiles of varied pulsed and chased endosome samples and their corresponding PNS fractions. Unlike marked difference(s) observed in the protein profiles of the PNS and RF, the lipid profiles of corresponding PNS and RF showed greater similarity indicating minor difference between the membrane lipid constituents of the intracellular organelles.

Not surprisingly, cholesterol showed up as the major lipid in all RF and PNS samples. The former is well known as a major constituent of mammalian membranes and possible component of 'raft lipids'. Besides, free cholesterol in the membrane is required for endocytosis of clathrin-independent cargo proteins and plays significant role in membrane ruffling and macropinocytosis (141). Incidentally, the level of cholesterol in the early endosomes (15'/30') and late endosomes (15'/60') was comparable. However, the 60'/0' pulsed cells showed significantly higher levels of cholesterol. The reason behind this is not clear. Interestingly this preparation evolved from a long pulse, presumably encompassed the entire plethora of endosome classes along the pathway – a fact that is indicated by the detection of both early and late endosome markers Rab 5, Rab 7 and LAMP-1, respectively. It may be speculated that unique class of endosomes with perhaps higher constituent cholesterol present in this preparation may have contributed to this observation. Another interesting observation was the considerable increase of PC during endosome maturation, evidenced by more than 5 fold increase of the former in the 15'/60' (and 60'/0' pulsed cells) compared to the 15'/0' pulsed cells. High PC content in late endosome membranes have also been observed by Kobayashi *et al.* who reported that the internal membranes of late endosomes is composed primarily of PC, a lipid concentrated in the fluid membrane domains of the endosome (107). The author also reported lysobisphosphatidic acid (LBPA) as a major late endosome membrane lipid (107, 108). LBPA is found at high concentrations on the internal membranes of late endosomes and lysosomes, where it plays an important role in the degradation of glycolipids and the transport of membrane proteins and lipids (79). The presence of the same could not be confirmed due to lack of LBPA standard in the current program. However, a major unidentified

lipid with migration behavior similar to that reported for LBPA was observed specifically in the late endosome sample. It is noteworthy that many cellular trafficking events like vesiculation, tubulation, membrane fusion and pinching off of endocytic vesicles are dependent on lipids which bring about the sharp changes in membrane curvature (88). Some of the other important lipids that were selectively enriched along the endocytic pathyway are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS) and spingomyelin (SM) whereas the level of free fatty acids decreased during endosome maturation. Higher levels of PE, SM and PS have been reported in late endosomes (107). SM preferentially associates with cholesterol and raft lipids. It is reported to contribute to membrane stability and to participate in endocytosis (233). However the accurate significance of increased levels of these lipids and concomitant decreased levels of free fatty acids in endosome maturation is not known and further research is required to explain these findings.

The next phase of the analysis was focused on resident macrophages and macrophages of thioglycolate background activated by inflammatory stimulus like LPS. Although thioglycolate (TG) stimulated macrophages are regularly used to overcome the limitation of obtaining adequate number of macrophages in native peritoneal lavages, the precise mechanism of TG elicitation is not fully clear. Analysis of TG elicitation have indicated that although TG recruits large number of macrophages to the site of administration, the latter are neither activated nor do they show enhanced endocytic or microbiocidal activity inside endosomes/phagosomes (100). Morphologically TG elicited cells look different from both resident as well as activated macrophages (45). Several reports indicate TG elicitation might actually reduce microbiocidal activity (42, 100, 115, 201). Interestingly, both functions were induced by Brewer's thioglycolate medium and not other thioglycolate preparations (115).

In contrast to thioglycolate, bacterial components such as lipopolysaccharide from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, zymosan (ZYM) from fungus or bacterial DNA bind to various toll-like receptors

(TLR) and potently activate macrophages (99, 158). The activated macrophages subsequently produce anti-inflammatory mediators like IL-1 receptor antagonist (IL-1Ra), soluble TNF-RII, IL-10 and TGF-β which regulate the production of inflammatory mediators. Along with inflammatory mediators, pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and chemokines also clear the bacterial infection. Although the impact of this total process on endocytosis is not known, it is not inconceivable that endocytic functions are affected by potent inflammatory stimuli. At least in two such cases interferon Y and LPS has been reported to suppress pinocytosis but stimulate phagocytosis in macrophages (46, 226) while at higher concentrations LPS enhanced pinocytosis (158).

In the present report, the noticeable absence of late endosome marker Rab 7 even after 40 min chase and significant association of the early endosome marker Rab 5 in the LPS stimulated U937 cells vs prominent association of Rab 7 in thioglycolate elicitated control cells (concomitant with insignificant levels of Rab 5 signal) indicates slower rate of endosome maturation in the LPS stimulated cells. The pattern agrees with that observed in Western blot analysis of endosomes from primary cultures from LPS treated animals which expressed significantly less Rab 7 and more Rab 5 than their thioglycolate treated or resident counterparts. Lennon-Dumenil *et al.* reported similar results in dentritic cells (DCs) and peritoneal macrophages where a substantial down-regulation of phagosome maturation was observed in response to 5 min LPS (0.1μg/ml) stimulation at 37°C; such a delay in phagolysosomal fusion was explained by LPS mediated decrease in the rate of transport along the endocytic axis (116). However, the reason for the apparent slower rate of endosome biogenesis in LPS treated macrophages in the present report is not clear. In line with the observed results, LPS stimulation also shifted the proteolytic activity of the studied proteases. All the three cathepsins were found to be active along the entire length of the endocytic pathway. This intracellular redistribution was observed after 18 h of LPS stimulation. The increased activity can be explained by changes in the endocytic pH and/or microarchitecture of the endocytic compartments as well as the endogenous proteolytic activators/inhibitors that regulate the protease functions. Such an increased activity of endocytic proteases was observed in dendritic cells, where

addition of LPS led to accumulation and activation of Cathepsin S and B further upstream in the endocytic pathway with accelerated degradation of a model antigen (114). These reports notwithstanding, further studies are required to understand the results in the present investigation; possibly delayed maturation in conjunction with increased proteolytic activity might help in the breakdown of relatively stubborn antigenic intermediates along the endocytic route in the antigen presenting macrophages.

In context with the role of cathepsins in endosome biogenesis, it is important to mention about the activation states of the proteases under different stimuli. Interestingly, several of them exist in their proforms. The detection of cathepsin proforms *per se* is not unusual, indeed most endosome or lysosome associated proteases are synthesized as inactive proforms that exhibit an N-terminal propiece occupying the active site that prevents autoactivation (37). However, it is increasingly evident that the stimulus and mechanism of activation of such proteases play a significant regulatory role in these endosomal enzymes (37). Although the stimulus and mechanism of activation of endosomal proteases are not fully understood and is conceivably unique for different types of proteases – the acidic milieu of mature endosomes acts as a general stimulus for activation. The activities of these enzymes are also set by the environment in which they function, for example pH conditions and the presence of small competitive inhibitors. The present work provided ample indication for stimulus specific association and activation state for the three proteases studied. A significant portion of the proteases present in the short pulsed resident macrophages were in their inactive proforms; hence it can be safely assumed that resident macrophages were proteolytically less active than their elicited counterparts. In comparison to the resident cells, majority of the proteases in LPS stimulated macrophages existed in their active forms. An exception was Cathepsin D which existed in both pro as well as mature forms. Further, Western blot analysis strongly confirmed that unlike the other proteases the level of Cathepsin D was fairly similar in LPS stimulated and control macrophages indicating its stable association with nearly all endocytic vesicles studied which otherwise showed dramatic changes in morphology and proteomic pool. Smitha *et al.* showed that proteolytic activity of

macrophages increased with stress; which was concomitant to higher levels of pro-Cathepsin D conversion to the processed active form (197). Likewise, the mRNA and consequent protein product of Cathepsin D was upregulated in intestinal macrophages from Crohns disease and induced acute colitis while inflammatory conditions and stress induced transcription and post-translational processing of Cathepsin D (89). However, such enhanced expression in response to inflammatory stimulus was not observed in the present case.

The considerable heterogeneity in endosomal types and functions along the endocytic pathway indicates analogous diversity in the endosome proteome. Literature suggests that a huge array of proteomic studies on endosomes and phagosomes have identified more than 140 different proteins (70, 160), many of which are post-translationally modified at specific stages. This adds to the complexity of type and functions of the protein components of endosomes. Since complete proteomic analysis of endosomes was beyond the scope of the present investigation, a one dimensional endosomal protein profile was carried out. The analysis revealed several interesting features. First, it demonstrated successfully the differences between the PNS and RF samples – that added to the battery of other evidences confirming the high degree of purification achieved. Second, comparison between endosomal populations isolated from three different states of stimulus, indicated interesting differences in the protein patterns. Two clusters of bands, one between 116 kDa - 55 kDa and the other between 46 kDa and 29 kDa showed differences in migration despite overall similarity in their patterns while ~81%, ~59% and ~67% of the total protein bands were common between the RF's of the resident, thioglycolate and LPS groups. Distinguishing feature in the protein profile of the resident macrophage systems was the significant presence of a ~29 kDa band. This protein was considerably over-expressed in endosomes from resident macrophage and less significantly present in the other two sets. Whereas the identity of this protein could not be confirmed in the present investigation, it is interesting to note that working with rat peritoneal macrophage Kiss *et al.* had shown earlier that a specific 29 kDa isoform of caveolin was selectively enriched in resident macrophages. Upon

elicitation this isoform became insignificant; however a new 20 kDa caveolin isoform was prominent in the thioglycolate elicited macrophages (103). In the present case, both of these bands were noted in the respective sets, a fact that strongly suggests that the 29 kDa band observed may be caveolin. Although further work is required to confirm if this band is indeed caveolin, it is interesting to speculate the reason behind the presence of caveolin like bands in resident marophages. Incidentally, caveolae are important membrane proteins present in endosome membranes. They are the principal components in caveolae mediated endocytosis (103). Using immunological tools Kiss *et al.* further demonstrated that the 29 kDa caveolin isoform (caveolin 2) was present in smaller vesicles and multivesicular bodies in the cytoplasm of resident cells, but was not directly related to caveolae formation. However, elicited cells which showed the presence of the 20 kDa caveolin isoform (caveolin 1) were associated with much higher numbers of caveolae. Together the authors concluded that the 29 kDa caveolin isoform is much less efficient in endocytosis but probably plays an as yet unidentified role specific in resident macrophages. In the present analysis a ~20 kDa band was indeed present in the endosome fraction of thioglycolate stimulated macrophages and not seen in either resident or LPS stimulated macrophages. The presence of the ~29 kDa protein at significantly higher levels in the endosome rich fraction also confirms that this protein is a part of vesicles actively involved in endocytosis. Although further work is imperative to find out the identity and role of this protein in resident macrophages, it is interesting to speculate the mechanism(s) of endocytosis of iron-dextran uptake in peritoneal macrophages. Considering pinocytosis as the mechanism of uptake (the size/structure relationship of iron-dextran and FITC-dextran does not entail phagocytosis as a likely mechanism), the current school suggests particle size to be a significant factor that dictates internalization mechanism: ~60 nm or smaller particles are preferentially internalized by caveolae, bigger particles ~120 nm in size use clathrin; whereas even larger particles (>1 μm) use macropinocytosis (44). In that context, electron microscopy of the iron-dextran showed uniform ~10 nm particles with electron dense core(s) surrounded by a comparatively thin electrolucent material. This is reminiscent of colloidal iron dextran core(s) surrounded by limiting layer of dextran: an observation also reported by Rodriguez-Paris *et al.* (174). Considering

this (~10 nm) to be the size of iron-dextran particles, larger aggregates between 30 - 50 nm were also frequent in such preparations. The wide range of sizes of such clusters would probably rationalize for all of the above mechanisms to be operative during iron-dextran endocytosis. Apart from particle size, use of 70 kDa FITC labelled dextran as an endocytic probe strongly supports macropinocytosis as one of the pinocytic mechanism (97).

In conclusion, the present study demonstrated for the first time, the successful application of 'magnetic affinity chromatography' for purification of endosomes from primary cultures, which are arguably more heterogeneous than studies reported in cell lines. Systematic analysis of the purified fractions using biochemical, microscopic and immunological tools confirmed that such preparations were remarkably free from other subcellular contaminants like ER, mitochondria or lysosomes. The technique was easily adaptable to different pulse-and-chase regimens that allowed accurate isolation of specific class(es) of endosomal vesicles. Together, the evidence makes a compelling case for magnetic purification technology for studying mammalian endosomes from complex primary cultures and adds to the existing growing pool of reports on the versatility of this technique in diverse cell systems (13, 151, 214). The studies provided consistent evidence, that despite inherent similarities between thioglycolate stimulated and resident macrophages, subtle differences between these systems exist and further showed an interesting delay in the maturation of LPS stimulated macrophage endosomes. Importantly, the successful adaptation of magnetic chromatography in primary cell lines promises the isolation of varied classes of receptosomes from such systems. Such studies should determine if different receptors target their respective ligands towards different compartments or same compartments with different kinetics. Finally, recent advances in proteomic technology together with the availability of pure endosomes has opened up exciting prospects for an in depth characterization of the endocytic pathway in the future.