CLEARANCE OF DYING AUTOPHAGIC CELLS OF DIFFERENT ORIGIN BY HUMAN PROFESSIONAL AND NON-PROFESSIONAL PHAGOCYTES

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

by

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Az elhaló sejtek eltakarítása nagyon fontos a kiegyensúlyozott szövet homeosztázis fenntartásában az emlős szervezetekben. Ha ez nem történik meg az gyulladáshoz és autoimmun betegségekhez vezethet. Bár sokat tudunk az apoptótikus és nekrotikus sejtek eliminálásáról, még mindig vannak nyitott kérdések az elhalt sejtek autofágián keresztül vagy autofágiával történő eliminálását illetően.

MCF-7 sejtekben autofágiás elhalás következik be tamoxifen kezelés hatására, amelyet *de novo* autofágiának is nevezünk, mivel a halált az autofágia indukálja. Ha nem tapadó

szubsztrátummal vonjuk be a sejteket, a sejthalál anoikis által következik be, miközben autofágia idéződik elő – amit itt autofágiával történő halálnak nevezünk (a sejthalált nem autofágia okozza) vagy anoikis-autofágiának megkülönböztetendő a *de novo*-tól. Mindegyik esetben az autofágiát monodansylcadaverine színezés, emelkedett LC3II protein szint és elektronmikroszkópos vizsgálat teszi láthatóvá.

Az élő sejtekben az autofágiát rendszerint nem érzékelik a szomszéd sejtek. Azonban a sejthalállal együtt történő előfordulása hozzájárulhat az elhalt sejtek fagociták által történő eltakarításához. Mind a *de novo* és anoikis-ből származó autofágiás elhaló sejteket bekebelezik a humán makrofágok és MCF-7 sejtek. Az autofágiának 3-methyladenine (3-MA)-nel történő gátlása megszünteti a *de novo* autofágián keresztül történő sejtek bekebelezését, de nem szünteti meg az anoikis által elhalókét.

A gén expresszió vizsgálatok azt mutattak, hogy míg mindkét típusú fagocita teljes repertoárját mutatta a phosphatidylserine felismerésnek és szignál útvonálanák, addig a makrofágok a *de novo* autofágiás sejtek bekebelezése során a potenciális calreticulin-mediált felismerés, felszin ’’letapogatás’’ és bekebelezést használják.

A pro-inflammatorikus citokinek makrofágokban történt LPS-indukált előidézése megelőzhető az elhaló autofág sejtekkel, hasonlóan az anoikis-autofág és apoptotikus sejtekhez. Azonban azt is megfigyeltük, hogy az autofágia által elhalt sejtek fagocitózisa pro-inflammatorikus válaszhoz vezet makrofágokban, melyet IL-1β és IL-6, TNFα, IL-8 és az anti-inflammatorikus cytokin IL-10 indukció és szekréció jellemez. Az IL-1β szekréció gátolható az autofágia 3-MA-val történő megelőzésével vagy blokkoló caspase-1 aktiválásával. Az eredmények azt sugallják, hogy a makrofágok belsejében az autofágia következtében elhaló sejtek aktiválhatják a NLR proteint a fagocitózis folyamata alatt.

Az adataink azt mutatják, hogy az autofágia és az anoikis következtében az autofágiával elhaló sejtek eltakarítása átfedő de ugyanakkor eltérő mechanizmussal történik a professzinális és nem professzionális fagocitákban.

1. SUMMARY

Clearance of dying cells is of great importance for maintaining balanced tissue homeostasis in mammalian organisms. Failure to do so may lead to inflammation and autoimmune diseases. Although we know quite a lot about the elimination of apoptotic and necrotic cells, there are still open questions on how cells dying through or with autophagy get eliminated.

MCF-7 cells undergo autophagic death upon tamoxifen treatment; this we consider here death through autophagy or *de novo* autophagy, since death is trigered by autophagy. When plated

on non-adhesive substratum these cells die by anoikis while inducing autophagy - what is considered here death with autophagy (cells' death is not caused by autophagy) or anoikic-autophagy to distinguish it from the *de novo* one. In each case autophagy was revealed by monodansylcadaverine staining, elevated LC3II protein levels and electron microscopy examination.

In living cells autophagy takes place usually unnoticed to the neighbours. However, its co-ocurrance with cell death may contribute to the clearance of these dying cells by recruited phagocytes. Both *de novo* and anoikis-derived autophagic dying cells were engulfed by human macrophages and MCF-7 cells. Inhibition of autophagy by 3-methyladenine (3-MA) abolished engulfment of cells dying through *de novo* autophagy, but not those dying through anoikis with autophagy. Blocking exposure of phosphatidylserine on both dying cell types inhibited phagocytosis by MCF-7 but not by macrophages. This means that when autophagy induces cell death it also contributes to the clearance of dying cells.

Gene expression profiling of 95 genes involved in the apopto-phagocytic system by TaqMan Low Density Array (TLDA) showed that while both types of phagocytes expressed full repertoire of the phosphatidylserine recognition and signaling pathway, macrophages could evolve during engulfment of *de novo* autophagic cells the potential of calreticulin-mediated recognition, tethering, tickling and engulfment processes. Different sets of genes (e.g. the phagocytosis receptors for asialoglycoprotein and oxidized LDL, the bridging molecule PTX3, the engulfment molecules GULP1 and RAC1) were upregulated in macrophages engulfing *de novo* autophagic as compared to anoikis-derived autophagic dying cells (e.g. the bridging molecule MFGE8, the engulfment gene RAC1). In MCF-7 cells, *de novo* autophagic cells induced the oxidized LDL receptor and ELMO1 engulfment gene, while anoikic-autophagic ones lead to elevated AXL receptor.

LPS-induced production of pro-inflammatory cytokines in macrophages could be prevented by the dying autophagic cells similarly to anoikic-autophagic and apoptotic cells. However, we also observed that phagocytosis of cells dying through autophagy leads to a pro-inflammatory response in macrophages characterized by the induction and secretion of IL-1β as well as IL-6, TNFα, IL-8 and the anti-inflammatory cytokine IL-10. The IL-1β secretion could be inhibited by preventing autophagy with 3-MA or blocking caspase-1 activation. The

results suggest that inside macrophages cells dying through autophagy can activate NLR family protein(s) during the phagocytosis process.

Our data show that cells dying through autophagy and those committing anoikis with autophagy may engage overlapping but distinct sets of clearance mechanisms in professional and non-professional phagocytes. Better understanding of the molecular mechanism of these phenomena may lead to more rational design of autophagy-based therapeutic interventions.

2. INTRODUCTION

2.1. Molecular elements of the apopto-phagocytic synapse

Close to 500 billion cells die each day in the human body - mainly by apoptosis – and they are either shed off or directly lost to the environment from body surfaces or efficiently removed by the apopto-phagocytic system without causing inflammation or scar formation (Grimsley et al. 2003; Gregory et al. 2004). The surface with complex molecular patterns and dynamic interactions between the dying and engulfing cells (macrophages and non-professional

phagocytes) is referred as the third synapse by analogy to those present in the nervous and immune (antigen presenting) system. The “anatomical” description of this synapse is probably almost complete (see the schematic description on Fig. 1) and it includes the large number of receptors and opsonins which bind to cellular ligands exposed during the various stages of apoptotic cell death (Savill et al. 2002). In spite of the remarkable progression in the field, we are still far from understanding the complex biochemical and regulatory processes which take place when dying cells of different origin are cleared from tissues.

One of the most intriguing aspects of the apopto-phagocytic system is the active and dynamic interrelationship between the dying and the engulfing cells. The apoptotic cells can induce migration of phagocytes by releasing lipid-derived attraction signals (Lauber et al. 2003). The engulfing cells are capable of influencing the fate of their neighbors by promoting either death or survival (Brown et al. 1999; Duffield et al. 2000; Hoeppner et al. 2001; Lobov et al. 2005; Pollard et al. 2004; Reddien et al. 2001; Szondy et al. 2003). This cooperative relationship between apoptotic cells and their phagocytes is even more significant in determining whether inflammation occurs or not in tissues with high apoptotic rate under normal or pathologic conditions.

2.2. Common elements between the apopto-phagocytic system and the innate immunity

None of the so far revealed elements of the apopto-phagocytic system seems to be specifically dedicated to the clearance of apoptotic cells. Cell surface receptors and the intermediate molecules participating in the phagocytic events are designed for other biological processes as well. Among these shared processes, the utilization of the innate immune system is particularly important for understanding the intimate relationship between the apopto-phagocytic system and the inflammatory response.

Invading pathogens are primarily recognized by a large number of pattern-recognition molecules including soluble factors. Many components of this innate immunity are also involved in the clearance of apoptotic cells (see Fig. 1) through an apoptotic cells associated molecular pattern (ACAMP), similarly to microbes being decorated by pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides (LPS) (Gregory et al. 2004). The multiplicity of ACAMP structures provides redundancy making the recognition of dead cells a fail-safe mechanism but it may also reflect early and late stages of the apoptotic cell surface which participate in distinct chronological events, such as recognition, binding (“tethering”), signaling (“tickling”) and engulfment during the clearance process.

Molecules of the innate immune response are particularly active in opsonization of apoptotic cells one such being proposed to be C1q preventing autoimmunity and maintaining self-tolerance by supporting efficient clearance of apoptotic material and actively modulating phagocyte function (Roos et al. 2004). However, it should be noted that while some PAMP receptors do participate in the clearance of apoptotic cells (e.g. CD14), most of them do not and the anti-inflammatory response of macrophages to ACAMPs is usually independent of the pro-inflammatory response to PAMP receptors due to a difference in their signaling through Toll-like receptors (TLRs). Necrotic cells, on the other hand, have been shown to engage TLR2 and induce pro-inflammatory and tissue repair genes (Li et al. 2001).

2.3. Clearance of apoptotic cells by non-professional phagocytes

In the early phase of investigations macrophages have been considered of being the main actors capable of engulfing apoptotic cells, but there are early papers providing morphological evidence of non-professional phagocytes "eating" adjacent cells dying by apoptosis. In the recent years increasing amount of evidence has suggested why it is very difficult to see apoptotic cells in tissue sections. Very likely, the efficient and fast removal of the daily formed apoptotic cells by their adjacent neighbors taking place even before they show morphological features of apoptosis can be held responsible for it. This view proposes the macrophages being important in the clearance of dying cells in the circulation and in places where the apoptotic rate is so high that the non-macrophage system can not cope with the high load of corpses (Gregory et al. 2004). In support of this hypothesis, it has been shown that mice containing no macrophages develop normally and are capable of removing dying corpses by the less efficient mesenchymal cells during embryogenesis without inducing an inflammatory response (Wood et al. 2000). In spite of the high ongoing rate of apoptosis in several of their organs (just like in normal mice), there is no sign of excess apoptotic cells in tissues. Unless these mice receive daily antibiotic treatment though, they succumb rapidly to bacterial infections due to absence of macrophages and neutrophils in them.

Clearance of dying corpses by neighboring viable cells not born to be phagocytes is becoming a well recognized phenomenon during tissue remodeling. Many cell types of different dermal origin have been "convicted" of being "guilty" for engulfing apoptotic (Fadok et al. 2001; Henson et al. 2001; Thomspon et al. 1995), necrotic (Cocco et al. 2001) cells as well as those undergoing anoikis or autophagy (Petrovski et al. 2007). The extensive list of non-professional phagocytes includes, but is not limited to fibroblasts, kidney mesangial cells,

testis Sertoli Cells, ovarian thecal cells, smooth muscle cells, endothelial and epithelial cells, hepatocytes and mesenchymal cells (for citations see Gregory et al. 2004). Some widely used cell lines such as 3T3, HeLa, Jurkat, COS7 (Majái and Petrovski et al. 2006), MCF-7 and HepG2 cells (unpublished results) have been found to engulf apoptotic cells in culture systems. It is very likely that all cell types can do this to a certain extent if and when the need for their "cannibalism" arises.

The mechanism of non-professional recognition, signaling and engulfment of apoptotic and necrotic cells is being increasingly studied and at the present not well defined. Naturally, basic results obtained by studying engulfment in the nematode *Caenorhabditis elegans*, which do not have macrophages, can be all considered as part of typical non-macrophage mechanisms. Genetic studies of the removal of apoptotic cell corpses by *C. elegans* have found numerous molecules on the phagocytic cell encoding transmembrane receptor: *ced-1* (its mammalian analogue being the scavenger receptor of endothelial cells (SREC) involved in tethering and initiation of signaling for uptake (Chung et al. 2000; Zhou et al. 2001), *ced-7,* (its mammalian analogue being ABCA-1 transporter on both apoptotic and phagocytic cells (Moynault et al. 1998)), *ced-2, 5, 7, 10, 12* (their mammalian being CrkII, DOCK180, ABC-1, Rac-1, ELMO, respectively, involved in signaling, cytoskeletal reorganization, ruffling, membrane extension and fusion during engulfment (Hamon et al. 2000; Leverrier et al. 2001; Tosello-Trampont et al. 2001)). All the latter molecules function during the engulfment process irrespective whether the phagocytosing cell is macrophage or non-professional phagocyte.

In this regard, the molecular changes on the surface of apoptotic cells that "flag" recognition and removal are unanimous for both professional and non-professional phagocytes. Many groups have studied these changes including but not limited to the exposure of specific carbohydrates binding to phagocyte lectins (Duvall et al. 1985), thrombospondin (Savill et al. 1992), collectins (Ogden et al. 2001) or complement products (iC3b) (Mevorach et al. 1998) all playing roles in formation of bridges between apoptotic cells and the phagocyte. Loss of phospholipid asymmetry and surface exposure of phosphatidylserine (PS) on apoptotic cells is required for their phagocytosis performed by fibroblasts (Fadok et al. 2001).

While we know that maturation of monocytes to macrophages is a prerequisite for their recognition of apoptotic cells, it is not clear what regulatory mechanisms make fibroblasts, epithelial and other cells capable of doing the same. Apparent differences have been observed

in the engulfment of apoptotic cells by professional and non-professional phagocytes (Parnaik et al. 2000): the latter act slower, recognize late stage apoptotic cells more, digest the corpses slower (Wood et al. 2000). This may reflect a need for "educating" these cells perhaps by apoptotic cells for the unusual task of phagocytosis.

Non-macrophage cellular systems, such as epithelial cells can regulate the function of other cells locally through paracrine actions coupled to the production of cytokines like IL-1 (Agace et al. 1993; Stadnyk et al. 1994). The "amateur" phagocytic cells are important source of cytokines including their pro-inflammatory response to endotoxins. For example, production of the pro-inflammatory cytokines IL-1β, IL-8, TNF-α and MIP-2 has been shown in mammary epithelial cells stimulated by LPS (Monks et al. 2005). Therefore, it is important to clarify how apoptotic cells regulate the inflammatory response of non-professional phagocytes while they recognize and engulf apoptotic cells. The few data available so far show that apoptotic cells, unlike necrotic cells, do not provide pro-inflammatory signals for these cells and can downregulate the expression of pro-inflammatory cytokines induced by LPS (Cvetanovic et al. 2006; Monks et al. 2005). Furthermore, apoptotic cells can elicit an anti-inflammatory response inducing the secretion of TGFβ in these cells. This means that the non-macrophage cells also posses or can acquire the recognition and signaling mechanisms which are needed to regulate their pro- and anti- inflammatory responses upon meeting apoptotic cells.

Indeed, lack of adequate regulation of the apopto-phagocytic system in some malignancies may lead to unwanted inflammation which promotes tumor growth and this may provide an explanation why chemotherapy-induced tumor cell death sometimes inversely ends up in tumor progression instead of regression (Reiter et al. 1999; Simamura et al. 2001). In the sequential events of carcinogenesis loss of pro-apoptotic genes (which is very likely reflected in disturbed phagocytosis of dead cells) may be followed by loss of autophagy-based cell death shifting the tumor tissue toward the frequent appearance of necrotic type of cell death and the inflamed tumors often have been considered the best growing tumors. Based on these data restoration or activation of apoptosis and their proper phagocytosis in tumors may be beneficial in anti-cancer therapy as it would pull the leverage away from necrosis and inflammation towards the anti-inflammatory dominant clearance of apoptotic tumor cells (Nelson et al. 2004).

2.4. Autophagic dying cells

In mammalian organisms various forms of cell death may occur (Kroemer et al. 2005). Besides the widely known apoptosis or type I ‘programmed cell death’ and necrosis, cells can undergo anoikis and autophagic cell death as well which have been described relatively less. Anoikis is induced in cells after their detachment from the extracellular matrix switching on apoptotic signaling pathways (Gilmore et al. 2005). Type II PCD or autophagic cell death has been observed during embryonic development (Bursch et al. 1996; Bursch et al. 2000). It is one of the caspase-independent forms of cell death, though its caspase-dependent element has been also described (Levine et al. 2005).

Since the first description of autophagy in 1966 (De Duve et al. 1966) numerous studies have described it as a survival mechanism under poor nutritional conditions or birth-related starvation. It is now clear that this process has a dual role (Codogno et al. 2005; Qu et al. 2003).On one hand, autophagy is a degradative mechanism for long-lived proteins and damaged organelles through the auto-phago-lysosomal pathway or serves as a survival pathway preventing or delaying apoptosis (Amaravadi et al. 2007; Abedin et al. 2007). On the other hand, it provides possibility of self-destruction for cells (Baehrecke et al. 2003; Bursch et al. 1996; Bursch et al. 2000). In *Drosophila*, autophagy can be hormonally controlled by ecdysone; through inhibition of a class-I phosphatidyl-inositol 3-kinase (PI3K) pathway organs, such as the fat body, are eliminated at the end of larval stage (Baehrecke et al. 2003; Baehrecke et al. 2005). MCF-7 cells can also be induced to undergo autophagy and cell death through the initiated autophagic response by treatment with the anti-estrogen tamoxifen which increases the intracellular ceramide level and eliminates the inhibitory effect of class-I PI3K pathway (Scarlatti et al. 2004). Expression of death-associated protein kinase (DAPK) and its death related protein kinase-1 (DRP-1) in MCF-7 and various other cell lines can induce autophagy and caspase-independent cell death (Inbal et al. 2002). TNF-related apoptosis-inducing factor (TRAIL) was found to mediate induction of autophagy in an *in-vitro* model of mammary gland acinar morphogenesis (Mills et al. 2004). When caspase-8 was inhibited and the *atg7* and *beclin-1* expression was knocked down by RNA interference death through autophagy was blocked in mouse L929 cells (Yu et al. 2004). In embryonic fibroblasts with homozygous deletion of both *bax* and *bak* with RNAi against *atg5* and *beclin* could prevent autophagic cell death (Shimizu et al. 2004). It is still an open question whether autophagic death can occur in cells with intact apoptotic machinery; it has been suggested that in most cases autophagy is secondary to apoptosis or necrosis without a causal role in the death process itself (Levine et al. 2005).

To our present knowledge the final fate and clearance mechanism of cells either dying through autophagy (killed by autophagy) or by other mechanisms with associated autophagy have not been clarified. While cell surface changes and markers for phagocytic recognition of cells undergoing apoptosis have been extensively researched (Savill et al. 2002), no such surface changes have been implicated for engulfment of autophagic cells. Furthermore, while the inflammatory response has been well studied during clearance of apoptotic and necrotic cells, nothing is known about the response induced in phagocytes during engulfment of autophagic dying cells. Different danger signals (bacteria, uric acid and ATP) (Fritz et al. 2006, Martinon et al. 2006) have been shown to induce Nod-like receptors (NLRs) and the inflammasome in mammalian cells. How autophagic dying cells affect the production of inflammatory cytokines and relate to the known inflammatory pathways remains to be determined.

2.5. Aims of the studies

1. To set up a system to study how cells dying through or with autophagy are phagocytosed.

2. To learn the molecular mechanisms involved in the clearance of autophagic dying cells.

3. To clarify which genes are induced during phagocytosis of autophagic dying cells using gene array technology.

4. To investigate whether cytokines are induced and secreted when autophagic dying cells are engulfed.

3. MATERIALS AND METHODS

Cell culture and treatments

Dulbecco's modified Eagle's medium (DMEM), poly(2- hydroxyethylmethacrylate) (poly-HEMA), tamoxifen (α-(4-f3-N-dimethylaminoethoxy)- phenyl- α'-ethyl-trans-stilbene]; TAM) and 3 -methyladenine (3-MA) were purchased from Sigma (Steinheim, Germany); CFDA-SE, carboxyfluoresceindiacetate-succinimidyl ester and CMTMR, 5-(and-6)-(((4-chloromethyl)benzoyl) amino)tetramethylrhodamine were purchased from Molecular Probes

(Leiden, Netherlands); recombinant annexin-V was kindly provided by Dr. Peter Zavodszky (Hungarian Academy of Sciences); caspase-1 inhibitor (zYVAD-FMK) was purchased from BioVision Int. (Brussels, Belgium); plastic tissue culture flasks were purchased from TPP (Trasadingen, Switzerland). MCF-7 human breast cancer cells were kindly provided by Dr. W. Bursch, Medizinische Universität Wien, Austria; culture conditions were described in detail previously (Bursch et al. 1996). Briefly, cells were grown as a monolayer in DMEM supplemented with 10% fetal calf serum (FCS), L-glutamine (300 mg/L) and penicillin/streptomycin antibiotics at 37°C in an atmosphere of 5% CO_2. Cells were detached from the substrate using trypsin/EDTA (0.05:0.02%). Seven days before the beginning of an experiment cells were steroid-withdrawn (according to Gill et al. 1987). Cells were plated in plastic tissue culture flasks at a density of $7.5x10^3/cm^2$ and the culture medium was replaced by DMEM containing 3% charcoal-stripped-FCS (DCC). Twenty four hours later the cells were treated with TAM; for treatment, freshly prepared dilutions in DMSO/ethanol (1:1, v:v) were added directly to the medium. Controls were treated with DMSO/ethanol. For the induction of anoikis, cells were plated on poly-HEMA covered dishes over a 7 day period in either 10% FCS or 3% DCC with or without TAM (i.e.10% FCS+poly-HEMA, 3% DCC+poly-HEMA, 3% DCC+TAM+poly-HEMA). Apoptosis was induced by UV irradiation - 5, 10 and 20 minutes, 45 mJ/(cm2.min) - and the cells were left to die for 8 hours before harvesting.

Assays of cell death and autophagy

Cell death was assessed by the Annexin-V-FITC Apoptosis Detection Kit (MBL, Woburn, MA) according to manufacturer's recommendations. In brief, collected cells ($1\text{-}5x10^5$) suspended in 1X binding buffer were incubated at room temperature for 5 minutes with Annexin-V-FITC or Annexin V and propidium iodide (PI), then proportion of stained Annexin-V+ and Annexin-V+PI+ cells was determined by FACS analysis on BD Bioscience flow cytometer. Autophagy was assessed by detection of autophagic vacuoles with monodansylcadaverin (MDC) according to the method of Biederbick et al. 1995. In brief, cells were incubated with 0.05 mM MDC (60 minutes, 37°C), cytospun on glass slides (5 minutes, 500 rpm) using Shandon Cytospin 3 Cytocentrifuge, followed by fixation in 4% PBS buffered paraformaldehyde (15 minutes, pH 7.4), then washing twice with PBS and counterstaining with PI (1 µg/ml in PBS). Since MDC-fluorescence bleaches quickly, quantitative determination of MDC-positive cells followed immediately the preparation on a Carl-Zeiss Fluorescent microscope (356 nm excitation/545 nm filters).Whenever inhibition of autophagy was carried out with 3-methyladenine, the treatment preceded peaking of autophagy by 2 days.

Electron microscopy

Samples were fixed in 0.1 M sodium cacodylate-buffered, pH 7.4, and 2.5% glutaraldehyde solution for 2 hours and then rinsed (3 times,10 min) in 0.1 M sodium cacodylate buffer, pH 7.4, and 7.5% saccharose and postfixed in 1% OsO_4 solution for 1 hour. After dehydration in an ethanol gradient [70% ethanol (20 min), 96% ethanol (20 min), 100% ethanol (2 times, 20 min)], samples were embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Philips CM 10 microscope (Philips Electronic Instruments, Mahwah, NJ) at 80 kV.

Antibodies and immunoblotting

Anti-LC3 polyclonal antibody raised against a synthetic peptide that corresponds to the N-terminal 14 amino acids of human and mouse LC3 with an additional cysteine (H_2N-PSDRPFKQRRSFADC-$CONH_2$), was prepared by Eurogentec (Seraing, Belgium) in rats and affinity purified on an immobilized peptide-Sepharose column. Cell lysates were separated on a NuPAGE 12% Bis-Tris polyacrylamide gel (Invitrogen) and transferred to an Immobilon-P Transfer Membrane (Millipore, Bedford, MA; pore size 0.45 μm). Membranes were blocked in Tris buffered saline containing 0.05% Tween-20 (TBS-T) and 5% non-fat dry milk (BioRad) for 1 hour. After blocking, membranes were probed overnight at 4°C with a primary rat anti-LC3 polyclonal antibody in antibody dilution buffer (TBS-T containing 1% non-fat dry milk), followed by 1 hour incubation with a rabbit anti-rat peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark) for 1 hour at room temperature. Peroxidase activity was detected with SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce, Rockford, IL) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

Phagocytosis assay

Human monocytes were isolated from "buffy coats" of healthy blood donors on Ficoll-PaqueTM Plus (Amersham Biosciences) gradient and a magnetic separation using CD14 human microbeads (Miltenyi Biotec, Auburn, CA), after which human macrophages were obtained through a five day differentiation using 5 ng/mL Macrophage Colony Stimulating Factor (MCSF). Non-dying MCF-7 cells acting as phagocytes were plated in serum-free medium 24 hours before phagocytosis. Dying cells were fed to engulfing cells when in their culture autophagy peaked: at day 4 for *de novo* autophagic and day 6 for anoikic-autophagic cells. If inhibition with 3-MA was investigated, it was added to cultures 2 days prior to the peaking of

autophagy. Inhibition of phagocytosis was carried out by pre-treating 10^5 dying cells with 10μg recombinant annexin-V for 30 minutes at 37 °C and maintaining this treatment throughout the phagocytosis assay. In addition, MCF-7 cells UV irradiated for 20 minutes and turning apoptotic 8 hours after irradiation were used for engulfment. The phagocytes, macrophages and non-dying MCF-7 cells, were stained with 7.5μM CMTMR for 16 and 24 hours, respectively, before starting the assay. The cells to be engulfed were stained with 12.5μM CFDA for 16 or 24 hours prior to the phagocytosis assay according to whether or not they were fed to macrophages or non-dying MCF-7 cells, respectively, then washed twice in PBS before being added to the phagocytes. The ratio of phagocytes and cells to be engulfed was set at 1:5. The phagocytosis assay was started when the cells to be engulfed were added to the appropriate phagocytes and kept together for appropriate time (1, 12 or 24 hours). The assay was carried out at either 4°C when phagocytes bind but do not engulf dying cells and at 37°C when phagocytes bind and/or engulf them. The whole cell mixture was collected by scraping the cells, centrifuging, washing twice in PBS and fixing in 1% PBS buffered paraformaldehyde (pH 7.4). The net phagocytosis rate was determined by FACS analysis as percent phagocytic cells that have engulfed (positive for both CMTMR and CFDA) at 37°C minus the observed percent of double positive cells at 4°C.

RNA preparation and TaqMan Real-Time-PCR

Total cellular RNA was isolated from human monocyte derived macrophages as well as from MCF-7 cells using TRIzol Reagent (Life Technologies) at the indicated time point of the phagocytic assay after washing away non-engulfed dead cells thoroughly pre-designed TaqMan probe and primer sets for target genes were obtained from Applied Biosystems (Foster City, CA). The sets were factory-loaded into the 384 wells of TaqMan Low Density Array (TLDA). Array format was customized with two replicates per target gene and for each biological sample two parallel analysis were carried out. TaqMan low-density arrays were used in a two-step RT-PCR process. First strand DNA was synthesized from 1 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). Q-PCR reactions and analysis were then carried out in TLDAs using TaqMan Universal Master Mix and ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). 100 μl sample volume per port (4 ports/sample) was used with 1 ng/μl cDNA concentrations. Expression levels of target genes were normalized to 18S rRNA as endogenous control. Thermal cycling conditions were as follows: 50°C for 2 min, 94°C for 10 min, 97°C for 30 s, and 59.7°C for 1 min, 40 repeats. Gene expression values were calculated based on the ΔΔCt method, where one sample was designated as calibrator, through which all other samples were analyzed. ΔCt represents the threshold cycle (Ct) of the target minus that of

18S rRNA and ΔΔCt represents the ΔCt of each target minus that of the calibrator. Relative quantities (RQ or fold changes) were determined using the equation where relative quantity equals $2^{-\Delta\Delta Ct}$.

Cytokine release assay

Macrophages differentiated according to the protocol above were either stimulated or not with 0.5 μg/mL LPS for 30 min prior to starting a phagocytosis assay. The samples were then incubated with autophagic dying cells for either 1 or 12 hours. After the 1 hour co-incubation period, the non-ingested dying cells were removed and the macrophages were incubated in fresh media without serum for additional 17 hours; in the case of 12 hours co-incubation, the cells remained together for the whole length of the phagocytosis assay. The supernatants in each case were collected and analyzed for the presence of IL-8, IL-6, IL-1β, IL-10, TNF-α, IL-12p70 using the Human Inflammation BD Cytometric Bead Array(CBA) (BD Biosciences). IL-1β inhibition studies with the specific caspase-1 inhibitor (zYVAD-FMK) at 50μM concentration was carried out for 30 minutes prior and throughout the phagocytosis assay.

4. RESULTS

4.1. Induction of autophagy in MCF-7 cells by tamoxifen treatment and through anoikis

Induction of autophagy, and through that cell death, by 3% DCC followed by tamoxifen treatment in MCF-7 mammary epithelial cells has been previously described by (Bursch et al. 2000) and demonstrated here using both monodansylcadaverin (MDC) uptake into autophagic vacuoles (AVs) and transmission electron microscopy (TEM) to identify autophagic cells. As shown on Fig. 2a, the number of MDC-positive *de novo* autophagic cells has peaked at day 4 under such conditions and autophagic vacuolization could be observed by TEM (Fig. 2 -b2).

Anoikis initiated by detachment of cells from the extracellular matrix (ECM) *in vitro* has been introduced as a model for studying the mechanism of cell death in the absence of ECM-derived signals *in vivo*. It is generally accepted that cells of epithelial and endothelial origin are more sensitive to anoikis than fibroblasts (Gilmore et al. 2005). Our MCF-7 cells underwent anoikis in 10% FCS of culture when they were not allowed to attach to the plate. However, we have also observed that they showed signs of autophagy at the day 6 of incubation: detachment of MCF-7 cells induced by poly-HEMA coated plates resulted in occurrence of autophagic vacuoles as detected by MDC staining (Fig. 2a) and TEM (Fig. 2 -b3). Combining detachment with either 3% DCC or 3% DCC plus TAM treatment accelerated the autophagic response to occur 2 days earlier than the time of autophagy in attached cells with the same treatment. All treatment regimens (3% DCC+TAM, 10% FCS+poly-HEMA, 3% DCC+poly-HEMA, 3% DCC+TAM+poly-HEMA) affected the time course but not the maximal degree of the autophagic response (about 70% of MCF-7 cells were affected in each case, Fig. 2a); an example of the MDC positive dot-like structures representing AVs in the cytoplasm of cells is shown (day 2, 3%DCC and tamoxifen treated anoikic MCF-7 cells, Fig. 2-c1). Typical absence of chromatin condensation in the nucleus of these cells and numerous autophagic vacuoles with double membranes containing digested materials were also seen by TEM (Fig. 2-c2).

Time-dependent development of autophagy was also supported by findings of Western blot analysis of LC3 expression in case of *de novo* autophagic (3%DCC+TAM) and anoikic-autophagic (10% FCS+poly-HEMA) cells (Fig. 2d). In autophagic MCF-7 cells LC3II (the autophagosomal membrane bound form of LC3 (Kabeya et al. 2000)) became detectable at day 4, then increased up to day 7 in parallel with the observed pattern of MDC staining. In the anoikic-autophagic cells, the expression of LC3II appeared at day 1 and 2, then showed a decrease from day 4 to day 7; this does not necessarily mean that autophagy was downregulated knowing that LC3 may itself undergo degradation by lysosomal enzymes.

4.2. Occurrence of cell death in *de novo* autophagic and anoikis-derived autophagic cells

After peaking of autophagy in *de novo* autophagic cells at day 4 (3%DCC+TAM), the number of dying cells peaked at day 5 as assessed by their positivity for annexin-V or double positivity for annexin-V and PI (Fig. 3a). When counted by fluorescence microscopy, more than 95% of the dying cells were MDC positive at this time point suggesting that autophagy was directly related to cell death. Inhibition of autophagy in these cells at day 4 by the autophagy inhibitor 3-MA (Seglen et al. 1982) almost completely abolished the MDC-positive staining and lead to a

significant drop in the number of annexin-V and annexin-V as well as PI positive cells (Fig. 3b). The percent of dying cells in anoikic-autophagic cells was twice as much than in case of *de novo* autophagy, death occurred earlier and peaked not in association with the peaking of autophagy (Fig. 3a). Inhibition of autophagy in these cells at day 6 with 3-MA almost completely abolished the MDC-positive staining as in *de novo* autophagic cells and lead to a decrease in the percentage of annexin-V or annexin-V and PI positive cells (Fig. 3c). For comparison, percentage of annexin-V or annexin-V and PI stained cells following UV-induced apoptosis is also presented (Fig. 3a); in this case cell death was not influenced by treatment with 3-MA.

4.3. Both *de novo* and anoikis-derived dying autophagic cells are engulfed by macrophages and non-dying MCF-7 cells

While phagocytosis of apoptotic and necrotic cells has been extensively studied, no data are available how cells dying through autophagy or those in which death is accompanied with autophagy are removed from tissues. Macrophages and MCF-7 cells acting as professional and non-professional phagocytes, respectively, could engulf dying *de novo* autophagic or anoikic-autophagic cells with increasing number of phagocytes containing cell corpses over a 24 hour time period (Fig. 4a-d). As flow cytometric analysis shows macrophages engulfed anoikic-autophagic cells more efficiently, comparable to phagocytosis of UV irradiated apoptotic MCF-7 cells, than they did *de novo* autophagic dying cells (Fig. 4b,d): at the 24th hour of co-incubation about 32% of them contained anoikis derived dead cells while only 15% of them showed phagocytosis of dying *de novo* autophagic cells. MCF-7 cells, however, engulfed dying *de novo* autophagic cells much more efficiently than either anoikic-autophagic or apoptotic ones, reaching an average phagocytosis frequency of 32% at the same time point.

To confirm the results obtained by flow cytometry the uptake of dying autophagic cells was also investigated by fluorescence confocal microscopy using a newly developed three-color technique. The latter was specifically designed for detecting co-localization of engulfed autophagic MCF-7 cells stained with both CFDA (green) and MDC (blue) within the CMTMR (red) colored phagocytosing cells (Fig. 5a-c). Confocal microscopy confirmed the intra-cytoplasmic localization of the engulfed *de novo* autophagic or anoikic-autophagic dead cells being double stained with CFDA and MDC within either macrophages or non-dying MCF-7 cells. Under the experimental conditions used, usually one non-dying MCF-7 cell acting as an 'eater' engulfed one autophagic or anoikicautophagic cell. Counting under the microscope it was determined that an average of 21% of macrophages and 42% of engulfing MCF-7 cells contained cell corpses of *de*

novo autophagic origin after a 24 hours co-incubation (Fig. 5c). Almost all of the engulfed cells were autophagic (87-90% positive for MDC staining) and all of the eaten cells could be stained by PI, that is, by definition they were dying autophagic cells.

4.4. Autophagic component of cell death and appearance of phosphatidylserine on dead cells differentially influence phagocytosis

Inhibition of autophagy by 3-MA, applied 2 days prior to peaking of autophagy and harvesting these cells for the phagocytic assay, almost completely abolished the engulfment of *de novo* autophagic cells by both macrophages and MCF-7 cells. On the contrary, treatment with 3-MA did not affect significantly the engulfment of anoikicautophagic cells by either macrophages or MCF-7 cells (Fig. 6a, b). Treatment of the dying *de novo* autophagic and anoikic-autophagic cells with recombinant annexin-V before and throughout the phagocytic assay, that is blocking PS on the surface of dying cells, could inhibit significantly only engulfment by non-dying MCF-7 cells and not by macrophages. The insert in Fig. 6 summarizes the differential effects of 3-MA and annexin-V on the phagocytosis of the two kinds of autophagic cells by the two types of engulfing cells.

4.5. Expression profile of apopto-phagocytic genes in macrophages and MCF-7 cells

To gain an insight into the potential molecular mechanisms mediating phagocytosis by macrophages and MCF-7 cells quantitative gene expression analysis was carried out of these cells. The genes analyzed on a TaqMan Low Density Array to determine their level of expression can be categorized into the following functional categories: phagocytosis receptors, cell surface molecules, bridging molecules: signal transducers, engulfment proteins, effector molecules, transcription factors, inflammatory regulators and cytokines (see Fig. 7 and Table 1). These genes have been selected to cover most of the so far described molecular elements which may participate in the clearance of dead cells. The basal expression levels were determined in relation to the abundant 18S RNA and have been presented in Fig. 7 in a way that the increments of the horizontal bars represent one order of magnitude increase in the range of relative expression and the size of the bar at each gene indicates where the actual value of relative expression (average of three repeated experiments) fell. The data from the gene expression profiling clearly show that both macrophages and MCF-7 cells are well equipped for phagocytosis of dying cells. In macrophages, almost all studied genes of the various functional groups were expressed and the following ones have very high basal expression levels: calreticulin, the scavenger receptors CD68 and MSR1, the low density lipoprotein-related protein 1 (LRP1), integrins α_x and β_2 (phagocytosis

receptors), annexin A5, the C1QA bridging molecule and TGFβ (Fig. 7). Interestingly, many of the studied and expressed phagocytic genes were also expressed in MCF-7 cells. The exceptions, with very low expression levels in comparison to macrophages, include the C1Q receptor 1, MSR1, the oxidized low density lipoprotein 1 receptor (OLR1), integrin α_x among the phagocytosis receptors, the adenosine receptor A3 (ADORA3), the formyl peptide receptor like 1 (FPRL1), the bridging molecules C1QA and ICAM3, the inflammatory regulators caspase 1 and 5, NALP12 and the cytokines IL6, IL10, IL12B, IL23A. Calreticulin, annexin A5 and calpain 1 showed high basal expression in these cells.

4.6. Clearance of autophagic and anoikic-autophagic cells initiates distinct transcriptional responses in macrophages and MCF-7 cells

The time point chosen for gene expression profiling of macrophages and MCF-7 cells while they were consuming dying cells as compared to their resting state was 12 hours. Change of mRNA level was considered significant when the average value of relative expression repeatedly (at least in two biological samples, both analyzed in two parallel runs) exceeded 2.5 times the average expression level in controls, either increasing or decreasing (+ or -). It is also indicated in Fig. 7 when the change of expression was more than 5.0 times (++ or --) and 10 times (+++ or ---) as compared to controls. The engulfment of *de novo* autophagic cells elicited different response in the two types of phagocytes. In macrophages, the asialoglycoprotein receptor 1 (ASGR1), adenosine receptor 2A (ADORA2A), the bridging molecule PTX3, phospholipase IPLA27, the engulfment molecules GULP1 (which was not expressed in the resting cells) and RAC1, the interferon regulatory factors (IRFs) 1 and 8, the cytokines IL-6, IL12B, IL23A and TNFα were induced. ADORA3, the platelet activating factor receptor (PTAFR), the bridging molecules C3 and ICAM 3, the inflammatory regulators BIRC1 and NALP12 were downregulated in macrophages eating *de novo* autophagic cells. MCF-7 cells showed increased expression of annexin-1 and the engulfment molecule ELMO1. Both phagocytes upregulated OLR1, IRF4 and IL6 to a significant degree in response to the dying *de novo* autophagic cells.

Macrophages eating anoikic-autophagic cells upregulated C1Q receptor 1, ADORA1 and ADORA2A, (while downregulating several phagocytosis receptor and surface molecules including CD68, MSR1, LRP1, integrins, ADORA3. annexin-1 and the platelet/endothelial cell adhesion molecule), the bridging molecules C4B and MFGE8 (while downregulating C2, C3 and ICAM3), the signaling molecule protein kinase 2, the engulfment protein RAC1 (while downregulating ELMO 1), DNASE1 (while downregulating DNASE2), the IRF 4 (while

downregulating IRF8), the pro-inflammatory caspase 5 (while downregulating NALP12) and the cytokine IL6. MCF-7 cells responded to anoikic-autophagic cells by up regulating the AXL receptor tyrosine kinase.

The question may arise whether the elevated mRNA levels observed in engulfing cells could come from the eaten cells. This is very unlikely, since the overlapping up regulated genes in two different types of phagocytes eating the same kind of dead cells do not show systematic "cross-contamination". In addition, analysis of the gene expression changes in the *de novo* autophagic (Day 4) and anoikic-autophagic (Day 6) cells (the two kinds of dying cells used as targets of phagocytosis) as compared to untreated MCF-7 cells clearly shows that the genes being upregulated in either macrophages or the engulfing MCF-7 cells are not upregulated in the two kinds of dying cells (Fig. 7).The exceptions are ASGR1 and IL6 in both kinds of dying cells, and OLR1 in *de novo* autophagic cells. However, other upregulated genes in dying cells (ITGAX, ICAM3, ALOX12) do not have increased levels in the engulfing phagocytes, which again argue against the possible influence of cross-contamination on the results.

4.7. Clearance of autophagic and anoikic-autophagic cells evokes pro-inflammatory response in macrophages

Gene expression profiling of macrophages consuming *de novo* or anoikic-autophagic dying cells showed upregulation of the cytokine genes studied by TLDA (Figs. 7, 8a). Having found this, it was important for us to know whether this upregulation was accompanied by corresponding cytokine secretion; therefore, we decided to take further investigations regarding this issue.

Macrophages pre-stimulated for 30 minutes with LPS and then challenged to engulf *de novo* or anoikic-autophagic cells for 1 hour had increased secretion of the pro-inflammatory cytokines IL-6, TNFα, IL-8 and the anti-inflammatory cytokine IL-10 (Fig. 8b). It is well known that apoptotic cells are able of downregulating the inflammatory response (Cvetanovic et al. 2006). Our question arose whether the autophagic dying cells are capable of doing this or not.

De novo autophagic cells themselves do not lead to secretion of the above mentioned inflammatory cytokines and they can prevent the inflammatory response induced by LPS in human macrophages (Fig. 9). On the other hand, anoikic-autophagic cells do lead to secretion of inflammatory cytokines, exceptions being TNFα, IL-8 and IL-10 where certain degree of suppression could be observed.

The pattern of IL-1β expression and secretion was different than the one from the other inflammatory cytokines (Fig. 10a, b). In this case, pre-stimulation with LPS could not produce an inflammatory response as also found by other groups (Martinon et al. 2006). *De novo* autophagic cells alone, however, could lead to significant increase in the secretion of IL-1β. This response was rather rapid at both protein secretion and gene expression levels, ocurring mostly after the first hour of the assay. Anoikic autophagic cells, on the other hand, were found to be somewhat pro-inflammatory leading to a small increase in the IL-1β secretion during their engulfment.

A possible explanation of the IL-1β phenomenon could be that engulfment of *de novo* autophagic cells can initiate induction of the inflammasome within the macrophages which further leads to caspase-1 as well as IL-1β activation. For this reason, we tested caspase-1 inhibition with specific inhibitor (zYVAD-FMK) and indeed, we could block the secretion of IL-1β by the macrophages. As a control, uric acid was used to induce IL-1β secretion in macrophages as described earlier by Martinon et al. (2006). When 3-MA was used to inhibit the autophagy in the *de novo* autophagic cells, IL-1β secretion was also inhibited, therefore proving that autophagy itself is crucial in the initiation of this cytokine's secretion. Supernatants from *de novo* autophagic cells could not induce IL-1β secretion, therefore, the engulfment itself and not secreted molecules released by the autophagic cells leads to IL-1β induction and secretion. (Fig. 10c).

5. DISCUSSION

5.1. Clearance of cells dying through autophagy

Clearance of dying cells is of great importance for maintaining balanced tissue homeostasis in mammalian organisms. Insufficient phagocytosis of dead cells may lead to inflammation and autoimmune diseases (de Almeida et al. 2005). Molecular mechanisms participating in the clearance of apoptotic cells by macrophages acting as professional phagocytes has been described in detail and, to a lesser extent, this can be said about those mediating engulfment of dead cells by

non-professional phagocytes (Fadok et al. 2000; Fadok et al. 2001; Grimsley et al. 2003; Krieser et al. 2002; Monks et al. 2005). It has been suggested that autophagy as a mechanism of cell death may have been developed because in some forms of programmed cell death the availability of engulfing cells is insufficient for the clearance of dead cells and autophagy has been evoked for elimination of excess cells by their own lysosomes (Levine et al. 2005; Shimizu et al. 2004). This would imply that cells dying though autophagy or with autophagy are not removed from tissues by phagocytosis. In our studies we could clearly demonstrate this is not the case: autophagic MCF-7 cells of different origin are recognized and eaten by both differentiated macrophages and non-dying MCF-7 cells. Almost all engulfed cells were MDC positive. Since all of them were also stained by PI, we may conclude that the eaten cells were dead or dying autophagic cells and not cells which use autophagy to survive.

An equally important question is whether the engulfed MCF-7 cells died through autophagy (that is autophagy killed the cells) or by apoptosis during which autophagy was switched on, but it did not contribute to killing of cells. It has been demonstrated that the autophagy pathway is capable of killing cells when the apoptosis machinery is not intact (Shimizu et al. 2004; Yu et al. 2004), but only a few examples are available to prove that autophagy is an important death pathway in cells with intact apoptosis machinery (Reef et al. 2006). Based on several indirect evidences it has been proposed that active cell death is induced through autophagy in tamoxifen treated MCF-7 cells (Bursch et al. 1996); one of the evidences is the inhibition of both autophagy and cell death by 3-MA, a compound which can specifically block the sequestration step in macro-autophagocytosis (Seglen et al. 1982) inhibiting class III phosphatidylinositol-kinase activity (Petiot et al. 2000). Since in our experiments 3-MA also inhibited macrophage and MCF-7 cell mediated phagocytosis of dying *de novo* autophagic cells, it can be suggested that molecular events linked to autophagy and death are involved in specific surface changes which are important in subsequent recognition and elimination of these dying cells.

5.2. Clearance of cells dying with autophagy

We have also described here a novel way of inducing autophagy, namely through initiation of anoikis, which could facilitate autophagy even in the *de novo* autophagic cells. In case of anoikis cells actually die through apoptosis with coincident autophagy, which has no causative role in the death process itself. How can an anoikis-initiated death response switch on autophagy and why does anoikis accelerate tamoxifen-induced autophagy? The answer, which certainly requires further investigation, may be found in understanding the complex regulatory interactions among

elements of the apoptotic and autophagic pathways. Examples for the induction of apoptosis with the help of autophagy regulators, such as the *p53*-induced *dram* and the calpain-generated cleavage product of *atg5*, have been described (Crighton et al. 2006; Yousefi et al. 2006). It is also possible that elements of the apoptosis machinery, while killing cells, may initiate autophagy to fasten self elimination even before phagocytosis; in our case loss of integrin-dependent survival pathways may lead to induction of death effectors which in turn converge onto mediators of autophagy, such as *Atg5*, *Atg7*, and *beclin-1*. Nevertheless, in our experiments cell death occurred much earlier in anoikic-autophagic cells than the appearance of autophagy, inhibition of autophagy by 3-MA in these anoikic-autophagic cells have resulted in less inhibition of cell death than in case of *de novo* autophagic cells and, most importantly, did not have an effect on the engulfment of these anoikic-autophagic cells by either of the phagocytic cell types. Therefore, we have described clearance of autophagic dying cells in two kinds of paradigm; one in which cells died mainly through autophagy, generating specific cell surface changes for recognition and engulfment, and another, where cell death occurred with concomitant autophagy which is not the main inducer of death and does not contribute to specific cell surface changes for removal by phagocytes.

5.3. Macrophages and non-professional phagocytes use distinct mechanisms for engulfment

How does the direct involvement of autophagy in the death process influence the clearance mechanisms used by engulfment cells? Our results show that non-professional phagocytes, the non-dying MCF-7 cells, are much more efficient in taking up *de novo* autophagic cells than anoikic-autophagic ones, that is they can recognize the difference between death through autophagy and death associated with autophagy. Dying cells present on their surface several "eat-me" signals facilitating their engulfment (Lauber et al. 2004). One such common "eat-me" signal for dead cell removal by phagocytes is the externalized PS (Wu et al. 2006). Recombinant annexin-V, which binds PS exposed on the surface of dying cells, could block the engulfment of dying *de novo* autophagic cells by MCF-7 cells (Fig. 6a, b); this clearly suggests that whatever specific changes are induced by autophagy on the surface of these dying cells - making them edible for MCF-7 cells (see inhibition by 3-MA) - is directly or indirectly linked to PS. During development, autophagy in dying cells may be crucial to generate sufficient ATP for PS exposure as demonstrated in PCD associated with morphogenesis (Qu et al. 2007). Though the need for autophagy-dependent ATP production in triggering PS exposure could be observed only in a three-dimensional culture setting, this mechanism may also be utilized in starvation induced autophagic death of MCF-7 cells cultured in monolayers. On the other hand, uptake of the *de*

novo autophagic cells by macrophages could not be inhibited by recombinant annexin-V indicating that PS-independent cell surface changes are also induced in cells dying through autophagy.

This means that macrophages, as compared to MCF-7 cells, must have additional recognition and signaling system(s) available for the initiation of efficient phagocytosis of these cells. What surface changes on *de novo* autophagic cells may initiate PS-dependent and PS-independent recognition by phagocytes? A handful of proteins have been shown to undergo internalization from the plasma membrane following the induction of autophagy (Baricault et al. 1995; Tagawa et al. 1999). To our present knowledge, however, no specific cell surface changes have been described which would make autophagic cells edible for phagocytes. Recently, calreticulin appearing on the surface of the dying cells was found to initiate clearance of apoptotic cells and the authors have suggested that calreticulin acts together with PS for achieving optimal phagocytic clearance of cells (Dini et al. 2000). Changing carbohydrate composition of glycoproteins (Oldenborg et al. 2000) and exposure of DNA on dying cells (Palaniyar et al. 2004) have been proposed as alternative mechanisms to PS for recognition of dead cells (Oldenborg et al. 2000). Phagocytes may also use a number of receptors not specifically designed for recognition of dead cells and also rely on the presence or absence of CD47 (integrin-associated protein) to distinguish dead from alive or self from foreign (Palaniyar et al. 2004). Further studies are required to determine whether these alternative “eat me” signals or others are exposed on the surface of cells dying through autophagy.

Autophagy in cells dying through anoikis did not contribute to clearance of these cells by either MCF-7 cells or macrophages. However, the uptake of these cells by MCF-7 was inhibited when PS was covered on the cells surface, supporting the proposition that PS may contribute to distinct recognition patterns on dying cells. The anoikic-autophagic cells were very efficiently eaten, actually more efficiently than *de novo* autophagic ones, by macrophages in a PS-independent manner. Since this was not related to the appearance of autophagy (could not be inhibited by 3 -methyladenine) the PS-independent recognition and signaling system in this case must be different from the one utilized by cells dying through autophagy.

5.4. Possible molecular elements in the clearance of autophagic cells by macrophages and non-professional phagocytes

Molecular elements used for the clearance of dead cells are highly conserved and strongly interrelated with components of the innate immune system (Savill et al. 2002). It has been

suggested that different surface receptors on the phagocytes are involved in tethering (recognition and binding of cell corpses) and tickling (internalization and activation of downstream signaling) processes, leading via at least two major pathways to activation of Rac which is obligatory for uptake of dead cells (Gardai et al. 2005; Henson et al. 2006). In case of PS recognition, at least one tethering and one internalization receptor is needed for efficient clearance (Wu et al. 2006); tethering is co-opted by integrins or integrin-associated proteins (e.g. CD36) working in contact with the bridging molecule MFGE8. Tickling is mediated by *Gas* or protein S through one of the tyrosine kinase receptors (MERTK, AXL or TYRO3) leading to activation of *Rac* engaging TRIO, *RhoG*, DOCK1 80, *CrkII* and ELMO. Exposed calreticulin is recognized by collectins and ficolins (such as C1QA, PTX) serving as bridging molecules, then the opsonized dead cells are bound to phagocytosis receptors on the surface, including calreticulin in conjunction with LRP1 (which signals to *Rac* through GULP); as an alternative, thrombospondin is the bridging molecule and integrin α_v or calreticulin serve as receptors. We have used a low density gene array approach to learn which of the above and other previously described molecular participants in the clearance mechanisms are expressed in the two types of phagocytic cells. Furthermore, comparing the basal expression levels and the ones induced while the phagocytes were actually eating the dying cells, we could obtain some clues what particular pathways may be involved in the engulfment of the two kinds of autophagic dying cells by the two types of phagocytes.

As discussed above, MCF-7 cells used mainly the PS recognition and signaling pathway for phagocytosis of dying cells. Indeed, various elements of this pathway are expressed by this cell type at significant levels, including the bridging molecules MFGE8, protein S and *Gas*, the integrin and CD36 tethering receptors, the tickling tyrosine kinase receptors AXL, MERTK and TYRO3, and all the engulfment proteins required for *Rac* activation (TRIO, *RhoG*, DOCK1 80, *CrkII* and ELMO) through this pathway. It is very likely that the expressed complement factors and scavenger receptors also contribute to the efficient uptake of dying cells. It is remarkable that the expression of none of the studied genes showed decrease during the engulfment of either kind of dying cells. The more efficient uptake of *de novo* autophagic cells as compared to anoikic-autophagic ones may be related to the specific changes on the surface or inside of the engulfed dying cells inducing distinct transcriptional responses, such as upregulation of OLR1, ANXA1 and ELMO 1. On the other hand, induction of AXL may be linked to surface changes which are unrelated to autophagy but have importance for the uptake by MCF-7 cells of anoikic-autophagic cell corpses in a PS-dependent way.

In macrophages, the molecular elements of the PS recognition and signaling pathway are also highly expressed; one may assume that it is utilized for engulfment of both kinds of autophagic dying cells under normal circumstances. Our gene array data show, however, that the calreticulin-mediated pathway of dead cell clearance is also available for efficient phagocytosis of the *de novo* autophagic cells and this may explain why phagocytosis of the dying autophagic cells is not blocked when PS is covered on the surface of dying cells. Calreticulin is highly expressed in both the dying and phagocytic cells and may become exposed on the surface, C1QA and LRP1 mRNA are at high concentrations in macrophages, PTX and GULP are specifically induced during the uptake of this kind of dying cells, in conjunction with the strong induction of *Rac* expression. Macrophages can engulf anoikis autophagic dying cells in a PS-independent way more efficiently than *de novo* ones, but the calreticulin-C1Q-LRP-GULP pathway is not available because GULP is not induced by the anoikic-autophagic cells (see the gene expression profile in Fig. 7). Again, the difference in phagocytosis efficiency may be related to specific changes on the surface of anoikic-autophagic cells leading to utilization of alternative pathways in macrophages, such as those which involve the highly expressed scavenger receptors, the specifically induced C4B and C1QR1, the signaling molecule PTK2 and DNASE I. Interestingly, macrophages eating anoikic-autophagic dying cells are the ones which show the most significant downregulation of several phagocytosis genes. One explanation for this could be related to the heterogeneity of the differentiated macrophage population, of which only a proportion is capable to recognize and engulf dying cells under even in the most optimized conditions (authors' unpublished observations); it is possible that while the engulfing cells are engaged in the phagocytosis process, the engulfment genes are downregulated in the non-phagocytic population.

5.5. Induction and secretion of cytokines in macrophages engulfing dying autophagic cells

Our gene array profiling of engulfing macrophages has revealed the surprising induction of the pro-inflammatory cytokine genes IL6, TNFα, IL12B and IL23A by both kinds of dying cells; these data could be confirmed by measuring significant amounts of secreted IL-6, TNFα, IL-8 and IL-10 in the culture fluid of phagocytosing macrophages (Petrovski et al. 2007) (Figs. 7, 8b). In case of anoikic-autophagy this may be explained by the presence of late apoptotic cells, which have been shown to be pro-inflammatory (Patel et al. 2006) in high proportion at the 6th day of anoikis (see per cent of annexin-V and PI positive cells in Figure 3a); in case of *de novo* autophagy, however, this is a novel finding and no known explanation exists yet for its occurence.

LPS induction of the pro-inflammatory cytokines was found to decrease in the presence of *de novo* autophagic cells (Figs. 9, 10a) similarly to apoptotic cells (Monks et al. 2005; Cvetanovic et al. 2006). Another similarity to apoptotic cells, which require contact between dying and engulfing cells to regulate the macrophage inflammatory response (Lucas et al. 2006), is the need of contact as well during engulfment of *de novo* autophagic cells for downregulation of the LPS response. Like apoptotic cells, where exposure of PS on the surface of dying cells causes induction of the anti-inflammatory response, it is likely that autophagic dying cells also use exposed PS for downregulation of the inflammatory response ether through bridging and PS-dependent receptors (Cvetanovic et al. 2006) or by inducing TGFβ (Freire-de-Lima et al. 2006).

A more surprising finding from our studies is the specific gene induction and secretion of the pro-inflammatory cytokine IL-1β by macrophages engulfing *de novo* autophagic cells but not anoikic-autophagic ones (Fig. 10). This activation and secretion of IL-1β is probably independent of the so far known external LPS-Toll-like receptor (TLR) induction pathway since *de novo* autophagic cells could block the external LPS-induced response (Fig. 10a). In further support of a putative internal pathway induced by the engulfed *de novo* autophagic cells is our study with caspase-1 inhibitor and thus blockage of the conversion of pro- IL-1β to its active secreted form in absence of external LPS stimulation (Fig. 10c). Our present findings suggest involvement of nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs) (Franchi et al. 2006) located inside the macrophages during engufment of *de novo* autophagic cells. Very likely, similar to other 'danger signals' known to induce NLRs and the inflammasome components such as ATP, crystals of monosodium urate (MSU), calcium prophosphate dihydrate (CPPD) originating from damaged or dead cells as well as different bacterial pathogens (Yasunori et al. 2006), *de novo* autophagic cells can somehow induce expression and secretion of IL-1β, too. Whether this IL-1β response itself induces further neighboring macrophages in a paracrine mode to produce other pro-inflammatory cytokines or there is an undiscovered pathway for induction of these cytokines upon engulfment of *de novo* autophagic cells remains unknown.

We propose here a model for the mechanism, we think, IL-1β gets induced and secreted by macrophages (Fig. 11). Probably cells dying through autophagy expose autophagy-related pro-inflammatory signals on their surface since inhibition of autophagy by 3-MA and contact inhibition with conditional medium from *de novo* autophagic cells could block the IL-1β secretion (Fig. 10c). This may have significance when autophagic death occurs in tumor cells, such as MCF-7 cells, and the inflammatory response can promote tumor growth and angiogenesis (Degenhardt et al. 2006; Lawrence et al. 2007). It has been suggested that expressed

PS on dying cells has a major function in the generation of anti-inflammatory effect; cells dying through autophagy probably use this mechanism to downregulate TLR-dependent pro-inflammatory response. Engagement of calreticulin with collectins stimulate pro-inflammatory mediator production (Gardai et al. 2007; Henson et al. 2006; Obeid et al. 2007). Since molecular elements of the calreticulin-dependent recognition and engulfment pathway (calreticulin-C1Q-LRP-GULP pathway) were induced in macrophages engulfing *de novo* autophagic cells, it is possible that signaling through this pathway participates in the induction of pro-inflammatory cytokine production (Fig. 8a). Our results show that either through a calreticulin-dependent or a so far unknown mechanism the engulfment of *de novo* autophagic cells can initiate assembly of the inflammasome and thereby activate caspase-1 with subsequent cleavage and secretion of the parallely induced IL-1β. Another possible alternative would be that the pro-inflammatory response of macrophages to dying autophagic cells may also be connected to the observed induction of adenosine receptors, particularly ADORA2A in the engulfing cells. Adenosine receptor A_{2A} is a potent regulator of macrophage functions, including Fc receptor mediated phagocytosis and pattern recognition receptor-mediated cytokine production (Hasko et al. 2006). However, since supernatants from *de novo* autophagic cells could not induce IL-1β secretion, it is very unlikely this alternatively applies here.

All these possibilities add a new angle to the already complicated connections between autophagy and cancer: the conflicting pro-survival and pro-death functions of autophagy in cancer cells (Levine et al. 2007) may also be manifested in the opposing anti-inflammatory and pro-inflammatory effects mediated by autophagy. Better understanding of the molecular mechanism of these phenomena may lead to more rational design of autophagy-based therapeutic interventions in disease. Ongoing investigations in our laboratory are aimed at clarifying whether the pro-inflammatory response of macrophages to engulfed autophagic dying cells is restricted to cancer cells and to learn what molecular mechanisms are involved in its induction.

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7. FIGURE AND TABLE LEGENDS

Figure 1. Molecules involved in the apopto-phagocytic synapse. $\alpha_v\beta_{3/5}$ vitronectin receptor integrins; ABCA-1, ATP-binding cassette transporter A1; ACAMPs, apoptotic cell-associated molecular patterns; ASGP-R, asyaloglycoprotein receptor; β2GPI, β2 glycoprotein I; β2GPI-R, β2GPI-receptor; β2-integrins (CR3, CR4); C1q, first component of complement; CHO, carbohydrate; CRP, C-reactive protein; Del-1, developmental endothelial locus-1; Gas-6, growth arrest specific gene-6; iC3b, inactivated complement fragment C3b; ICAM-3 (CD50), intercellular adhesion moleculae-3; Lox-1, oxidized low density lipoprotein receptor 1; MER,

myeloid epithelial reproductive tyrosine kinase; MFG-E8, milk fat globule epidermal growth factor-8; Ox-PL, oxidized phospholipids; PS, phosphatidylserine; PSR, putative PS receptor; SHPS-1, Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1; SR-AI, scavenger receptor AI; SR-BI, scavenger receptor BI; TSP-1, thrombospondin-1.

Figure 2. Autophagy in MCF-7 cells following various treatments. (**a**) Percentage of MCF-7 cells with MDC-positive autophagic vacuoles; (○) underwent heavy autophagy by day 4; (▽), (▼) and (□) showed shift to autophagy by day 6, 4 and 2, respectively; 1000 cells in each of triplicate cultures were counted; the total number of MDC-positive cells are expressed as percentage of total cells scored. Means of three experiments are shown. Vertical bars indicate S.D.; when not shown, it is smaller than the symbols. (*) time of peaking autophagy assessed by MDC positivity. (**b1-3**) Transmission electron microscopy of control cells (**b1**), as well as day 4, attached, 3% DCC and TAM treated cells (**b2**) and day 6 anoikic cells in 10% FCS (**b3**) showing numerous double membraned autophagic vacuoles containing digested materials. (**c1**) Fluorescent microscopy with MDC/PI and (**c2**) transmission electron microscopy of day 2, 3% DCC and TAM treated anoikic cells. Note the autophagic vacuoles in the cytoplasm (in blue) surrounding the nucleus (in red). (**d**) Western blot analysis of the LC3 expression in anoikic-autophagic (▽) and autophagic cells (○). Positive control (C) was obtained from C2C12 cells undergoing autophagy and showing bands for both LC3-I and LC3-II.

Figure 3. Cell death and the influence of 3-MA in autophagic, anoikic and apoptotic MCF-7 cells. (**a**) Percent of annexin-V and both annexin-V and PI positive cells under the shown treatments as assessed by FACS analysis. (**b**) Effect of 10 mM 3-methyladenine treatment on autophagy and cell death of day 4 autophagic and day 6 anoikic-autophagic cells (**c**) as assessed by the presence of MDC, annexin-V and/or PI positive cells. Note that *de novo* autophagic cells were fed on 3% DCC for 7 days prior to the indicated ‘0‘ time point. At this starting point, anoikic-autophagic as well as anoikis-3% DCC cells with or without TAM showed less than 0.04% cell death. Means of three experiments are shown. Vertical bars indicate S.D.; when not shown it is smaller than the symbol.

Figure 4. Flow cytometric analysis of the phagocytosis of dying cells. (**a**) Macrophages and (**c**) MCF-7 cells stained with CMTMR engulfing either autophagic or anoikic-autophagic cells stained with CFDA. The phagocytic assays were performed at 4°C at which binding occurs without engulfment and parallely at 37°C at which both binding and engulfment occur. The macrophages that have not phagocytosed are in the upper left quadrant and the ones that have are in the right upper quadrant; dying cells have been gated out from the lower left and right quadrants. Time of co-incubation in each case was 12 hours. (**b**) Macrophages and (**d**) MCF-7 cells were challenged to engulf *de novo* autophagic (■), anoikic-autophagic (□), or UV irradiated apoptotic (▨) MCF-7 cells. Bars represent mean of four independent experiments and S.D.

Figure 5. Detection of the phagocytosis of autophagic cells by confocal microscopy. (**a1-3**) Macrophage stained with CMTMR (red) that had engulfed *de novo* autophagic cell stained with CFDA (green) and MDC (blue) are co-localized. (**a3**) Phase contrast image of the same cells as in (**a1-2**). Non-dying MCF-7 cells stained with CMTMR (red) that have engulfed either *de novo* autophagic stained with both MCD (blue) and CFDA (green) (**b**) or anoikic-autophagic cells (**c**) stained with CFDA (green). Note the orthogonal views of phagocytosis in (**b**) and (**c**) as well as the yet uneaten cells (green) in the proximity. (**d**) Percent of both CFDA and MDC positive cells being engulfed by CMTMR positive cells in (**a**) and (**b**) as counted under a fluorescent microscope. In all cases the time of co-incubation of dying and engulfing cells was 24 hours. Means of three separate experiments are presented with their S.D.

Figure 6. Phagocytic capacity of (**a**) macrophages and (**b**) MCF-7 cells when challenged to engulf *de novo* autophagic or anoikic-autophagic MCF-7 cells (□), when the autophagic and anoikic-autophagic cells were pretreated by 3-methyladenine 2 days before autophagy peaked (■) and when phagocytosis was carried out in the presence of recombinant annexin-V, 10μg per 10^5 cells(▨). The insert shows when 3-methyladenine and recombinant annexin-V influence uptake of dying cells by the two types of phagocytes. Bars represent mean values of three independent experiments and their S.D. * $P<0.05$; ** $P<0.01$ using Student's *t*-test.

Figure 7. Gene expression levels in macrophages and MCF-7 cells and their changes during phagocytosis .The basal expression levels were determined in 3 independent biological samples and each was analyzed in two parallel runs in which each genes had two technical replicates. Based on the average of the obtained result (the variation coefficient was less than 20% in each case) the relative expression of a particular gene in macrophages and MCF-7 cells is indicated. The increments of the horizontal bars represent one order of magnitude higher range of relative expression (related to 18S RNA) and the size of the bar at each gene indicates into which range the actual expression data (average of three repeated experiments) fall. In case of analyzing gene expression changes during phagocytosis, change of mRNA level was considered significant when it repeatedly (at least in two biological samples, both analyzed in two parallel runs each with two replicates per gene) exceeded 2.5 times the average expression level of the controls, either increasing or decreasing (+ or -). It is also indicated the table when the change of expression was more than 5.0 times (++ or --) and 10 times (+++ or ---) as compared to controls. The nomenclature of the genes studied is in compliance with the HUGO classification; [1]Mer tyrosine kinase family, [2]scavanger receptors, [3]integrins

Figure 8. IL-1β cytokine release assay. **(a)** Macrophages engulfing *de novo* autophagic (AU) or anoikic autophagic (AN-AU) cells for either 1 hour and 17 hours dying-cells-free secretion period. **(b)** Gene expression after 1, 2, 4 and 6 hours after the 1 hour co-incubation with AU cells. The expression levels were analyzed in two parallel runs in which each gene had two technical replicates. Data are mean±SEM. Relative expression was determined compared to Human 36b RNA. **(c)** The same macrophages as in **(a)** engulfing *de novo* autophagic cells and their IL-1β secretion during the 1st hour of engulfment (white semi-bar) and during the 17 hours dying-cells-free secretion period (white semi-bars). Whenever stimulated with 0.5 µg/mL LPS, it was carried out for 30 min prior to starting the phagocytosis assay. Pretreatment of *de novo* autophagic cells with 3-methyladenine took place 2 days before autophagy peaked. Conditional medium from *de novo* autophagic cells at the time of their use was used for the supernatant studies. Caspase-1 inhibition with (zYVAD-FMK) at 50µM concentration was carried out for 30 minutes prior and throughout the phagocytosis assay. Bars represent mean values of three independent experiments and their S.D.

Figure 9. Gene expression and cytokine secretion analysis after 12 hours phagocytosis of dying autophagic cells. **(a)** TaqMan RT-PCR gene expression analysis in control macrophages (open bars) and those phagocytosing dying autophagic cells (closed bars). The expression levels were analyzed in two parallel runs in which each gene had two technical replicates. Data are mean$\pm$SD of three biological experiments. Relative expression was determined compared to 18S RNA ($x10^9$). * Expression is not detectable. **(b)** Cytometric bead array analysis of secreted cytokines by macrophages (M) while engulfing dying autophagic cells (AU); values represent average of three independent experiments.

Figure 10. Human inflammatory cytokine release assay. Macrophages engulfing *de novo* autophagic (AU) or anoikic autophagic (AN-AU) for 1 hour and 17 hours dying-cells-free secretion period. Whenever stimulated with 0.5 μg/mL LPS took place, it was carried out for 30 min prior to starting the phagocytosis assay. Bars represent mean values of three independent experiments and their S.D.

Figure 11. Proposed model for the mechanism of IL-1β cytokine release by macrophages. Cells dying through autophagy (AU) can block Toll-like receptor (TLR) dependent pro-inflammatory response but may initiate the activation of the inflammasome in macrophages. AU, *de novo* autophagic MCF-7 cells; CLR, calreticulin; NALP, NACHT-LRR-PYD-containing protein; PS, phosphatidylserine; TLR, Toll-like receptor.

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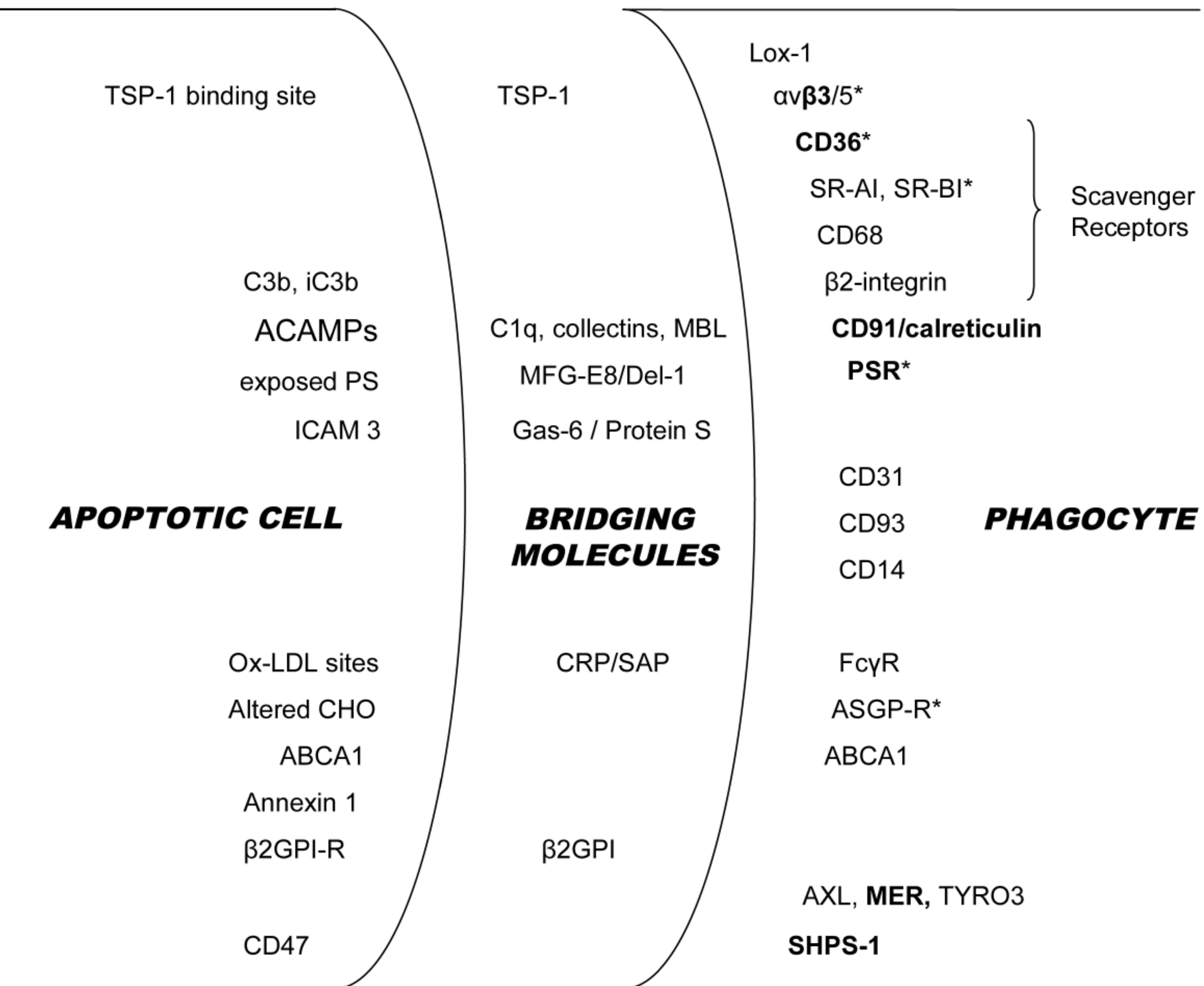
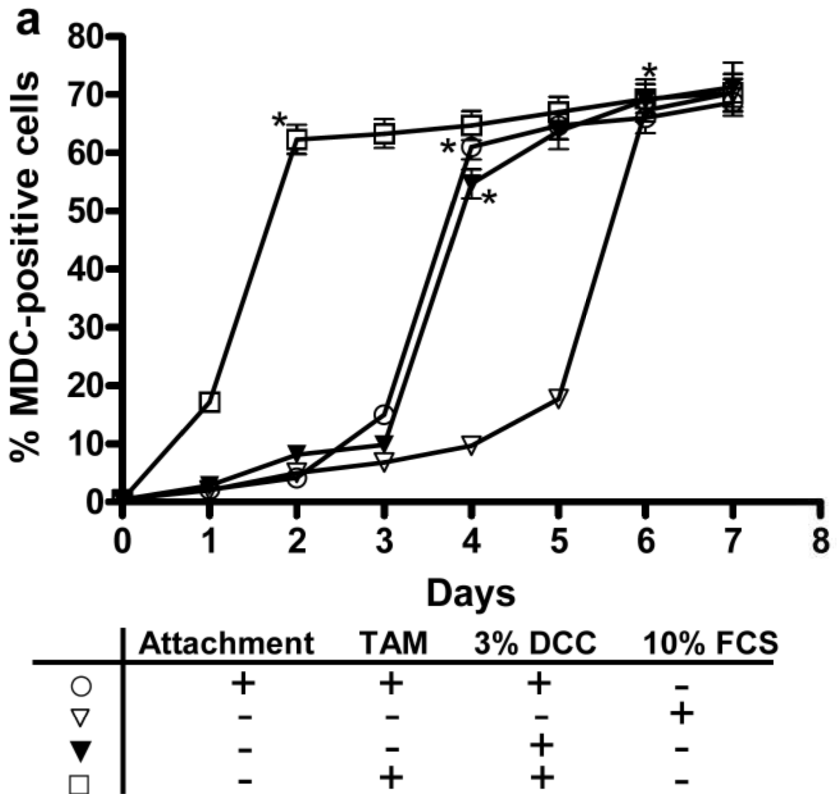
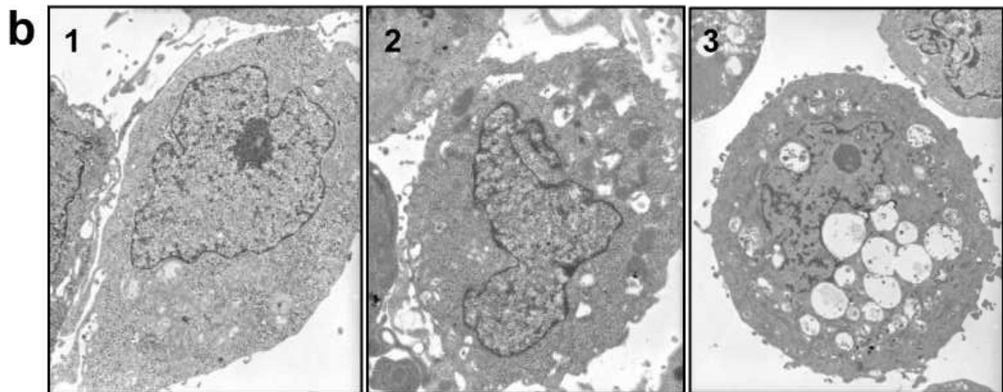
9. FIGURES AND TABLES

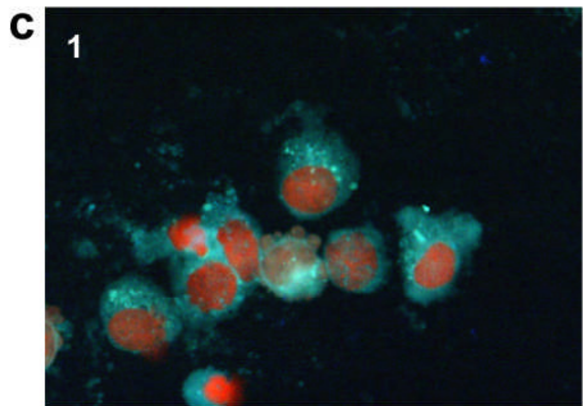


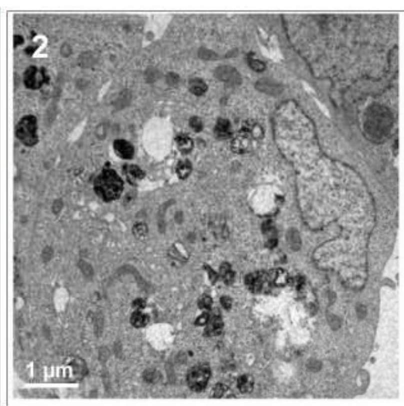
(*) Receptors found on non-professional phagocytes
Receptors suggested of being involved in the regulation of the inflammatory response are shown in bold

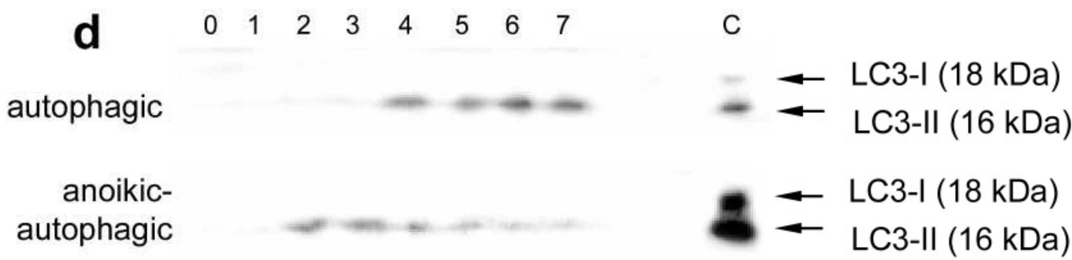
Fig.1











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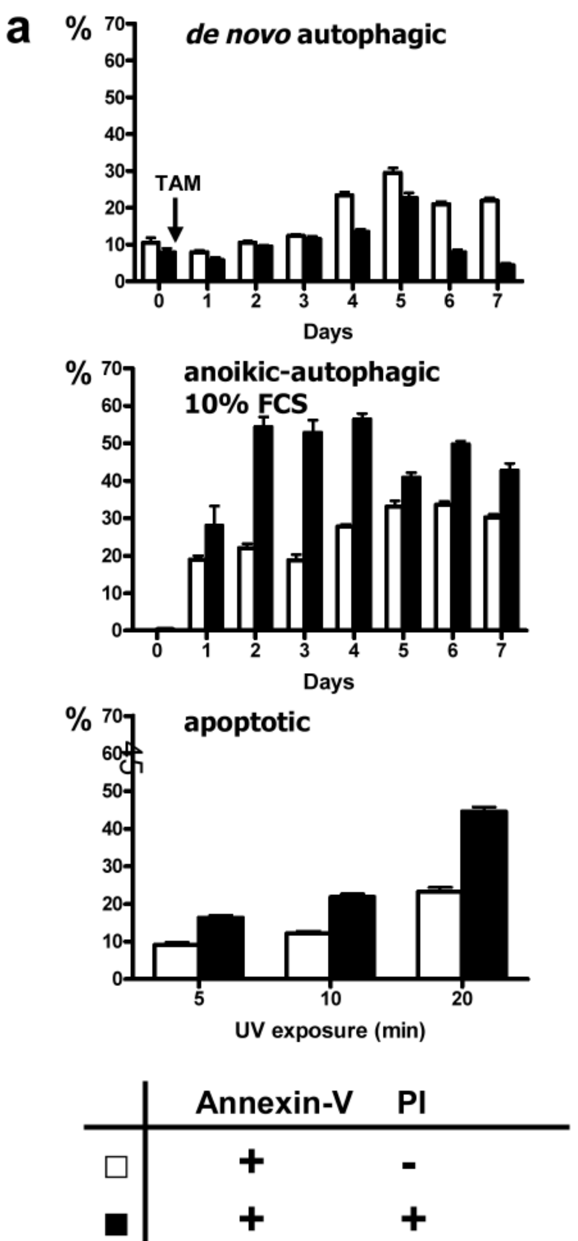

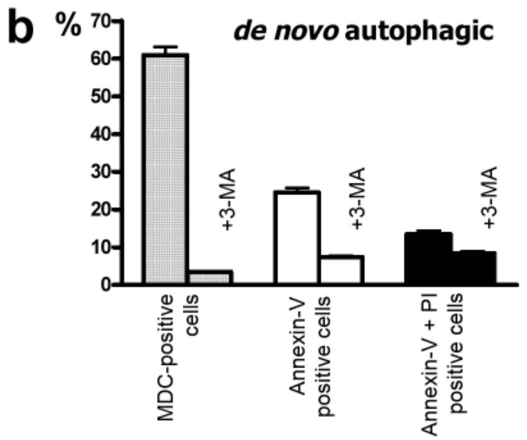

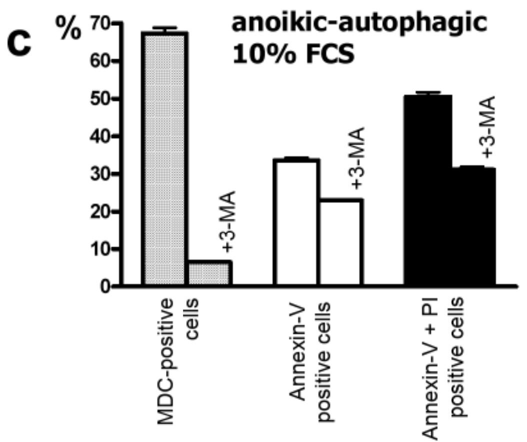

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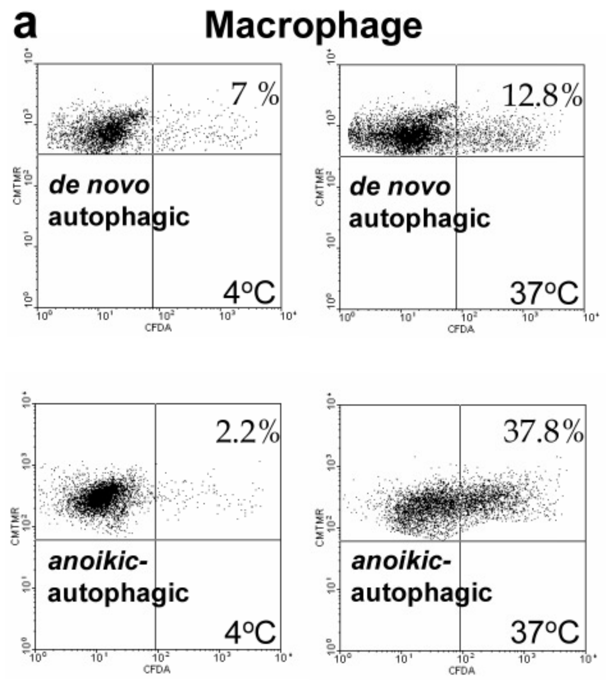
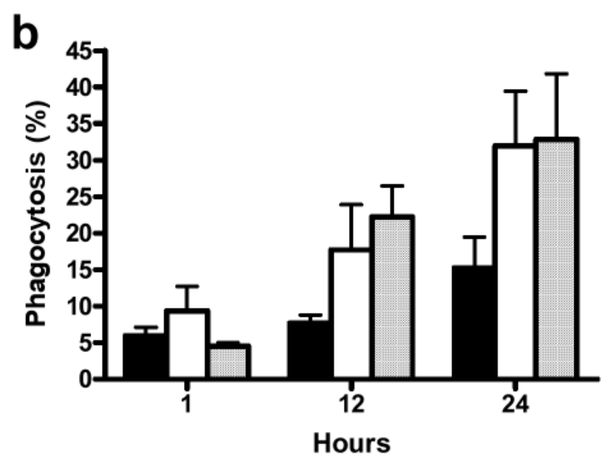
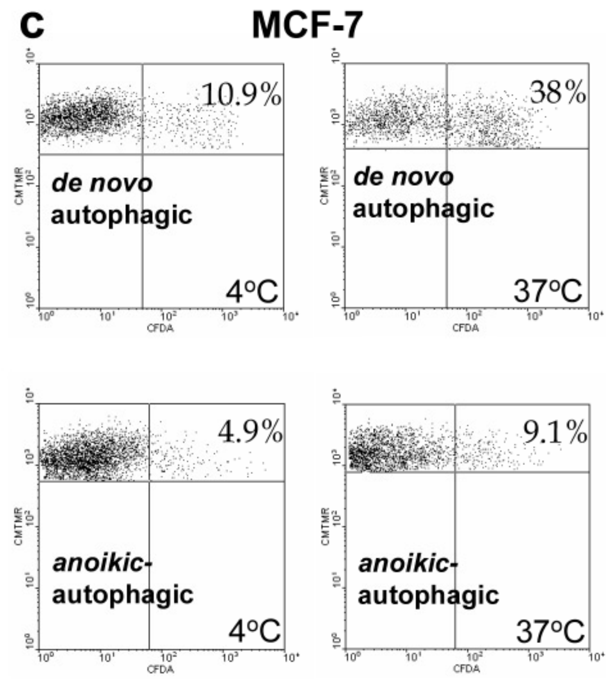






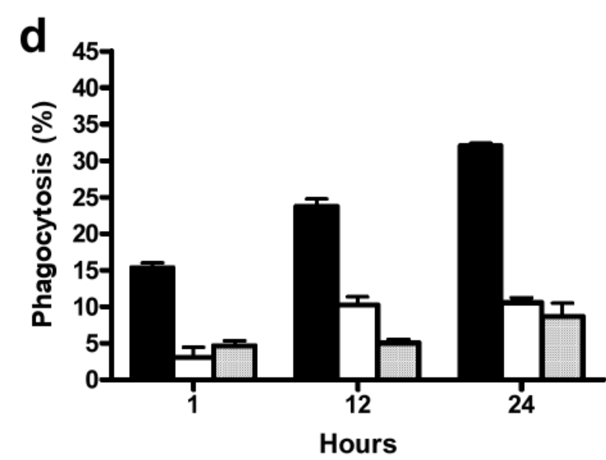
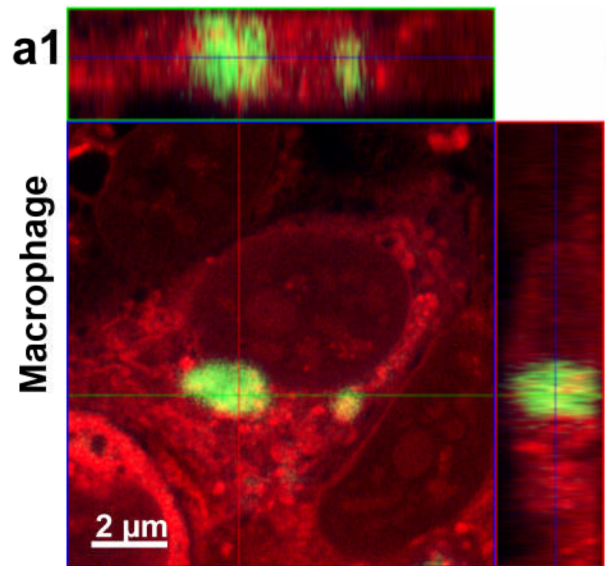
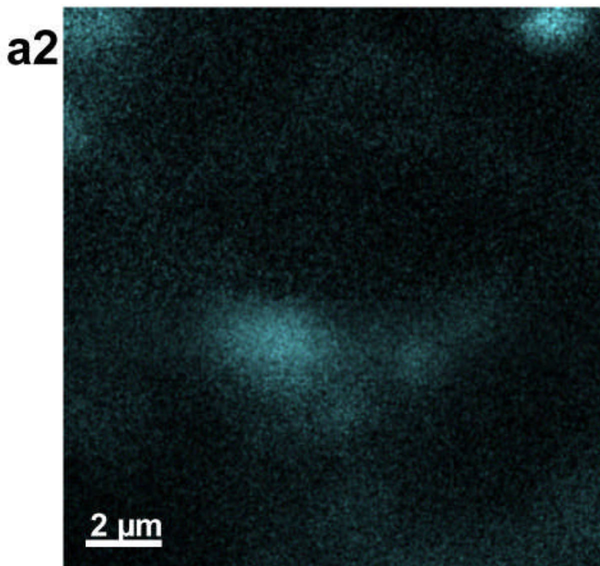
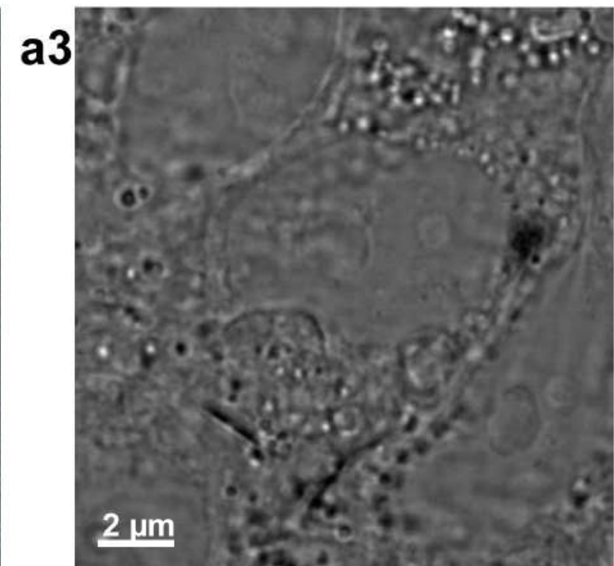
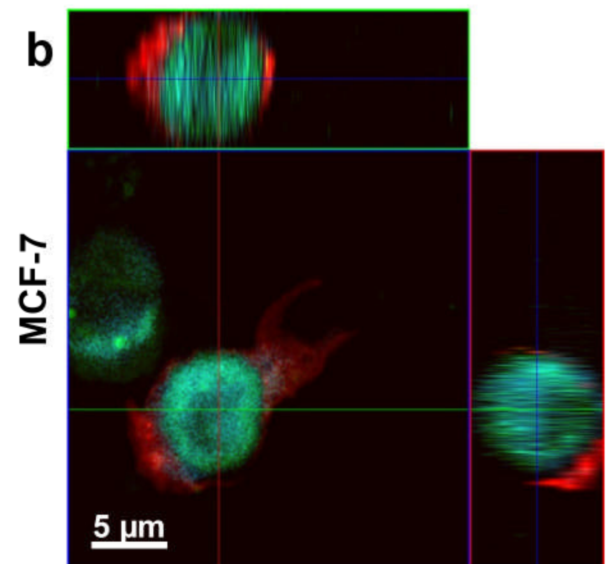


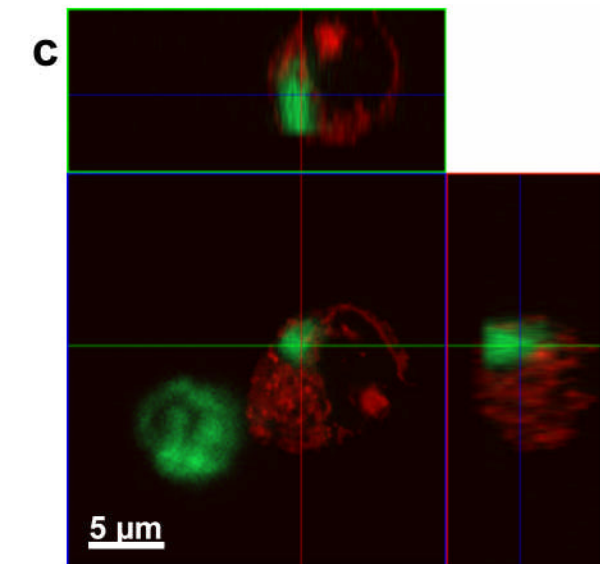
Fig.4









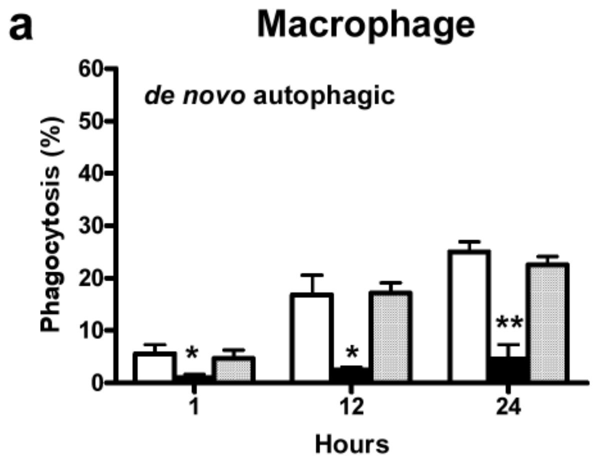


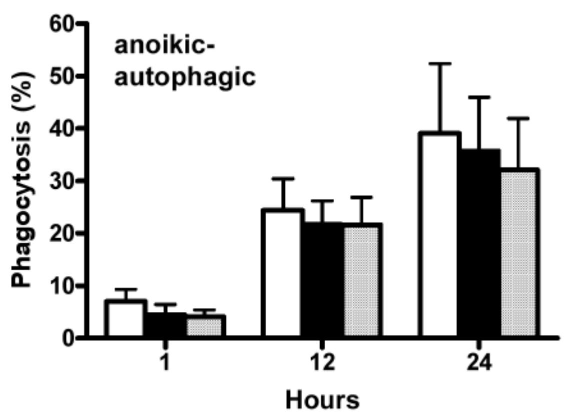
d

Cells	Macrophage + MCF-7 (autophagic)	MCF-7 + MCF-7 (autophagic)
% phagocytosis	20.7±2.5%	41.7±2.1%
% MDC positive	18±1%	37.7±1.5%
% PI positive	20.7±2.5%	41.7±2.1%

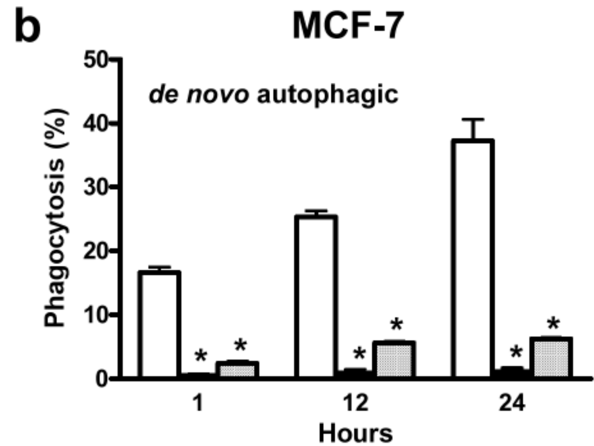
Fig.5

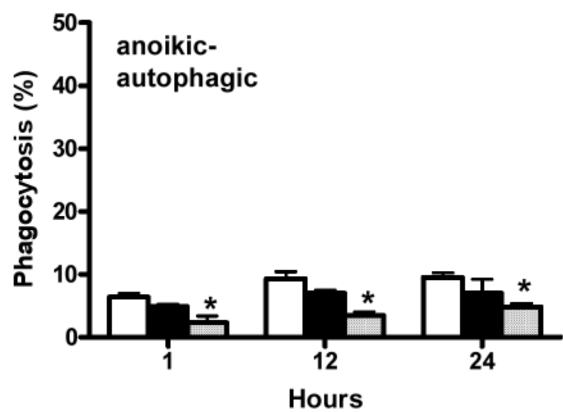
a Macrophage





b MCF-7





Eater	Treatment	*De novo* Autophagic	Anoikic-autophagic
MΦ	3-MA	+	-
	Annexin-V	-	-
MCF-7	3-MA	+	-
	Annexin-V	+	+

□ Control

■ 3-methyladenine

▨ Annexin-V

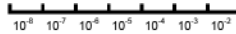
Fig.6

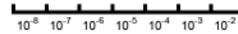
Fig. 7

Category	Gene description	Phagocytosing Macrophage			Phagocytosing MCF-7			Dying MCF-7	
		Control	Autophagic	Anoikic- autophagic	Control	Autophagic	Anoikic- autophagic	Autophagic	Anoikic- autophagic
Phagocytosis receptors/surface molecules	ADORA1			++					-
	ADORA2A		+++	+++					
	ADORA3		--	--					
	ASGR1		+++					+++	+++
	C1QR1			+					
	CALR								
	CD14								
	CD47								-
	CD68			-					+
	FCGR2B								---
	FPRL1								
	IL4R								
	ITGAM			--				-	---
	ITGAV								
	ITGAX			--				+++	+++
	ITGB2			-				--	-
	ITGB3								--
	ITGB5								
	LRP1			-					
	MSR1			--					
	PECAM1			--					
	PTAFR		-					-	-
	PTDSR								
	PTGER2						-	-	---
	PTPNS1					-		-	-
	PTX3		+++						---
	SCARB1								
	TGFBR1								
Bridging molecules	ANXA1			-					-
	ANXA5								
	APOH								
	C1QA								
	C2			--				---	
	C3		-	-				-	-
	C4B			+					
	CRP								
	EDIL3								
	GAS6								-
	ICAM3		-	--				+++	
	MFGE8			+					
	PROS1					+			-
	RAP1A								
	THBS1								--
Engulfment genes	BCAR1								
	CRK								
	DOCK1								-
	ELMO1			--		++			
	ELMO2								
	GULP1		+++						
	RAC1		+++	+++					
	RHOG								
	TRIO								

	Gene								
Signalling	ALOX12							+++	+++
	ALOX5							-	
	AXL						+	---	
	IPLA2(GAMMA)		+						-
	MERTK							--	
	OLR1		++	++		+++		+++	
	PTK2			+					
	TYRO3								-
Effectors	ABCA1							-	---
	CAPN1								
	CAPN2								
	DNASE1			+					
	DNASE2			-					
	TGM2							-	
Nuclear receptors	CXXC1								-
	GRLF1								
	NFKB1								-
	PPARG			---					-
Autophagy	APG12L								
	APG16L								
	APG5L								
	BECN1								
	MAP1LC3A								
IRFs	IRF1		++						
	IRF4		++	+		+++		+++	
	IRF5								
	IRF7								
	IRF8		+	---			-		---
Inflammatory agents	BIRC1		-						---
	CARD15							-	-
	CARD4								
	CASP1								
	CASP5			+++					
	CIAS1								
	NALP12		--	---					
	PYCARD								-
Cytokines	IL10								
	IL12B		+++	+++					
	IL18								
	IL23A		+++	+++					
	IL6		+++	+		+++		+++	+++
	TGFB1								
	TNF		+++	+				-	

Rel.expression level compared to 18S RNA:





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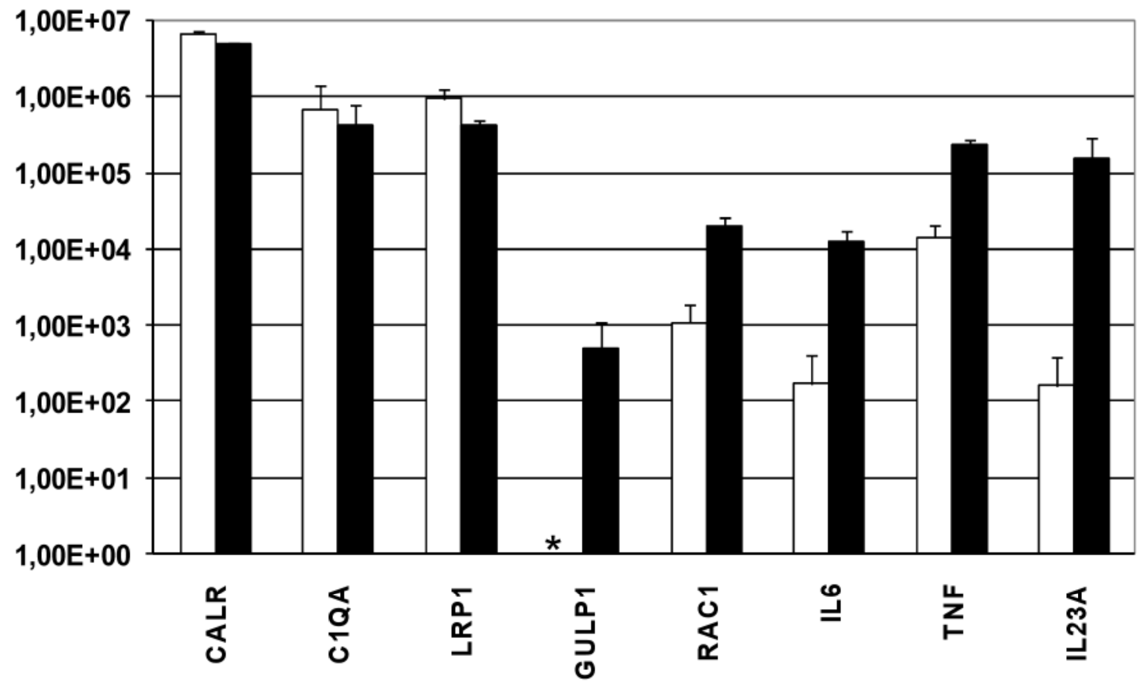
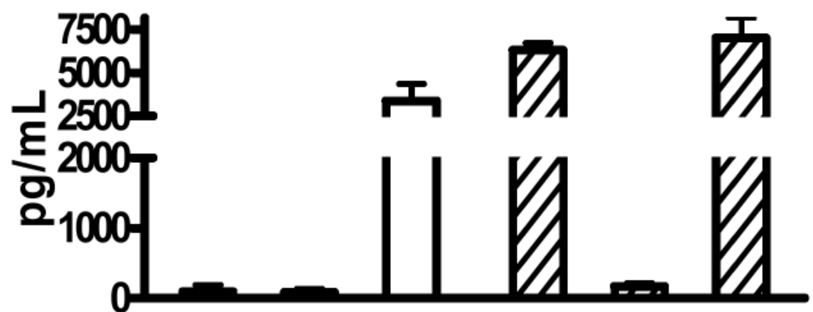
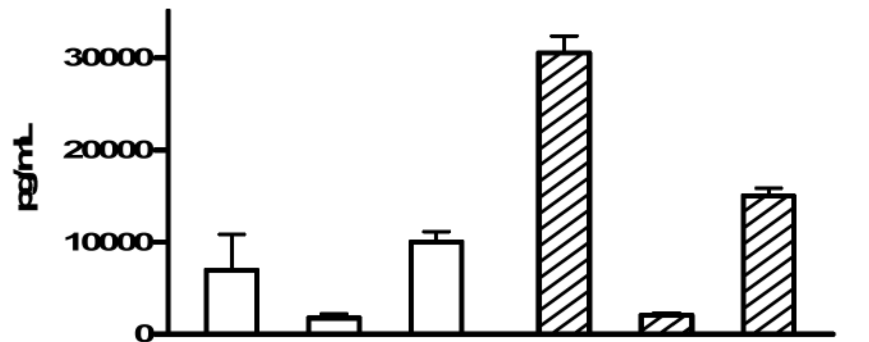


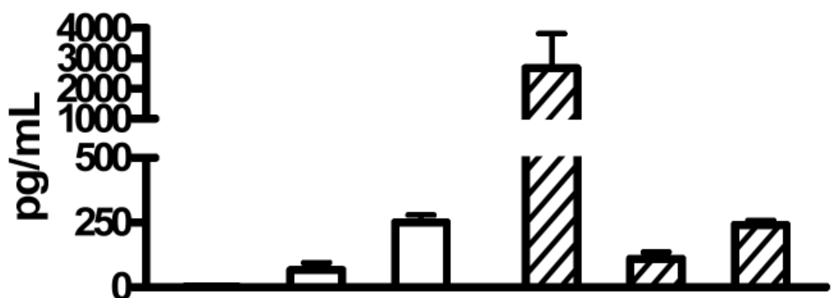
b

	IL-6	TNFα	IL-8	IL-10
	pg/mL			
M	1.4	0.5	692.5	0.4
M + AU	746.2	263.6	28669.4	186.2

Fig.8







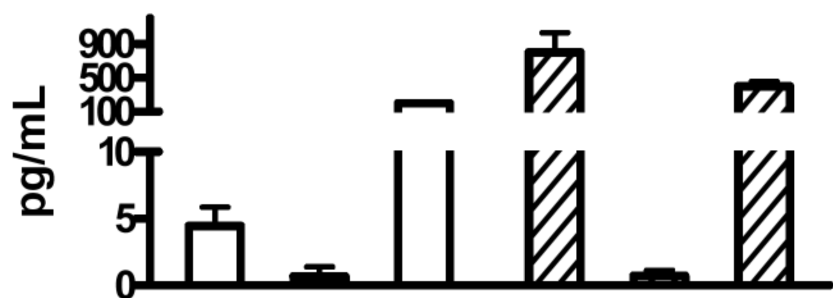


Fig.9

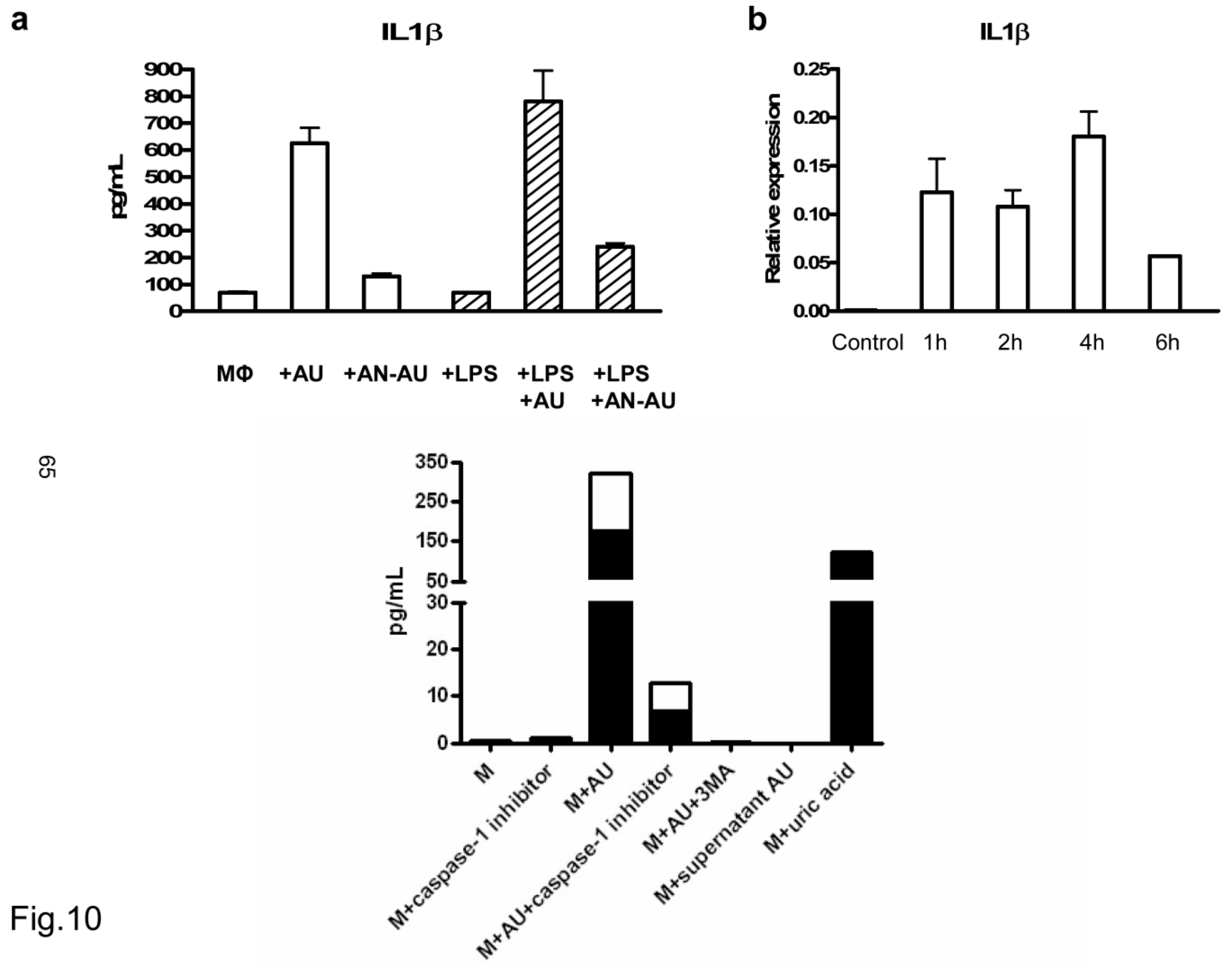


Fig.10

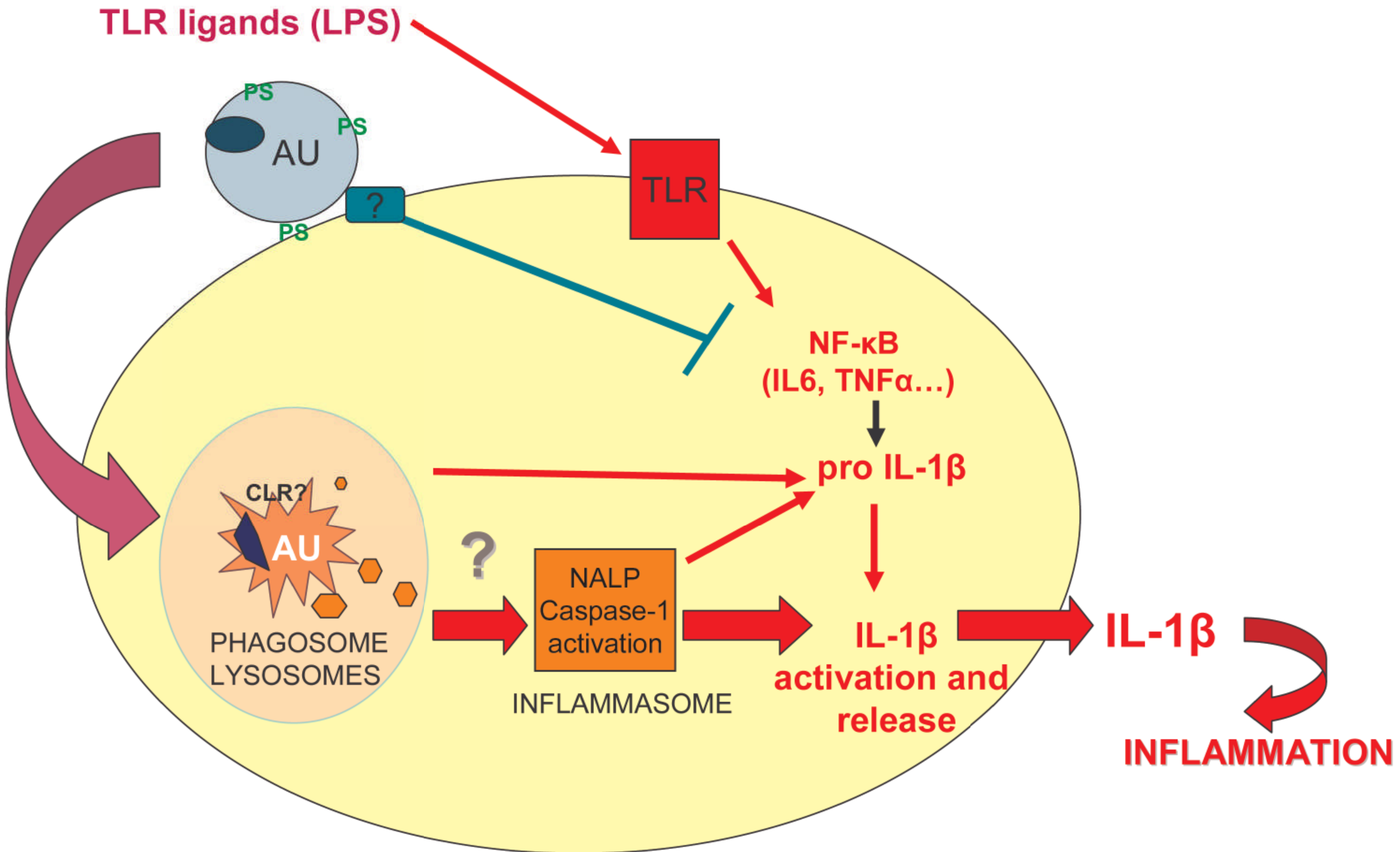


Fig.11

Table 1. Description and list of genes analyzed by TaqMan Real-Time-PCR

Category	Gene description	
Phagocytosis receptors/ surface molecules	ASGR1	asialoglycoprotein receptor 1
	AXL	AXL receptor tyrosine kinase
	MERTK	c-mer proto-oncogene tyrosine kinase
	TYRO3	TYRO3 protein tyrosine kinase
	C1QR1	complement component 1, q subcomponent, receptor 1
	CALR	calreticulin
	CD14	CD14 antigen
	CD68	CD68 antigen
	MSR1	macrophage scavenger receptor 1
	OLR1	oxidised low density lipoprotein (lectin-like) receptor 1
	SCARB1 (CD36)	scavenger receptor class B, member 1
	ITGAM	integrin, alpha M (complement component receptor 3, alpha)
	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
	ITGAX	Homo sapiens integrin, alpha X (antigen CD11C (p150), alpha polypeptide)
	ITGB2	integrin, beta 2 (antigen CD18 (p95)
	ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
	ITGB5	integrin, beta 5
	LRP1	low density lipoprotein-related protein 1 (alpha-2-macroglobulin receptor)
Cell surface molecules	ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1
	ADORA1	adenosine A1 receptor
	ADORA2A	adenosine A2a receptor
	ADORA3	adenosine A3 receptor
	ANXA1	annexin A1
	ANXA5	annexin A5
	CD47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)
	FCGR2B	Fc fragment of IgG, low affinity IIb, receptor for (CD32)
	FPRL1	formyl peptide receptor-like 1
	IL4R	interleukin 4 receptor
	PECAM1	platelet/endothelial cell adhesion molecule (CD31 antigen)
	PTAFR	platelet-activating factor receptor
	PTDSR	phosphatidylserine receptor
	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa
	TGFBR1	transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa)
Bridging molecules	APOH	apolipoprotein H (beta-2-glycoprotein I)
	C1QA	complement component 1, q subcomponent, alpha polypeptide
	C2	complement component 2
	C3	complement component 3
	C4B	complement component 4B
	CRP	C-reactive protein, pentraxin-related
	EDIL3	EGF-like repeats and discoidin I-like domains 3
	GAS6	growth arrest-specific 6
	ICAM3	intercellular adhesion molecule 3
	MFGE8	milk fat globule-EGF factor 8 protein
	PROS1	protein S (alpha)
	PTX3	pentraxin-related gene, rapidly induced by IL-1 beta
	RAP1A	RAP1A, member of RAS oncogene family
	THBS1	thrombospondin 1
Signaling	ALOX12	arachidonate 12-lipoxygenase
	ALOX5	arachidonate 5-lipoxygenase
	IPLA2(GAMMA)	intracellular membrane-associated calcium-independent phospholipase A2 gamma
	PTK2	PTK2 protein tyrosine kinase 2
	PTPNS1	protein tyrosine phosphatase, non-receptor type substrate 1
Engulfment genes	BCAR1	breast cancer anti-estrogen resistance 1
	CRK	CDNA FLJ38130 fis, clone D6OST2000464
	DOCK1	dedicator of cytokinesis 1
	ELMO1	engulfment and cell motility 1 (ced-12 homolog, *C. elegans*)
	ELMO2	engulfment and cell motility 2 (ced-12 homolog, *C. elegans*)
	GULP1	GULP, engulfment adaptor PTB domain containing 1
	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
	RHOG	ras homolog gene family, member G (rho G)
	TRIO	triple functional domain (PTPRF interacting)
Effectors	CAPN1	calpain 1, (mu/I) large subunit
	CAPN2	calpain 2, (m/II) large subunit
	DNASE1	deoxyribonuclease II, lysosomal
	DNASE2	deoxyribonuclease I
	TGM2	tissue transglutaminase-2
Nuclear receptors	CXXC1	CXXC finger 1 (PHD domain)
	GRLF1	glucocorticoid receptor DNA binding factor 1
	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
	PPARG	peroxisome proliferative activated receptor, gamma
Inflammatory agents	BIRC1	Baculoviral IAP repeat-containing protein 1
	CARD15	caspase recruitment domain family, member 15
	CARD4	caspase recruitment domain family, member 4
	CASP1	CARD only protein
	CASP5	caspase 5, apoptosis-related cysteine protease
	CIAS1	cold autoinflammatory syndrome 1
	NALP12	NACHT, leucine rich repeat and PYD containing 12
	PYCARD	apoptosis-associated speck-like protein containing a CARD
Cytokines	IL6	interleukin 6 (interferon, beta 2)
	IL10	interleukin 10
	IL12B	interleukin 12B (natural killer cell stimulatory factor 2, p40)
	IL18	interleukin 18 (interferon-gamma-inducing factor)
	IL23A	interleukin 23, alpha subunit p19
	TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)
	TNF	tumor necrosis factor (TNF superfamily, member 2)

SUPPLEMENT 1.

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Short review

Inflammation and the apopto-phagocytic system

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Abstract

Although under normal conditions many cells die daily mainly by apoptosis in human tissues, inflammation does not occur. The redundant function of a relatively large number of molecules are available to recognize changes occurring on the surface of apoptotic cells, to opsonize the dead cells and to engulf the apoptotic cells previously opsonized or not. Several components of the innate immune system are utilized in this process, mainly soluble factors which bind to the distinct molecular pattern of apoptotic cells. These cells, unlike necrotic ones, do not induce the expression of inflammatory cytokines in phagocytic cells, they can even inhibit such a response and engage an active signaling process to elicit a direct anti-inflammatory effect. The molecular details of these signaling processes have not been clarified yet. Both professional and "amateur" cells can engulf apoptotic cells and mediate an anti-inflammatory action. Disturbance of these processes have significant roles in development of autoimmune diseases and highly malignant tumors.
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Keywords: Apoptosis; Macrophages; Non-professional phagocytes; Innate immunity; Pro-inflammatory and anti-inflammatory cytokines; Signaling; Autoimmunity; Tumors

1. Introduction

In the human body close to 500 billion cells die each day – mainly by apoptosis – and the huge number of dead cells are either lost directly to the environment from body surfaces or continuously removed by a remarkably efficient apopto-phagocytic system while inflammation and scar formation do not occur [1,2]. The surface with complex molecular patterns and dynamic interactions between the dying and engulfing cells (macrophages, immature dendritic cells (DCs) and non-professional phagocytes) is often called the third synapse by analogy to the synapses in the nervous and immune (antigen presenting) system. The "anatomical" description of this synapse is probably almost complete (see the schematic description on Fig. 1) and it includes the large number of receptors and opsonins which bind to cellular ligands exposed during the various stages of apoptotic cell death [3]. However, in spite of the remarkable progression in the field, we are still far from understanding the complex biochemical and regulatory processes which take place when dying cells are cleared from tissues.

One of the most intriguing aspects of the apopto-phagocytic system is the active and dynamic interrelationship between the dying and the engulfing cells. The apoptotic cells can induce migration of phagocytes by releasing lipid-derived attraction signals [4]. The engulfing cells are capable of influencing the fate of their neighbors by promoting either death or survival [5–11]. This cooperative relationship between apoptotic cells and their phagocytes is even more significant in determining whether inflammation occurs or it does not in tissues with high apoptotic rate under normal or pathologic conditions. In this paper the encounter between the apopto-phagocytic and inflammatory systems will be reviewed with particular emphasis on three closely related issues, namely (i) what elements of innate immunity are involved in the clearance of apoptotic cells; (ii) how apoptotic cells can suppress the inflammatory response; and (iii) how inflammation is prevented when the dead cells are cleared by non-professional phagocytes, for example by parenchymal or epithelial cells of various tissues.

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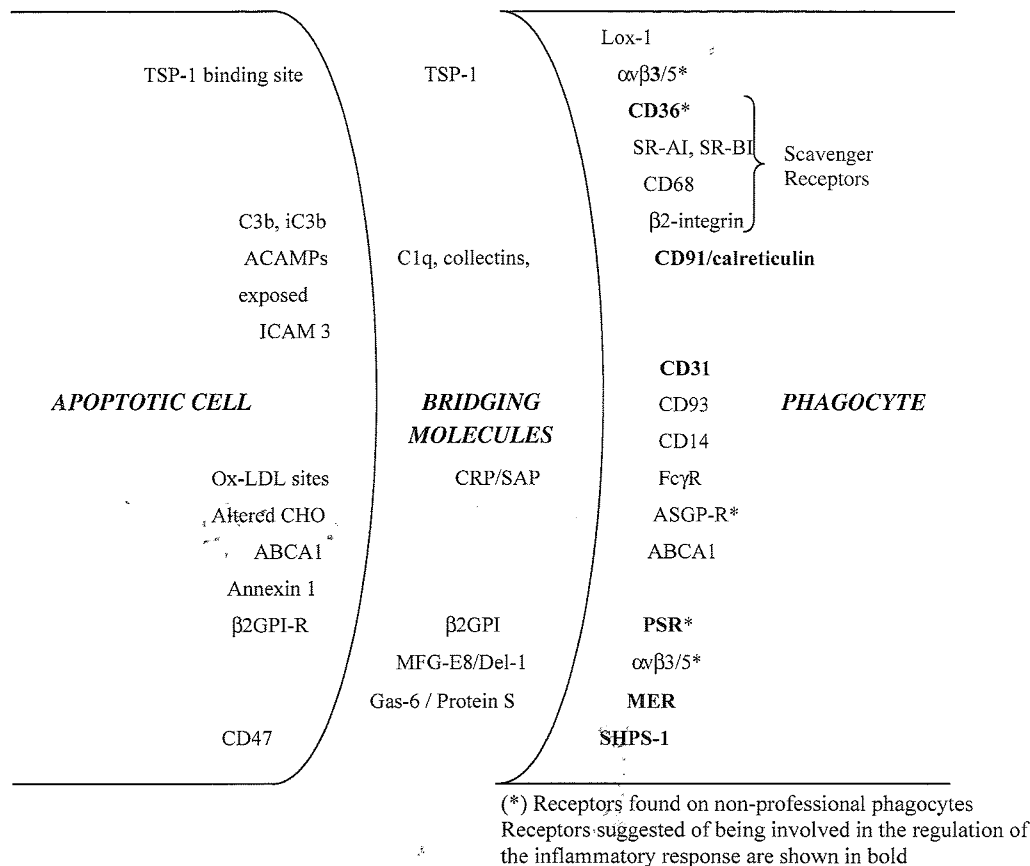

Fig. 1. Molecules involved in the apopto-phagocytic synapse. αvβ3/5 Vitronectin receptor integrins; ABCA-1, ATP-binding cassette transporter A1; ACAMPs, apoptotic cell-associated molecular patterns; ASGP-R, asyaloglycoprotein receptor; β2GPI, β2 glycoprotein I; β2GPI-R, β2GPI-receptor; β2-integrins (CR3, CR4); C1q, first component of complement; CHO, carbohydrate; CRP, C-reactive protein; Del-1, developmental endothelial locus-1; Gas-6, growth arrest specific gene-6; iC3b, inactivated complement fragment C3b; ICAM-3 (CD50), intercellular adhesion moleculae-3; Lox-1, oxidized low density lipoprotein receptor 1; MER, myeloid epithelial reproductive tyrosine kinase; MFG-E8, milk fat globule epidermal growth factor-8; Ox-PL, oxidized phospholipids; PS, phosphatidylserine; PSR, putative PS receptor; SHPS-1, Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1; SR-AI, scavenger receptor AI; SR-BI, scavenger receptor BI; TSP-1, thrombospondin-1.

2. Common elements between the apopto-phagocytic system and the innate immunity

None of the so far revealed elements of the apopto-phagocytic system seems to be specifically dedicated to the clearance of apoptotic cells. Changes on the surface of dying cells involve elimination, modification or translocation of molecules which have well established roles in living cells. Cell surface receptors and the intermediate molecules participating in the phagocytic events are designed for other biological processes as well, such as functional organization of the extracellular matrix, wound healing, tissue repair, lipid metabolism or others. Among these shared processes the utilization of the innate immune system is particularly relevant to our understanding of the intimate relationship between the apoptotic-cell clearance and the inflammatory response.

Invading pathogens are primarily recognized by a large number of pattern-recognition molecules including soluble factors (complement proteins, members of the collectin and pentraxin family), calreticulin, C-type lectins, CD14 and Toll-like receptors (TLRs). Many components of this innate immunity are also involved in the clearance of apoptotic cells (see Fig. 1) as they present conserved molecular patterns—apoptotic cells associated molecular pattern (ACAMP), similarly to microbes which are decorated by pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides (LPS), to innate immune molecules [2]. It is likely that molecular groupings forming repetitive structures and topological associations in Annexin-I-, phosphatidylserine (PS)-, and CD43-rich distinct domains on cells undergoing apoptosis are among the critical determinants of ACAMP. The multiplicity of ACAMP structures provides redundancy making the recognition of dead cells a failsafe mechanism but it may also reflect early and late stages of the apoptotic cell surface which participate in distinct chronological events, such as recognition, binding ("tethering"), signaling ("tickling") and engulfment during the clearance process.

Molecules of the innate immune response are particularly active in opsonization of apoptotic cells; actually, it has been proposed that C1q and other opsonins prevent autoimmunity and maintain self-tolerance by supporting the efficient clearance of apoptotic material, as well as by actively modulating phagocyte function [12]. However, it should be noted that while some PAMP receptors do participate in clearance of apoptotic cells (e.g. CD14), most of them do not and the response of

macrophages to ACAMPs is usually independent of PAMP receptors. Furthermore, the response of macrophages to PAMPs is pro-inflammatory but to ACAMP it is anti-inflammatory. The reason of this crucial difference is related to signaling through TLRs: while engagement of PAMPs by TLRs leads to a full-blown inflammatory response, there is no TLR response to ACAMP, that is the various TLRs do not recognize ACAMPs. On the other hand, necrotic cells have been shown to engage TLR2 and induce pro-inflammatory and tissue repair genes [13].

3. Suppression of inflammation by apoptotic cells

3.1. Apoptotic cells, unlike necrotic cells, do not induce inflammatory response

Clearance of apoptotic cells has an important role in tissue remodeling and resolution of inflammation protecting tissue from exposure to the inflammatory and immunogenic contents of dying cells. In contrast to apoptosis, necrosis is characterized by loss of membrane integrity, swelling and disintegration leading to the release of cellular contents. Macrophages discriminate innately between cells that have undergone a physiological death and those that have suffered a pathological form of death. Recognition of these two classes of dying cells occurs via distinct and non-competitive mechanisms [14]. The lysed necrotic cells or apoptotic cells undergoing secondary necrosis can initiate an inflammatory response in macrophages since they can release proteases, inflammatory eicosanoids, granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein 2 (MIP-2), IL-8, tumor necrosis factor (TNF)-α and they can engage TLR2 (see above). The DNA-binding protein HMGB-1, which elicits a pro-inflammatory response in macrophages through TLR2 and TLR4, can also leak out of necrotic but not of apoptotic cells [15,16]. Therefore, leakage of macromolecules should be prevented during the apoptotic process to avoid inflammation; one mechanism for this is the induction and activation of the protein cross-linking enzyme, transglutaminase (TG) 2 in apoptotic cells [17]. The major mechanism to prevent secondary necrosis is the recognition and fast removal of early phase apoptotic cells [2]. However, even cells undergoing secondary necrosis may not necessarily be pro-inflammatory either because of biochemical mechanism sequestering pro-inflammatory molecules inside dying cells or as the result of the anti-inflammatory effects of the coexisting apoptotic cells. Indeed, there is ample evidence to show that apoptotic cells do not only fail to induce but can actively suppress the release of pro-inflammatory cytokines from macrophages which engulf them.

3.2. How apoptotic cells suppress inflammation?

Early studies demonstrated that apoptotic cells actively suppress an inflammatory response, and not just fail to provide inflammatory signals. The presence of thymocyte-derived apoptotic cells during monocyte activation increased their secretion of the anti-inflammatory and immunoregulatory cytokine IL-10 and decreased production of the pro-inflammatory cytokines such as TNF-α, IL-1 and IL-12. [18] Later, these results were reproduced using apoptotic neutrophils instead of apoptotic lymphocytes [19]. Fadok et al. had shown that phagocytosis of apoptotic neutrophils by human macrophages inhibited the production of IL-1β, IL-8, IL-10, GM-CSF, TNF-α, as well as leukotriene C4 and tromboxane B2 trough an autocrine/paracrine mechanism and increased the production of TGF-β1, prostaglandin E2 and platelet activating factor [20]. Moreover, TGF-β neutralizing antibodies largely reversed the inhibitory effect of apoptotic cell uptake and exogenous TGF-β1 down-regulated the synthesis of the chemokines IL-10 and TNF-α [21]. There are convincing in vivo data that TGF-β1 released by macrophages has an anti-inflammatory effect in inflamed peritoneum and lung [22]. Using the LPS lung inflammation model it was demonstrated that surfactant protein A has a role in induction of alveolar macrophage TGF-β1 release thereby promoting the resolution of acute inflammation within alveolar space [23]. All these data led to the conclusion that suppression of inflammatory response by apoptotic cells in macrophages is mediated mainly by an indirect mechanism through the release of TGF-β1 [24]. In contrast to this it was found by Cvetanovic and Ucker that the initiation of anti-inflammatory modulation occurs as a direct consequence of the interaction of apoptotic target cells with macrophages and without involvement of TGFβ [14]. Nevertheless, the high importance of TGFβ in the clearance of apoptotic cells has been strengthened by the observation that lack of TG2, the cross-linking enzyme which is involved in the biochemical maturation of TGFβ, compromises phagocytosis of apoptotic cells and the anti-inflammatory response to the dying cells [9,25].

The signaling mechanisms that determine the anti-inflammatory mediator release are far from being completely understood. The process appears to be related to the appearance of phosphatidylserine on the surface of apoptotic cells and recognition of PS by macrophages through a PS receptor was suggested to be the dominant element in the release of TGF-β [26]. While a PS receptor may exist, the one described by Fadok et al. has turned out not to be a real PS receptor mediating uptake of apoptotic cells [26,27]. Knock out mice with deletion of this putative PS receptor and defects in phagocytosis of apoptotic cells have been shown to be double knock out animals and when only this putative PS receptor was deleted, the phagocytosis defect disappeared. Interestingly, the double knockout mice were impaired in pro- and anti-inflammatory signaling after stimulation with apoptotic cells [28]. Antibody ligation experiments indicate that CD36 and vitronectin receptor, and bridging protein TSP1 can suppress monocyte/macrophage inflammatory responses by stimulating TGF-β release [18,29]. The endogenous ligand, Annexin-I, inducible by glucocorticoids as well, seems to be capable of inducing IL-10 production and IL-12 synthesis inhibition [30]. A direct suppressive signaling could arise through the tyrosine inhibitory domain of CD31 and kinase domain of MER via Gas-6 [3]. Another signaling pathway which does not require soluble factors and can suppress the pro-inflammatory response is the ligation of Src homology 2 domain-bearing protein tyrosine phosphatase substrate-1 (SHPS-1) [31].

It is important to realize that a maturation process must take place before macrophages can recognize and remove dead cells and display a concomitant anti-inflammatory response [32,33]. This suggests that gene expression regulation is involved in preparing macrophages to these tasks. Glucocorticoids are the most effective anti-inflammatory and immunosuppressive agents. They have been shown to inhibit in macrophages the expression of cytokines, adhesion molecules, and enzymes involved in the inflammatory process. Glucocorticoids act by binding to the glucocorticoid receptor, that upon activation, translocates to the nucleus and either transactivates or transrepresses gene expression. Inhibition of pro-inflammatory transcription factors such as adhesion protein (AP-1), nuclear factor of activated T cells (NFAT) and nuclear factor kappa B (NF-κB) is thought of being a major action of glucocorticoids [34]. Although treatment of maturing macrophages with glucoroticoids results in a large increase in the efficiency of macrophages to engulf dying neutrophils [33], there is no evidence that the anti-inflammatory effects of apoptotic cells and glucocorticoids are interrelated. Our recent results have clearly demonstrated the involvement of PPARγ in the regulation of both the engulfment and the anti-inflammatory response (Majai et al., manuscript in preparation).

Not only the phagocyting cells can release anti-inflammatory cytokines, but also the cells that are dying by apoptosis can release cytokines with direct immunosuppressive properties. It was shown that apoptotic T cells release TGF-β1 which is not only latent but also bioactive and is localized within the intracellular membrane bound compartments including mitochondria [35]. Administration to the eye of antigen-bearing lymphocytes showed that the observed tolerance depends on the ability of dying cells to secrete IL-10 [36].

In contrast to previous studies which suggested an anti-inflammatory effect of apoptotic cells, Iyoda and Kobayashi showed that injection of apoptotic cells into the peritoneal cavity induced the expression of an inflammatory chemokine, MIP-2, and infiltration of neutrophils; anti-MIP-2 antibodies suppressed this infiltration significantly [37]. They also showed that macrophage mediated uptake and digestion of apoptotic thymocytes was accelerated upon coculturing them with neutrophils and the latter are recruited for acceleration of apoptotic-cell clearance in tissues with high apoptosis rate [38].

3.3. Loss of the anti-inflammatory response to apoptotic cells leads to diseases

Apoptotic cells also can suppress the inflammatory response elicited by PAMPs through the TLRs. The combination of apoptotic cells and ligands for TLR2, 4, and 9 mount cytokine responses that differ importantly from those elicited by either class of stimulus alone. TLR ligands induced early secretion of TNF-α, MIP-1α, and MIP-2 with later secretion of IL-10, IL-12, TGF-β1; apoptotic cells alone stimulated TGF-β1 secretion only. The combination of apoptotic cells and TLR ligands enhanced early secretion of TNF-α, MIP-1α, and MIP-2 and increased late TGF-β1 secretion, while suppressing late TNF-α, IL-10, IL-12 by a mechanism which could nevertheless be overridden by IFNγ [39]. These results point to the possibility that inflammatory diseases initiated by microbial pathogens are influenced, very likely attenuated, by apoptosis occurring in the infected tissues and lack of this influence may contribute to a serious outcome in the pathologic response. In some pathologic conditions like chronic granulomatous disease (CGD) phagocytes are severely compromised in their ability to produce anti-inflammatory mediators such as PGD2 and TGF-β1 during clearance of apoptotic debris and invading pathogens, contributing to persistence of inflammation in CGD [40].

The clearance of apoptotic cells has a role not only in tissue homeostasis, but provides also a source of antigens for immune tolerance and activation. Macrophages' anti-inflammatory and immunosuppressive tendencies in responding to apoptotic cells make them a potentially powerful regulator of adaptive immune responses, including autoimmune and anti-tumor responses. It is known that immature DCs are capable of engulfing apoptotic cells by a mechanism involving CD36, PS receptor and integrins. This results in down-regulation of IL-12 as well as some markers of DC activation, such as CD86, and release of TGF-β1. However, if antibodies that can bind and opsonize apoptotic cells are present, ligation of Fc receptors will result in DC maturation and production of immunostimulatory cytokines. Also, passive release of HMGB-1, box1, uric acid, and heat shock proteins from necrotic cells have a potential role in stimulating inflammation through the NF-κB mediated pathway. Dendritic cells are unique among phagocytes in being capable of presenting antigenic peptides derived from dying cells on MHC I and MHC II molecules for recognition by $CD8^+$ T cells. In the absence of $CD4^+$ T cell help, dendritic cells that cross-present antigens to $CD8^+$ T cells result in tolerance by a deletion mechanism. By contrast, the ability to activate $CD8^+$ T cells depends on the presence of antigen specific $CD4^+$ T cell help. Once activated, these $CD8^+$ T cells return to the site of inflammation and can destroy target cells [41]. This process can be beneficial in the development of tumor immunity, but severely pathogenic when it targets self. Defective clearance of apoptotic cells is often associated with autoimmune diseases: mice with deleted C1q, MER tyrosine kinase, TG2 or MFG-E8 have high titres of autoantibodies and develop autoimmune syndromes [42–45]. In TG2 knock out mice even neutrophils show up in tissues where a high rate of apoptosis has been initiated [9]. On the other hand, lack of CD14 does not lead to autoimmune disease despite of extensive persistence of apoptotic cells in tissues. It has been suggested that defective apoptotic-cell clearance plays a primary role in autoimmune disease pathogenesis only under circumstances when the clearance deficiency is accompanied by a defect in the regulation of anti-inflammatory response by apoptotic cells as it seems to be the case in C1q, MER tyrosine kinase, TG2 or MFG-E8 knock out mice [2].

4. Clearance of apoptotic cells by non-professional phagocytes

In the early phase of investigations macrophages have been considered of being the main actors capable of engulfing apoptotic cells. However, even in the early papers there are mor-

phological evidences of non-professional phagocytes "eating" adjacent cells dying by apoptosis. In recent years more and more evidences have been collected to support the notion that perhaps the reason why it is very difficult to see apoptotic cells in tissue sections is the efficient and fast removal of the daily formed apoptotic cells by their adjacent neighbors even before they show morphological features of apoptosis. According to this view, macrophages are important in the clearance of dying cells in the circulation and in places where the apoptotic rate is so high that the non-macrophage system cannot cope with the highload of corpses [2]. Mice without macrophages develop normally and are capable of removing dying corpses by the less efficient mesenchymal cells during embryogenesis without inducing an inflammatory response [46]. In spite of the high ongoing rate of apoptosis in several of their organs (just like in normal mice), there is no sign of excess apoptotic cells in tissues. These mice although born alive, rapidly succumb to bacterial infections in the absence of macrophages and neutrophils unless they receive daily antibiotic treatment.

Removal of dying corpses by neighboring viable cells not born to be phagocytes is becoming a well recognized process during tissue remodeling. Many cell types of different dermal origin have been "convicted" of being "guilty" for engulfing apoptotic [24,47,48], necrotic [49] cells as well as those undergoing anoikis or autophagy (our own unpublished findings). The extensive list of non-professional phagocytes includes, but is not limited to fibroblasts, kidney mesangial cells, testis Sertoli cells, ovarian thecal cells, smooth muscle cells, endothelial and epithelial cells, hepatocytes and mesenchymal cells (for citations see [2]). Some widely used cell lines such as 3T3, HeLa, Jurkat, COS7 (see Fig. 2), MCF-7 and HepG2 cells (from our own experiments) have been found to engulf apoptotic cells in culture systems. It is very likely that all cell types can do this to a certain extent if and when the need for "cannibalism" arises.

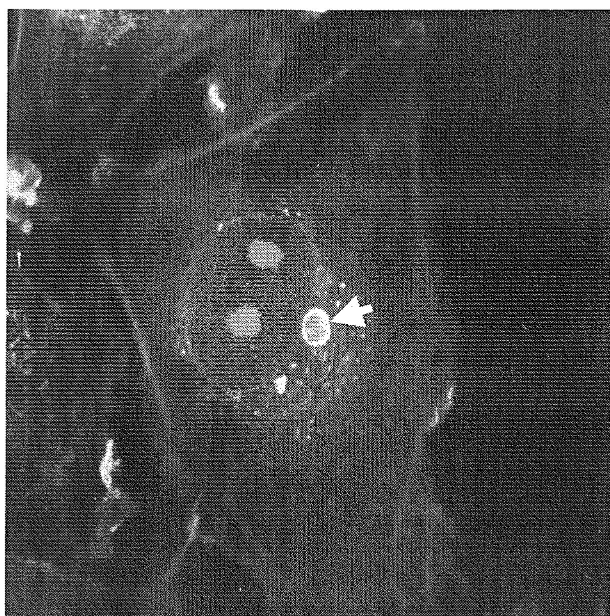

Fig. 2. Attached COS7 cell stained with phalloidin-FITC/Propidium iodide that engulfed remnant of anoikic COS7 cell stained with propidium iodide (24 h coincubation time).

The mechanism of non-professional recognition, signaling and engulfment of apoptotic and necrotic cells is being increasingly studied and at the present not well defined. Naturally, basic results obtained by studying engulfment in the nematode *Caenorhabditis elegans*, which do not have macrophages, can be all considered as part of typical non-macrophage mechanisms. Genetic studies of the removal of apoptotic cell corpses by *C. elegans* have found on the phagocytic cell *ced*-1 encoding a transmembrane receptor similar to the mammalian scavenger receptor of endothelial cells (SREC) involved in tethering and initiation of signaling for uptake of the target cell [50,51]. *Ced*-7 encodes a protein similar to the mammalian ABCA-1 transporter on both the apoptotic and phagocytic cell and is also required for phagocytosis [52]. The description of signaling molecules involved in the engulfment process have also been built upon studies in *C. elegans* (*ced*-2, 5, 7, 10, 12) and by analogy the corresponding mammalian molecules have been found, respectively (CrkII, DOCK180, ABC-1, Rac-1, ELMO) [53–55]. All the latter molecules function in cytoskeletal reorganization, ruffling, membrane extension and fusion of the membrane during the engulfment process irrespective whether they function in macrophages or non-professional phagocytes.

The changes on the surface of apoptotic cells that "flag" recognition and removal are unanimous for both professional and non-professional macrophages. Many groups have studied these including but not limited to the exposure of specific carbohydrates binding to phagocyte lectins [56], thrombospondin [57], collectins [58] or complement products (iC3b) [59] all playing roles in formation of bridges between apoptotic cells and the phagocyte. Loss of phospholipid asymmetry and surface exposure of PS on apoptotic cells is required for their phagocytosis performed by fibroblasts [60]. Gas-6 is one of the soluble proteins able to bind PS on the target cell bridging it to MER, member of the receptor tyrosine kinase family, on the phagocyte [61]. Different non-professional phagocytic cell lines express varying receptors for target interaction: fibroblasts have $\alpha_V\beta_3$, $\alpha_V\beta_5$ and CD36; kidney mesangial cells show $\alpha_V\beta_3$; testis Sertoli and ovarian thecal cells exhibit SR-B1 (scavenger receptor); endothelial cells display lectins; smooth muscle cells and epithelial cells expose PS receptors [2]. Mouse mammary epithelial cells eat apoptotic cells using the same receptors utilized by professional phagocytes as well, including the putative PS receptor, CD36, the vitronectin receptor $\alpha_V\beta_3$ and CD91 [62]. As more and more data are collected, it is becoming increasingly obvious that there are no unique molecular tools available for the non-macrophage system to ingest apoptotic cells; they just take advantage of the same molecular repertoire which is used by the professionals. However, while we know that maturation of monocytes to macrophages is a prerequisite for their recognition of apoptotic cells, it is not clear what regulatory mechanisms make fibroblasts, epithelial and other cells capable of doing the same. Apparent differences also have been observed in the engulfment of apoptotic cells by professional and non-professional phagocytes [63]: the lat-

ter are slower, recognize late stage apoptotic cells more, digest the corpses slower [46]. This may reflect a need for "educating" these cells perhaps by apoptotic cells for the unusual task of phagocytosis.

It is now clear that non-macrophage cellular systems, such as epithelial cells can regulate the function of other cells locally through paracrine actions coupled to the production of cytokines like IL-1 [64,65]. The "amateur" phagocytic cells are important source of cytokines including their pro-inflammatory response to endotoxins. For example, production of the pro-inflammatory cytokines IL-1β, IL-8, TNF-α and MIP-2 has been shown in mammary epithelial cells stimulated by LPS [62]. Therefore, it is important to clarify how apoptotic cells regulate the inflammatory response of non-professional phagocytes while they recognize and engulf these apoptotic cells. The few data available so far show that apoptotic cells, unlike necrotic cells, do not provide pro-inflammatory signals for these cells and can down-regulate the expression of pro-inflammatory cytokines induced by LPS ([62], and personal communication of David Ucker). Furthermore, apoptotic cells can elicit an anti-inflammatory response inducing the secretion of TGFβ in these cells. This means that the non-macrophage cells also posses or can acquire the recognition and signaling mechanisms which are needed to regulate their pro- and anti-inflammatory responses upon meeting apoptotic cells.

Non-professional and professional phagocytes exposed to apoptotic cells are reprogrammed for secretion of growth and survival factors like VEGF and promote the growth of surrounding endothelial and epithelial cells [66]. This is particularly important in the complex processes of wound healing which require the collaborative efforts of several cell lineages, their proliferation, migration, matrix building and contraction capabilities. The wound healing response also involves a regulated inflammatory response. The presence of apoptotic cells and their phagocytosis with the concomitant release of growth and survival factors are crucial elements of the proper healing process. Importantly, mice without macrophages are also to repair wounds with time course similar to wild-type siblings and without raising a pathologic inflammatory response; the dead cells are engulfed by stand-in phagocytic fibroblasts which seem capable of controlling the inflammatory events in wounds [67]. Lack of adequate regulation of the apopto-phagocytic system in some malignancies may lead to unwanted inflammation which promotes tumor growth and this may provide an explanation why chemotherapy-induced tumor cell death sometimes inversely ends up in tumor progression instead of regression [68,69]. In the sequential events of carcinogenesis loss of pro-apoptotic genes (which is very likely reflected in disturbed phagocytosis of dead cells) may be followed by loss of autophagy-based cell death shifting the tumor tissue toward the frequent appearance of necrotic type of cell death and the inflamed tumors often have been considered the best growing tumors. Based on these data restoration or activation of apoptosis and proper phagocytosis of dead cells in tumors may be beneficial in anti-cancer therapy as it would pull the leverage away from necrosis and inflammation towards the anti-inflammatory dominant clearance of apoptotic tumor cells [70].

5. Concluding remarks

For a long time elimination of the high number of apoptotic cells in the body has been considered a passive phenomenon with the sole purpose of getting rid of the cell corpses as fast as possible. It is clear now that naturally dying cells have important duties while passing away—beyond the obvious utilization of their biochemical components for the benefit of the whole organism. They are recognized and engulfed by both professional and non-professional phagocytes and this recognition is not only assisted by molecular elements of the innate immune system but the apoptotic cells influence functions of innate immunity modulating its inflammatory response. In addition, the apoptotic cells have a unique feature: they can induce a direct anti-inflammatory response through a so far unrevealed signaling system which may be considered as an unconventional and ubiquitous innate immunity distinguishing life from effete. It looks that the continuous appearance of apoptotic cells in tissues (cell turnover) dampens inflammatory cytokines and their frequent occurrence in inflamed tissues helps to dissolve the acute inflammatory response to prevent long term tissue damage. Apoptotic cells provide a rich source of antigens for dendritic cells and this way for the adaptive immune response and when these antigens are presented self tolerance develops with the concomitant help of the anti-inflammatory response induced by the dying cells. The balance in the number of apoptotic and necrotic cells is crucial for the development of a responding adaptive immunity including the naturally developing immune response to pathogens and the pathologic conditions in autoimmunity and tumor development. While the significance and major characteristics of the apopto-phagocytic system have been outlined there are many unanswered questions. What is the exact nature of the molecular pattern recognized by phagocytic cells before they respond both by engulfment and regulatory signals for the pro- and anti-inflammatory cytokine genes? Are soluble molecules involved in the regulatory process? Which gene regulatory systems are involved in changing the cytokine pattern secreted by the phagocytic cells? How non-professional phagocytes acquire their capacity to engulf apoptotic cells and to regulate inflammatory process? What is the relative importance of macrophages and non-macrophage phagocytes in the elimination of cell corpses and to keep the inflammatory system quiet? How the adaptive immune system can utilize best the continuous interference of apoptotic cells with pathogens or tumor antigens? The large interface between living and dying cells still have many secrets to reveal and new information to be obtained for better understanding and treatment of major diseases.

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SUPPLEMENT 2.

Petrovski, G., G.Zahuczky, K.Katona, G.Vereb, W.Martinet, Z.Nemes, W.Bursch, and L.Fésüs. 2007. Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes. *Cell Death. Differ.* 14:1117-1128.

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Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes

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MCF-7 cells undergo autophagic death upon tamoxifen treatment. Plated on non-adhesive substratum these cells died by anoikis while inducing autophagy as revealed by monodansylcadaverine staining, elevated light-chain-3 expression and electron microscopy. Both *de novo* and anoikis-derived autophagic dying cells were engulfed by human macrophages and MCF-7 cells. Inhibition of autophagy by 3-methyladenine abolished engulfment of cells dying through *de novo* autophagy, but not those dying through anoikis. Blocking exposure of phosphatidylserine (PS) on both dying cell types inhibited phagocytosis by MCF-7 but not by macrophages. Gene expression profiling showed that though both types of phagocytes expressed full repertoire of the PS recognition and signaling pathway, macrophages could evolve during engulfment of *de novo* autophagic cells the potential of calreticulin-mediated processes as well. Our data suggest that cells dying through autophagy and those committing anoikis with autophagy may engage in overlapping but distinct sets of clearance mechanisms in professional and non-professional phagocytes.

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In mammalian organisms various forms of cell death may occur. Apoptosis or type I 'programmed cell death' (PCD), upon activation, proceeds via 'extrinsic' (death receptor activation) and 'intrinsic' (mitochondrial) pathways, both converging at the level of caspase-3.[1] Anoikis is induced in cells after their detachment from the extracellular matrix (ECM) switching on apoptotic signaling pathways.[2] Necrosis, also known as unorganized way of dying or bioenergetic catastrophe, has been recently shown to be 'programmed' under certain conditions.[3] Type II PCD or autophagic cell death has been observed during embryonic development.[4,5]

Since the first description of autophagy in 1966,[6] numerous studies have described it as a survival mechanism under poor nutritional conditions. It is now clear this process has a dual role.[7] By contrast, autophagy is a degradative mechanism for long-lived proteins and damaged organelles through the auto–phago–lysosomal pathway and on the other, it provides possibility of self-destruction for cells.[4,5,8] In *Drosophila*, autophagy can be hormonally controlled by ecdysone; through inhibition of a class-I phosphatidyl-inositol 3-kinase (PI3K) pathway organs, such as the fat body, are eliminated at the end of larval stage.[8,9] MCF-7 cells can also be induced to undergo autophagy and cell death by treatment with the anti-estrogen tamoxifen, which increases the intracellular ceramide level and eliminates the inhibitory effect of class-I PI3K pathway.[10] Expression of death-associated protein kinase and its death-related protein kinase-1 in MCF-7 and various other cell lines can induce autophagy and caspase-independent cell death.[11] When caspase-8 was inhibited and the *atg7* and *beclin-1* expression knocked down by RNA interference, death through autophagy was blocked in mouse L929 cells.[12] In embryonic fibroblasts with homozygous deletion of both *bax* and *bak,* RNA interference against *atg5* and *beclin* could prevent autophagic cell death.[13]

The fate of dead cells depends on their localization within the body. Macrophages acting as professional phagocytes are capable of engulfing apoptotic cells undergoing type I PCD without inducing inflammatory response.[14,15] Typical examples of this clearance are found during embryonic development when a large number of apoptotic cells are removed[16] and during clearance of apoptotic granulocytes in inflammation.[17] Many cells of different origin, including epithelial cells, can act as non-professional phagocytes and engulf apoptotic cells.[18,19,20,21] The list of key molecular players involved in these phagocytic interactions, often referred to as the 'third synapse', is surprisingly extensive suggesting there are several redundant molecular mechanisms for efficient and fast removal of dying and dead cells.[22]

To our present knowledge the final fate and clearance mechanism of cells dying either through autophagy or by other

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Abbreviations: AV, autophagic vacuoles; CFDA-SE, carboxyfluoresceindiacetatesuccinimidyl ester; CMTMR, 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine; DAPK, death-associated protein kinase; DRP-1, death-related protein kinase-1; FITC, fluorescein isothiocyanate; LC3, light-chain-3; MCSF, macrophage colony stimulating factor; MDC, monodansylcadaverin; PCD, programmed cell death; PI3K, phosphatidyl-inositol 3-kinase; poly-HEMA, poly(2-hydroxyethylmethacrylate); PS, phosphatidylserine; PI, propidium iodide; TAM, tamoxifen ([α-(4-β-N-dimethylaminoethoxy)-phenyl-α'-ethyl-trans-stilbene]); TEM, transmission electron microscopy; TLDA, TaqMan low-density array; TNF, tumor necrosis factor

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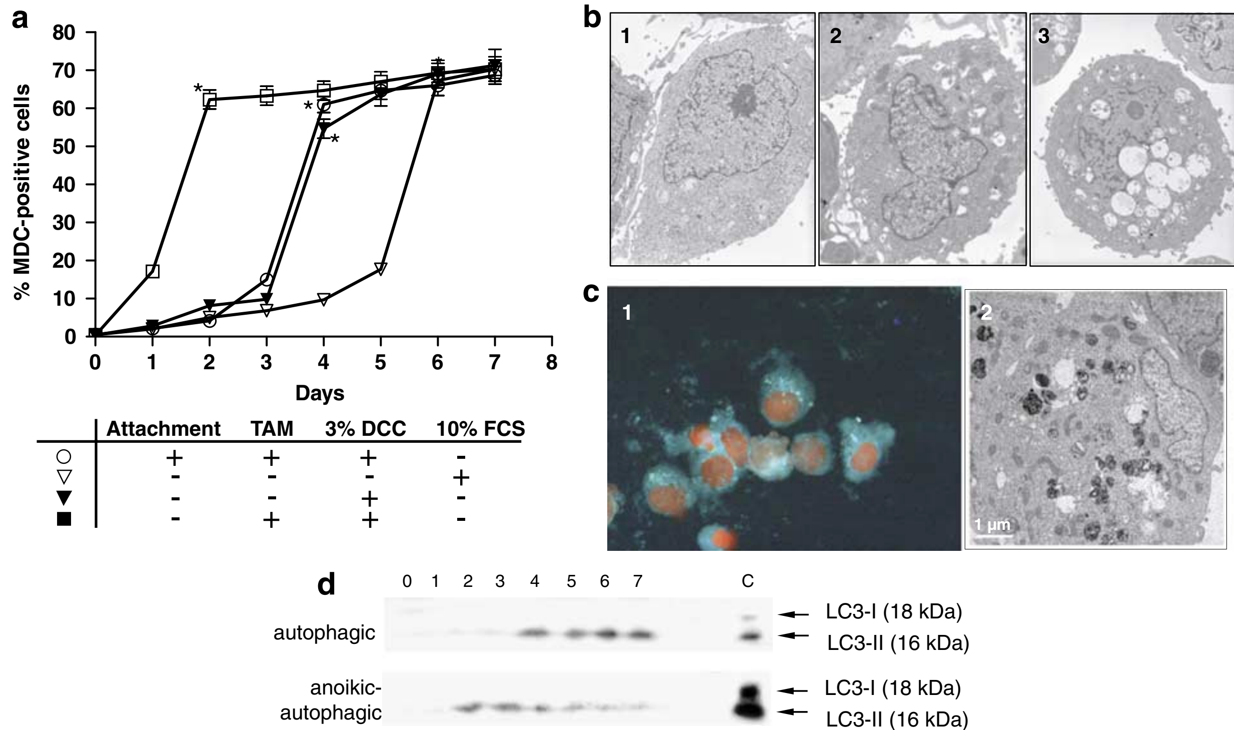


Figure 1 Autophagy in MCF-7 cells following various treatments. (**a**) Percentage of MCF-7 cells with MDC-positive AVs; (○) underwent heavy autophagy by day 4; (▽), (▼) and (□) showed shift to autophagy by days 6, 4 and 2, respectively; 1000 cells in each of triplicate cultures were counted; the total number of MDC-positive cells are expressed as percentage of total cells scored. Means of three experiments are shown. Vertical bars indicate s.d.; when not shown, it is smaller than the symbols. (*) time of peaking autophagy assessed by MDC positivity. (**b1–3**) Transmission electron microscopy of control cells (**b1**), as well as day 4, attached, 3% DCC and TAM- treated cells (**b2**) and day 6 anoikic cells in 10% FCS (**b3**), showing numerous double-membraned AVs containing digested materials. (**c1**) Fluorescent microscopy with MDC/PI and (**c2**) TEM of day 2, 3% DCC and TAM-treated anoikic cells. Note the AVs in the cytoplasm (in blue) surrounding the nucleus (in red). (**d**) Western blot analysis of the LC3 expression in anoikic-autophagic (▽) and autophagic cells (○). Positive control (C) was obtained from C2C12 cells undergoing autophagy and showing bands for both LC3-I and LC3-II

mechanisms with associated autophagy have not been clarified. We have initiated a series of experiments, in which type II cell death was induced through autophagy in MCF-7 cells by tamoxifen treatment (*de novo* autophagy),[4] and we studied the engulfment of these cells by professional and non-professional phagocytes, that is human macrophages and MCF-7 cells, respectively. Furthermore, we studied the clearance of MCF-7 cells showing autophagy following initiation of death through anoikis (anoikic-autophagy), a newly recognized phenomenon described here. We show here that both *de novo* autophagic and anoikis-derived dying autophagic MCF-7 cells are engulfed by macrophages and MCF-7 cells. Their phagocytosis is executed through distinct mechanisms and low-density gene array studies have revealed that the expression of genes related to the engulfment process is differentially regulated by the two kinds of dying cells.

Results

Induction of autophagy in MCF-7 cells by tamoxifen treatment and through anoikis. Induction of autophagy, and through that cell death, by 3% Dulbecco's modified Eagle's medium (DMEM) containing 3% charcoal-stripped-fetal calf serum (FCS) (DCC) followed by tamoxifen treatment in MCF-7 mammary epithelial cells has been previously described by Bursch *et al.*[4] and demonstrated here using both monodansylcadaverin (MDC) uptake into autophagic vacuoles (AVs) and transmission electron microscopy (TEM) to identify autophagic cells. As shown in Figure 1a, the number of MDC-positive *de novo* autophagic cells peaked at day 4 under such conditions and autophagic vacuolization could be observed by TEM (Figure 1-b2).

Anoikis initiated by detachment of cells from the ECM *in vitro* has been introduced as a model for studying the mechanism of cell death in the absence of ECM-derived signals *in vivo*. Our MCF-7 cells underwent anoikis in 10% FCS of culture when not allowed to attach to the plate. However, we have also observed they showed signs of autophagy at day 6 of incubation: detachment of MCF-7 cells induced by poly(2-hydroxyethylmethacrylate) (poly-HEMA) coated plates resulted in occurrence of AVs as detected by MDC staining (Figure 1a) and TEM (Figure 1-b3). Combining detachment with either 3% DCC or 3% DCC + tamoxifen ((α-(4-β-*N*-dimethylaminoethoxy)-phenyl-α'-ethyl-*trans*-stilbene)) (TAM) treatment accelerated the autophagic response to occur 2 days earlier than the time of autophagy in attached cells with the same treatment. All treatment regimens (3% DCC + TAM, 10% FCS + poly-HEMA, 3% DCC + poly-

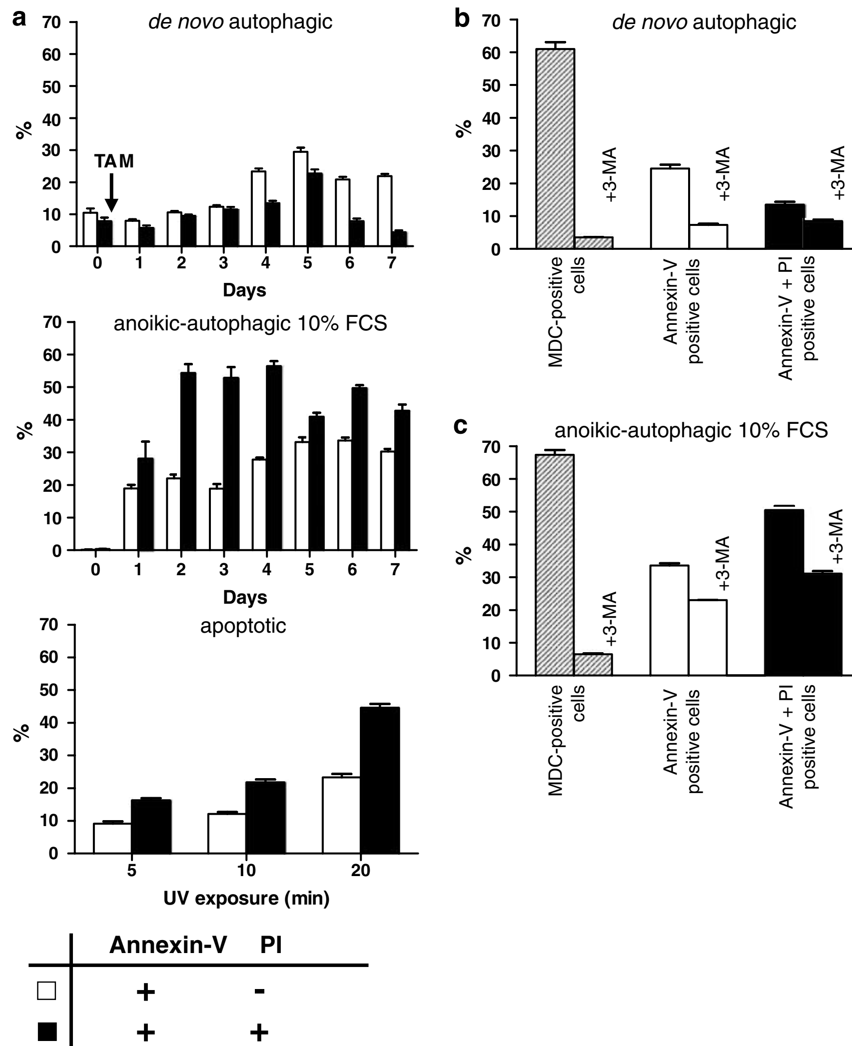


Figure 2 Cell death and the influence of 3-MA in autophagic, anoikic and apoptotic MCF-7 cells. (**a**) Percent of annexin-V and both annexin-V and PI-positive cells under the shown treatments as assessed by FACS analysis. (**b**) Effect of 10 mM 3-MA treatment on autophagy and cell death of day 4 autophagic and day 6 anoikic-autophagic cells (**c**) as assessed by the presence of MDC, annexin-V and/or PI-positive cells. Note that *de novo* autophagic cells were fed on 3% DCC for 7 days before the indicated '0' time point. At this starting point, anoikic-autophagic as well as anoikis-3% DCC cells with or without TAM showed less than 0.04% cell death. Means of three experiments are shown. Vertical bars indicate s.d.; when not shown it is smaller than the symbol

HEMA, 3% DCC + TAM + poly-HEMA) affected the time course but not the maximal degree of the autophagic response (about 70% of MCF-7 cells were affected in each case, Figure 1a); an example of the MDC-positive dot-like structures representing AVs in the cytoplasm of cells is shown (day 2, 3% DCC + TAM + poly-HEMA treated MCF-7 cells, Figure 1-c1). Typical absence of chromatin condensation in the nucleus of these cells and numerous AVs with double membranes containing digested materials were also seen by TEM (Figure 1-c2).

Time–dependent development of autophagy was also supported by findings of Western blot analysis of light-chain-3 (LC3) expression in case of *de novo* autophagic (3% DCC + TAM) and anoikic-autophagic (10% FCS + poly-HEMA) cells (Figure 1d). In autophagic MCF-7 cells LC3-II (the autophagosomal membrane-bound form of LC3[25]) became detectable at day 4, then increased up to day 7 in parallel with the observed pattern of MDC staining. In the anoikic-autophagic cells, the expression of LC3-II appeared at days 1 and 2, then showed a decrease from day 4 to day 7; this does not necessarily mean that autophagy was down-regulated knowing that LC3 may itself undergo degradation by lysosomal enzymes.

Occurrence of cell death in *de novo* autophagic and anoikis-derived autophagic cells. After peaking of autophagy in *de novo* autophagic cells at day 4 (3% DCC + TAM), the number of dying cells peaked at day 5 as assessed by their positivity for annexin-V$^+$ or annexin-V$^+$ propidium iodide (PI)$^+$ (Figure 2a). When counted by fluorescence microscopy, more than 95% of the dying cells were MDC positive at this time point (data not shown) suggesting that autophagy was directly related to cell death.

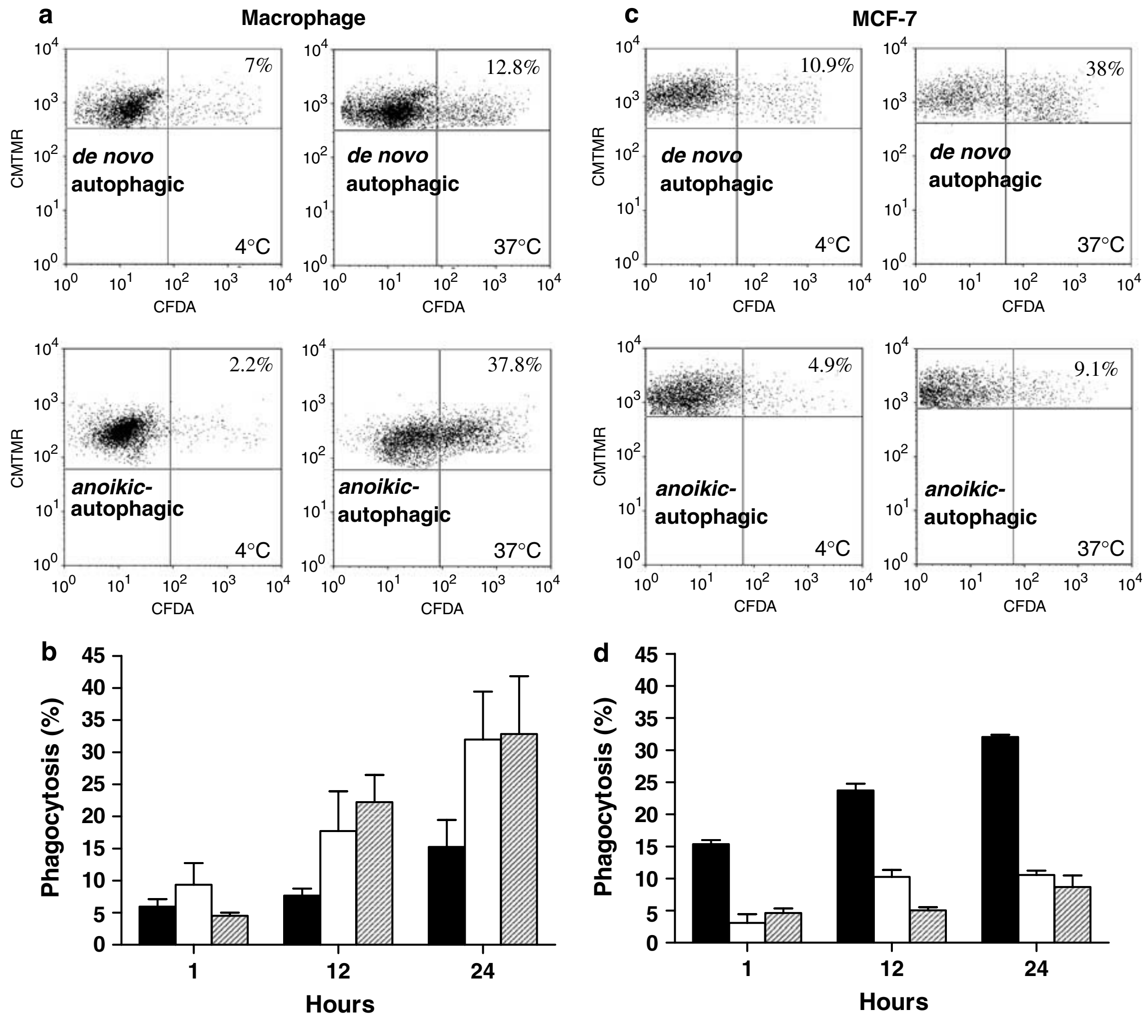
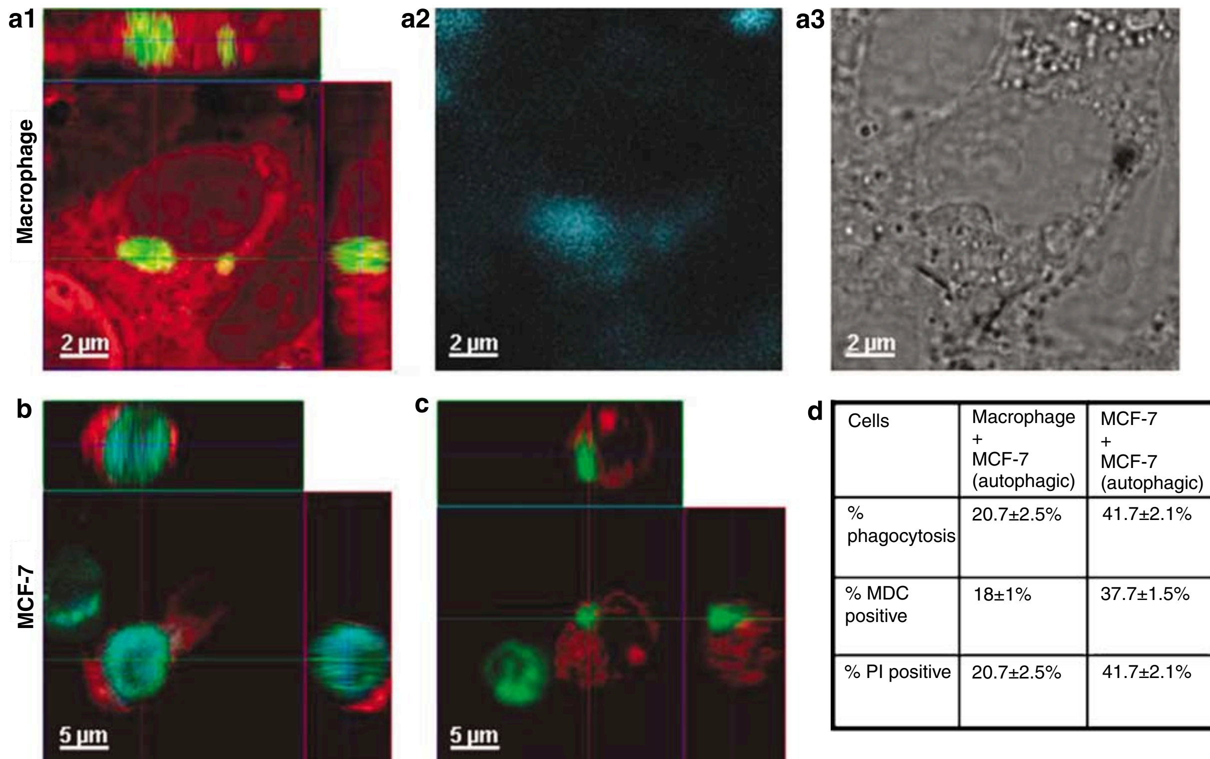


Figure 3 Flow cytometric analysis of the phagocytosis of dying cells. (**a**) Macrophages and (**c**) MCF-7 cells stained with CMTMR engulfing either autophagic or anoikic-autophagic cells stained with CFDA. The phagocytic assays were performed at 4°C at which binding occurs without engulfment, and parallels at 37°C at which both binding and engulfment occur. The macrophages that have not phagocytosed are in the right upper left quadrant and the ones that have are in the right upper quadrant; dying cells have been gated out from the lower left and right quadrants. Time of coincubation in each case was 12 h. (**b**) Macrophages and (**d**) MCF-7 cells were challenged to engulf *de novo* autophagic (■), anoikic-autophagic (□), or UV irradiated apoptotic (▨) MCF-7 cells. Bars represent mean of four independent experiments and s.d.

Inhibition of autophagy in these cells at day 4 by the autophagy inhibitor 3-methyladenine (3-MA) almost completely abolished the MDC-positive staining and led to a significant drop in the number of annexin-V^+ and annexin-V^+PI^+ cells (Figure 2b). The percentage of dying cells in anoikic-autophagic cells was twice as much as in *de novo* autophagy; death occurred earlier and peaked not in association with the peaking of autophagy (Figure 2a). Inhibition of autophagy in these cells at day 6 with 3-MA almost completely abolished the MDC-positive staining as in *de novo* autophagic cells and led to a decrease in the percentage of annexin-V^+ or annexin-V^+PI^+ cells (Figure 2c). For comparison, percentage of annexin-V^+ or annexin-V^+PI^+ stained cells following UV-induced apoptosis is also presented (Figure 2a); in this case cell death was not influenced by treatment with 3-MA (not shown).

Both *de novo* and anoikis-derived dying autophagic cells are engulfed by macrophages and non-dying MCF-7 cells. Although phagocytosis of apoptotic and necrotic cells has been extensively studied, no data are available how cells dying through autophagy or those in which death is accompanied with autophagy are removed from tissues. Our macrophages and MCF-7 cells could engulf dying *de novo* autophagic or anoikic-autophagic cells with increasing number of phagocytes containing cell corpses over a 24 h time period (Figure 3a–d). As flow cytometric analysis shows, compared to phagocytosis of UV-irradiated apoptotic MCF-7 cells, macrophages engulfed anoikic-autophagic cells more efficiently, than they did *de novo* autophagic dying cells (Figure 3b and d): at 24 h of coincubation about 32% of them contained anoikis-derived dead cells, whereas only 15% of them showed phagocytosis of dying *de novo* autophagic cells. MCF-7 cells, however,



Cells	Macrophage + MCF-7 (autophagic)	MCF-7 + MCF-7 (autophagic)
% phagocytosis	20.7±2.5%	41.7±2.1%
% MDC positive	18±1%	37.7±1.5%
% PI positive	20.7±2.5%	41.7±2.1%

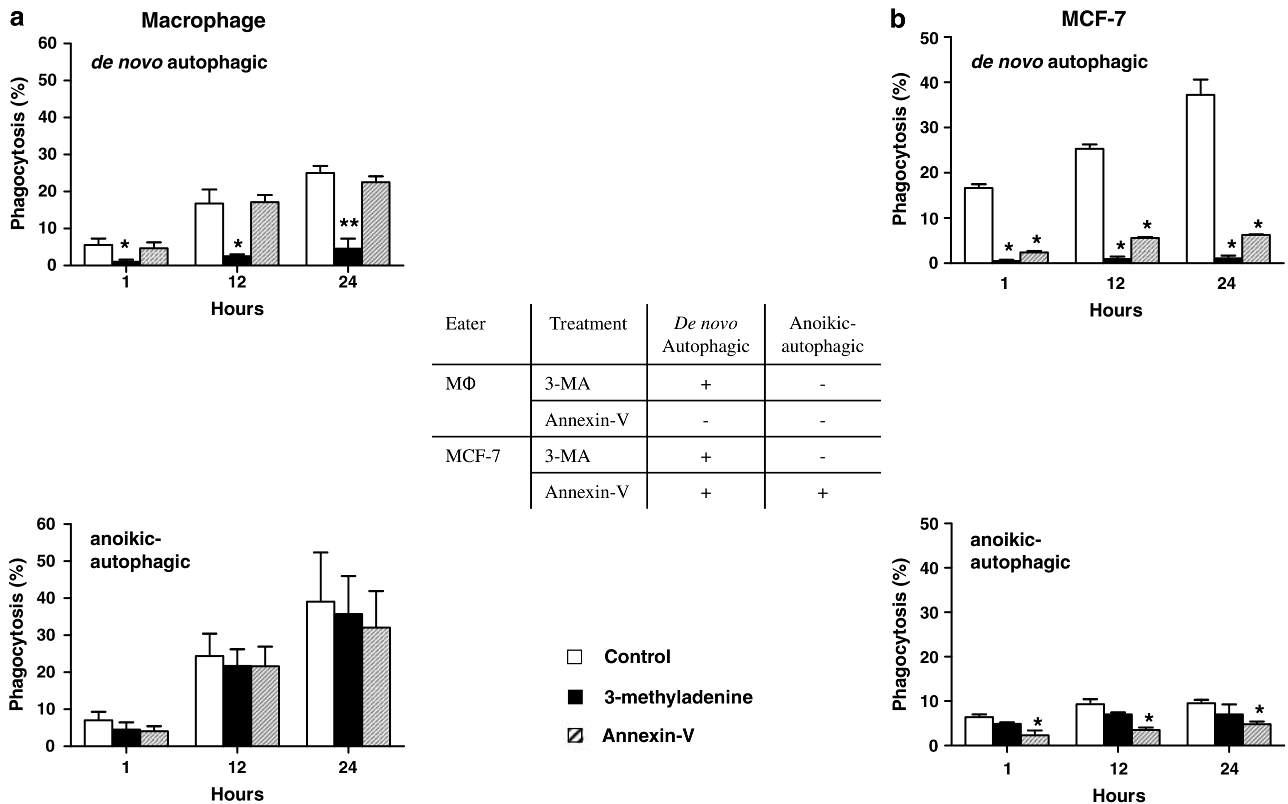
Figure 4 Detection of the phagocytosis of autophagic cells by confocal microscopy. (**a1–3**) Macrophage stained with CMTMR (red) that had engulfed *de novo* autophagic cell stained with CFDA (green) and MDC (blue) are co-localized. (**a3**) Phase-contrast image of the same cells as in (**a1–2**). Non-dying MCF-7 cells stained with CMTMR (red) that have engulfed either *de novo* autophagic stained with both MCD (blue) and CFDA (green) (**b**) or anoikic-autophagic cells (**c**) stained with CFDA (green). Note the orthogonal views of phagocytosis in (**b**) and (**c**) as well as the yet uneaten cells (green) in the proximity. (**d**) Percentage of both CFDA and MDC-positive cells being engulfed by CMTMR-positive cells in (**a**) and (**b**) as counted under a fluorescent microscope. In all cases, the time of co-incubation of dying and engulfing cells was 24 h. Means of three separate experiments are presented with their s.d.

engulfed dying *de novo* autophagic cells much more efficiently than either anoikic-autophagic or apoptotic ones, reaching an average phagocytosis frequency of 32% at the same time point.

To confirm the results obtained by flow cytometry the uptake of dying autophagic cells was also investigated by fluorescence confocal microscopy using a newly developed three-color technique. The latter was specifically designed for detecting colocalization of engulfed autophagic MCF-7 cells stained with both carboxyfluoresceindiacetate (CFDA) (green) and MDC (blue) within the 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) (red) colored phagocytosing cells (Figure 4a–c). Confocal microscopy confirmed the intracytoplasmic localization of the engulfed *de novo* autophagic or anoikic-autophagic dead cells being double stained with CFDA and MDC within either macrophages or non-dying MCF-7 cells. Under the experimental conditions used, usually one non-dying MCF-7 cell acting as 'eater' engulfed one autophagic or anoikic-autophagic cell. Counting under the microscope determined that an average of 21% of macrophages and 42% of engulfing MCF-7 cells contained cell corpses of *de novo* autophagic origin after 24 h coincubation (Figure 4c). Almost all of the engulfed cells were autophagic (87–90% positive for MDC staining) and all of the eaten cells could be stained by PI, that is, by definition they were dying autophagic cells.

Autophagic component of cell death and appearance of phosphatidylserine on dead cells differentially influence phagocytosis. Inhibition of autophagy by 3-MA, applied 2 days before peaking of autophagy, and harvesting these cells for the phagocytic assay, almost completely abolished the engulfment of *de novo* autophagic cells by both macrophages and MCF-7 cells. On the contrary, treatment with 3-MA did not affect significantly the engulfment of anoikic-autophagic cells (Figure 5a and b). Treatment of the dying *de novo* autophagic and anoikic-autophagic cells with recombinant annexin-V before and throughout the phagocytic assay that is blocking phosphatidylserine (PS) on the surface of dying cells, could inhibit significantly only engulfment by non-dying MCF-7 cells and not by macrophages. The inset in Figure 5 summarizes the differential effects of 3-MA and annexin-V on the phagocytosis of the two kinds of autophagic cells by the two types of engulfing cells.

Expression profile of apopto–phagocytic genes in macrophages and MCF-7 cells. Quantitative gene expression analysis of macrophages and MCF-7 cells was



Eater	Treatment	*De novo* Autophagic	Anoikic-autophagic
MΦ	3-MA	+	-
	Annexin-V	-	-
MCF-7	3-MA	+	-
	Annexin-V	+	+

Figure 5 Phagocytic capacity of (**a**) macrophages and (**b**) MCF-7 cells when challenged to engulf *de novo* autophagic or anoikic-autophagic MCF-7 cells under uninhibited conditions (□), when the autophagic and anoikic-autophagic cells were pretreated by 3-MA 2 days before autophagy peaked (■) and when phagocytosis was carried out in the presence of recombinant annexin-V 10 μg per 10^5 cells (▨). The inset shows when 3-MA and recombinant annexin-V influence uptake of dying cells by the two types of phagocytes. Bars represent mean values of three independent experiments and their s.d. $^*P<0.05$; $^{**}P<0.01$ using Student's *t*-test

carried out to gain insight into the potential molecular mechanisms mediating phagocytosis by these cells. The studied genes have been selected to cover most of the so far described molecular elements, which may participate in the clearance of dead cells. The data from the gene expression profiling clearly show that both macrophages and MCF-7 cells are well equipped for phagocytosis of dying cells. In macrophages, almost all studied genes of the various functional groups were expressed and the following ones have very high basal expression levels: calreticulin, the scavenger receptors CD68 and MSR1, the low-density lipoprotein-related protein 1 (LRP1), integrins α_x and β_2 (phagocytosis receptors), annexin A5, the C1QA-bridging molecule and transforming growth factor -β (Table 1). Interestingly, many of the studied and expressed phagocytic genes were also expressed in MCF-7 cells. The exceptions, with very low expression levels in comparison to macrophages, include the C1Q receptor 1, MSR1, the oxidized low-density lipoprotein 1 receptor (OLR1), integrin α_x among the phagocytosis receptors, the adenosine receptor A3 (ADORA3), the formyl peptide receptor like 1, the bridging and surface molecules C1QA and ICAM3, respectively, the inflammatory regulators caspase 1 and 5, NALP12 and the cytokines interleukin-6 (IL)-6, IL-10, IL-12B, IL-23A. Calreticulin, annexin A5 and calpain 1 showed high-basal expression in these cells.

Clearance of autophagic and anoikic-autophagic cells initiates distinct transcriptional responses in macrophages and MCF-7 cells. Gene expression profiling of macrophages and MCF-7 cells while they were consuming dying cells as compared with their resting state was done at 12 h. The engulfment of *de novo* autophagic cells elicited different response in the two types of phagocytes. In macrophages, the asialoglycoprotein receptor 1 (ASGR1), ADORA2A, the bridging molecule PTX3, phospholipase IPLA2γ, the engulfment molecules GULP1 (which was not expressed in the resting cells) and RAC1, the cytokines IL-6, IL-12B, IL-23A and tumor necrosis factor (TNF-α) were induced. ADORA3, the platelet-activating factor receptor, the bridging and surface molecules C3 and ICAM 3, respectively, the inflammatory regulators BIRC1 and NALP12 were downregulated in macrophages eating *de novo* autophagic cells. MCF-7 cells showed increased expression of annexin-1 and the engulfment molecule ELMO1. Both phagocytes upregulated OLR1 and IL-6 to a significant degree in response to the dying *de novo* autophagic cells.

Macrophages eating anoikic-autophagic cells upregulated C1Q receptor 1, ADORA1 and ADORA2A, (whereas down-regulating several phagocytosis receptor and surface molecules including CD68, MSR1, LRP1, integrins, ADORA3, annexin-1, ICAM3 and the platelet/endothelial cell adhesion

molecule), the bridging molecules C4B and MFGE8 (whereas downregulating C2 and C3), the signaling molecule protein kinase 2, the engulfment protein RAC1 (whereas downregulating ELMO 1), DNASE1 (whereas downregulating DNASE2), the proinflammatory caspase 5 (whereas downregulating NALP12) and the cytokine IL-6. MCF-7 cells responded to anoikic-autophagic cells by upregulating the AXL receptor tyrosine kinase.

The question may arise whether the elevated mRNA levels observed in engulfing cells could come from the eaten cells.

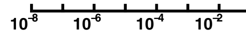
Table 1 Gene expression levels in macrophages and MCF-7 cells and their changes during phagocytosis

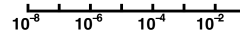
Category	Gene description	Macrophage	Phagocytosing macrophage		MCF-7	Phagocytosing MCF-7		Dying MCF-7	
		Control	With autophagic	With anoikis-autophagic	Control	With autophagic	With anoikis-autophagic	Autophagic	Anoikis-autophagic
Phagocytosis (tethering/tickling) receptors	ASGR1		+++					+++	+++
	AXL[1]						+	−−−	
	MERTK[1]							−−	
	TYRO3[1]								−
	C1QR1			+					
	CALR								
	CD14								
	CD68[2]			−					+
	MSR1[2]			−−					
	OLR1[2]		++	++		+++		+++	
	SCARB1[2] (CD36)								
	ITGAM[3]			−−				−	−−−
	ITGAV[3]								
	ITGAX[3]			−−				+++	+++
	ITGB2[3]			−				−	−
	ITGB3[3]								−−
	ITGB5[3]								
	LRP1			−					
Cell surface molecules	ABCA1							−	−−−
	ADORA1			++					−
	ADORA2A		+++	+++					
	ADORA3		−−	−−					
	ANXA1			−		+			−
	ANXA5								
	CD47								−
	FCGR2B								−−−
	FPRL1								
	ICAM3		−	−−				+++	
	IL4R								
	PECAM1			−−					
	PTAFR		−					−	−
	PTDSR								
	PTGER2							−	−−−
	TGFBR1								
Bridging molecules	APOH								
	C1QA								
	C2			−−				−−−	
	C3		−	−				−	−
	C4B			+					
	CRP								
	EDIL3								
	GAS6								−
	MFGE8			+					
	PROS1								−
	PTX3		+++						−−−
	RAP1A								
	THBS1								−−

Table 1 Continued

Category	Gene description	Macrophage	Phagocytosing macrophage		MCF-7	Phagocytosing MCF-7		Dying MCF-7	
		Control	With autophagic	With anoikis-autophagic	Control	With autophagic	With anoikis-autophagic	Autophagic	Anoikis-autophagic
Signaling	ALOX12							+++	+++
	ALOX5							−	
	IPLA2(GAMMA)		+						−
	PTK2			+					
	PTPNS1							−	−
Engulfment proteins	BCAR1								
	CRK								
	DOCK1								−
	ELMO1					++			
	ELMO2			−−					
	GULP1		+++						
	RAC1		+++	+++					
	RHOG								
	TRIO								
Effectors	CAPN1								
	CAPN2								
	DNASE1			+					
	DNASE2			−					
	TGM2							−	
Transcr. factors	CXXC1								−
	GRLF1								
	NFKB1								−
	PPARG			−−−					−
Inflammatory regulators	BIRC1		−						−−−
	CARD15							−	−
	CARD4								
	CASP1								
	CASP5			+++					
	CIAS1								
	NALP12		−−	−−−					
	PYCARD								−
Cytokines	IL6		+++	+		+++		+++	+++
	IL10								
	IL12B		+++	+++					
	IL18								
	IL23A		+++	+++					
	TGFB1								
	TNF		+++	+				−	

Relative expression levels:





The basal expression levels were determined in 3 independent biological samples and each was analyzed in two parallel runs in which each gene had two technical replicates. Based on the average of the obtained result (the variation coefficient was less than 20% in each case), the relative expression of a particular gene in macrophages and MCF-7 cells is indicated. The increments of the horizontal bars represent one order of magnitude higher range of relative expression (related to 18S RNA) and the size of the bar at each gene indicates into which range the actual expression data (average of three repeated experiments) fall. In case of analyzing gene expression changes during phagocytosis, change of mRNA level was considered significant when it repeatedly (at least in two biological samples, both analyzed in two parallel runs each with two replicates per gene) exceeded 2.5 times the average expression level of the controls, either increasing or decreasing (+ or −). It is also indicated in the table when the change of expression was more than 5.0 times (++ or −−) and 10 times (+++ or −−− as compared to controls. The nomenclature of the genes studied is in compliance with the HUGO classification; [1]Mer tyrosine kinase family, [2]scavanger receptors, [3]integrins

This is very unlikely, as the overlapping upregulated genes in two different types of phagocytes eating the same kind of dead cells do not show systematic 'cross contamination'. In addition, analysis of the gene expression changes in *de novo* autophagic (day 4) and anoikic-autophagic (day 6) cells as compared to untreated MCF-7 cells clearly shows the genes being upregulated in either macrophages or engulfing MCF-7 cells are not upregulated in the two kinds of dying cells (Table 1). The exceptions are ASGR1 and IL-6 in both kinds of dying cells, and OLR1 in *de novo* autophagic cells. However,

other upregulated genes in dying cells (ITGAX, ICAM3, ALOX12) do not have increased levels in the engulfing phagocytes.

Discussion

Insufficient clearance of dying cells may lead to inflammation and autoimmune diseases. Molecular mechanisms participating in the clearance of apoptotic cells by macrophages have been described in detail and, to a lesser extent, this can be said about non-professional phagocytes.[14,18,19,26,27] It has been suggested that autophagy as a mechanism of cell death may have been developed because in some forms of PCD the availability of engulfing cells is insufficient for the clearance of dead cells and autophagy has been evoked for elimination of excess cells by their own lysosomes.[13] In the present study we clearly demonstrate this is not the case: autophagic MCF-7 cells of different origin are recognized and eaten by both differentiated macrophages and non-dying MCF-7 cells. As almost all engulfed cells were MDC^{+} and all were PI^{+}, we may conclude that the eaten cells were dead or dying autophagic cells, and not cells that use autophagy to survive.

An equally important question is whether the engulfed MCF-7 cells died through autophagy (i.e. autophagy killed the cells) or by apoptosis during which autophagy was switched on, but did not contribute to killing of the cells. Only a few examples are available to prove that autophagy is an important death pathway in cells with intact apoptosis machinery.[28] On the basis of several indirect evidences it has been proposed that active cell death is induced through autophagy in tamoxifen-treated MCF-7 cells;[5] one of the evidences is the inhibition of both autophagy and cell death by 3-MA, a compound that can specifically block the sequestration step in macro-autophagocytosis inhibiting class III phosphatidylinositol-kinase activity. As in our experiments 3-MA also inhibited macrophage and MCF-7 cell-mediated phagocytosis of dying *de novo* autophagic cells, it can be suggested that molecular events linked to autophagy and death are involved in specific surface changes, which are important in subsequent recognition and elimination of these dying cells.

We have also described here a novel way of inducing autophagy through initiation of anoikis, which could facilitate autophagy even in *de novo* autophagic cells. In case of anoikis, cells actually die through apoptosis with coincident autophagy, which, has no causative role in the death process itself. How anoikis-initiated death response switches on autophagy and why anoikis accelerates tamoxifen-induced autophagy certainly requires further investigation. Examples for the induction of apoptosis with the help of autophagy regulators, such as the *p53*-induced *dram* and the calpain-generated cleavage product of *atg5*, have been described.[29,30] It is also possible that elements of the apoptosis machinery, while killing cells, may initiate autophagy to fasten self-elimination even before phagocytosis; in our case loss of integrin-dependent survival pathways may lead to induction of death effectors' which in turn converge onto mediators of autophagy, such as *Atg5*, *Atg7* and *beclin-1*. Nevertheless, inhibition of autophagy by 3-MA in these anoikic-autophagic cells have resulted in less inhibition of cell death than in *de novo* autophagic cells and, most importantly, did not have an effect on the engulfment of these anoikic-autophagic cells by either of the phagocytic cell types. Therefore, we have described clearance of autophagic dying cells in two kinds of paradigm; one in which cells died mainly through autophagy, generating specific cell surface changes for recognition and engulfment, and another, where cell death occurred with concomitant autophagy, which is not the main inducer of death and does not contribute to specific cell surface changes for removal by phagocytes.

Our results show that non-professional phagocytes, the non-dying MCF-7 cells, are much more efficient in taking up *de novo* autophagic cells than anoikic-autophagic ones; that is, they can recognize the difference between death through autophagy and death associated with autophagy. Dying cells present on their surface several 'eat-me' signals facilitating their engulfment, one such being externalized PS. Recombinant annexin-V, which binds PS exposed on the surface of dying cells, could block the engulfment of dying *de novo* autophagic cells by MCF-7 cells (Figure 5a and b); this clearly suggests that whatever specific changes are induced by autophagy on the surface of these dying cells – making them edible for MCF-7 cells (see inhibition by 3-MA) – is directly or indirectly linked to PS. On the other hand, uptake of *de novo* autophagic cells by macrophages could not be inhibited by recombinant annexin-V, indicating that PS-independent cell surface changes are induced in cells dying through autophagy. To our present knowledge, no specific cell surface changes have been described that would make autophagic cells edible for phagocytes. A handful of proteins have been shown to undergo internalization from the plasma membrane following the induction of autophagy.[31,32] Recently, calreticulin appearing on the surface of apoptotic cells was found to initiate their clearance acting together with PS for achieving optimal phagocytic clearance of cells.[33] Changing carbohydrate composition of glycoproteins[34] and exposure of DNA on dying cells[35] have been proposed as alternative mechanisms to PS for recognition of dead cells. Further studies are required to determine whether these alternative 'eat me' signals or others are exposed on the surface of cells dying through autophagy.

Autophagy in cells dying through anoikis did not contribute to clearance of these cells by either MCF-7 cells or macrophages. However, the uptake of these cells by MCF-7 was inhibited when PS was covered on the cell surface. The anoikic-autophagic cells were very efficiently eaten by macrophages in a PS-independent manner. As this was not related to the appearance of autophagy (could not be inhibited by 3-MA), the PS-independent recognition and signaling system in this case must be different from the one utilized by cells dying through autophagy.

Molecular elements used for the clearance of dead cells are highly conserved and strongly interrelated with components of the innate immune system.[15] It has been suggested that different surface receptors on the phagocytes are involved in tethering (recognition and binding of cell corpses) and tickling (internalization and activation of downstream signaling) processes, leading via at least two major pathways to activation of *Rac*, which is obligatory for uptake of dead

cells.[36] In case of PS recognition, at least one tethering (integrins or integrin-associated proteins, e.g. CD36) and one internalization receptor (bridging molecule MFGE8) are needed for efficient clearance. Tickling is mediated by *Gas* or protein S through one of the tyrosine kinase receptors (MERTK, AXL or TYRO3) leading to activation of *Rac* engaging TRIO, *RhoG*, DOCK180, *CrkII* and ELMO. Exposed calreticulin is recognized by collectins and ficolins (such as C1QA, PTX) serving as bridging molecules; then the opsonized dead cells are bound to phagocytosis receptors on the surface, including calreticulin in conjunction with LRP1 (which signals to *Rac* through GULP); as an alternative, thrombospondin is the bridging molecule and integrin α_v or calreticulin serve as receptors. We have used a low-density gene array approach to learn which of the above and other previously described molecular participants in the clearance mechanisms are expressed in the two types of phagocytic cells.

As discussed above, MCF-7 cells used mainly the PS recognition and signaling pathway for phagocytosis of dying cells. Indeed, various elements of this pathway are expressed by this cell type at significant levels, including the bridging molecules MFGE8, protein S and *Gas*, the integrin and CD36 tethering receptors, the tickling tyrosine kinase receptors AXL, MERTK and TYRO3, and all the engulfment proteins required for *Rac* activation (TRIO, *RhoG*, DOCK180, *CrkII* and ELMO) through this pathway. It is very likely the expressed complement factors and scavenger receptors also contribute to the efficient uptake of dying cells. It is remarkable that the expression of none of the studied genes showed a decrease during the engulfment of either kind of dying cells. The more efficient uptake of *de novo* autophagic cells as compared with anoikic-autophagic ones may be related to the specific changes on the surface or the inside of the engulfed dying cells inducing distinct transcriptional responses, such as upregulation of OLR1, ANXA1 and ELMO1. On the other hand, induction of AXL may be linked to the uptake of anoikic-autophagic cell corpses in a PS-dependent way.

In macrophages, the molecular elements of the PS recognition and signaling pathway are also highly expressed; one may assume that it is utilized for engulfment of both kinds of autophagic dying cells under normal circumstances. Our gene array data show, however, that the calreticulin-mediated pathway of dead cell clearance is also available for efficient phagocytosis of *de novo* autophagic cells, and this may explain why phagocytosis of dying autophagic cells is not blocked when PS is covered on their surface. Calreticulin is highly expressed in both the dying and phagocytic cells and may become exposed on the surface; C1QA and LRP1 mRNA are at high concentrations in macrophages; PTX and GULP are specifically induced during uptake of this kind of dying cells, in conjunction with the strong induction of *Rac* expression. Macrophages can engulf anoikic-autophagic dying cells in a PS-independent way more efficiently than *de novo* ones, but the calreticulin-C1Q-LRP-GULP pathway is not available because GULP is not induced by the anoikic-autophagic cells (see the gene expression profile in Table 1). Again, the difference in phagocytosis efficiency may be related to specific changes on the surface of anoikic-autophagic cells leading to utilization of alternative pathways in macrophages, such as those which involve the highly expressed scavenger receptors, the specifically induced C4B and C1QR1, the signaling molecules PTK2 and DNASE1. Interestingly, macrophages eating anoikic-autophagic dying cells are the ones showing most significant downregulation of several phagocytosis genes; it is possible that while the engulfing cells are engaged in the phagocytosis process, the engulfment genes are downregulated in the non-phagocytic population.

Our gene array profiling of engulfing macrophages has revealed surprising induction of the proinflammatory cytokine genes IL-6, TNF-α, IL-12B and IL-23B by both kinds of dying cells. In the case of anoikic autophagy this may be explained by the presence of late apoptotic cells, which have been shown to be proinflammatory,[37] in high proportion at day 6 of anoikis (see percent of annexin-V$^+$PI$^+$ cells in Figure 2a). Regarding cells dying through autophagy, they may expose autophagy-related pro-inflammatory signals on their surface; this may have significance when autophagic death occurs in tumor cells and the inflammatory response can promote tumor growth.[38] It has been suggested that expressed PS on dying cells has a major function in the generation of an anti-inflammatory effect, whereas engagement of calreticulin with collectins stimulate pro-inflammatory mediator production.[36] As molecular elements of the calreticulin-dependent recognition and engulfment pathway were induced in macrophages engulfing *de novo* autophagic cells, it is possible that signaling through this pathway participates in the induction of pro-inflammatory cytokine production. The pro-inflammatory response of macrophages to dying autophagic cells may be also connected to the observed induction of adenosine receptors, particularly ADORA2A in the engulfing cells. ADORA2A is a potent regulator of macrophage functions, including Fc receptor-mediated phagocytosis and pattern recognition receptor-mediated cytokine production.[39] Detailed studies are in progress in our laboratory to clarify the molecular mechanism and general implication of cytokine induction in macrophages.

Materials and Methods

Cell culture and treatments. The list of compounds used throughout the experiments can be found in the Supplementary file. MCF-7 human breast cancer cells were kindly provided by Dr W Bursch, Medizinische Universität Wien, Austria; culture conditions were described in detail previously.[5] Briefly, cells were grown as a monolayer in DMEM supplemented with 10% FCS, L-glutamine (300 mg/l) and penicillin/streptomycin antibiotics at 37°C in an atmosphere of 5% CO_2. Seven days before beginning an experiment cells were plated at density of $7.5 \times 10^3/cm^2$ in DCC according to Bardon *et al.*[23] Twenty-four hours later the cells were treated with freshly prepared TAM in dimethyl sulfoxide/ethanol (1:1, v:v) added directly to the medium. For the induction of anoikis, cells were plated on poly-HEMA covered dishes over a 7 day period in either 10% FCS or 3% DCC with or without TAM (i.e. 10% FCS + poly-HEMA, and 3% DCC + poly-HEMA, 3% DCC + TAM + poly-HEMA). Apoptosis was induced by UV irradiation – 5, 10 and 20 min, 45 mJ/(cm^2 min) – and the cells were left to die for 8 h before harvesting.

Assays of cell death and autophagy. Cell death was assessed by the Annexin-V-fluorescein isothio-cyanate Apoptosis Detection Kit (MBL, Woburn, MA, USA) according to manufacturer's recommendations; proportion of stained Annexin-V$^+$ and Annexin-V$^+$PI$^+$ cells was determined by fluorescence activated cell sorter (FACS) analysis on BD Bioscience flow cytometer. Autophagy was assessed by detection of AVs with MDC according to the

method of Biederbick et al.[24] and described in more detail in the Supplementary file. Whenever inhibition of autophagy was carried out with 3-MA, the treatment preceded peaking of autophagy in 2 days.

Electron microscopy. Samples were fixed in 0.1 M. sodium cacodylate-buffered, pH 7.4 and 2.5% glutaraldehyde solution for 2 h and then rinsed (three times, 10 min) in 0.1 M sodium cacodylate buffer, pH 7.4 and 7.5% saccharose and postfixed in 1% OsO_4 solution for 1 h. After dehydration in an ethanol gradient (70% ethanol (20 min), 96% ethanol (20 min), 100% ethanol (two times, 20 min)), samples were embedded in Durcupan ACM. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined in a Philips CM 10 microscope (Philips Electronic Instruments, Mahwah, NJ, USA) at 80 kV.

Antibodies and immunoblotting. Anti-LC3 polyclonal antibody was prepared in rats by Eurogentec (Seraing, Belgium, see Supplementary file). Cell lysates were separated on NuPAGE 12% Bis–Tris polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) and transferred to Immobilon-P Transfer Membrane (Millipore, Bedford, MA, USA; pore size 0.45 μm). Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 5% nonfat dry milk (BioRad) for 1 h, probed overnight at 4°C with primary rat anti-LC3 polyclonal antibody in TBS-T containing 1% nonfat dry milk and incubated for 1 h with rabbit antirat peroxidase-conjugated secondary antibody (DAKO, Glostrup, Denmark) at room temperature. Peroxidase activity was detected with SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Pierce, Rockford, IL, USA) using a Lumi-Imager (Roche Diagnostics, Mannheim, Germany).

Phagocytosis assay. Human monocytes were isolated from 'buffy coats' of healthy blood donors on Ficoll–Paque Plus (Amersham Biosciences) gradient and magnetic separation using CD14 human microbeads (Miltenyi Biotec, Auburn, CA, USA). Human macrophages were obtained through a 5-day differentiation using 5 ng/ml macrophage colony-stimulating factor. Non-dying MCF-7 cells acting as phagocytes were plated in serum-free medium 24 h before phagocytosis. Dying cells were fed to engulfing cells when in their culture autophagy peaked: at day 4 for *de novo* autophagic and day 6 for anoikic-autophagic cells. Inhibition with 10 μg recombinant annexin-V was carried on 10^5 dying cells (30 min before phagocytosis assay, 37°C) and maintained throughout the assay. In addition, MCF-7 cells UV irradiated for 20 min and turning apoptotic 8 h after irradiation were used for engulfment. The phagocytes, macrophages and non-dying MCF-7 cells, were stained with 7.5 μM CMTMR for 16 and 24 h, respectively, before starting the assay. Accordingly, the cells to be engulfed were stained with 12.5 μM CFDA for 16 or 24 h before the phagocytosis and washed twice in phosphate-buffered saline (PBS) before being added to the phagocytes. The ratio of phagocytes and cells to be engulfed was set at 1 : 5. The phagocytosis assay started when the cells to be engulfed were added to the appropriate phagocytes and kept together for either 1, 12 or 24 h. The assay was carried out at either 4°C when phagocytes bind but do not engulf dying cells, and at 37°C when phagocytes bind and/or engulf them. The whole-cell mixture was collected by scraping the cells, centrifuging, washing twice in PBS and fixing in 1% PBS-buffered paraformaldehyde (pH 7.4). The net phagocytosis rate was determined by FACS analysis as percent phagocytic cells that have engulfed (positive for both CMTMR and CFDA) at 37°C minus the observed percent of double-positive cells at 4°C.

RNA preparation and TaqMan real-time RT-PCR. Total cellular RNA was isolated from human monocyte-derived macrophages as well as from MCF-7 cells using TRIzol Reagent (Invitrogen Life Technologies) at the indicated time point of the phagocytic assay after washing away non-engulfed dead cells thoroughly. Pre-designed, factory-loaded 384- well TaqMan low-density array (Applied Biosystems, Foster City, CA, USA) was used to determine the level of expression of genes grouped and listed in Table 1. Two replicates per target gene and two parallels per biological sample were carried out. Expression levels of target genes were normalized to 18S rRNA as endogenous control. Gene expression values were calculated based on the $\Delta\Delta C_t$ method, where one sample was designated as calibrator, through which all other samples were analyzed. ΔC_t represents the threshold cycle (C_t) of the target minus that of 18S rRNA and $\Delta\Delta C_t$ represents the ΔC_t of each target minus that of the calibrator. Relative quantities (RQ or fold changes) were determined using the equation where relative quantity equals $2^{-\Delta\Delta C_t}$ (average of three repeated experiments).

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SUPPLEMENT 3.

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Addendum

Phagocytosis of Cells Dying through Autophagy Evokes a Pro-Inflammatory Response in Macrophages

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autophagy, cell death, phagocytosis, calreticulin, pro-inflammatory response

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Addendum to:

Clearance of Dying Autophagic Cells of Different Origin by Professional and Non-Professional Phagocytes

G. Petrovski, G. Zahuczky, K. Katona, G. Vereb, W. Martinet, Z. Nemes, W. Bursch and L. Fésüs

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ABSTRACT

Autophagy as a natural part of cellular homeostasis usually takes place unnoticed by neighboring cells. However, its co-occurrence with cell death may contribute to the clearance of these dying cells by recruited phagocytes. Autophagy associated with programmed cell death has recently been reported to be essential for presentation of phoshatidylserine (PS) on the cell surface (Qu et al. 2007) that has a key role in the clearance of apoptotic cells. Recently, we have demonstrated that upon triggering cell death by autophagy in MCF-7 cells, the corpses were efficiently phagocytosed by both human macrophages and non-dying MCF-7 cells. Death as well as engulfment could be prevented by inhibiting autophagy. Based on our data, two molecular mechanisms have been proposed for the uptake of cells which die through autophagy: a PS-dependent pathway which was exclusively used by the living MCF-7 cells acting as non-professional phagocytes, and a PS-independent uptake mechanism that was active in macrophages acting as professional phagocytes. Several lines of evidence suggest that macrophages utilize calreticulin-mediated recognition, tethering, tickling and engulfment processes. Phagocytic uptake of cells dying through autophagy by macrophages leads to a pro-inflammatory response characterized by the induction and secretion of IL-6, TNFα, IL-8 and IL-10.

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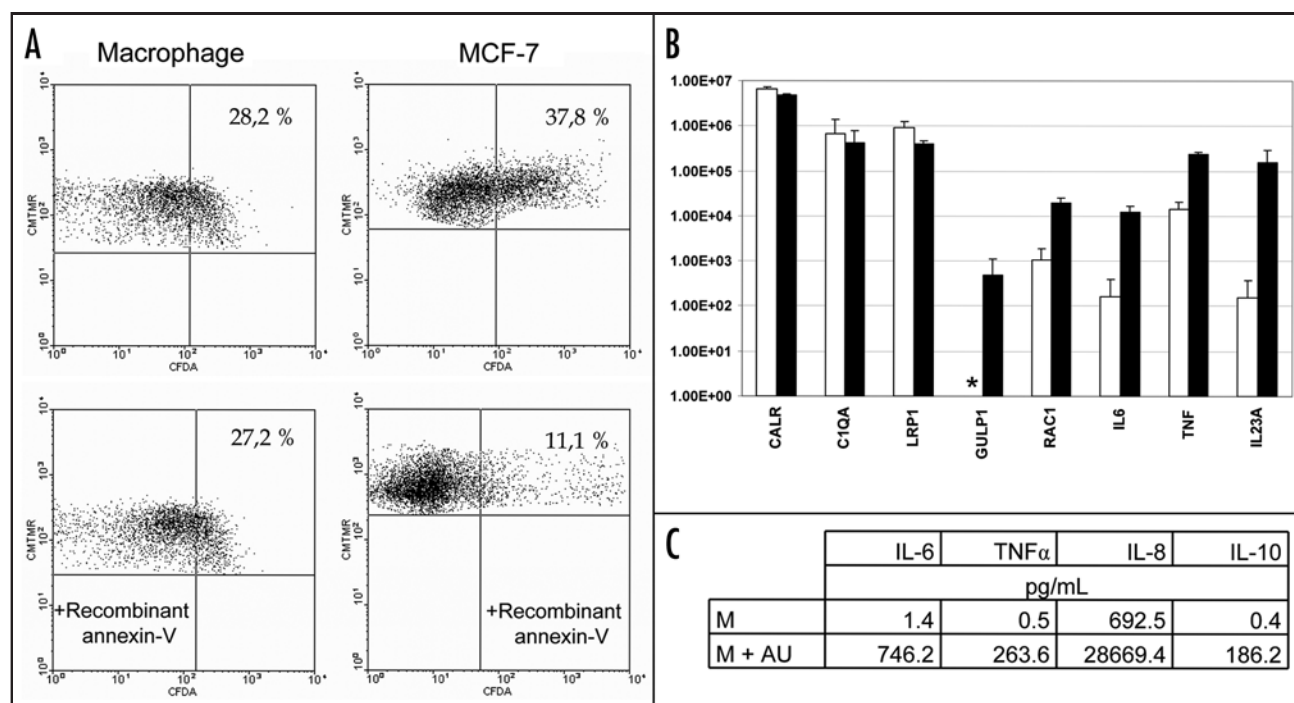
Autophagy has several distinct physiological functions in mammalian organisms.[1] It is a survival mechanism under nutrient- or birth-related starvation. A low level of constitutive autophagy plays a role in the clearance of misfolded proteins and protein aggregates. This can serve as an active defense mechanism to clear toxic mutant proteins and invasive pathogens. In all these cases, the autophagic machinery works inside cells in such a way that cells with autophagy are usually not specifically noticed by their neighbors. This may not be the case, however, when autophagy is associated with cell death.

Autophagy may occur in dying cells and this is most pronounced in developmental cell elimination processes. The term type II programmed cell death has been applied to distinguish autophagic cell death from apoptosis.[2] However, depending on the specific conditions, autophagy can have divergent roles. In some circumstances it serves as a survival pathway preventing or delaying apoptosis.[3,4] On the other hand, autophagy induces apoptosis when the death signals reach cells deficient in functioning pro-apoptotic proteins[5,6] or when the autophagic response is so excessive as to lead to degradation of apoptosis inhibitors and crucial cell components.

Cells dying through either apoptosis or necrosis are recognized, engulfed and degraded by either professional or non-professional phagocytes, and whereas necrotic cells usually provoke an inflammatory response, apoptotic cells are not pro-inflammatory and even downregulate inflammation.[7,8] Until recently it has not been clarified how dying autophagic cells are removed from tissues and whether autophagy may contribute to the efficient clearance of dying cells. In a recent study[9] we demonstrate that when MCF-7 cells are triggered to die through autophagy by starvation and tamoxifen treatment, the dying cells are efficiently engulfed by human monocyte-derived macrophages as well as non-dying MCF-7 cells. When autophagy is inhibited in dying MCF-7 cells by 3-methyladenine, both death and phagocytosis can be prevented. This means that autophagy not only induces cell death but can also contribute to the development of cell surface changes, which mediate recognition by phagocytes. We have also observed autophagy in MCF-7 cells dying through anoikis; however, in this case inhibition of autophagy does not prevent death and phagocytosis, suggesting that co-occurrence of death and autophagy does not necessarily mean involvement of the latter in the generation of "eat me" signals.



	IL-6	TNFα	IL-8	IL-10
	pg/mL			
M	1.4	0.5	692.5	0.4
M + AU	746.2	263.6	28669.4	186.2

Figure 1. Flow cytometric, gene expression and cytokine secretion analysis after 12 hours phagocytosis of dying autophagic cells. (A) Macrophages and MCF-7 cells stained with CMTMR engulfing dying autophagic MCF-7 cells stained with CFDA9. The macrophages that have not phagocytosed are in the upper left quadrant and the ones that have are in the upper right quadrant; dying cells have been gated out from the lower left and right quadrants. Recombinant annexin was added at a concentration of 10 mg per 10^5 cells (B) TaqMan RT-PCR gene expression analysis in control macrophages (open bars) and those phagocytosing dying autophagic cells (closed bars). The expression levels were analyzed in two parallel runs in which each gene had two technical replicates. Data are mean ± SD of three biological experiments. Relative expression was determined compared to 18S RNA (x10^9). *Expression is not detectable. (C) Cytometric bead array analysis of secreted cytokines by macrophages (M) while engulfing dying autophagic cells (AU); values represent average of three independent experiments.

As in apoptotic cells, exposure of phosphatidylserine (PS) on the cell surface is one of the changes induced when autophagy triggers cell death, as demonstrated by our finding that uptake of starvation- and tamoxifen-treated dying cells is inhibited when annexin-V (blocking exposed PS) was present during phagocytosis by non-dying MCF-7 cells (Fig. 1A). This means that the non-professional phagocytes are equipped with molecular elements of the PS-dependent uptake pathway, but do not have alternative ways for phagocytosis of autophagic dying cells.

It remains to be clarified how autophagy triggers not only death in starving MCF-7 cells but also the exposure of PS on the surface of these dying cells. Autophagy in dying cells during development may be crucial to generate sufficient ATP for PS exposure as it was demonstrated in programmed cell death associated with morphogenesis.[10] Though the need for autophagy-dependent ATP production in triggering PS exposure could be observed only in a three-dimensional culture setting, this mechanism may also be utilized in starvation-induced autophagic death of MCF-7 cells cultured in monolayers.

Uptake of dying autophagic cells by macrophages could not be inhibited by annexin-V (Fig. 1A) suggesting involvement of at least one other molecular mechanism. It has been shown recently, that in addition to the PS-dependent processes, calreticulin-mediated recognition, tethering and tickling leads to PS-independent Rac activation, which is obligatory for uptake of dead cells. We have obtained some indirect evidence that the calreticulin engulfment pathway is utilized in macrophages engulfing dying autophagic cells. A high level of calreticulin expression was observed both in dying autophagic MCF-7 cells and macrophages by TaqMan RT-PCR, suggesting that calreticulin is available and may be utilized in the uptake process on the surface of both, as suggested by others.[11] Exposed calreticulin is recognized by collectins and ficolins, such as C1QA and PTX, then the opsonized dead cells are bound to the phagocytosis receptors, including the calreticulin receptor, in conjunction with LRP1, which signals to Rac through GULP. C1QA and LRP1 are highly expressed in macrophages, and PTX3, RAC1 and GULP1 are strongly induced during uptake of dying autophagic cells (Fig. 1B). The latter is particularly interesting since there is no basal GULP1 expression in differentiated macrophages and its induction by the dying autophagic cells completes the calreticulin pathway.

Calreticulin-dependent uptake of dying cells can provoke a pro-inflammatory response;[11,12] indeed, we could observe induction of IL-6, TNFα, IL-12B and IL-23A mRNA in macrophages taking up dying autophagic cells (Fig. 1b); these data could be confirmed by measuring significant amounts of secreted IL-6, TNFα, IL-8 and IL-10 in the culture fluid of phagocytosing macrophages (Fig. 1C). The possibility that MCF-7 cancer cells dying through autophagy could convey pro-inflammatory signals for macrophages is intriguing since autophagy occurring in apoptosis-deficient tumors has been considered to be a tumor suppressor mechanism by preventing metabolic stress-induced necrosis and the subsequent inflammatory response which favors tumor progression.[13] According to our results there are circumstances, for example during phagocytosis of cells triggered to die by autophagy as presented here, when a pro-inflammatory response is generated by autophagic cells themselves in macrophages. The inflammatory response evoked by cells dying through autophagy, especially if it occurs in tumor-associated

macrophages,[14] can favor tumor progression by promoting cell proliferation and angiogenesis. This possibility adds a new angle to the already complicated connections between autophagy and cancer: the conflicting pro-survival and pro-death functions of autophagy in cancer cells[15] may also be manifested in the opposing anti-inflammatory and pro-inflammatory effects mediated by autophagy. Better understanding of the molecular mechanism of both phenomena may lead to more rational design of autophagy-based therapeutic interventions. Ongoing investigations in our laboratory are aimed at clarifying whether the pro-inflammatory response of macrophages to engulfed autophagic dying cells is restricted to cancer cells and to learn which signaling pathways are involved in its induction.

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