

Execution and elimination of apoptotic cells

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Abbreviations

Act D	actinomycin D
ATP	adenosine triphosphate
BMDM	bone marrow derived macrophages
CHAPS	3-[(3-cloamidopropyl)-dimethylammonio]propanesulfate
CHX	Cycloheximide
CTL	cytotoxic lymphocytes
DEVD-afc	Asp-Glu-Val-Asp-aminotrifluoromethylcoumarine
DIV	days in vitro
DMSO	dimethylsulfoxide
DTT	dithiothreitol
E.coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylglycol-bis(β -aminoethylether)tetraacetic acid
HMDM	human monocyte derived macrophages
HSC	heat shock cognate
HSP	heat shock protein
HT	heat denatured
IFN	interferon
Ig	immunoglobuline
IL	interleukin
iNOS	inducible NO synthase
LAL	limulus ameocyte lysate
LBP	LPS binding protein
LDH	lactat dehydrogenase
l.e.	low endotoxin
LPS	lipopolysaccharide
LTA	lipoteichoic acid
M-CSF	macrophage colony stimulating factor
MTT	3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide
NAD	nicotinamide adenine dinucleotide
NF κ B	nuclear factor kappa B
NO	nitric oxide
NPC	non-parenchymal cells
PBS	phosphate buffered saline
PBR	benzodiazepine receptor
PDE	phosphodiesterase
PGE	prostaglandin E
PI	phagocytotic index
PKC	protein kinase C
SDS	sodium dodecylsulfate
STS	staurosporine
TB	trypan blue
TLR	toll like receptor
TMR	tetramethylrhodamine
TNF	tumour necrosis factor
zVAD-fmk	z-Val-Ala-DL-Asp-fluormethylketone

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1 INTRODUCTION

1.1 Modes of cell death

1.1.1 Apoptosis

Apoptosis is a greek word, describing the falling of leaves from trees in autumn. This process is not the result of damage, but a regulated process occurring in annual rhythm. In analogy the term apoptosis was also used to define programmed cell death that was found to follow a conserved morphological pattern in various tissues and cell types (Kerr *et al.*, 1972) from the nematode *Caenorhabditis elegans* to mammals (Horvitz, 1999; Metzstein *et al.*, 1998).

Apoptosis plays important roles in animal development and tissue homeostasis and is therefore referred to as physiological cell death. The need to remove unwanted or injured cells can be explained by the strategy of metazoan animals to produce more cells than are eventually needed. In some cases it seems to be more appropriate to produce large numbers of cells and select them afterwards, instead of taking care for a perfect generation and a long life span. Estimations exist that a human body consists of 10^{14} cells and has a daily turnover of 10^{12} cells (Kerr *et al.*, 1972), representing one percent and indicating the short life span of many cells in the human body.

Apoptosis is an active cellular process, which follows an intrinsic suicide program and ends with a systematic destruction and removal of the cell. The identification of the proteins involved in execution and regulation of this process revealed an unusual class of cysteine proteases, now termed caspases (for cysteine aspartase). The activation of these caspases is followed by the coordinated degradation of structural and functional components followed by morphological changes like cell shrinkage, detachment from neighbouring cells and chromatin condensation (Oberhammer *et al.*, 1993; Wyllie, 1980).

Beside this physiological role of apoptosis, it can also occur in pathological situations in a wide variety of diseases, including cancer, AIDS, ischaemic stroke and many neurodegenerative disorders like Parkinson`s, Alzheimer`s and Huntington`s disease (Mattson, 2000).

Cancer for examples may occur when mutated cells are able to bypass or stop the apoptotic program and in non-infected CD4⁺ cells of AIDS patients apoptosis is induced by HIV components (Roshal *et al.*, 2001).

1.1.2 Alternative forms

The caspases execute apoptosis in programmed cell death. Nevertheless some cases of caspase independent controlled cell death are known now and can be taken as indication that cells may die a programmed cell death by several ways. This subject of programmed cell death was reviewed by Leist (Leist and Jaattela, 2001).

For example cells can survive the activation of caspases (Jaattela *et al.*, 1998; Lacana *et al.*, 1997; Wright *et al.*, 1997), indicating that other mechanisms should exist to kill these

cells. On the other hand apoptosis like cell death can occur in the absence of caspase activation (Foghsgaard *et al.*, 2001; Lavoie *et al.*, 1998; Rabinovitch, 1983) or the induction of apoptosis can result in a non-apoptotic death under some circumstances (Leist *et al.*, 1997; Leist *et al.*, 1999a; Vabulas *et al.*, 2002). As apoptotic cells are known to influence the macrophage response, one should also ask the question how cells that are dying these 'alternative' death programs can influence macrophage recognition and response.

1.1.3 Necrosis

Necrosis is characterised by the loss of membrane integrity and the release of intracellular contents, which may cause an inflammatory response (Searle *et al.*, 1982). The induction of an inflammatory response provides an important defence mechanism against tissue damage and invading microorganisms. Mediators of this protection are infiltrating neutrophils and macrophages, which can phagocytose tissue debris and microorganisms.

The fact that neutrophils are detected within areas of tissue necrosis was judged as an evidence for the inflammatory properties of necrotic cells (Searle *et al.*, 1982). How the recruitment and induction of inflammation occurs is not known (Green and Beere, 2000; Savill, 1997), but chemokines seem to play an important role (DeVries *et al.*, 1999; Ebnet *et al.*, 1996; Lentsch *et al.*, 1998; Tessier *et al.*, 1997).

Another important factor in induction of inflammation is the high mobility group 1 (HMGB1) protein (Abraham *et al.*, 2000; Andersson *et al.*, 2000; Wang *et al.*, 1999). Released exclusively by necrotic cells after cell lysis and not after induction of apoptosis, this protein is able to promote inflammation. Monocytes produce the inflammatory cytokine TNF α after stimulation with necrotic cells, but fail when apoptotic cells or necrotic Hmgb1⁻ cells are used (Scaffidi *et al.*, 2002).

Necrosis takes place exclusively in pathological settings, for example after exposure to high concentrations of endogenous or exogenous toxins or high intensities of pathological insults (Leist and Nicotera, 1997; Leist and Nicotera, 1998). A further characteristic is the fast and uncontrolled progression, when compared with apoptosis. Therefore it was also described as accidental necrosis or cell lysis, which just can be prevented by the removal of the stimulus. Cellular oedema and absence of zeiosis are often associated with necrosis (Leist and Jaattela, 2001).

Interestingly, necrosis and apoptosis, often described as counterparts, can also be observed simultaneously in some settings. During the occlusion of vessels, cells with a necrotic morphology are found in the centre of tissue damage (anoxic region). These regions are surrounded by apoptotic cells (hypoxic region), the so-called penumbra (Leist *et al.*, 1995). This may serve as a further example for the notion that the intensity of the insult has an influence on the prevalence of apoptosis and necrosis (Bonfoco *et al.*, 1995).

One of the molecular switches between apoptosis and necrosis is represented by the energy levels available after induction of cell death. Cells challenged with a suitable stimulus died by apoptosis in the presence of ATP, whereas ATP depleted cells exhibited typical necrosis features after stimulation with the same cell death inducer (Eguchi *et al.*, 1997; Leist *et al.*, 1997; Leist *et al.*, 1999a; Leist *et al.*, 1999b).

1.2 Interactions of phagocytes and apoptotic cells

The very last and perhaps most important step in programmed cell death is the removal of the dying cells (Ren and Savill, 1998). This is best studied for apoptosis. The recognition and uptake of apoptotic cells is done by different kinds of macrophages and also by neighbouring cells, so called semi-professional phagocytes. The interactions of macrophages with apoptotic cells and especially the recognition mechanisms are of special interest. The research in this field already described several receptors on the surface of macrophages and ligands on apoptotic cells, and new ones are still found.

1.2.1 Surface changes on the apoptotic cell

In order to take up an apoptotic cell, a phagocyte has to recognize this cell by a ligand that is not present on a healthy cell. One could also imagine a negative selection, where a specific ligand is always present on vital cells, but missing on apoptotic cells (Brown *et al.*, 2002).

Ligands fulfilling the first criterion are phosphatidylserine in the outer leaflet of the plasma membrane, changes in the pattern of glycosylation of cell surface proteins, and surface charge (Aderem and Underhill, 1999; Li *et al.*, 1999; Raff, 1998). Recognition by the phagocyte normally occurs before lysis of the apoptotic cell. Therefore surface changes on apoptotic cells should be early processes during apoptosis.

Beside PS exposure, as an early signal evidence is mounting that the complexity on the surface of apoptotic cells is increasing during the ongoing of apoptosis (Pradhan *et al.*, 1997).

Phosphatidylserine exposure

One of the most characteristic surface changes on apoptotic cells is the exposure of phosphatidylserine (PS) on their cell surface. In vital cells an asymmetric distribution, with PS at the inner leaflet of the plasma membrane, is maintained by an aminophospholipid translocase (Bever *et al.*, 1999; Daleke and Lyles, 2000). PS exposure at the outer leaflet during apoptosis seems often to involve a calcium-dependent activation of phospholipid scramblase. This enzyme moves phospholipids bi-directional across the membrane, resulting in the rapid increase of PS on the surface during apoptosis (Sims and Wiedmer, 2001).

At least four homologs of the scramblase are found in human and mouse, but their exact function during apoptosis could not be described yet (Sims and Wiedmer, 2001). Interestingly, it could be shown that scramblase 1 is phosphorylated by PKC δ during cell death and this kinase can be cleaved during apoptosis by caspase-3 resulting in a fragment with increased activity (Datta *et al.*, 1997; Frasch *et al.*, 2000; Pongracz *et al.*, 1999).

In all mammalian cells undergoing apoptosis the phospholipid asymmetry is lost, except for some tumour cell lines (Fadeel *et al.*, 1999; Fadok *et al.*, 2001c). This circumstance was detected so reliable that PS exposure is used as a marker of apoptosis. PS appears on the surface quite early during apoptosis, before nuclear changes and the lysis of the plasma membrane are observed (Chan *et al.*, 1998; Zhuang *et al.*, 1998). Interestingly, PS is

detected after the loss of mitochondrial membrane potential, giving rise to the view that mitochondria could release factors that may mediate the translocation (Chan *et al.*, 1998). PS exposure is shown by binding of fluorescence labelled annexin V, a calcium-dependent phospholipid-binding protein with high affinity for PS (Vermes *et al.*, 1995). Nevertheless, the specific events leading to PS exposure are not clear yet. These events are associated with the activation of caspases (Martin *et al.*, 1996), but it was shown that PS exposure can also occur by disturbing the calcium-homeostasis. The effect is observed in the absence of caspase activation and nuclear changes like chromatin condensation (Verhoven *et al.*, 1999).

Carbohydrate changes

Another class of surface changes observed on the surface of apoptotic cells are changes of membrane carbohydrates. These changes can induce the recognition and removal of apoptotic cells (Dini *et al.*, 1992; Duvall *et al.*, 1985).

In some publications a lectin-like activity was suggested, other authors discussed alterations of the surface charge as an effect of carbohydrate moieties on apoptotic cells (Morris *et al.*, 1984; Savill *et al.*, 1989). However, specific sugars on apoptotic cells were not identified. Interestingly, the removal of apoptotic cells (peripheral blood lymphocytes) in the liver seems to be facilitated by increased expression of mannose, N-acetylgalactosamine and D- galactose (Dini, 2000; Falasca *et al.*, 1996).

Oxidation

Another modification of apoptotic cells that can influence recognition by macrophages was observed with the help of a monoclonal antibody that recognizes oxidized forms of choline-containing phospholipids. This antibody binds only to the surface of apoptotic cells and inhibits uptake by macrophages (Chang *et al.*, 1999). These results indicated that the redistribution of phosphatidylserine during apoptosis is followed by its oxidation (Kagan *et al.*, 2000; Kagan *et al.*, 2002; Tyurina *et al.*, 2000).

CD31

CD31, also known as platelet-endothelial cell adhesion molecule-1, is not a typical ligand for apoptotic cells. Interestingly, this ligand prevents attachment and ingestion of viable cells by transmitting 'detachment' signals and can change its function on apoptotic cells. This results in tethering of apoptotic cells to phagocytes and could facilitate recognition and uptake (Brown *et al.*, 2002).

1.2.2 The recognition by the phagocyte

Phosphatidylserine receptor

PS expression present on the surface of most apoptotic cells appears to be also a critical element in the recognition and uptake. Several potential recognition receptors like scavenger receptors (Fadok *et al.*, 1998c; Shimaoka *et al.*, 2000; Terpstra and van Berkel, 2000), LDL-receptors (Fadok *et al.*, 1998a; Sambrano and Steinberg, 1995) or CD14 (Devitt *et al.*, 1998) were discussed. In addition some soluble proteins, like Gas-6 (Ishimoto *et al.*, 2000) or MFG-E8 (Hanayama *et al.*, 2002) that can recognize and bind PS on apoptotic cells were discussed.

Recently, a further PS-receptor was found that shows stereospecific binding to PS interestingly. An antibody against this receptor can inhibit uptake of apoptotic cells, exclusively by activated macrophages. Furthermore, transfection of non-phagocytosing cells with this receptor enables them to recognize and take up apoptotic cells (Fadok *et al.*, 2000).

Lectins

Lectin-like receptors are known to be involved in the cell-cell contact and their involvement can be recognized quite easily because addition of sugars can inhibit binding by interactions with lectins. In this way the binding of apoptotic cells to macrophages was inhibited by addition of N-acetyl glucosamine (Duvall *et al.*, 1985). Further studies stressed the importance of lectin-like receptors especially in cases where apoptotic cells are recognized by semi-professional phagocytes (Dini *et al.*, 1992; Dini *et al.*, 1995; Hall *et al.*, 1994).

Integrins, thrombospondin and CD36

The $\alpha_v\beta_3$ integrin is known to mediate adhesion to the extracellular matrix (Pytela *et al.*, 1985), but is also involved in the recognition of apoptotic cells (Savill *et al.*, 1992). Thrombospondine is a protein secreted by macrophages and other cells that can form a molecular bridge between apoptotic cells and macrophages via $\alpha_v\beta_3$ and CD36 (Savill *et al.*, 1992), but their ligand on the apoptotic cell remains still unknown. Another bridging molecule that connects $\alpha_v\beta_3$ integrin and PS on apoptotic cells was identified as MFG-E8 (Hanayama *et al.*, 2002).

The uptake of apoptotic cells could be inhibited by specific tetrapeptides which bind to $\alpha_v\beta_3$ integrin, PS-liposomes and an antibody against CD36 (Fadok *et al.*, 1998c). Therefore CD36 was suggested to be a PS-receptor (Tait and Smith, 1999).

A homologous protein to CD36 in *Drosophila* called Croquemort was demonstrated to play an important role in the removal of apoptotic cells in the fly (Franc *et al.*, 1999).

CD14

CD14 is a soluble or glycosphosphoinositole-linked protein and involved in the LPS signal transduction via toll-like receptor 4. Another function seems to be the recognition of apoptotic lymphocytes. An antibody for CD14 was able to inhibit phagocytosis of apoptotic cells (Devitt *et al.*, 1998). Interestingly, binding of this antibody on apoptotic cells could inhibit LPS signalling measured as TNF α release. This observation suggests that the same regions of CD14 are responsible for LPS signalling and recognition of apoptotic cells (Devitt *et al.*, 1998). On the other hand apoptotic cells also reduce TNF α production via the release of TGF β (Fadok *et al.*, 1998b) and therefore it is still open whether the binding sites are identical.

Other receptors

In addition to the mentioned receptors above many others are known to act as surface receptors for the recognition of apoptotic cells. A monoclonal antibody against scavenger receptor-A was able to inhibit the uptake of apoptotic thymocytes and comparable defects have also been present in scavenger receptor-A null mice (Platt *et al.*, 1996).

Another candidate is an ABC-transporter, a structural homologue of Ced-7, that seems to be required in uptake of apoptotic cells. This ABC1-transporter is a transmembrane protein, suggesting that it is also involved in the signalling processes necessary for uptake (Luciani and Chimini, 1996).

1.2.3 Macrophages as executioner of apoptosis

Phagocytes are responsible for the removal of apoptotic cells. In addition, new studies indicated that they are also involved in the initiation or execution of apoptosis. These findings stress the importance of the macrophage during apoptosis, especially with respect to the initiation and the fact that most of the apoptotic changes take place after the cell has been already engulfed by the phagocyte (Ichas *et al.*, 1997; Nakamura *et al.*, 1997; Nakamura *et al.*, 1995).

These indications received from studies in *C. elegans*, which described a positive feedback loop between the engulfment machinery in phagocytotic cells and the cell death machinery in apoptotic cells. Mutations that block engulfment were able to enhance the ability of partial loss of function mutation of pro-apoptotic genes to rescue cells destined to die by phagocytosis (Hoeppner *et al.*, 2001; Reddien *et al.*, 2001).

In a number of other cases macrophages can induce cell death. Cytotoxicity of macrophages was described to contribute to tumour elimination by releasing TNF α , TRAIL or NO (Griffith *et al.*, 1999; Keller *et al.*, 1990) or *in vivo* towards vascular endothelial cells during capillary regression in the rat eye (Diez-Roux and Lang, 1997; Lang and Bishop, 1993). Further examples for the initiation of apoptosis by macrophages are the killing of myofibroblast-like mesangial cells, when co-cultured with macrophages (Duffield *et al.*, 2000) or experiments with the developing rat eye, where a depletion of macrophages influences the formation of the lens (Lang and Bishop, 1993).

The new fact that feedback loops can exist during this process was not known. Now it could be speculated that activation of macrophages might be a reaction on factors of apoptotic cells, inducing pathways that are normally activated by eat-me signals on the surface of apoptotic cells (Conradt, 2002).

1.2.4 Immunomodulation by phagocytosis of dying cells

Phagocytosis by macrophages or neighbouring cells is the final event in many cells undergoing apoptosis (Savill *et al.*, 1993; Wyllie *et al.*, 1980). Removal of these cells occurs before lysis and prevents the release of potentially toxic and immunogenic intracellular contents into the surrounding tissue. Therefore, phagocytosis of apoptotic cells by macrophages has been suggested to be a process that does not lead to production of inflammatory mediators. In further studies the question was asked whether this silent process is the result of a passive lack of response or more an active suppression of proinflammatory mediator production (Fadok *et al.*, 1998b).

1.2.4.1 Suppression of inflammation

Whereas phagocytosis of bacteria or zymosan results in the release of inflammatory mediators by macrophages, the engulfment of apoptotic cells has no influence (Kerr *et al.*, 1972; Stern *et al.*, 1996) on these mediators and rather contributes to an anti-inflammatory environment (Fadok *et al.*, 1998b; McDonald *et al.*, 1999). After incubation with apoptotic cells stimulation with LPS, *E.coli* or zymosan results in an increase of anti-inflammatory and in a decrease of inflammatory cytokines. After phagocytosis of apoptotic neutrophils by macrophages a decrease of their production of IL-1 β , IL-8, IL-10, GM-CSF, TNF α , leukotriene C₄ and thromboxan B₂ was observed. On the other hand, an increase of TGF- β , prostaglandin E₂, and platelet-activating factor was seen. These factors seem to be responsible for the effect of apoptotic cells, as their addition in the absence of apoptotic cells also inhibited pro-inflammatory cytokine production. In addition, the inhibition of TGF- β and PAF restored the LPS induced cytokine production in macrophages after phagocytosis of apoptotic cells (Fadok *et al.*, 1998b).

In the same study a further indication was found that the receptors involved in recognition and/or uptake determine the response by the macrophage. Opsonisation of apoptotic cells with immunoglobulin G results in recognition of the antibodies via the Fc-receptor. This pre-treatment of apoptotic cells had no effect on the lipopolysaccharide induced production of inflammatory mediators, in contrast to the effects seen when apoptotic cells were added without previous opsonisation.

A comparable result was seen with apoptotic eosinophils, where Fc-receptor mediated uptake was stimulatory and the one of apoptotic cells had no effect on macrophage response (Fadok *et al.*, 1998b; Stern *et al.*, 1996).

With regard to chemokines an interesting finding was the suppression of Mip-1 α (Mip, macrophage inflammatory protein) and Mip-2, both known as chemoattractants for neutrophils. Their decreased production could result in an inhibition of neutrophil recruitment to inflammatory sites. In addition, the release of MCP-1 (MCP, monocyte chemotactic protein) is increased and could contribute to the recruitment of macrophages into the inflamed site and the resolution of inflammation (Fadok *et al.*, 2001a).

The question was asked which ligands and receptors are responsible for the establishment of the anti-inflammatory environment (Fadok *et al.*, 2001a; Fadok *et al.*, 2000; Henson *et al.*, 2001). First indication for an interesting ligand on apoptotic cells came from experiments, where phosphatidylserine-containing liposomes were used to mimic the effects of apoptotic cells. These experiments were successful, suggesting PS, a classical marker for apoptotic cells, as one important ligand for the recognition by macrophages and the induction of an anti-inflammatory environment (Savill, 1997). In further experiments a PS-receptor on macrophages was identified and antibodies against this receptor were also able to mimic the effects of apoptotic cells as measured by the production of TGF β by the macrophages (Savill, 1997).

Nevertheless, one could imagine a situation, where phagocytosis might not be sufficient or even impaired. In these situations apoptotic cells could release factors that may contribute to organ damage (Hentze *et al.*, 2001; Jahr *et al.*, 2001; Renz *et al.*, 2001). For pharmacological intervention it could be therefore interesting to promote the resolution of inflammation by stimulating the nonphlogistic phagocytosis of apoptotic cells (Godson *et al.*, 2000; Liu *et al.*, 1999).

1.2.4.2 Induction of immunity

When macrophages recognize apoptotic cells and are stimulated subsequently with LPS the production of inflammatory mediators like TNF is downregulated by anti-inflammatory mediators like TGF β and PGE $_2$ (Henson *et al.*, 2001).

Especially the influence of apoptotic cells on dendritic cells is of interest, because phagocytosis of apoptotic tumour cells can activate cytotoxic lymphocytes to kill these tumour cells (Henson *et al.*, 2001).

Another example that impaired phagocytosis of apoptotic cells could be an important factor in the pathogenesis of systemic autoimmune diseases, like systemic lupus erythematosus, was presented in mice lacking the c-mer membrane tyrosine kinase (Cohen *et al.*, 2002). This protein is believed to bind to the protein Gas6, which in turn bind to PS (Chen *et al.*, 1997) and in this way recognises apoptotic cells. Therefore, c-mer-deficient mice are characterised by an impaired clearance of infused apoptotic cells and the development of progressive lupus-like autoimmunity (antibodies against chromatin, DNA, and IgG) (Cohen *et al.*, 2002).

These results seem to contradict the observation that apoptotic cells cannot stimulate dendritic cells to induce expression of antigen-presenting and co-stimulatory molecules on dendritic cells, whereas necrotic cells can and in this way strongly stimulate the activation of T cells (Basu *et al.*, 2000; Sauter *et al.*, 2000; Steinman *et al.*, 2000). It was shown that heat shock proteins released from lysed cells can bind to CD91 on antigen presenting cells and thereby stimulate cytotoxic T cells (Basu *et al.*, 2001).

The uptake of apoptotic cells was also suggested to be tolergenic. For this reason apoptosis could be a possibility for tumour cells or intracellular pathogens to avoid immune surveillance (Fadok *et al.*, 2001b; Green and Beere, 2000; Steinman *et al.*, 2000).

1.3 The diverse functions of Hsp72

1.3.1 Introduction

The heat shock protein (Hsp) 72 protein, also named Hsp70, is one member of the Hsp70 family. The human Hsp70 family encompasses a group of highly related proteins. In the human genome at least eleven genes are known which encode these proteins. They are found in all major subcellular compartments. Beside the cognate forms some inducible forms are known. These forms are an indication of the importance of the proteins in the stress response (Tavaria *et al.*, 1996).

In first reports an increase in the protein synthesis of some proteins was seen after heat-induced puffing of fruit fly salivary gland chromosomes (Tissieres *et al.*, 1974). These findings gave rise to the abbreviation Hsp for **heat shock proteins**.

Beside heat many other stress situations like cold, osmotic imbalance, toxins, heavy metals, irradiation, viral infections and pathophysiological signals such as cytokines and eicosanoids can trigger the induction of these proteins (Lindquist and Craig, 1988; Welch, 1993).

1.3.2 Hsp72 is a chaperon

As mentioned before human cells contain several Hsp70 family members. The focus of this work was put on the highly stress inducible protein Hsp70 (corresponds to Hsp72). Under normal conditions Hsp70 functions as an ATP dependent molecular chaperon by assisting the folding of newly synthesized polypeptides, the assembly of multiprotein complexes, the transport of proteins across cellular membranes (Beckmann *et al.*, 1990; Chiang *et al.*, 1989; Murakami *et al.*, 1988; Shi and Thomas, 1992) and the degradation of proteins (Hayes and Dice, 1996). Molecular chaperones are defined as 'proteins that assist the correct non-covalent assembly of other protein-containing structures *in vivo* but are not permanent components of these structures when they are performing their normal biological function' (Ellis, 1996).

Under stress conditions the induction of Hsp70 synthesis enhances the ability of the cell to cope with increased concentrations of unfolded and denatured proteins (Nollen *et al.*, 1999). The cell-stress response is an evolutionarily ancient, ubiquitous and essential mechanism for cell survival. This is reflected in the conservation of the protein structure of the molecular chaperones throughout living organisms (Gething, 1997; Lindquist and Craig, 1988). In cultured cells the potential of Hsp70 to inhibit cellular death processes was demonstrated (Jaattela and Wissing, 1993; Jaattela *et al.*, 1992; Jaattela *et al.*, 1998; Mosser *et al.*, 1997). Other data suggested an important protective role for Hsp70 also *in vivo* reviewed by Jäättelä (Jaattela, 1999b).

1.3.3 Hsp72 and apoptosis

The protective function of Hsp70 can be attributed to the prevention of protein aggregation and promotion of protein disaggregation by catalysing the refolding of damaged or

denatured proteins (Gething and Sambrook, 1992; Parsell and Lindquist, 1993; Parsell *et al.*, 1993) and thus is explained by the chaperone activity of protein. Several other studies have further shown that Hsp70 is able to modulate the engagement and/or progression of apoptosis induced by a wide variety of stimuli (Jaattela, 1999a). This modulation was also seen after destruction of the chaperone activity of Hsp70 by deletion of the ATPase domain, when cells were stimulated with non-denaturing apoptotic stimuli like TNF α or staurosporine (Jaattela *et al.*, 1998). It therefore follows that Hsp70 may directly influence apoptotic signalling pathways. Further arguments for this point of view are the interaction of Hsp70 with JNK1 (c-Jun N-terminal kinase 1). This contact is followed by the suppression of JNK activation and JNK-mediated cell death (Buzzard *et al.*, 1998; Park *et al.*, 2001). In detail JNK suppression was reported to occur by Hsp72-mediated stimulation of JNK phosphatase that inactivates the stress kinase after protein-damaging treatments and in this way prevents apoptosis (Meriin *et al.*, 1999). Similar results were obtained in systems with TNF induced apoptosis. Furthermore, this inhibition was also seen with a mutated Hsp70EEVD. This form is characterised by a deletion of the four C-terminal amino acids, which are essential for the chaperone function (Gabai *et al.*, 2002).

Another influence of Hsp70 on the apoptotic signalling was shown via binding directly to apoptosis protease activating factor-1 (Apaf-1) and thereby the prevention of procaspase9 recruitment to the apoptosome (Beere *et al.*, 2000; Saleh *et al.*, 2000) and the interaction with apoptosis inducing factor (AIF) in Apaf^{-/-} cells (Ravagnan *et al.*, 2001). In both cases Hsp70 protected against the induction of cell death. Nevertheless, Hsp70 failed to interact with the CARD motif of Apaf-1 in the absence of ATP, an indication that the chaperone activity is critical to this interaction. On the other hand the ATP-binding domain seemed to be dispensable for AIF binding and inhibition (Ravagnan *et al.*, 2001).

1.3.4 Hsp72 on the surface of cells

Heat shock proteins are normally localised in the cytoplasm and cellular organelles. Interestingly, Hsp70 was found on the cell surface of cancer cells (Ferrarini *et al.*, 1992; Multhoff *et al.*, 1995) and on the membrane of T cells undergoing apoptosis (Poccia *et al.*, 1996). Little is known about the cellular pathways leading to Hsp70 expression on the cell surface. In many studies Hsp70 was shown to be highly immunostimulatory in the pathology of several autoimmune diseases and in the antitumour immune response *in vivo* and *in vitro*. Therefore, it was suggested that the surface expression of Hsp70 in sarcoma cells correlates with the lysis of these cells mediated by NK cells (Multhoff *et al.*, 1995). Interestingly, Hsp70 expression was not found on normal cells, indicating that Hsp70 could be a tumour-specific target structure for immunocompetent effector cells.

Indeed, in human colon carcinoma cells (CX2) Hsp70 was found on 60 % of the cells and the Hsp70-positive cells were sensitive to lysis mediated by adherent non-MHC-restricted effector cells, indicating that Hsp72 might act as a tumour-specific recognition structure for a distinct NK cell population (Multhoff *et al.*, 1997). This could be confirmed by studies with a 14-mer peptide that is part of the C-terminal domain of Hsp70 and was able to stimulate proliferation and the cytolytic activity of NK cells against Hsp70-expressing tumour cells. Therefore, the role of NK cell receptors *in vivo* might be an NK cell-mediated elimination of stressed cells, including tumour cells expressing HSP70 on their cell surface (Multhoff *et al.*, 2001).

1.3.5 Hsp72 in adaptive immunity

Another role of Hsp72 among the already described ones is the one it plays in eliciting a specific immune response against tumour-derived and viral antigens (Srivastava *et al.*, 1986). Hsp70 preparations from cancer cells elicited immunity to these cells, but preparations from normal tissues did not (Udono and Srivastava, 1993). In further studies it could be shown that the immunogenicity of tumour-derived heat shock proteins resulted from peptides non-covalently associated with the Hsps. These peptides originated from degradation of proteins in the corresponding cells and represent a unique collection of all the peptides generated in a cell, tumour or virus-infected tissue that occurs *in vivo*. These Hsp-peptide complexes were used successfully for the therapy of a variety of cancers (Tamura *et al.*, 1997).

The success of Hsp70-peptide complexes in activating T cells was explained by cross-priming. An antigen-expressing cell might not directly stimulate the T cells that recognize that antigen. This is observed with cancer cells and their major histocompatibility complex (MHC) class I molecules presenting also peptides originated from their mutated proteins. The antigen must often be transferred from the antigen-expressing cell to a specialised immune cell that then stimulates the naive T cell. This transfer can be observed after infection followed by cell lysis. Then antigen-presenting cells can bind and take up Hsp70-peptide complexes via the CD91 receptor and cross-present these specific peptides to CD4⁺ and CD8⁺ T cells. The same events are supposed to occur when Hsp70-peptide complexes are used for cancer therapy (Blachere *et al.*, 1997).

Exogenous antigens taken up by antigen presenting cells (APC) are normally presented via MHC class II molecules. Interestingly, the Hsp70-peptide complexes are also introduced into the MHC presentation pathway and therefore can generate a powerful CD8⁺ T cell response (Yewdell, 1999). For more details on Hsp70 in adaptive immunity, Srivastava wrote an excellent review (Srivastava, 2002).

1.3.6 Hsp72 and toll-like receptor 4

In patients with autoimmune diseases increased levels of antibodies against the inducible form of Hsp70 (= Hsp72) were found. For this reason it was speculated that Hsp70 can be found in the extracellular milieu. Exogenous Hsp70 was shown to act as a cytokine to human monocytes by stimulating an inflammatory transduction cascade that resulted in the release of interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumour necrosis factor α (TNF α) (Asea *et al.*, 2000b). An immediate release of Hsp70 into the circulation was seen after coronary artery bypass grafting (Dybdahl *et al.*, 2002). By definition, cytokines are proteins secreted by cells with regulatory effects on other cells (Janeway and Travers, 1997). The term chaperokine was introduced to define the dual function of Hsp70 as a chaperone and a cytokine (Asea *et al.*, 2000a). Nevertheless, the pathways of a regulated release of Hsp70 are still crucial.

The signal transduction of Hsp70 could be shown to act via at least two pathways. One is independent on CD14 but dependent on intracellular calcium and another one depends on CD14 plus intracellular calcium in human monocytes (Asea *et al.*, 2000b). CD14 is a glycosylphosphatidylinositol-anchored membrane protein that also exists in a soluble form but lacking transmembrane and intracellular signalling domains. The function of CD14 as

a co-receptor for LPS signalling involves the toll-like receptors (TLR) 2 and 4 (Politorak *et al.*, 1998; Yang *et al.*, 1998) and suggested that toll-like receptors participate in the response to Hsp70.

In a mouse model the chaperokine effect of Hsp72 is transduced via a Ca^{2+} and $\text{NF}\kappa\text{B}$ -dependent pathway. This study further demonstrated that the peptide-binding pocket of the chaperone was not involved in binding to the putative Hsp70 receptor (Asea *et al.*, 2000b). Hsp70 was therefore suggested to be a 'danger signal' that can result in augmentation of the immune response, activation of cytotoxic lymphocytes (CTL) and T helper functions.

A potential receptor for Hsp70 on human macrophages was characterised by binding studies (Sondermann *et al.*, 2000) and identified as the previously known α_2 -macroglobulin or CD91 (Binder *et al.*, 2001). Recently Hsp70 was defined as an endogenous stimulus for the Toll/IL-1 receptor signal pathway that engages TLR2 and TLR4 (Dybdahl *et al.*, 2002; Vabulas *et al.*, 2002).

2 AIMS OF THIS STUDY

Cell recognition, engulfment and digestion are the final steps in the process of apoptosis. Phagocytosis of apoptotic cells is known to be fast, efficient and non-inflammatory. Most interestingly it could be shown that this last step in apoptosis has an active anti-inflammatory effect. This effect could be explained in part by the existence of phosphatidylserine (PS) on the surface of apoptotic cells and a PS-receptor on macrophages that can influence the release of anti-inflammatory cytokines.

The present study was designed to:

- Set up a homologous system to investigate the effects of dying cells by:
 - I. Developing an *in vitro* co-culture model with primary murine macrophages and dying cells that is capable of monitoring the immunomodulating effects by the alterations in cytokine levels.
 - II. Establishing of cell death models to obtain dying cells from the BALB/c thymoma cell line S49.1 or primary thymocytes.
 - III. Establishing a highly sensitive phagocytosis assay for fluorescence labelled *Escherichia coli* (*E.coli*) particles to describe the effects of apoptotic cells on macrophages. This assay has to fulfill the criteria outlined by Stossel (Stossel, 1977) for quantitative phagocytosis assays.
- Describe the extracellular activity of caspase-3 released by apoptotic cells and after massive apoptosis in human samples.
- Investigate the function of extracellular Hsp70, that is known to be released from necrotic cells as a possible 'danger signal' by its potential to stimulate macrophages to release pro-inflammatory cytokines. Further experiments should help to describe the potential contamination of the protein solution with lipopolysaccharide (LPS).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Laboratory equipment and technical devices

Centrifuges: Biofuge fresco and Megafuge 1.0 R (Heraeus Instruments, Hanau, Germany). **ELISA-Reader:** SLT Spektra (SLT Labinstruments, Crailsheim, Germany). **Digital camera:** Hamamatsu Digital Camera C 4742-95, (Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany). **Fluorimeter:** Microplate Fluorescence Reader FL 600 (Deelux Labortechnik, Gödenstorf, Germany). **Imaging camera:** Dage-72 CCD camera (Dage-MTI, Michigan City, IN, USA). **Incubator:** Model BB 6220 (Heraeus Instruments, Hanau, Germany). **Laminar Flow:** LaminAir[®] HB 2448 (Heraeus Instruments, Fellbach, Germany). **Luminometer:** 1250 and Display 1250 (Wallac-ADL GmbH, Freiburg, Germany). **Microscopes:** Leitz DM IRB, Leitz DM IL (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany). **Pipettes:** Eppendorf (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). **Spectrophotometric analyser:** Eppendorf ACP 5040 (Netheler & Hinz GmbH, Hamburg, Germany).

3.1.2 Chemicals and antibodies

Bachem Biochemica GmbH, Heidelberg, Germany: z-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk)

Bender & Hobein GmbH, Heidelberg, Germany: Pierce BCA protein assay reagent.

Biomol, Hamburg, Germany: Asp-Glu-Val-Asp-aminotrifluoromethylcoumarine (DEVD-afc), Hsp70 (ESP-755, low endotoxin and SPP-755).

Boehringer Mannheim (Roche), Mannheim, Germany: Annexin V fluorescein-conjugated.

Calbiochem-Novabiochem GmbH, Schwalbach, Germany: calphostin C from *Cladosporium cladosporioides*.

Metalon, Wustenhofen, Germany: lipopolysaccharide (LPS) from *Salmonella abortus equi*.

Molecular Probes Europe BV, Leiden, Netherlands: calcein-AM, *E.coli* bioparticles, Hoechst 33342, SYTOX green, Alexa Fluor 594 anti rat IgG (H+J).

Pharmingen, Hamburg, Germany: OptEIA[™] Set: mouse TNF α (mono/mono), mouse IL-10 (mono/mono)

Serotec GmbH, Dusseldorf, Germany: rat anti mouse F4/80 antigen, MCAP497

Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany: actinomycin D (ActD), 3-[(3-cloamidopropyl)-dimethylammonio]-propanesulfate (CHAPS), cycloheximide (CHX), dimethylsulfoxide (DMSO), dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), ethylglycol-bis(β -aminoethylether) tetraacetic acid (EGTA), ionomycin, sodium dodecylsulfate (SDS), staurosporine (STS), TMB liquid substrate solution, Triton X-100, trypan blue 0.4 %, Tween 20.

3.1.3 Cell lines

Jurkat human T cell lymphoma clone E6-1 was obtained from the American tissue culture collection (ATCC no. TIB-152, Rockville, MD, USA)

S49.1 mouse lymphoma from BALB/c was obtained from the American tissue culture collection (ATCC no. TIB-28, Rockville, MD, USA)

Mouse fibroblast cell line L929csf was a kind gift from J. Barsig, Altana Pharma, Konstanz.

3.1.4 Cell culture material

RPMI 1640 with glutamine (FG 1215) and fetal calf serum was bought from Biochrom KG (Berlin, Germany). Penicillin, streptomycin and trypsin were purchased from Gibco BRL Life Technologies (Eggenstein, Germany).

Cell culture flasks and plates were obtained from Costar GmbH (Bodenheim, Germany) and Greiner GmbH (Nurtingen, Germany).

Cell inserts and cell strainers were purchased from Beckton Dickenson Labware (Franklin Lakes, NJ, USA).

3.1.5 Animals

Pregnant BALB/c mice were obtained from the animal unit of the University of Konstanz. Mice pups were used 1-2 days post partum.

For the preparation of BMDM, male BALB/c mice, six weeks old, were obtained from the animal unit of the University of Konstanz.

C3H/HeJ and C3H/HeN mice were obtained from Charles River (Schweinfurt, Germany).

The mouse strains C57Bl/10ScCr and C57Bl/10ScSn were a kind gift from Mrs. Freudenberg, MPI Immunology (Freiburg, Germany).

All mice were maintained under controlled conditions (22 °C, 55 % humidity, 12 h day/night rhythm) and fed a standard laboratory chow prior to the experiments.

3.2 Methods

3.2.1 Preparation and culturing of bone marrow derived macrophages

Mice were killed by cervical dislocation. Femurs and tibias of the hind legs were dislocated aseptically. Bone marrow cells were isolated by flushing the bones with RPMI 1640 medium with glutamine [2 mM] supplemented with penicillin [10000 U/ml] and streptomycin [10 mg/ml]. After repeated gentle aspiration through a 10 ml pipette the single cell suspension was left for one minute to separate remaining bone and tissue leftovers by sedimentation. The cells were centrifuged for 5 min at 190 x g, and the pellet was resuspended in 500 µl 0.17 M ammonium chloride to lyse erythrocytes and incubated for 1 min at room temperature (RT). To stop the incubation 9.5 ml medium was added, the cell suspension was centrifuged again and the pellet resuspended in medium. Cells were cultured at an initial density of 10⁵ cells/ml on 94/16-mm microbiology plates in 20 ml RPMI 1640 with glutamine supplemented with 10 % heat-inactivated FCS, penicillin/streptomycin and 20 % macrophage colony stimulating factor (M-CSF)-containing L929 supernatants for 6-9 days. Differentiated bone marrow derived macrophages (BMDM) were harvested with cold phosphate buffered saline (PBS) without Ca²⁺ and Mg²⁺ and resuspended in medium without L929 supernatant.

3.2.2 Characterisation of BMDM

3.2.2.1 Immunostaining

BMDM were identified as macrophages with the help of the F4/80 antibody. BMDM were seeded in different cell densities on 13 mm coverslips. At day 5, 7 and 9 the supernatant was removed, the cells were washed with PBS and fixed by the addition of 4 % PFA for 5 minutes. After fixation the cells were washed with PBS and stored in PBS / 0.1 % sodiumazide at 4 °C until staining. Cells were blocked for 1 h with PBS / 0.1 % BSA and incubated with the primary antibody F4/80 (Serotec, MCAP 497) over night in a dilution of 1:100 [10 µg/ml]. This monoclonal rat anti mouse antibody F4/80 (IgG) recognizes a 160 kD cell surface glycoprotein on mouse macrophages. A secondary antibody, AlexaTM Fluor 594-conjugated anti-rat IgG (H+J) (Molecular Probes) was employed. This antibody was diluted 1:100 [20 µg/ml] in PBS and incubated with the cells for 1 h. Chromatin was counterstained with 250 ng/ml Hoechst 33342. Cells were mounted in Aqua Polymount. Images of stained cells were obtained by a fluorescence microscope equipped with a digital camera.

3.2.2.2 Functional test

BMDM were identified by their ability to engulf fluorescence labelled *E.coli* particles (Molecular Probes) to discriminate them from the non-phagocytosing fibroblasts, which could originate from tissue at the preparation.

BMDM cells were seeded at different cell densities in 96-well microtiter plates. At day five to nine *E.coli* particles were added in a final concentration of 2 µg/ml for 1 hour. Then cells were washed twice with PBS. To count all cells in the fluorescent microscope DNA was counterstained by addition of 250 ng/ml Hoechst 33342. After incubation for 5 minutes at 37 °C the Hoechst and tetramethylrhodamine (TMR) stained cells were counted. Per well 200 cells were counted and expressed as percentage TMR positive cells of total cell number (Hoechst positive cells).

3.2.3 Preparation of M-CSF containing L929csf supernatants

The supernatant of the mouse L929csf cell line was used as a source of M-CSF. These fibroblasts are the product of a stable transfection with a M-CSF cDNA. The cells were maintained in culture in RPMI 1640 with glutamine supplemented with 10 % heat inactivated serum and penicillin/streptomycin in 225 cm² culture flasks. 3 x 10⁶ cells were seeded in 65 ml medium per flask and cultivated at 37 °C and 5 % CO₂. After seven days the supernatant was harvested and centrifuged for 10 minutes at 500 x g and 4 °C to remove cell debris. The supernatant of several flasks was mixed in a beaker and stored in 50 ml tubes at -20 °C for several months.

3.2.4 Test of M-CSF containing supernatants in bone marrow cells

To test the differentiation potential of the obtained supernatants, bone marrow cells were added to 96-well microtiter plates (4 x 10⁵ cells /ml, 100 µl/well) in RPMI 1640 with 10 % heat inactivated FCS and penicillin/streptomycin. Further 100 µl/well M-CSF containing supernatant were added to obtain final concentrations between 5 and 30 %. The cells were cultured at 37 °C and 5 % CO₂ for 6 and 10 days. Medium was removed and 100 µl fresh medium without M-CSF supernatant were added. After addition of 10 µl MTT solution (5 mg/ml) the plates were put back for 90 minutes into the incubator. The final steps are described in the MTT assay below. The supernatant was used at the lowest saturating concentration.

3.2.5 Preparation of mouse thymocytes

Mice were killed by cervical dislocation, swamped with 70 % ethanol. Skin was removed and the chest was opened along the sternum. The thymus was removed with forceps and placed in a sterile 70 µm cell strainer in a 50 ml tube. The thymus was minced through the strainer using the pestle of a 5 ml syringe. The strainer was rinsed with 10 ml RPMI 1640 supplemented with heat inactivated fetal calf serum and penicillin/streptomycin and the tube was filled up to 50 ml with medium. The tubes were centrifuged for 8 minutes at 190 x g, the supernatant was removed and the cell pellet was resuspended in 10 ml medium.

3.2.6 Preparation of human monocyte-derived macrophages

Human monocytes were isolated as described by Gantner (Gantner *et al.*, 1997). Peripheral venous blood was drawn from volunteers. To prevent coagulation citrate (0.31 % w/v) was added. The citrate blood was diluted 1.6 fold with PBS and centrifuged at 220 x g at 20 °C for 20 min. The cell pellet was resuspended and layered on a Percoll gradient ($\rho=1.077$ g/ml). After centrifugation at 800 x g for 25 min the peripheral blood mononuclear cells (PBMC) were enriched in the interphase. This phase was separated and washed twice in elutriation medium (PBS, 2 % heat-inactivated human AB serum, 2 mM EDTA, 5 mM glucose [pH 7.4]). The cells were obtained by countercurrent centrifugal elutriation using a J2-MC centrifuge equipped with a JE-6b rotor at a flow rate of 39 ml x min^{-1} and a rotor speed of 3000 rpm.

Monocytes were plated at a density of 1×10^5 cells/well on 96-well culture dishes. After 1 h at 37 °C the non-adherent cells were removed by medium exchange with RPMI 1640 containing 10 % heat-inactivated human AB serum, penicillin (5000 IU/ml) and streptomycin (5 mg/ml).

Monocytes were differentiated to macrophages for 8-12 days in a volume of 200 μl /well on 96-well culture dishes. Medium was exchanged 5 days after plating and prior to experiments.

3.2.7 Apoptotic and necrotic triggers in S49.1

Apoptosis was triggered by pre-incubation with actinomycin D (0.4 $\mu\text{g}/\text{ml}$, 1 h) followed by addition of CD95 ligand (10 % N2A CD95-ligand supernatant, 16 h). S49.1 cells were incubated in RPMI 1640 medium containing 1 % fetal calf serum. After stimulation the cells were washed twice with RPMI 1640 containing 10 % heat inactivated fetal calf serum supplemented with penicillin [10000 IU/ml] / streptomycin [10 mg/ml].

Necrotic cells were obtained by two independent methods: first by incubating cells at 56 °C for 20 min induced hyperthermia. The second method was the freeze-thaw of the cells. Cell suspension was centrifuged at 190 x g for 5 min in a 15 ml PE tube and supernatant was removed. The pellet was frozen in liquid nitrogen for 1 min and then thawed at 37 °C in a waterbath.

After both treatments cells showed necrotic cell morphology, characterised by the uptake of trypan blue.

3.2.8 Viability assays

3.2.8.1 MTT assay for adherent cells

To detect cell growth we used a solution of the tetrazolium salt MTT stock [5 mg/ml in PBS]. This compound is reduced to a blue coloured product formazan within functional mitochondria in living cells. The concentration of formazan is proportional to the number of viable cells and was measured spectrophotometrically. The assay was performed in flat bottom 96-well microtiter plates. Cells were incubated with 0.5 mg/ml MTT in medium for 1 h in the incubator. The supernatant was removed and cells were lysed by addition (100 μl /well) of a lysing mixture (iso-propanol:formic acid, 95:5). Absorption was

measured at 560/690 nm in an ELISA-reader.

3.2.8.2 MTT assay for cell suspensions

For experiments with non-adherent cells a variant of the MTT assay described above was used. Cell suspensions were incubated with MTT solution as previously described. At the assay end-point, the volume per well was reduced to 50 μ l to reduce background signal. Then, cells were lysed by direct addition of solubilisation solution [100 μ l/well] (20 g SDS dissolved in a 100 ml mixture of distilled water:N,N-dimethyl-formamide, 1:1). Plates were wrapped with aluminium foil to protect from light and stored overnight at room temperature to dissolve the formazan. Absorption was measured at 560/690 nm with an ELISA-reader.

3.2.8.3 SYTOX/Hoechst assay

To discriminate between vital, apoptotic and necrotic morphology, cells were stained with the combination of the fluorescent dyes Hoechst 33342 and SYTOX by adding the dye 5 minutes prior to counting. The solution was added in a 200x solution in DMSO. Hoechst 33342 [500 ng/ml] is membrane permeant and stains all nuclei. SYTOX [500 nM] is cell membrane impermeant and stains lysed cells. Cells with condensed or fragmented nuclei were scored as apoptotic; lysed cells with non-condensed nuclei were scored as necrotic. For each data point more than 200 cells were counted in at least three different microscopic fields.

3.2.8.4 LDH assay

Lactate dehydrogenase (LDH) as a cytosolic marker was determined according to Bergmeyer (Bergmeyer, 1984). The LDH in the supernatant was expressed in percent of the total LDH. In brief, supernatants were collected and remaining cells were lysed by addition of PBS / 0.1 % Triton X-100. Supernatant and cell lysates were stored at 4 °C and measured within one week. Determination of LDH activity was performed in an Eppendorf ACP 5040 Analyser. The reduction of nicotinamide adenine dinucleotide (NAD) to NADH was followed at 340 nm.

3.2.9 Determination of protein content

The protein concentration of cell lysates was determined by the BCA (bicinchoninic acid) method of Pierce. 10 μ l of cell lysate were pipetted on a 96-well microtiter plate and incubated with 200 μ l of reagents mixture. After 30 minutes the BCA reaction was finished. The optical density was measured at 550 nm with an ELISA reader. Calibration was performed with BSA in a concentration range of 0.2 to 2 mg/ml.

3.2.10 ELISA measurement

3.2.10.1 Human TNF α

A TNF α ELISA was used to determine the concentrations in supernatants, using specific antibodies purchased from PharMingen (Hamburg, Germany). Samples were diluted 5 fold.

3.2.10.2 Mouse TNF α /IL-10

The mouse TNF α and IL-10 OptEIA™ kit from PharMingen (Hamburg, Germany) was used to determine TNF α in supernatants of mouse BMDM. Samples were not diluted. The detection limit was 25 pg/ml.

3.2.11 DEVD-afc cleavage assay

3.2.11.1 Using cell lysates

For analysis of intracellular DEVD-afc cleavage activity of cell pellets or monolayers were lysed by addition of a lysing buffer containing HEPES (25 mM, pH 7.5), MgCl₂ (5 mM), EGTA (1 mM), Triton X-100 (0.5 %), leupeptin (1 μ g/ml), aprotinin (1 μ g/ml) and AEBSF (1 mM). After lysis the samples were stored at -20 °C until analysis.

The tests were performed in 96-well microtiter plates. The substrate DEVD-afc was used at a final concentration of 40 μ M. The cleavage was followed by fluorescence measurement of afc with an excitation wavelength of 390 nm and emission wavelength of 505 nm. The DEVD-afc cleavage activity was calculated using calibration curves generated with free afc. One unit represents 1 mmol afc generated within 1 min by the indicated amount of protein.

3.2.11.2 Total activity per well

The assay of whole well (ww) activity was introduced to include the DEVD-afc activity of lysed cells. Lysed cells lose caspases into the medium. By lysing the cells directly within their original medium a ww activity could be measured. Cell lysis was achieved by addition of 50 μ l of ww-buffer (100 mM HEPES (pH 7.5), 25 mM MgCl₂, 5 mM EGTA, 2.5 % Triton X-100, 20 mM DTT, and 5 % AEBSF) to 200 μ l medium. The further steps were performed as described above.

3.2.12 Determination of NO production

Cells were induced to produce nitric oxide (NO) by addition of different stimuli for 24 h. Supernatants were stored at -20 °C until measurement. NO production was determined indirectly by measuring nitrite levels in the supernatant by the Griess reaction (Ding *et al.*, 1988; Green *et al.*, 1982). The reaction was performed in flat bottom 96-well microtiter plates by addition of 20 μ l of 1 % sulfanilamid solution in 1.2 M HCl and 20 μ l of 0.1 % naphthylethyldiamin solution in distilled water. Samples on the plates were mixed on an agitator for 5 minutes. The absorption was measured at 550 nm to the reference at 690 nm.

3.2.13 Fluorescence labelled *E.coli* phagocytosis assay

3.2.13.1 Quenching with trypan blue

BMDM were plated in 96-well tissue culture plates in 200 µl per well and allowed to adhere for at least 1 hour. Fluorescence labelled *E.coli* particles (Molecular Probes) were added at final concentrations ranging from 1 to 10 µg/ml. In some experiments, the *E.coli* particles were opsonised with 10 % BALB/c mouse serum, fetal calf serum or heat inactivated fetal calf serum at 37 °C for 30 min prior to use.

Phagocytosis was stopped by addition of trypan blue solution to quench the fluorescence of surface attached bacteria. Fluorescence was determined at 530 nm excitation and 590 nm emission wavelengths using a fluorescence microplate reader. Cells without bacteria and cells incubated for a very short time ($t \leq 1$ min) were used to determine the background fluorescence. Inhibition by low temperature or cytochalasin D was used to distinguish between binding and phagocytosis of bacteria in preliminary experiments.

3.2.13.2 Lysis with Triton X-100

To increase sensitivity of the phagocytosis assay another method was used. It was performed as described (Oben and Foreman, 1988; Oda and Maeda, 1986; Uff *et al.*, 1993) with the following modifications. Cells were plated in 96-well microtiter plates and allowed to adhere for 1 hour. Fluorescence labelled *E.coli* particles were added at different times at final concentrations from 1 to 10 µg/ml. Phagocytosis was stopped by washing the cells twice with PBS to remove non-phagocytosed bacteria. Cells were lysed by addition of 100 µl/well PBS + 0.1 % Triton X-100. Fluorescence was determined as described above.

3.2.14 Phagocytosis of apoptotic thymocytes

BMDM were seeded on 48-well microtiter plate (3.8×10^5 cells/ml, 300 µl/well). Macrophages were stained with DiI [2.5 µg/ml, 20 min] and washed three times with PBS for 5 min before fresh medium was added. Thymocytes were stimulated with dexamethasone [1 µM, 6 h] and stained with calcein-AM [2 µM]. Staining was performed 20 min before the washing of thymocytes to remove dexamethasone.

Apoptotic thymocytes (8×10^6 cells/ml, 150 µl/well) were co-incubated with BMDM and washed away by addition of 500 µl/well PBS after the indicated time. This washing step was repeated 5 times. Phagocytosis was determined as phagocytotic index (PI).

3.2.15 Immunomodulation by apoptotic cells

BMDM were plated in 48 or 96-well microtiter plates and allowed to adhere for 1 hour. The volume per well was 100 or 200 µl. Apoptotic and necrotic mouse thymocytes were obtained as described above. Cells were counted, cell density adjusted and morphology was controlled by SYTOX/Hoechst staining. Cells were added in 100 or 200 µl medium per well (2×10^6 cells/ml) depending on the plates that were used. Apoptotic primary thymocytes were used at a density of 1×10^7 cells/ml and added in 100 or 200 µl medium per well.

The cells were co-incubated for two hours and washed twice with PBS. Fresh medium was added and cells were allowed to rest for one hour. Then, macrophages were stimulated as described in detail at the respective experiments. After different incubation times supernatants were stored at -80 °C until determination of released cytokines and NO.

3.2.16 Statistical analysis

Experiments were run in duplicates and repeated at least three times with different cell preparations. The r values of linear regressions were performed with the GraphPad Prism[®] 3 software. Non-linear regression was also performed with the Prism software. Details are described at the respective graphs.

4 RESULTS

4.1 Differentiation and characterisation of BMDM

A culture system to study immunomodulation by apoptotic cells was established. The advantages in using BMDM for cell culture experiments are the high yield of primary cells and the possibility to investigate the effects of gene knockouts in mice.

4.1.1 Characterisation of M-CSF containing supernatants

To culture BMDM M-CSF is needed to differentiate bone marrow cells into macrophages. M-CSF is available as recombinant protein or in supernatant of the M-CSF transfected fibroblastic cell line L929csf. These cells release the M-CSF protein in an active form. To test the activity of the supernatants the MTT assay was used to characterise the number of bone marrow cells differentiated into macrophages. The reduction of MTT correlates with the cell number.

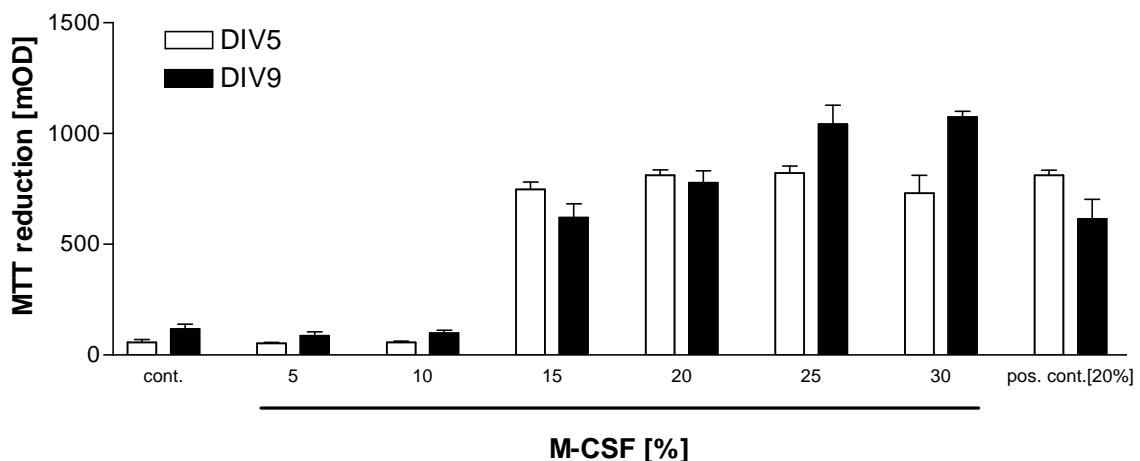


Figure 1: Characterisation of M-CSF containing supernatants with BMDM

Bone marrow cells (4×10^4 cells/well) were plated and exposed to different concentrations of M-CSF containing L929csf supernatants in a range from 0 to 30 percent (v/v). As positive control a previously tested M-CSF supernatant with a concentration of 20 % (v/v) was used. At the times indicated (DIV=days in vitro) the medium was removed and fresh one was added together with MTT. Cells were incubated for 1.5 h, lysed and the OD was determined. Data are means + SD of triplicate determinations.

We found that a concentration of 20 % M-CSF containing supernatant was sufficient for a maximum cell yield (Fig. 1). Even after nine days in culture this amount was still sufficient to allow optimal cell growth. After ten days in differentiation medium macrophages start to detach from substrate and get an apoptotic feature. The effect of additional M-CSF containing supernatant after ten days in culture was not tested.

4.1.2 Immunostaining

To characterise the purity of the mouse macrophages the MCA49 rat anti mouse F4/80 antibody (Serotec, Germany) was used. This antibody recognizes a 160 kDa glycoprotein expressed by murine macrophages on their surface. This antibody binds to macrophages from peritoneal cavity, lung, spleen, thymus, blood monocytes and to macrophages derived from bone marrow precursors in culture.

After five to nine days cells were fixed and stained with a TMR-labelled secondary antibody. By counterstaining with the chromatin dye Hoechst33342 and counting the blue nuclei in a fluorescence microscope, the cell number of a section was determined. The red, TMR-labelled cells were expressed in percent of total cell number. A fraction of 80 to 95 % of F4/80 positive cells was found (Fig. 2). This shows that differentiation of bone marrow cells with M-CSF results in a quite pure macrophage culture.

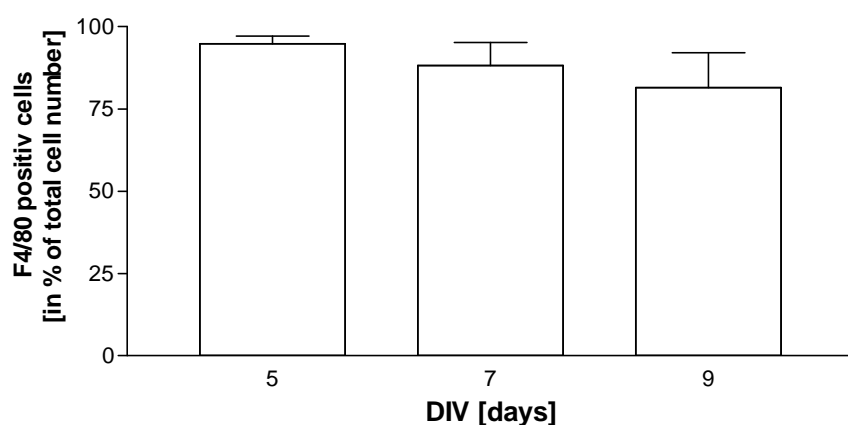


Figure 2: Immunostaining of fixed BMDM differentiated with M-CSF

Mouse bone marrow cells were incubated with 20 % M-CSF containing L929csf supernatant. After the time indicated the cells were fixed with 4 % paraformaldehyd, immunostained for F4/80, counterstained with the chromatin dye Hoechst 33342 and counted by fluorescence microscopy. Data are means + SD of triplicate determinations.

4.1.3 Functional characterisation

A further hallmark of macrophages is the phagocytosis of particles. In the literature, phagocytosis assays with IgG, IgM and complement opsonised polystyrene or latex particles are described. These opsonising proteins are specifically detected by surface receptors on macrophages. Here, fluorescence labelled *E.coli* particles were used to analyse phagocytosis by macrophages. To characterise the BMDM, fluorescence labelled (TMR) *E.coli* particles were added to the cells after different days *in vitro* and counterstained with Hoechst 33342.

Pictures were taken after one hour of incubation with a digital camera to avoid loss of visible cells by photo bleaching.

A range between 85 to 95 % cells positive for *E.coli* phagocytosis was determined. This part of the cell population stayed constant from 5 to 9 DIV. For the following experiments the BMDM between 5 to 9 DIV were used (Fig. 3).

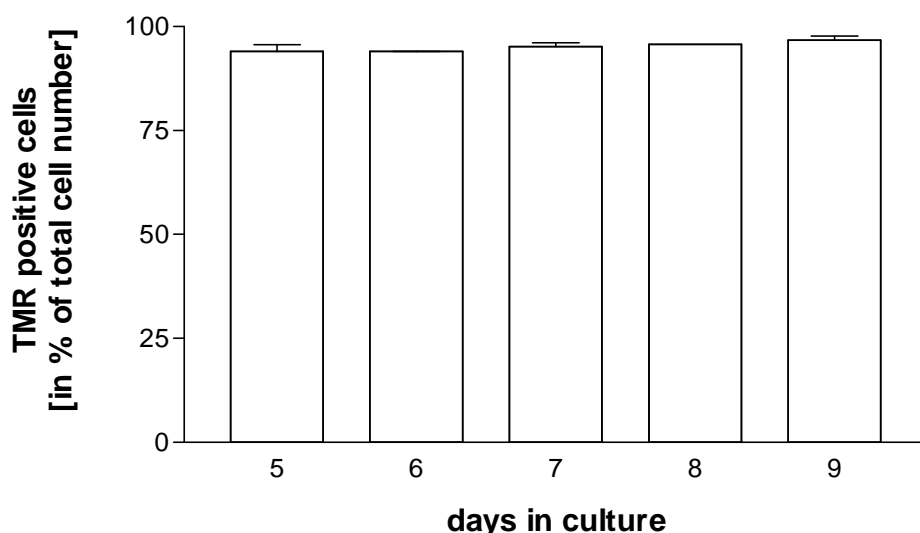


Figure 3: Functional characterisation of BMDM by *E.coli* phagocytosis

Mouse bone marrow cells were incubated with 20 % M-CSF containing L929csf supernatant. After the time indicated the cells were plated and *E.coli* particles were added. After 1 h phagocytosis cells were counterstained with the chromatin dye Hoechst 33342 and three digital images were taken per well with the help of fluorescence microscopy. Images were analysed by an image software at the computer. Data are means + SD of triplicate determinations.

4.1.4 Kinetic of cytokine release after stimulation with LPS

The release of cytokines by macrophages after stimulation with LPS is a well-characterised process. The pro-inflammatory cytokines interleukin-1 β (IL-1 β), IL-6 and TNF α were measured following stimulation of BMDM. The cytokine IL-10 was used as representative for the anti-inflammatory group of cytokines. It has previously been shown that in human macrophages phagocytosis of apoptotic cells inhibits the production of the cytokines IL-1 β , TNF α and IL-10 (Fadok *et al.*, 1998b).

For the design of further experiments it was necessary to characterise the time dependent release of these cytokines after stimulation of BMDM with LPS [1 μ g/ml].

The IL-1 β concentration increased slowly and reached detectable concentrations after 4 hours. Concentrations increased up to 24 hours. IL-6 was already found after 2 hours and reached plateau levels between 8 and 16 hours. The release of IL-10 was also already detectable after 2 hours, increased up to 8 hours and decreased afterwards.

RESULTS

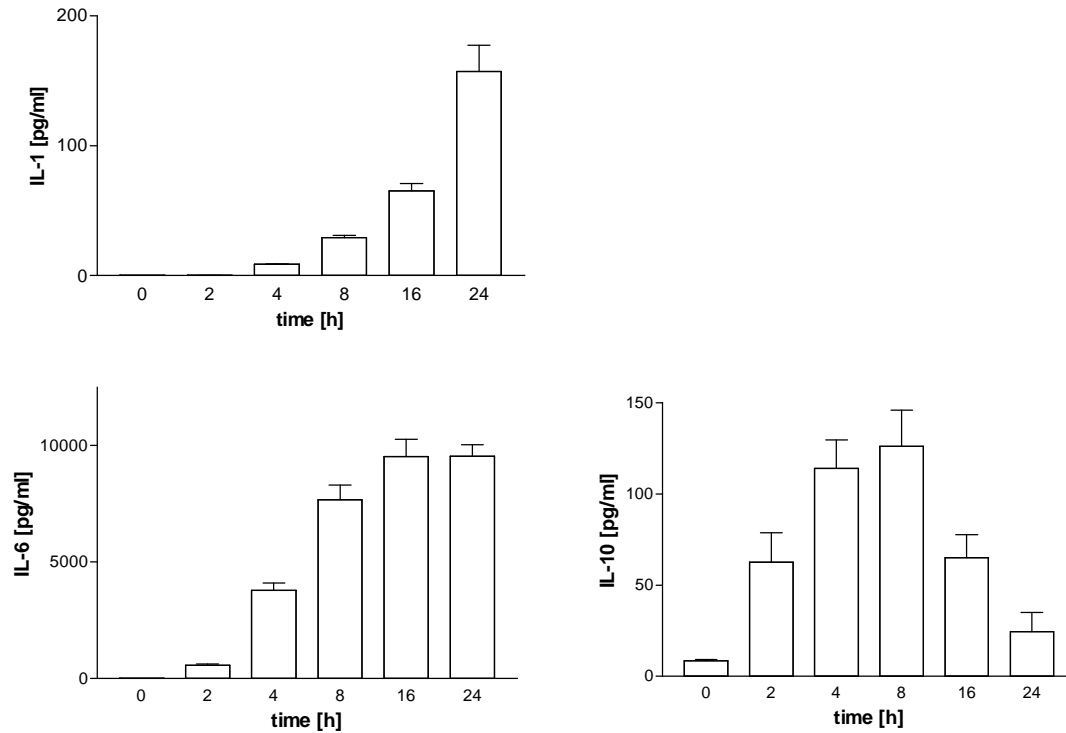


Figure 4: Cytokine release of BMDM after stimulation with LPS

BMDM were seeded on 96-well microtiter and challenged with LPS [100 ng/ml]. Supernatants were recovered after indicated times. Cytokine concentrations were determined by ELISA. Data shown are means + SD of duplicate determinations.

4.1.5 Effects of opsonisation of *E.coli* with mouse serum on phagocytosis by BMDM

An opsonisation of *E.coli* particles with autologous serum is known to enhance the uptake of these particles. Heparinised blood from BALB/c mice was centrifuged at 14000 x g for 10 min at 4 °C and serum was stored at -20 °C. *E.coli* particles were incubated with this serum [final concentration 10 % v/v] from BALB/c mice for 30 min at 37 °C prior to addition to the BMDM.

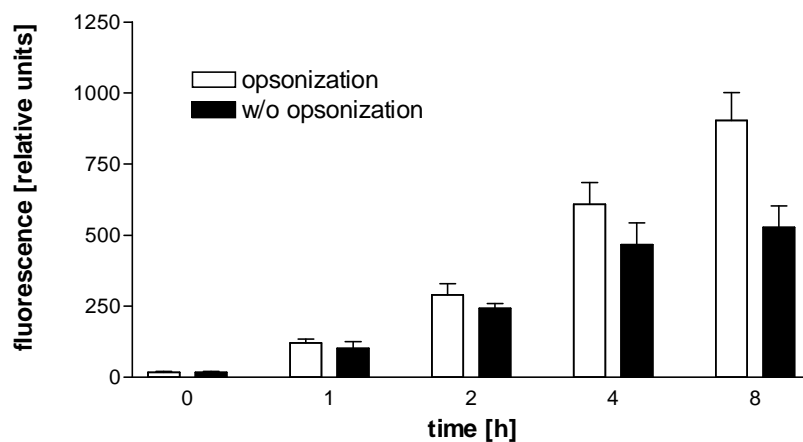


Figure 5: Effects of opsonisation of *E.coli* on phagocytosis by BMDM

BMDM were plated and fluorescence labelled *E.coli* particles were added. Bioparticles were opsonised for 30 min at 37 °C with mouse serum. Supernatants were removed after 2 h, cells were washed and uptake was determined by fluorescence measurement. Data are means + SD of triplicate determinations.

RESULTS

fluorescence measurement. Data are means + SD of triplicate determinations.

The opsonisation has no effect on phagocytosis after 1 and 2 hours incubation. Interestingly, after 4 and 8 hours a difference can be seen (Fig. 5), but this difference can also be explained by the influence of other effects. According to experiments with the phagocytosis of fluorescence labelled bioparticles presented here, uptake measured with this method only results in relevant data when the assay was stopped after two hours. The opsonisation of bioparticles facilitates the uptake via the Fc-receptor and complement receptors. The presented data indicate that also other receptors can be involved in the uptake and because the uptake via the Fc- and complement receptor is not dominant in our test system.

4.2 *E.coli* phagocytosis assay with BMDM

To assess phagocytosis an assay with fluorescence labelled *E.coli* particles was performed. In the literature several assays are discussed in detail. Here, attention was focused on two different principles to measure the uptake of *E.coli*. Phagocytosis can be described as a process of several steps. The first ones are recognition and attachment, followed by uptake and finally the digestion of the particle. To discriminate between uptake and attachment substances were introduced that possess the ability to quench extracellular fluorescence. This is an elegant way to measure solely the intracellular fluorescence.

In further experiments it was shown that this method had some drawbacks with respect to sensitivity and to controls necessary to fulfil the requirements for a quantitative phagocytosis assay described by Stoessel (Stoessel, 1977). Therefore, a second method was developed, involving lysis of the macrophages before the fluorescence was measured. In this work it was shown that the extracellular fluorescence is of minor importance for the measurement.

4.2.1 The trypan blue quenching assay

This assay uses trypan blue to quench extracellular fluorescence of bacteria attached at the surface of the macrophages. Trypan blue interacts with fluorescent probes in a way that they cannot emit fluorescent light any longer. Quenching is restricted to the surface of macrophages and thus on the attached particles only.

4.2.1.1 Quenching of TMR-fluorescence with trypan blue

Since the relation between TMR fluorescence and quenching with trypan blue was unknown the highest concentration of TMR labelled *E.coli* particles used in our tests was taken and the ability of different concentrations of trypan blue (TB) solution to quench the resulting fluorescence was determined. The quantity of quenching was expressed as percentage of decrease compared to the untreated control. Addition of TB at its highest concentration resulted in an inhibition of the fluorescent signal of 62 % compared to control without TB (Fig. 6). This result showed clearly that the extracellular fluorescence of attached particles has a high impact on the measured signal. Nevertheless a lower concentration of the quenching dye was finally taken. This was useful since the TB can enter even vital cells after prolonged incubation. A concentration of 250 µg/ml TB was taken for further experiments, resulting in an inhibition of 56 % when compared to the unquenched sample (Fig. 6).

RESULTS

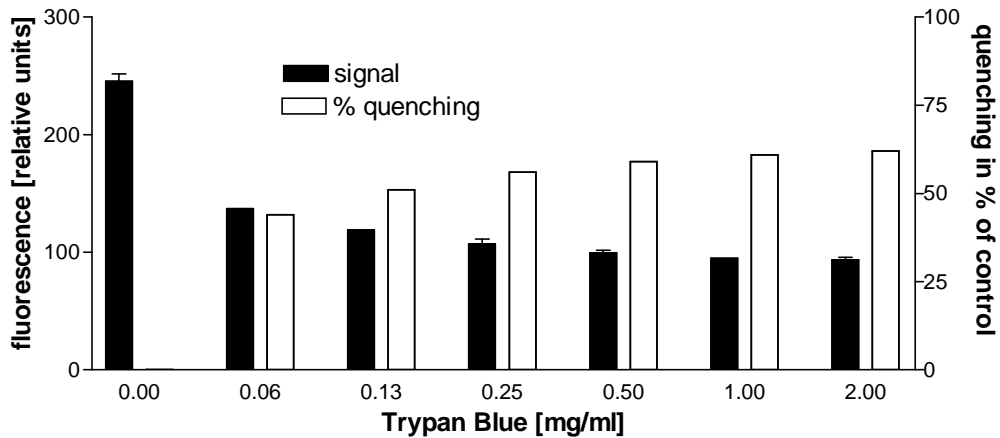


Figure 6: Quenching of extracellular TMR-fluorescence by different concentrations of trypan blue solution

BMDM were seeded on a 96-well microtiter plate and fluorescent *E.coli* particles were added at a concentration of 10 $\mu\text{g/ml}$. At the end of experiment the cells were washed twice to remove the unbound particles and 50 μl of quenching solution were added per well. Quenching was expressed in percent compared to the unquenched signal. Data are means + SD of triplicate determinations.

4.2.1.2 Quenching of BODIPY-fluorescence with trypan blue

To compare results from experiments with fluorescence labelled *E.coli* particles with another type of bioparticles BODIPY labelled zymosan particles were used as a second probe. The highest concentration of BODIPY labelled zymosan [5 $\mu\text{g/ml}$] was added to BMDM. After phagocytosis the cells were incubated with trypan blue solution [250 $\mu\text{g/ml}$].

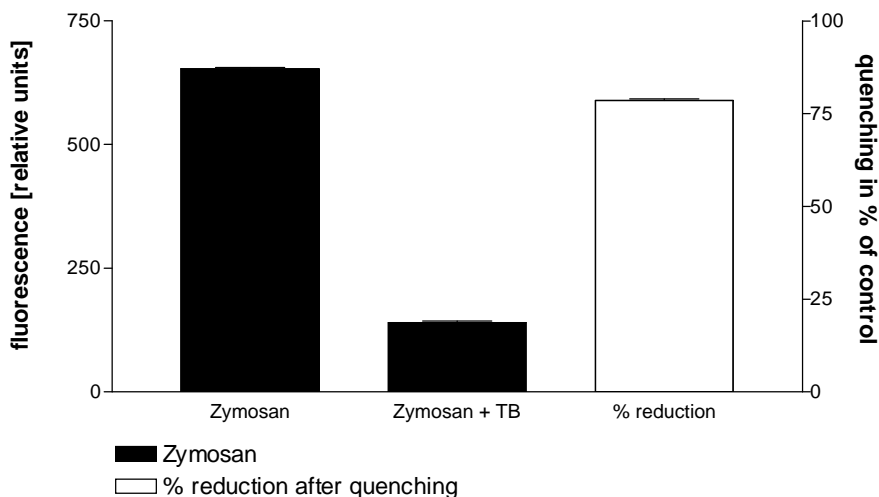


Figure 7: Quenching of extracellular BODIPY-fluorescence by trypan blue solution

BMDM were seeded on a 96-well microtiter plate and fluorescent zymosan particles were added at a concentration of 5 $\mu\text{g/ml}$. At the end of the experiment cells were washed twice to remove the unbound particles and 50 μl of quenching solution were added per well. Quenching was expressed in percent compared to the unquenched signal. Data are means + SD of duplicate determinations.

The addition of trypan blue solution resulted in a decrease of 80 % compared to the unquenched sample (Fig. 7). This observation compared with the effect on TMR fluorescence could be explained by the bigger overlap of absorption and excitation spectra from trypan blue and BODIPY. Another important parameter is the size of the particles.

Zymosan particles are much larger and it takes much longer for a phagocyte to internalise them completely. The rate of uptake is lower and fluorescent molecules can still be reached by trypan blue unless engulfment already has started.

4.2.1.3 Quenching capacity of Trypan Blue at different concentrations of BODIPY and TMR labelled bioparticles

To compare the quenching of BODIPY and TMR fluorescence the question was asked to which degree the signal was reduced when the bioparticles were incubated with trypan blue alone. The highest concentration of bioparticles that was used corresponded with the fluorescence signal after two hours of phagocytosis.

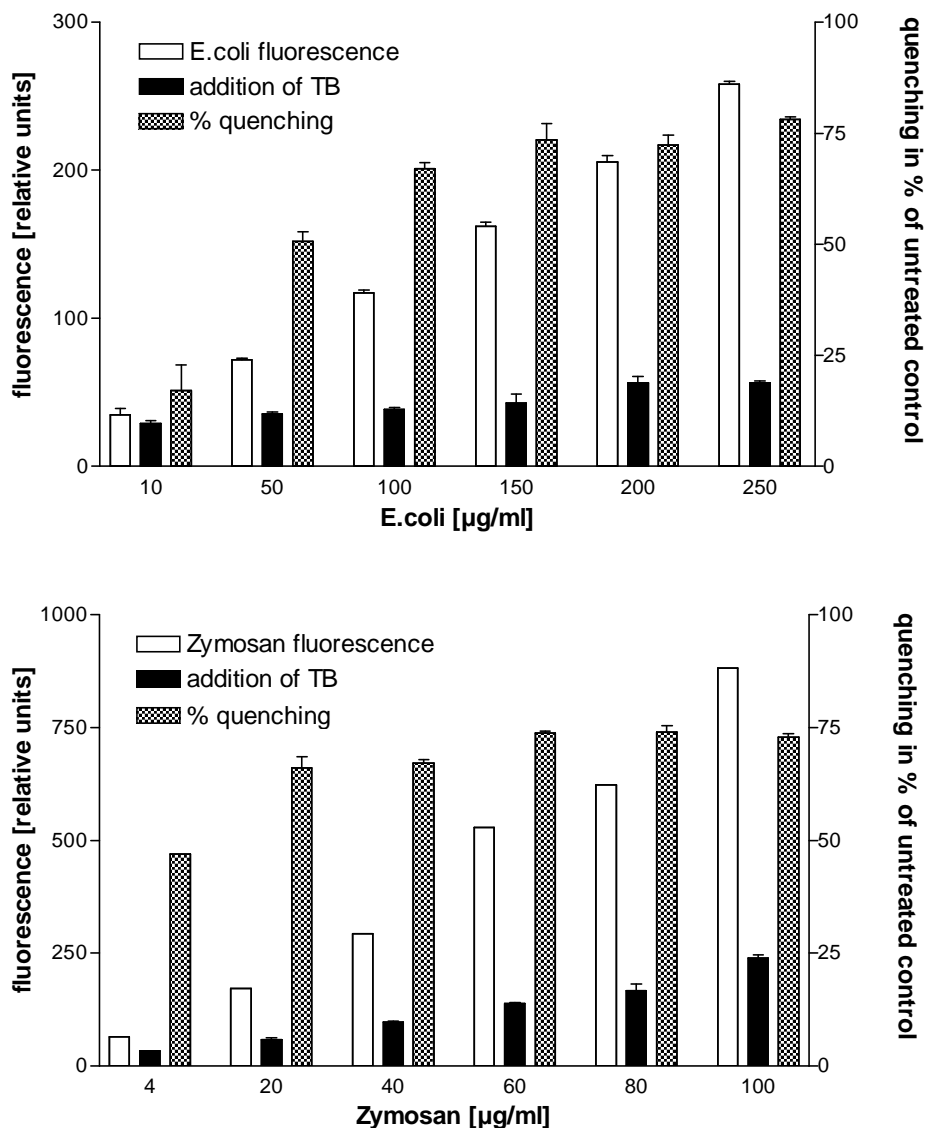


Figure 8: Quenching by trypan blue at different concentrations of BODIPY and TMR labelled bioparticles

Trypan blue solution [0.5 mg/ml] was pipetted [25 µl/well] on a 96-well microtiter plate. This solution was diluted with 25 µl bioparticle suspension to a final TB concentration of 250 µg/ml and the indicated concentrations of bioparticles. TMR fluorescence was measured at an excitation/emission wavelength of 530/590 nm, BODIPY fluorescence at 508/530 nm. Quenching was calculated by the difference of both signals at the respective bioparticle concentration. Data are means + SD of duplicate determinations.

It was found that quenching capacity of TB is similar in both kinds of bioparticles. Fluorescence was reduced to 75 % compared to the samples without TB. Interestingly, it was not possible to reduce the signal completely. Especially at the lowest concentrations the reduction was 25 % in TMR labelled particles compared to 50 % with the BODIPY labelled ones. The reason is the autofluorescence of the medium that was used. TB was only able to quench this signal partially (~ 25 %). Another interesting finding was that the concentrations of bioparticles necessary to achieve a value comparable to the one in the phagocytosis assay were much higher (Fig. 8).

After two hours the incubation with *E.coli* suspension [10 µg/ml] resulted in a signal that was as strong as the one determined with a concentration of 50 µg/ml. This was explained by different positions of the particles in the well at the time of measurement. After phagocytosis by macrophages the particles are very close to the bottom and can be excited very well. Under these circumstances the resulting fluorescence is much higher compared to the situation without cells, when the particles are distributed in 50 µl solution.

The stronger signal of BODIPY fluorescence compared to TMR at the same concentration was caused by the fit of excitation und emission filters used.

4.2.1.4 Time dependence of quenching

Quenching with trypan blue is regarded to be a fast process. But despite the favourable impact on fluorescent molecules trypan blue has cytotoxic effects. After prolonged incubation time it may pass the plasma membrane and exhibit cytotoxic effects. Intracellular trypan blue may result in further quenching and in this way to misleading results. Therefore, the measurement of phagocytotic activity observed within a quenching time up to one hour to examine this effect. For quenching a trypan blue concentration of 250 µg/ml was used.

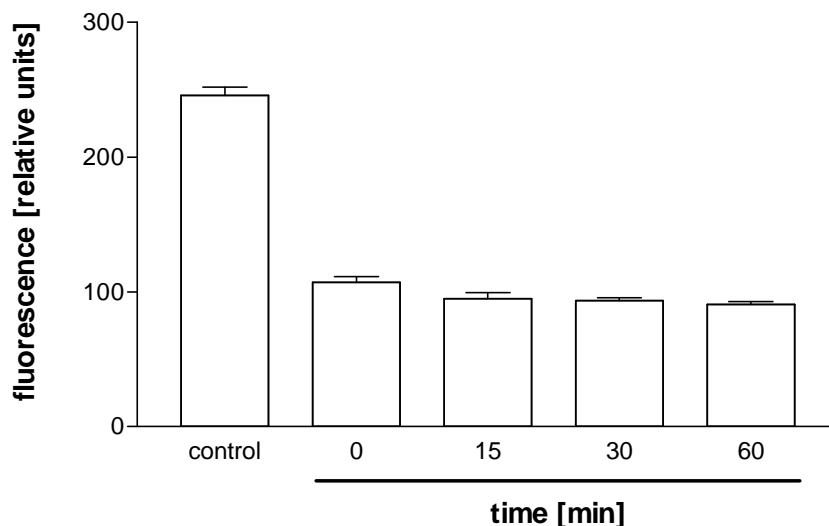


Figure 9: Intracellular quenching by trypan blue after prolonged incubation

BMDM were seeded on a 96-well microtiter plate and fluorescent *E.coli* particles were added at a concentration of 10 µg/ml. At the end of the experiment cells were washed twice to remove the unbound particles and 50 µl of trypan blue solution were added per well. Fluorescence was measured directly after addition of trypan blue solution and then at the indicated times. Data are means + SD of duplicate determinations.

In figure 9 it was shown that intracellular fluorescence was quite stable after addition of trypan blue dye. The decrease in fluorescence after 60 min was below 10 % compared with signal intensity measured directly after addition of trypan blue.

4.2.1.5 Kinetic of *E.coli* and Zymosan phagocytosis

Two criteria outlined by Stossel (Stossel, 1977) for a quantitative phagocytosis assay are the time depending saturability of the particle ingestion and the lack of apparent ingestion at zero time. These criteria were tested in experiments with BODIPY labelled zymosan and TMR labelled *E.coli* particles. Mouse BMDM were used as phagocytes.

In both experiments saturation was observed. Macrophages engulfing zymosan reached a maximum level after 10 h. The cells engulfing *E.coli* reached saturation already between 4 and 6 h (Fig. 10).

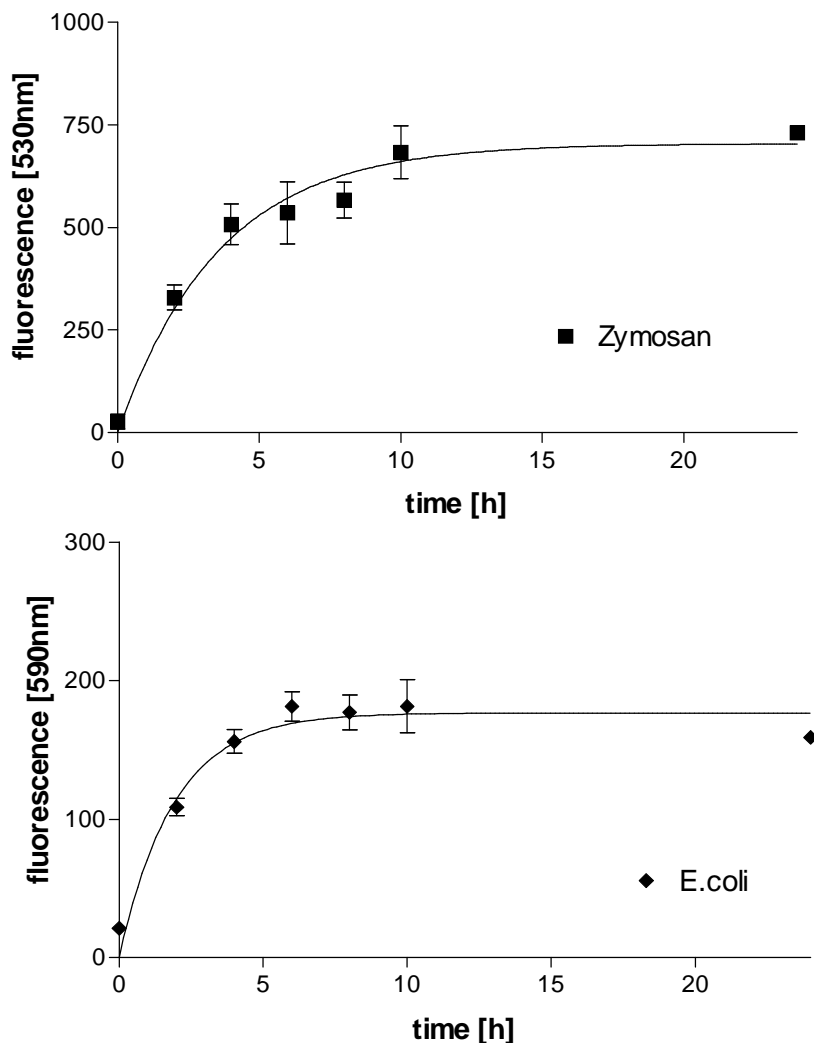


Figure 10: Kinetic of Zymosan and *E.coli* phagocytosis

BMDM were plated on 96-well microtiter plates. Zymosan [40 $\mu\text{g/ml}$] and *E.coli* [10 $\mu\text{g/ml}$] particle suspensions were added and removed by two washing steps at the indicated time. After quenching with trypan blue solution the emission was measured at the wavelength mentioned in the graphs. Lines drawn are the result of a nonlinear regression curve fit, calculated by the GraphPad Prism[®] software. Data are means \pm SD of triplicate determinations.

4.2.1.6 Inhibition of *E.coli* phagocytosis

The third criterion for a quantitative phagocytic assay defined by Stossel was complete inhibition of ingestion at 0 °C or in the presence of a metabolic inhibitor. Instead of these inhibitors the known phagocytosis inhibitor cytochalasin D (Parod and Brain, 1986) was used. BMDM were seeded on 96-well microtiter plates and were pre-incubated with cytochalasin [25 µM] for 5 hours before particles were added.

Cooling of the macrophages was started after the cells were attached at the substrate. After 1 hour in an ice water bath in a fridge the particles were added.

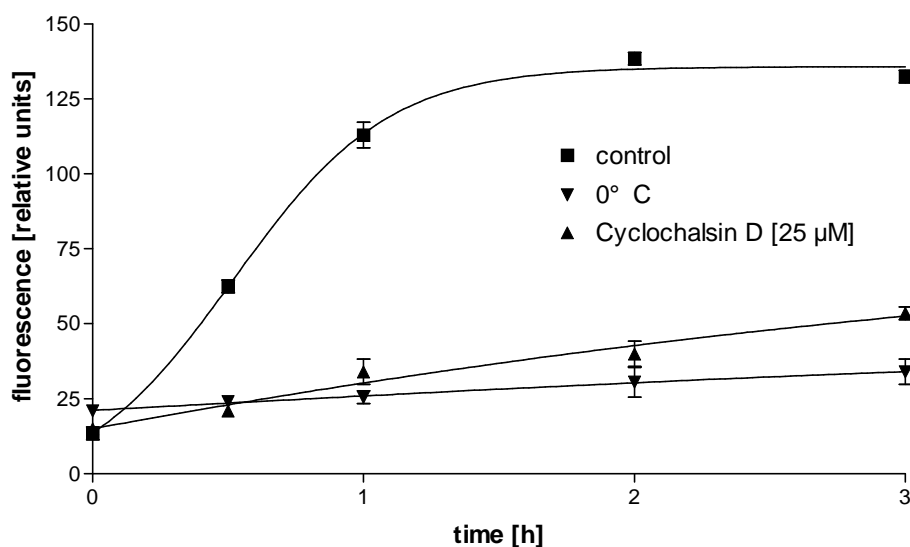


Figure 11: Inhibition of uptake of *E.coli* particles by 0 °C and cytochalasin D

Phagocytosis by BMDM was measured at different times after addition of *E.coli* particles [10 µg/ml]. Shown are means +/- SD. Lines drawn are the result of a nonlinear regression (curve fit) with boltzman sigmoidal equation, calculated by the GraphPad Prism® software. Data are means +/- SD of triplicate determinations.

An inhibition was observed by both treatments. The reduction was not due to a decrease in cell adherence or viability as tested by Sytox/Hoechst assay (data not shown).

After cytochalasin D pre-incubation the cells still take up some fluorescence. This could be explained by pinocytosis, a process unaffected by cytochalasin D. Incubation at 0 °C inhibited phagocytosis of *E.coli* particles completely (Fig. 11).

4.2.1.7 Influence of unlabelled *E.coli* on phagocytosis

In an additional experiment the influence of unlabelled particles on phagocytosis of the labelled *E.coli* was examined. We intended to show that phagocytosis of fluorescence labelled *E.coli* could be inhibited by an excess of unlabelled *E.coli* particles competing for the receptors responsible for recognition and uptake. Constant concentrations of labelled *E.coli* with increasing concentrations of the competitor were incubated.

As shown in figure 12 and in contrast to our speculation the highest concentration of the competitor (100x, fifth bar) resulted in the strongest signal. At all time points measured this signal is even stronger compared to the phagocytosis of labelled particles alone (4 µg/ml, second bar). The signal of the 1:1 and 1:10 mixture is as strong as the signal

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from labelled *E.coli* particles [4 µg/ml] without the unlabelled competitor. This relation is constant for 8 h of phagocytosis.

Interestingly, after 4 hours phagocytosis the signal from the incubation with a hundred fold higher concentration of the competitor (fifth bar) is even stronger compared to the incubation of a ten fold concentration of TMR labelled particles (40 µg/ml, first bar).

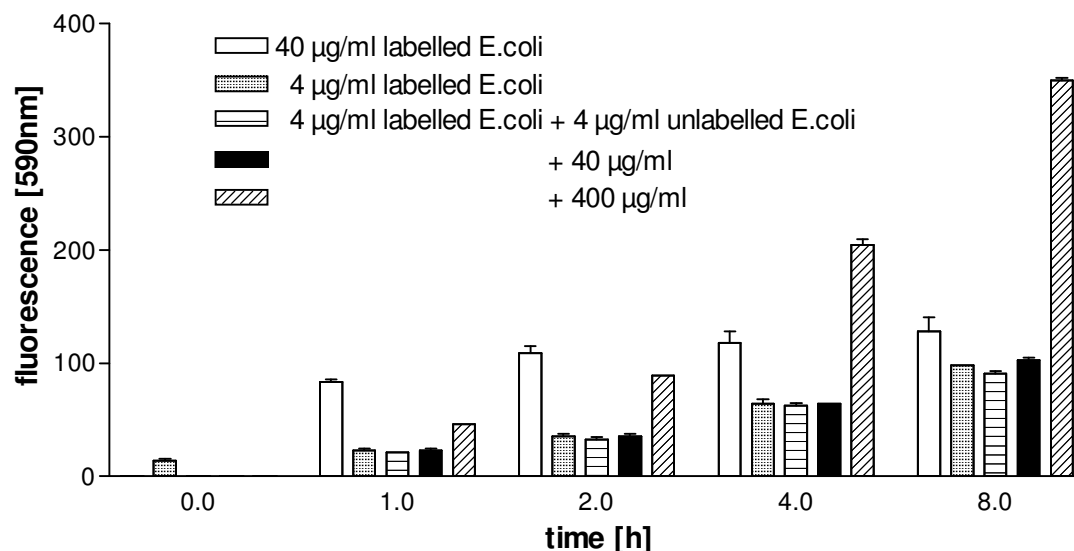


Figure 12: Competition experiment with fluorescence labelled and unlabelled *E.coli* particles

BMDM cells were incubated with 40 µg/ml and 4 µg/ml fluorescence labelled *E.coli* particles. Competition in the uptake of labelled and unlabelled *E.coli* particles was assessed. The labelled particles were mixed with the one, ten and hundred fold concentration of unlabelled particles. Data are means + SD of duplicate determinations.

One could speculate that the unlabelled *E.coli* particles do not compete with the labelled ones for the same receptors. This is unlikely because both are *Escherichia coli* particles from the K-12 strain and it is doubtful that the staining facilitates the uptake. At least one could not explain the observation with this theory.

Another explanation could be a 'self quenching' of the fluorescent molecules in the endosomes. The phagocytosis of the labelled *E.coli* particles resulted in great number of endosomes with high concentrations of the fluorescent molecules. The concentration of the fluorophores reached a level where the signal:concentration ratio was not linear any longer. When a ratio of 1 to 100 was used this situation was prevented. This argument would explain all observations seen in fig. 12.

To see a competition with unlabelled particles it was necessary to use a much lower concentration [1 µg/ml], to increase the concentrations of unlabelled *E.coli* [2000 x, 2 mg/ml] and an incubation time of 8 hours (data not shown).

4.2.2 The lysing assay

The competition experiment with the unlabelled *E.coli* particles suggested that the fluorescence signal can be significantly increased by the reduction of ‘self quenching’ of fluorescent molecules. We decided to investigate if this observation could be used in a modified assay to increase the signal to background ratio. This allows an optimal excitation of the fluorophores, since the molecules are then distributed in a volume where no ‘self quenching’ occurs.

4.2.2.1 Fluorescence quenching in macrophages

Initial experiments were performed to compare the TB quenching method with the new approach. Compared to the first method described, the cells were washed twice after addition of bioparticles to remove the unbound targets. The efficiency of this step was quite good compared with the quenching of TB without this first step. In figure 13 the fluorescence signal is measured after two hours of phagocytosis of BODIPY and TMR labelled *E.coli* particles. As shown before, TB quenches the fluorescence of attached particles significantly. But compared to the enormous increase after lysis of macrophages with 0.1 % Triton X-100 this difference is negligible.

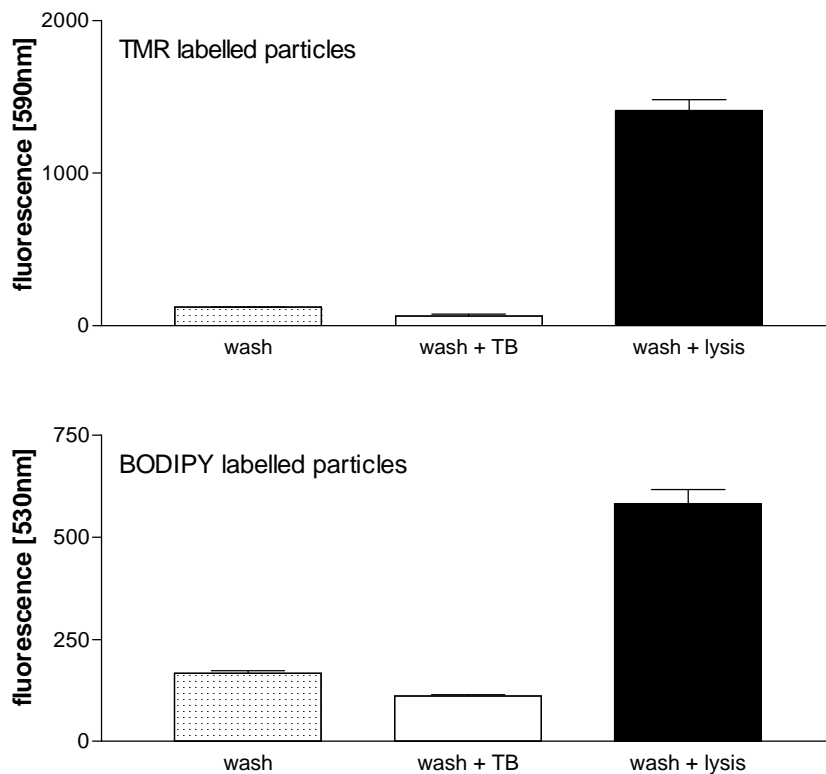


Figure 13: Fluorescence quenching in macrophages with two different fluorescent molecules

BMDM were incubated with 40 $\mu\text{g/ml}$ fluorescence labelled *E.coli* particles for two hours. The fluorescence was measured after two wash procedures (wash), wash and addition of TB (50 $\mu\text{l/well}$, wash + TB) and wash followed by cell lysis with Triton X-100 (0.1 %, wash + lysis). Data are means + SD of duplicate determinations.

4.2.2.2 Kinetic of *E.coli* phagocytosis

The kinetics of *E.coli* phagocytosis were also analysed with the new method. After different incubation times the fluorescence was measured. As can be seen in fig. 14 the saturation with the new method was reached much later compared with the other method. This can be explained by the improved measurement. The new method allowed a better correlation between the measured signal and the particles taken up into macrophages.

Surprisingly, the fluorescence signal of the lysed cells in this test was even much stronger compared with the fluorescence added at the beginning of the test. By the measurement of the fluorescence in the supernatants of the macrophages one could see that this signal increased in a way like the one obtained from the lysed macrophages.

This provoked the idea that the overall increased fluorescence was a result of the processing and digestion of the particles. The particles were stained so excessively that not all fluorophores at their surface could be excited. This excess of fluorophores is useful if one wants to follow the fate of the particles, but has its drawbacks when they are used in a quantitative phagocytosis assay.

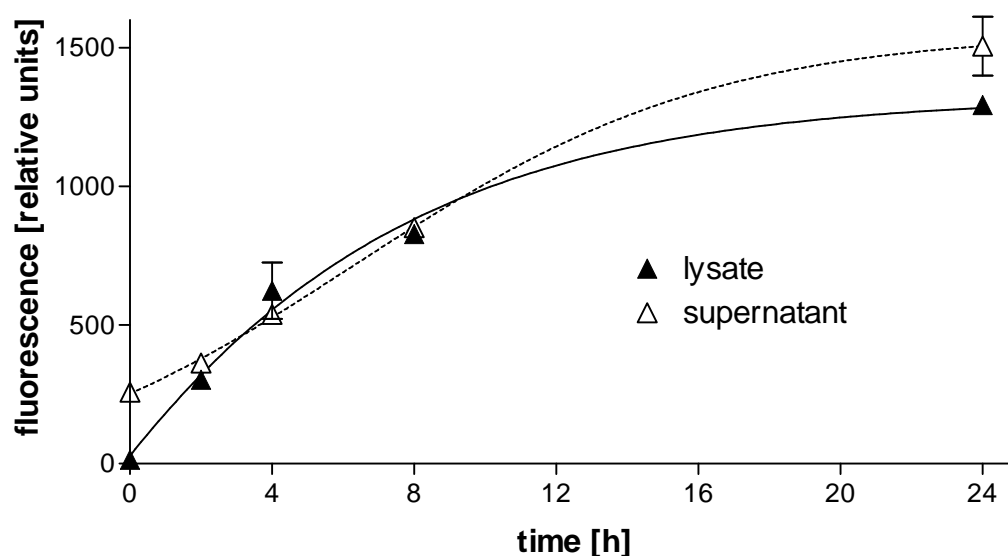


Figure 14: Phagocytosis of TMR labelled *E.coli* particles measured after lysis of the macrophages

BMDM were incubated with *E.coli* particles [10 $\mu\text{g}/\text{ml}$] and phagocytosis was measured after wash and lysis of the cells. The supernatant of the cells was taken and measured as well. Lines drawn are the result of a nonlinear regression (curve fit) with boltzman sigmoidal equation, calculated by the GraphPad Prism[®] software. Data are means \pm SD of triplicate determinations.

As can be seen in fig. 14 the total fluorescence (= lysate + supernatant) is already increases after two hours of phagocytosis.

One could criticise that this increase is a result of 'decolourisation' of the particles. But as was shown in the experiments with the inhibitors (fig. 16, p. 42) a dramatic increase in fluorescence was not observed.

4.2.2.3 Concentration signal relation of TMR labelled *E.coli*

To quantify the *E.coli* particles engulfed during phagocytosis a concentration signal relation was measured. The fluorescence was measured in 100 μl /well lysing buffer after agitation of the plates to resemble the conditions of the lysing assay. The samples were measured again four hours later to see if sedimentation of particles increased the fluorescence signal. The sedimentation increased the signal intensity for about 10 %. The weight of 3×10^9 *E.coli* particles is equivalent to 10 mg (information of the supplier). Therefore the concentration of 10 $\mu\text{g/ml}$ is equivalent to a particles concentration of 3×10^6 *E.coli* particles/ml (Fig. 15).

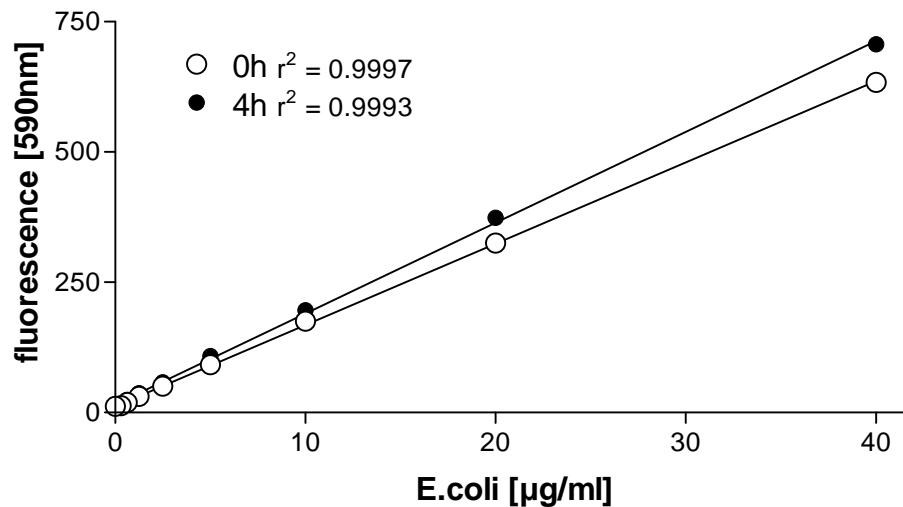


Figure 15: Concentration signal relation of TMR labelled *E.coli* particles

E.coli particles were diluted with lysis buffer (0.1 % Triton). Then triplicates with 100 μl /well were pipetted into a 96-well microtiter plate. The plate was agitated and measured afterwards. Lines drawn and r^2 values (goodness of fit) are the result of a linear regression calculated by the GraphPad Prism[®] software. Data are means \pm SD of duplicate determinations.

4.2.2.4 Inhibition of phagocytosis

To inhibit phagocytosis several different targets were chosen. In initial experiments the effects of 0 $^{\circ}\text{C}$ and of cytochalasin D in this system was tested with improved sensitivity again. These two treatments inhibited phagocytosis measured with the TB quenching method as shown previously (Fig. 11, p. 36).

As can be seen in fig. 16 both negative controls worked. The difference between positive and negative control can already be seen after 45 min. The increase during the first three hours showed a linear time dependent relation to the signal.

Compared to the phagocytosis assay performed before the improved assay showed a much better ratio between control and treated sample.

RESULTS

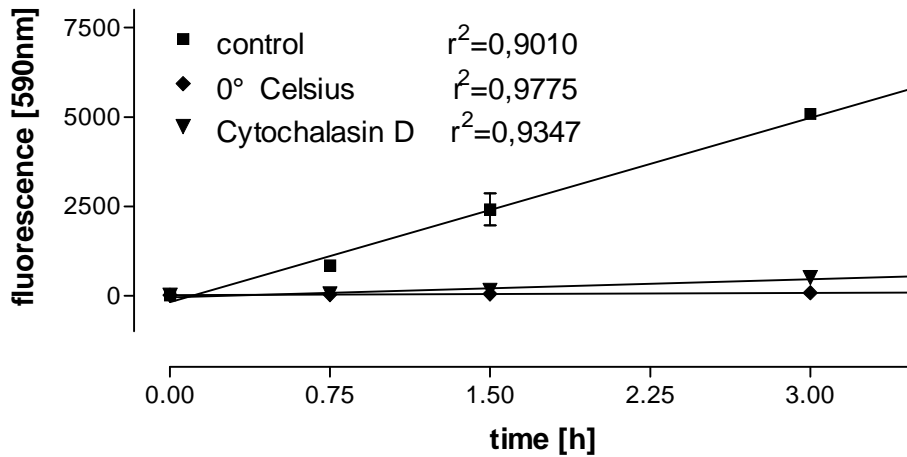


Figure 16: Inhibition of *E.coli* phagocytosis by BMDM

BMDM were precooled at 0 °C for 1 h or pre-incubated with 25 μM cytochalasin D for 16 h. *E.coli* particles [200 μg/ml] particles were added. After wash and lysis the fluorescence intensity was measured. Lines drawn and r^2 values (goodness of fit) are the result of a linear regression calculated by the GraphPad Prism® software. Data are means +/- SD of triplicate determinations.

As shown in fig. 17 a seven fold increase in the ratio was seen for the control (fluorescent bacteria). Comparable increases were seen with the other inhibition experiments. The ratio of 0.4 could be explained by the observation that the fluorescence measured with the hundred fold excess was higher compared to the control without the unlabelled particles (Fig. 12, p. 37). Altogether the results in fig. 17 show that the lysis method is an improvement for the measurement of *E.coli* phagocytosis.

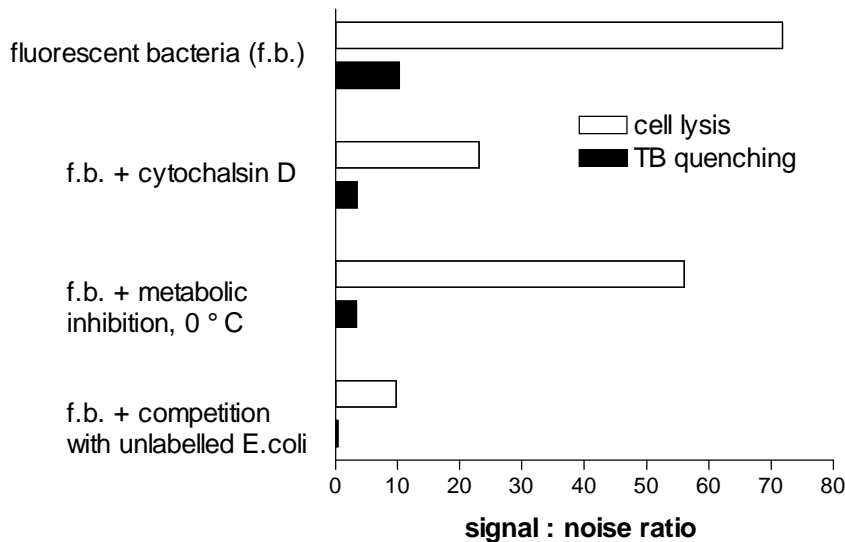


Figure 17: Background/signal (control) and positive control/treatment ratio from both assays

BMDM cells were pre-treated with the substances for one hour. Phagocytosis was stopped after two hours followed by wash and lysis. Ratio for fluorescent bacteria was calculated by dividing the fluorescence signal after two hours by the background signal at time zero. Ratio of the other treatments was calculated by dividing the value of the positive control (2 h of phagocytosis) by the value of the treated sample. Data are means +/- SD of duplicate determinations.

4.2.2.5 Different targets for the inhibition of phagocytosis

After description of these inhibitors the question was asked if other inhibitors were interfering with phagocytosis of *E.coli* particles. In these tests the incubation time was reduced to two hours. To get a brief overview the data were grouped in tab. 1. Data were expressed in percentage inhibition of untreated controls.

The substances tested have different targets. Gadolinium chloride is used to deplete Kupffer cells. A concentration of 200 μM GdCl_3 was necessary to see an inhibition of 38 % (data not shown). Without heating gadolinium chloride showed the formation of fine little crystal structures. Probably these structures competed with the labelled *E.coli* particles or just covered the receptors and prevented attachment. Treatment with a 1 mM concentration of GdCl_3 resulted in an inhibition of 87 %. It is likely that the observed effects were the result of a cytotoxic effect.

db-cAMP is a cAMP analogon that can easily cross the plasma membrane and has the same effect like an increased cAMP concentration, but had no effect on phagocytosis in our test.

Staurosporine (STS) and calphostin C are inhibitors of the protein kinase C (PKC). Calphostin C had a strong effect on the phagocytosis of *E.coli* (94.5 % inhibition), but also STS at a much lower concentration resulted in an obvious inhibition (38.7 %).

SB 203580 is an inhibitor of SAPK2/p38, PD 98059 a MEK-kinase inhibitor, both had no effect on phagocytosis. These proteins are representatives of the MAP-kinase signalling pathway.

substances	concentration [μM]	inhibition [%]
db-cAMP	2000	0
Gadolinium chloride	1000	87.3
Staurosporine	0.1	38.7
Calphostin C	5	94.6
SB 203580	1	14.3
PD 98059	1	14.9
Wortmannin	1	89.8
Ro5-4864	10	39.7
PK 11195	10	31.3
FK-506	1	20.8
Cyclosporin A	1	17.9
Taxol	1	14
Cytochalasin D	25	95.6

Table 1: Inhibition of phagocytosis by different inhibitors of signal transduction pathways

BMDM were pre-incubated with the substances for one hour, except of cytochalasin D with 4 h. Inhibition was calculated by dividing the signal of the samples by the signal of the untreated control, followed by subtraction of one. Phagocytosis was measured two hours after addition of the TMR labelled *E.coli* particles. Data are means of duplicate determinations.

Wortmannin is an inhibitor of the phosphatidylinositol-3 kinase (PI3K). It was shown that phagocytosis is a phosphatidylinositol-3-OH-kinase-dependent process in macrophages with myosin-X as a downstream target. A concentration of 1 μM wortmannin was sufficient to inhibit phagocytosis by 89.8 %.

Ro5-4864 is a peripheral benzodiazepine receptor (PBR) agonist, PK 11195 is a PBR antagonist. The PBR is associated with numerous biological functions, including the regulation of cellular proliferation, immunomodulation, anion transport, regulation of steroid genesis and apoptosis. Quite a high concentration of 10 μ M resulted in an inhibition of 39.7 % with Ro5-4864 and 31.3 % with PK11195.

FK-506 and cyclosporin A are both inhibitors of the protein phosphatase calcineurin. Both substances had no strong effect on phagocytosis.

Taxol and cytochalasin D can influence cytoskeletal dynamics in different ways. Taxol stabilizes microtubule assembly and cytochalasin D inhibits the assembly of actin microfilament. A concentration of 1 μ M taxol had no effect on phagocytosis. Cytochalasin D [25 μ M] inhibited phagocytosis by 95.6 % and was used as positive control.

4.2.3 Effects of kinase- and PDE inhibitors on phagocytosis and TNF α release

Kinase and phosphodiesterase (PDE) inhibitors represent two strategists that may influence the release of cytokines. With respect to inflammation these inhibitors influence the signal transduction pathways downstream of the toll-like receptor family.

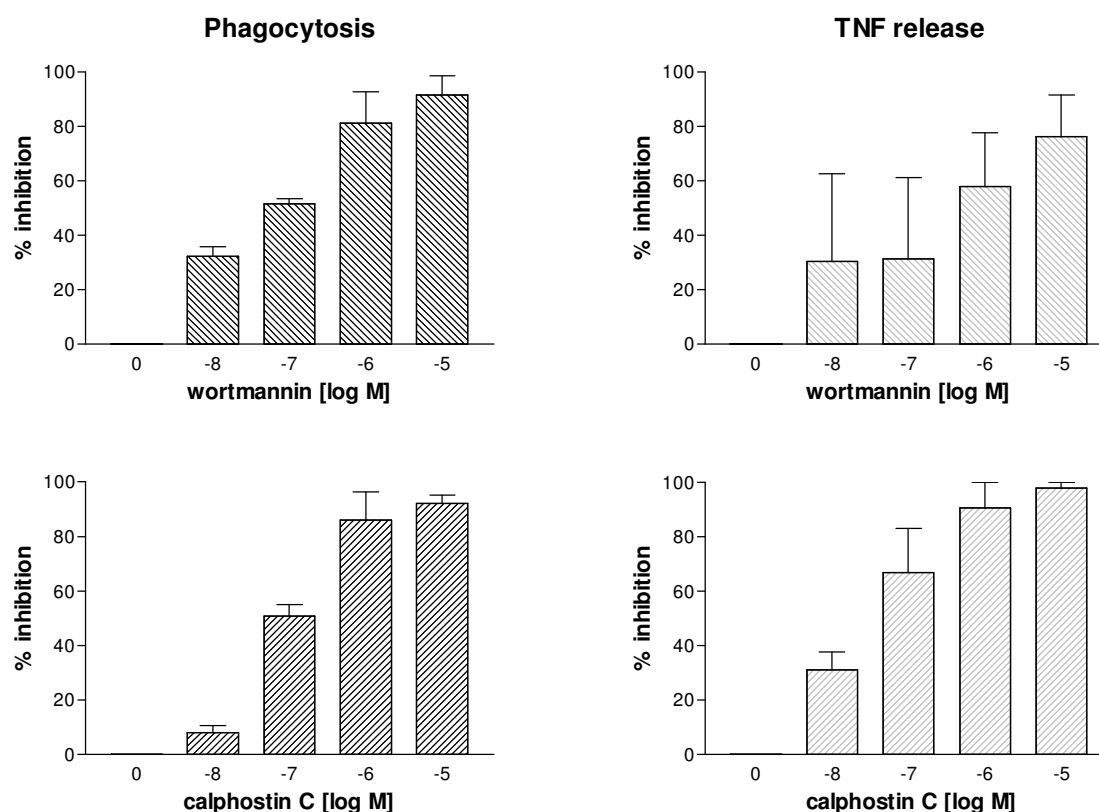


Figure 18: Inhibition of phagocytosis and TNF α release by wortmannin and calphostin C

BMDM were pre-incubated with the inhibitors for one hour followed by addition of TMR-labelled *E.coli*. After two hours of phagocytosis the supernatants were taken and stored at 20 °C until the TNF α concentration was determined. Cells were washed and lysed to measure phagocytosis. Data are means + SD of duplicate determinations.

RESULTS

These surface proteins can recognize inflammatory stimuli and transduce this signal into the cell resulting in activation of several kinase pathways and finally in induction of transcription of pro-inflammatory cytokines by translocation of nuclear factor kappa B (NF κ B) and other transcription factors. The inhibition of signal transduction by kinase or PDE inhibitors can result in a decrease of cytokine release. It was of interest if the two positive tested inhibitors of *E.coli* phagocytosis also had an effect on TNF α release.

As shown in fig. 18 calphostin C and wortmannin also inhibited the release of TNF α . These results indicate that PKC and PI3-kinase are not only involved in the phagocytosis of *E.coli* particles, but also play a role in the signalling pathway of lipopolysaccharide induced TNF α release.

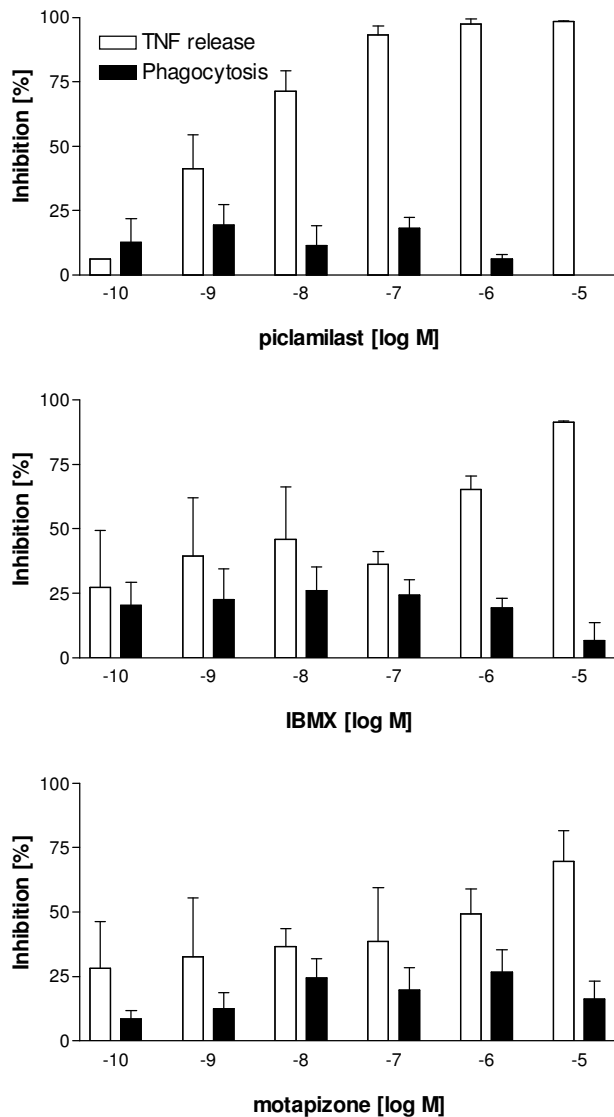


Figure 19: Effect of PDE inhibitors on phagocytosis and TNF α release

BMDM (6.5×10^4 /well) were pre-incubated for one hour with the inhibitors. The TMR labelled *E.coli* particles [$10 \mu\text{g/ml}$] were added and after 4 h supernatants were collected and stored at 20°C until measurement of TNF α concentration. Cells were washed, lysed and fluorescence was measured. Data are means + SD of duplicate determinations.

Another class of interesting TNF α synthesis influencing compounds are phosphodiesterase inhibitors. It was investigated if the stimulation by TMR-labelled *E.coli* particles and the

RESULTS

previously described TNF α release could be inhibited by pre-treatment with PDE inhibitors. Within the same experiment we wanted to clarify if PDE inhibitors also had an effect on phagocytosis.

The effect of PDE4 inhibitors in macrophages is an elevation of the cyclic AMP concentration. IBMX is an unselective PDE inhibitor, RP73401 (piclamilast) is a PDE 4 and motapizone, a PDE 3-selective inhibitor.

As shown in fig. 19 specific and unspecific inhibitors can prevent the *E.coli* induced TNF α release almost completely. A distinct reduction of *E.coli* phagocytosis was not seen with the specific or the unspecific inhibitor. We concluded that the treatment with anti-inflammatory PDE inhibitors has no negative effect on the beneficial phagocytosis of *E.coli* by macrophages.

4.3 Immunomodulation by dying cells

4.3.1 Induction of inflammatory mediators by different stimuli

For the investigation of immunomodulation a co-culture system with macrophages and thymocytes was used. The macrophages were co-incubated with apoptotic cells for a definite time and then stimulated with different stimuli. The reaction of these macrophages was compared to macrophages that were not pre-incubated with apoptotic cells. The observed effect, such as an increase or decrease of certain inflammatory mediators was termed immunomodulation.

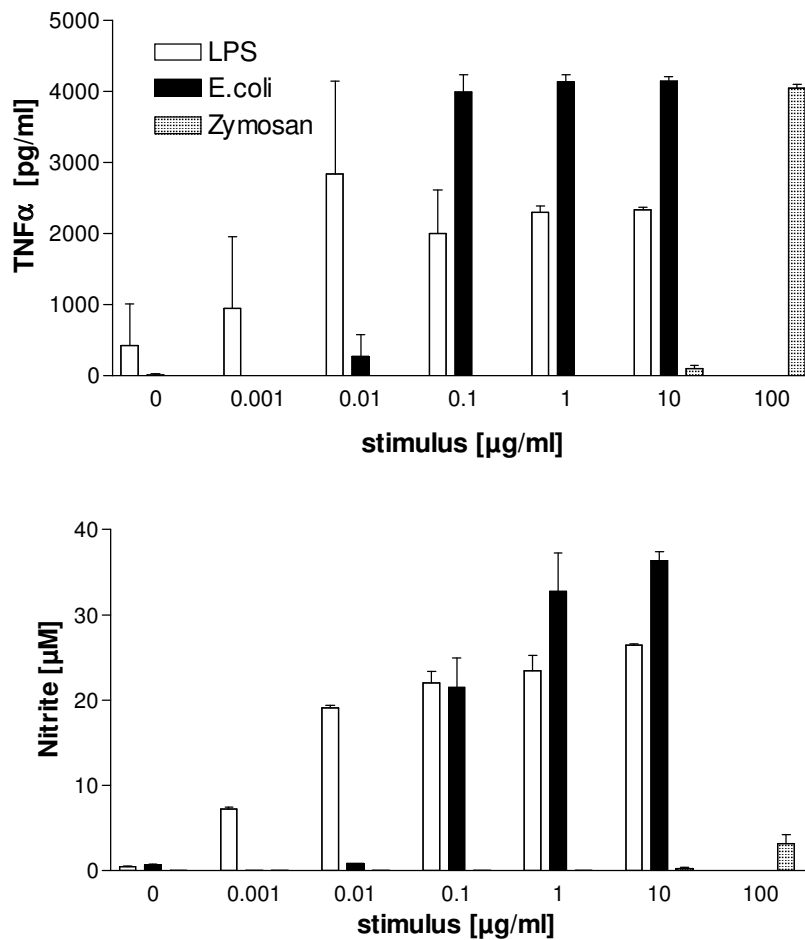


Figure 20: Release of TNF and NO in BMDM after stimulation with LPS, *E.coli*- and Zymosan-particles

BMDM were plated and stimulated with concentration range of different stimuli. Supernatants were recovered after 6 h for TNFα release and concentrations were determined by the TNFα ELISA. Samples for the NO determination were collected after 24 h and determined by the Griess assay. Data are means + SD of duplicate determinations.

In preliminary experiments the reaction of the macrophages, in this case bone marrow derived macrophages, on the stimuli LPS, *E.coli* particles and Zymosan particles was described by their release of TNFα and NO (Fig. 20).

The BMDM responded to all used stimuli. The amount of released mediators differed remarkably with respect to the stimuli, LPS being the most potent one, followed by *E.coli* particles and Zymosan.

4.3.2 Induction of cell death in primary thymocytes

One source of apoptotic targets were primary mouse thymocytes. These cells were stimulated with dexamethasone [$1 \mu\text{M}$] to induce apoptosis. The induction of apoptosis was characterised by DEVD-afc cleavage and by nuclear morphology after staining with the chromatin intercalating dyes SYTOX/Hoechst 33342.

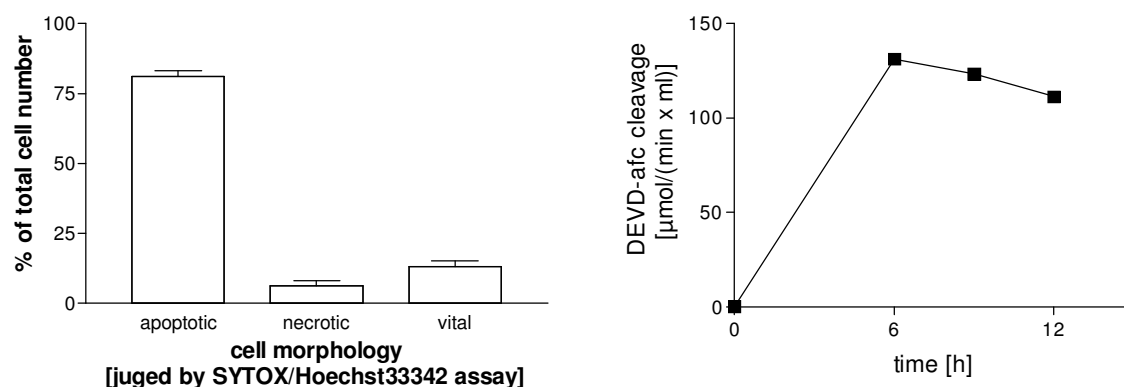


Figure 21: DEVD-afc cleavage and morphological characterisation of primary thymocytes after stimulation with dexamethasone

Primary thymocytes were stimulated with $1 \mu\text{M}$ dexamethasone in the presence of 1% FCS. Samples for the determination of DEVD-afc cleavage were taken at the indicated times and stored at -20°C until measurement. Cells were stained with SYTOX/H-33342 6 h after stimulation. At least 200 cells were counted per well. Two wells were counted per experiment. Data are presented as means + SD of three independent experiments.

Over 75% of the dexamethasone treated thymocytes showed apoptotic morphology after 6 h. Furthermore the number of necrotic cells was quite low. The DEVD-afc cleavage assay indicated that peak levels were reached in primary lymphocytes after six hours treatment with $1 \mu\text{M}$ dexamethasone (Fig. 21).

4.3.3 Uptake of apoptotic thymocytes

Since the recognition and uptake of apoptotic cells by macrophages was essential for the ongoing experiments the phagocytosis of apoptotic thymocytes by BMDM was investigated. BMDM and apoptotic thymocytes were stained with different fluorescent dyes to determinate phagocytosis. Apoptotic target cells were washed away after one hour and the uptake was described as a phagocytotic index (PI) with the help of a fluorescence microscope. The phagocytotic index was calculated by multiplying the percentage of phagocytosing cells with the average of ingested cells per phagocyte.

As shown in fig. 22 apoptotic thymocytes were taken up by BMDM very efficiently compared to control thymocytes. These control cells were used directly after preparation and represent a population with mainly vital thymocytes (> 95%). The low uptake

observed after co-incubation with control cells could be explained by cells dying as a result of the preparation.

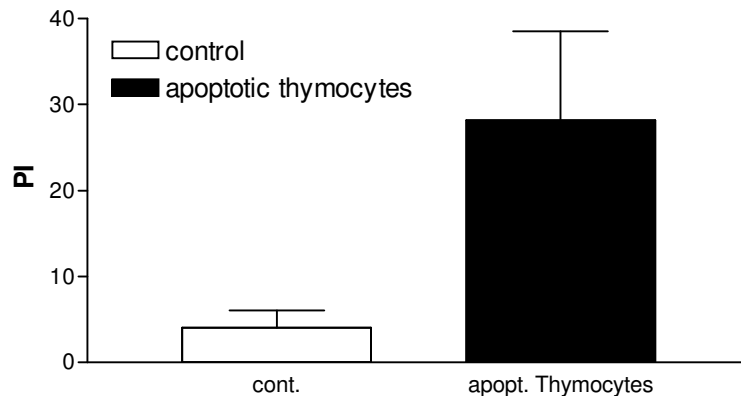


Figure 22: Phagocytosis of apoptotic thymocytes by BMDM

BMDM were stained with DiI and apoptotic thymocytes with calcein-AM [2 μ M]. BMDM were seeded on 48-well microtiter plate (3.8×10^5 cells/ml, 300 μ l/well). Thymocytes were stimulated with dexamethasone [1 μ M, 6 h]. Apoptotic thymocytes (8×10^6 cells/ml, 150 μ l/well) were co-incubated with BMDM and washed away by addition of 500 μ l/well PBS after 1 hour. This wash step was repeated 5 times. Phagocytosis was determined as phagocytotic index (PI). Data are means + SD of three independent experiment.

4.3.4 Modulation of macrophage response by dying thymocytes

The interest of this experiment was to set up a system where immunomodulation of macrophages by apoptotic cells could be observed. As mentioned above BMDM were used as source of macrophages. Primary thymocytes stimulated with dexamethasone were used as apoptotic target cells. Both cell types were co-incubated for two hours, and then non ingested apoptotic cells were washed away by several washing steps. Fresh medium was added and cells were allowed to recover for 30 min. These steps were followed by activation of the BMDM with different stimuli.

The influence of apoptotic cells was described by the measurement of inflammatory mediator TNF α . This cytokine was released by BMDM after stimulation with LPS and *E.coli* particles as shown before (P. 47). As a further stimulus lipoteichoic acid (LTA) was used.

The co-incubation with apoptotic cells resulted in a increase of TNF α . As shown in fig. 23 the measured TNF α concentration decreased after pre-incubation with apoptotic cells. This decrease was seen with all three different stimuli. Interestingly, the reduction was more pronounced after four and eight hours, when LPS or *E.coli* was used. When LTA was used the decrease was more distinct after 16 and 24 hours stimulation. This difference is perhaps due to the different receptors for LTA (toll-like receptor 2) and LPS (toll-like receptor 4).

RESULTS

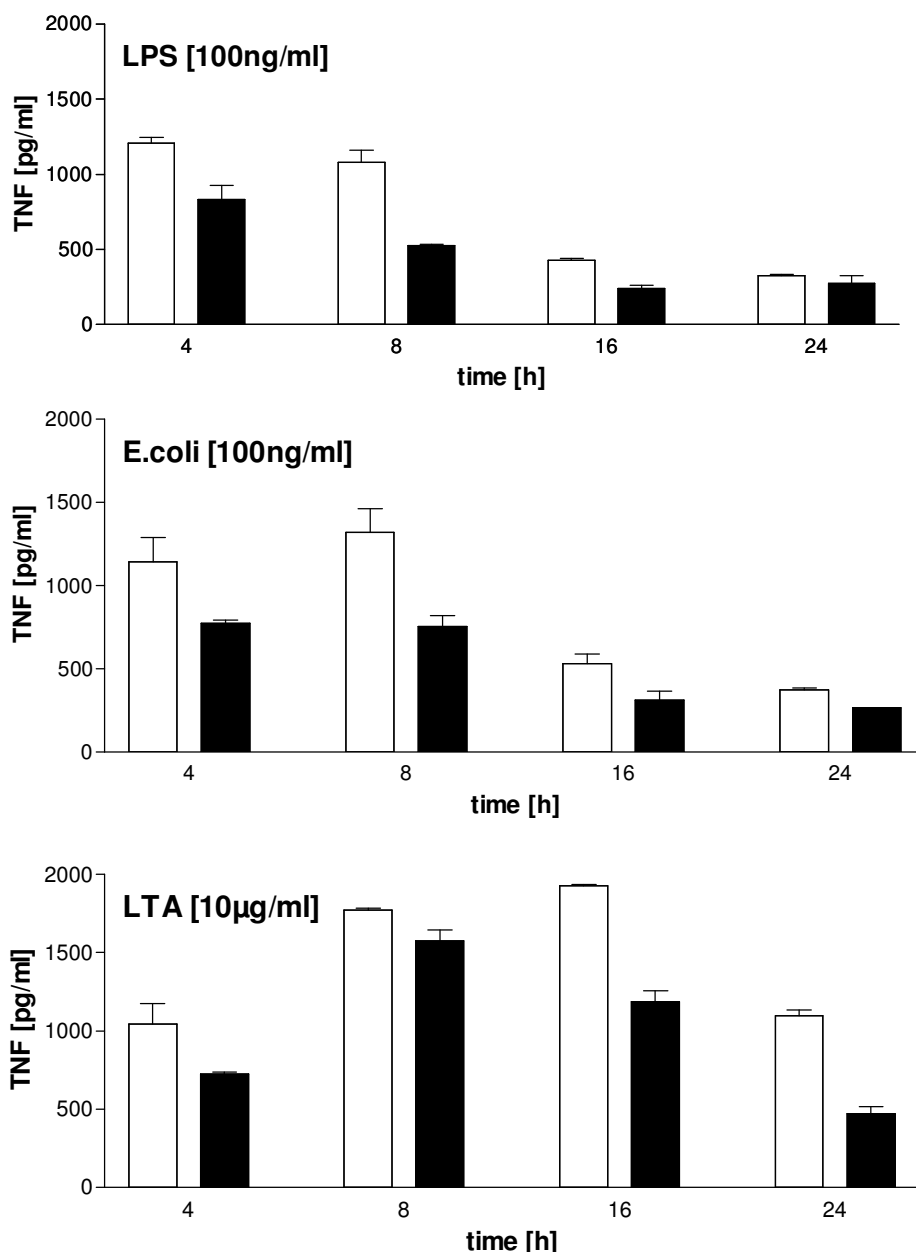


Figure 23: Immunomodulation by apoptotic thymocytes after co-incubation with BMDM followed by stimulation with LPS, *E.coli* particles or LTA

BMDM were seeded on 96-well microtiter plates and co-incubated with apoptotic thymocytes for 2 h. After this period BMDM were washed several times with PBS, fresh medium was added and cells were challenged with LPS [100 ng/ml], *E.coli* particles [10 ng/ml] or LTA [10 µg/ml]. Supernatants were recovered after 4 h. TNF α concentrations were determined by a TNF α ELISA. Data shown are means + SD of duplicate determinations.

In further experiments the influence that dexamethasone could have on TNF release was tested. Dexamethasone concentrations as low as 40 nM had no effect on the viability as determined by MTT assay or by TNF release (data not shown). In experiments presented here concentrations of dexamethasone higher than 40 nM are not likely because of the many washing steps performed.

To exclude that these effects were induced by cytotoxicity of apoptotic cells, the release of prostaglandin E₂ (PGE₂) was measured. This inflammatory mediator is known to be unregulated after co-incubation with apoptotic cells followed by stimulation with LPS (Fadok *et al.*, 1998b).

RESULTS

Data presented in fig. 24 showed an increase in PGE₂ concentrations after stimulation with LPS. Interestingly, the detected concentration after co-incubation with apoptotic thymocytes was even higher. Furthermore, co-incubation with apoptotic cells alone did not induce a detectable PGE₂ release.

These experiments contributed to the point of view that immunomodulation is an active process and not the result of a cytotoxic effect of apoptotic thymocytes.

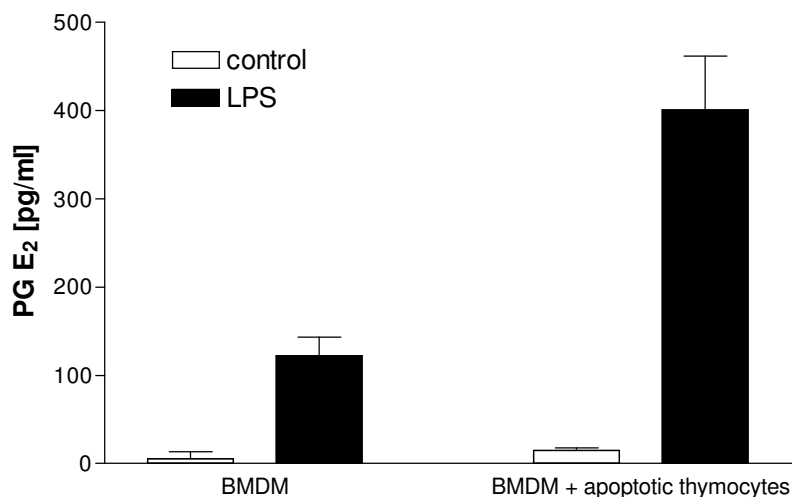


Figure 24: PGE₂ release of BMDM after co-incubation with apoptotic thymocytes followed by stimulation with LPS

BMDM were seeded on 96-well microtiter plates and co-incubated with apoptotic thymocytes for 2 h. After several washes to remove apoptotic cells, macrophages were stimulated with LPS [100 ng/ml]. Supernatants were recovered after 4 h. PGE₂ concentrations were determined with a PGE₂ CLIA. Data shown are means + SD of duplicate determinations.

4.3.5 Induction of cell death in S49.1 thymoma cell line

4.3.5.1 Actinomycin / CD95-ligand

As additional target for phagocytosis S49.1 cells, a BALB/c thymoma cell line, was used for further experiments. Apoptosis was induced in this cell line by stimulation with actinomycin D / CD95 ligand for 16 h. Cell death was characterised by DEVD-afc cleavage and SYTOX/Hoechst33342 assay. The suitable concentrations of CD95-ligand to induce cell death were tested with the MTT assay.

Caspase cleavage could be shown in S49.1 cell line after stimulation with ActD/CD95L. Interestingly, the cleavage activity still increases after 14 h. At this time a surplus of cells had apoptotic morphology and only some had uncondensed chromatin. The S49.1 cells lose their membrane integrity quite fast after apoptosis was induced. For the SYTOX/H33342 assay cells were even counted as apoptotic when they had lost their membrane integrity but a typical apoptotic chromatin condensation could be seen.

For the CD95L a concentration range was tested. A concentration of 10 % [v/v] could be shown to result in an optical density that could be compared with the cells after Triton-X100 lysis in the MTT assay (Fig. 25). For further experiments this concentration was used as sufficient to induce cell death in S49.1 cells with apoptotic morphology.

RESULTS

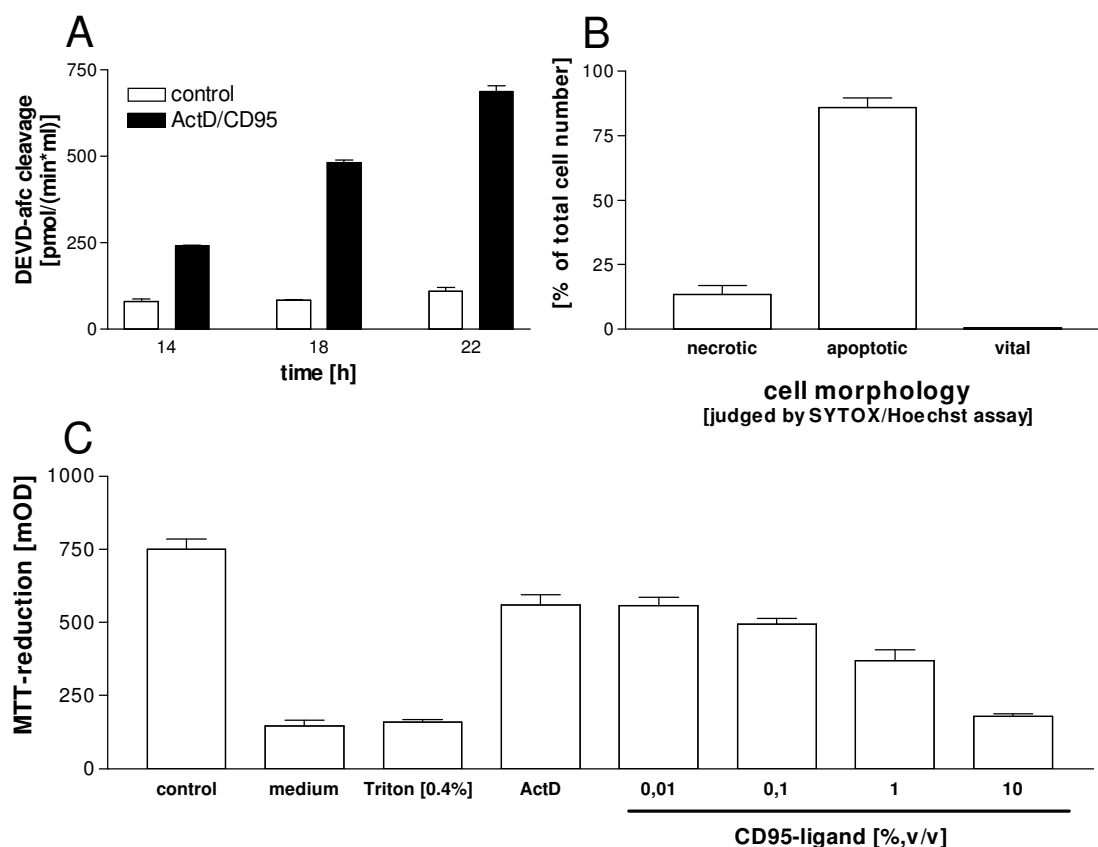


Figure 25: Induction of cell death by actinomycin D / CD95 ligand in S49.1 cells .

A: DEVD-afc cleavage in S49.1 after stimulation with ActD/CD95L. S49.1 cells [2×10^6 cells/ml, 100 μ l/well] were seeded on 96-well microtiter plates and stimulated with ActD [0.4 μ g/ml] and CD95L [10 %, v/v] for the indicated times. Data shown are means + SD of duplicate determination.

B: Morphology of S49.1 cells judged by SYTOX/Hoechst33342 assay after stimulation with ActD/CD95L. S49.1 cells were seeded in 96-well microtiter plates and stimulated with ActD [0.4 μ g/ml] and CD95L [10 %, v/v] for 16 h. 200 cells were counted per well. Data shown are means + SD of duplicate determination.

C: MTT-reduction of BMDM after stimulation with ActD/CD95L. S49.1 cells were seeded in 96-well microtiter plates and stimulated with ActD [0.4 μ g/ml] and CD95L [10 %, v/v] for 16 h. Fresh medium was added together with MTT-solution and incubated for two further hours. Data shown are means + SD of triplicate determinations.

4.3.5.2 Staurosporine

As an alternative for the induction of apoptosis in S49.1 cells staurosporine was used. Staurosporine is a protein kinase C inhibitor and was shown to induce apoptosis in primary mouse thymocytes within six hours. In S49.1 cells a concentration of 1 μ M was used to induce cell death. Compared to primary cells the cell line was more resistant to induction of apoptosis. Incubation time was increased up to 24 h to get a comparable percentage of apoptotic cells.

The results in fig. 26A showed that incubation with 1 μ M staurosporine for 24 h was sufficient to induce cell death in the same range compared to the positive control (Triton X-100, cell lysis). Staurosporine concentration of 10 μ M did not result in a significant increase of cell death.

Morphology of S49.1 cells was characterised with the SYTOX/Hoechst33342 assay. Compared to treatment with ActD/CD95L the percentage of necrotic and vital cells was higher after incubation with 1 μ M staurosporine. Since increasing incubation time resulted

RESULTS

in a higher number of necrotic cells incubation with 1 μM staurosporine for 24 h was used for further experiments (Fig. 26B).

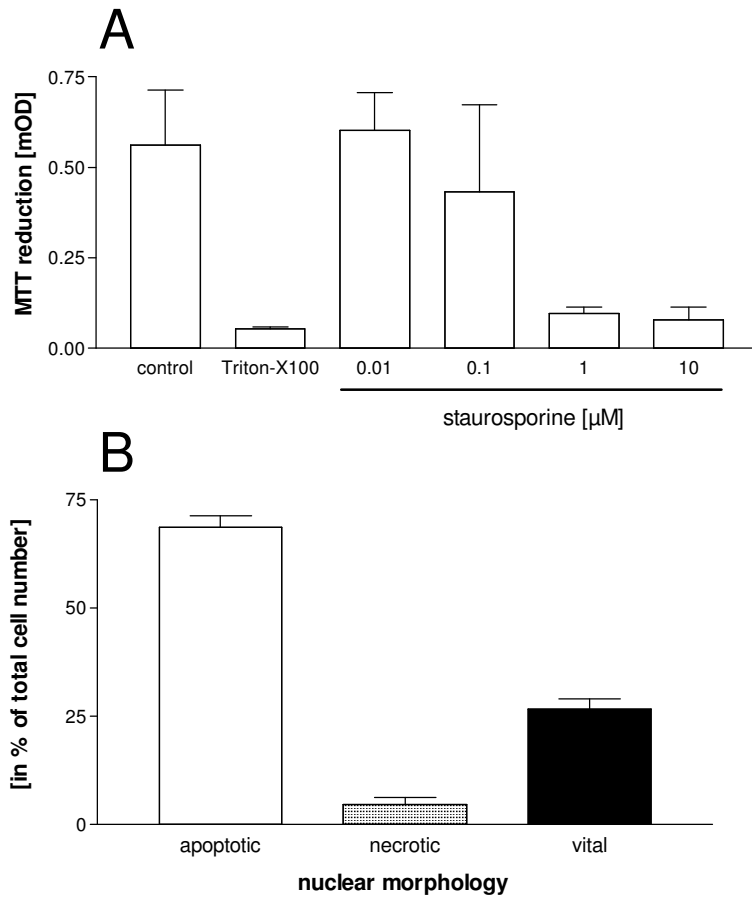


Figure 26: Induction of cell death with staurosporine in S49.1 cells

A: MTT-reduction of BMDM after stimulation with staurosporine. S49.1 cells were seeded in 96-well microtiter plates and stimulated with staurosporine for 24 h. Fresh medium was added together with MTT-solution and incubated for the following two hours. Triton X-100 was used as positive control inducing cell lysis and break down of the mitochondrial potential. Data shown are means + SD of triplicate determinations.

B: Morphology of S49.1 cells judged by SYTOX/Hoechst33342 assay after stimulation with ActD/CD95L. S49.1 cells were seeded in 96-well microtiter plates and stimulated with ActD [0.4 $\mu\text{g}/\text{ml}$] and CD95L [10 %, v/v] for 16 h. 200 cells were counted per well. Data shown are means + SD of duplicate determination.

4.3.5.3 Hyperthermia and freeze-thaw

Hyperthermia and freeze-thaw are standard methods to induce necrosis in cells. This mode of cell death could be described as a counterpart of the programmed cell death. The most prominent characteristic of this death mode is cell lysis and an uncondensed chromatin structure. SYTOX/Hoechst33342 assay and MTT assay were used to characterise these two methods to induce necrosis. Another elegant way to induce necrosis is the stimulation with staurosporine after ATP depletion in Jurkat T cells. Induction of necrosis in S49.1 cell was not possible by this treatment, because ATP depletion resulted in apoptosis.

Freeze-thaw and hyperthermia resulted in high numbers of necrotic cells (Fig. 27A). These cells were not able to reduce MTT (Fig. 27B) and no increase in caspase activation could be observed compared with untreated cells (data not shown).

RESULTS

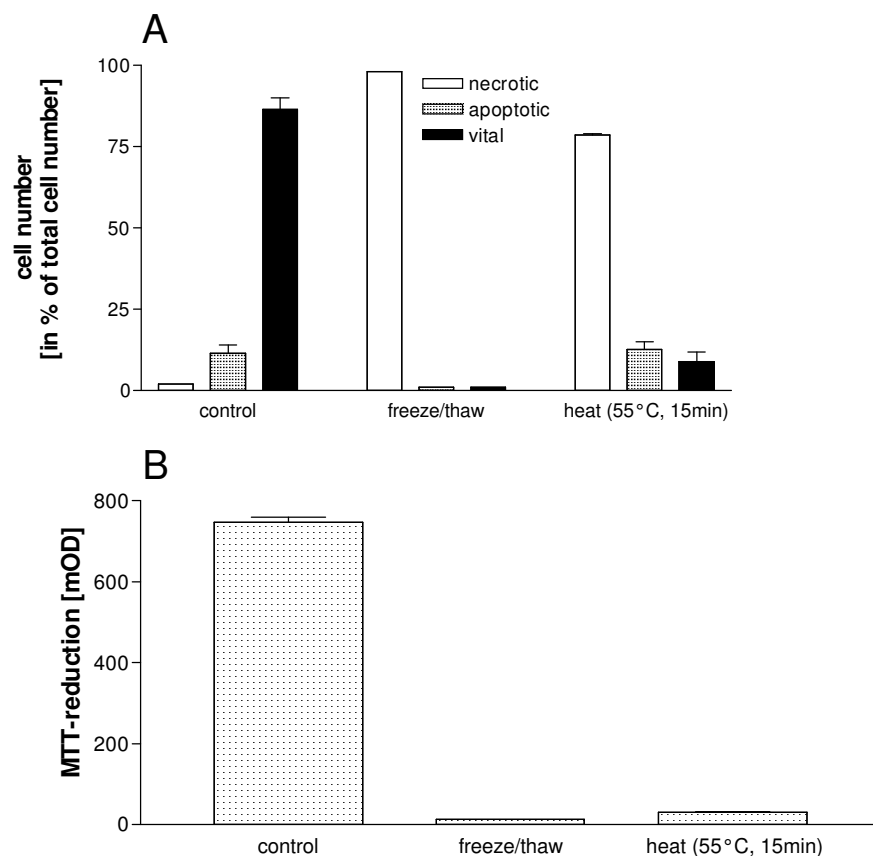


Figure 27: Induction of cell death by freeze-thaw and hyperthermia in S49.1 cells

A: Morphology of S49.1 cells judged by SYTOX/Hoechst33342 assay after treatment. S49.1 cells were frozen in liquid nitrogen and thawed in a 37 °C waterbath, which was repeated once. For hyperthermia cell suspension was put into a 55 °C waterbath for 15 min. 200 cells were counted per well. Data shown are means + SD of duplicate determinations.

B: MTT-reduction of BMDM after treatment. S49.1 cells were seeded in 96-well microtiter plates. Fresh medium was added together with MTT-solution and incubated for two hours. Data shown are means + SD of triplicate determinations.

4.3.6 Modulation of macrophage response by dying S49.1

Immunomodulation could be shown in BMDM in response to apoptotic primary mouse thymocytes. As a second model apoptotic mouse thymoma S49.1 cells were used. It was of interest to see if these transformed cells had a comparable influence on macrophage response like the primary cells.

Apoptotic S49.1 cells were co-incubated with BMDM for two hours, washed away and stimulated with LPS. LPS induced TNF α release of these BMDM was compared with the one of BMDM without contact to apoptotic thymocytes.

Co-incubation of BMDM with apoptotic S49.1 cells had comparable effects on macrophages response as previously seen with primary cells. During co-incubation with apoptotic cells no TNF α was detectable. Also co-incubation with apoptotic cells alone had no effect on TNF α release. The decrease of TNF α concentration after co-incubation with apoptotic cells followed by stimulation with LPS was comparable to the one seen before with primary thymocytes (Fig. 28A).

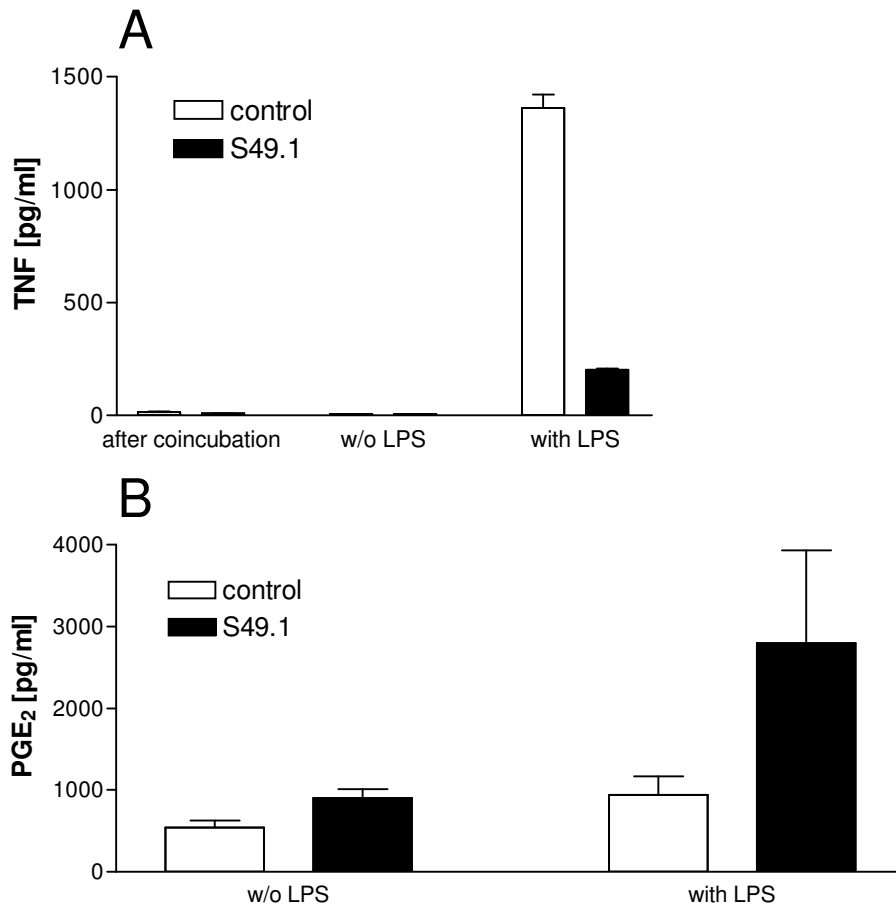


Figure 28: TNF α and PGE $_2$ response of BMDM after co-incubation with apoptotic cells followed by LPS stimulation

BMDM were pre-incubated with apoptotic S49.1 cells (ActD/CD95L) followed by stimulation with LPS [100 ng/ml]. **A:** Samples for TNF α measurement were taken after co-incubation with apoptotic cells and 6 h after stimulation with LPS. TNF α concentrations were measured by ELISA. Data are means + SD of duplicate determinations. **B:** Samples for PGE $_2$ measurement were taken 6 h after stimulation with LPS. PGE $_2$ concentrations were measured by a CLIA. Data are means + SD of duplicate determinations.

PGE $_2$ release was measured as a further inflammatory mediator. Concentrations of PGE $_2$ increase after co-incubation with apoptotic cells and served as positive control to exclude cytotoxic effects by apoptotic cells. Basal PGE $_2$ release could be seen in control cells, but after co-incubation with apoptotic cells followed by stimulation with LPS increased PGE $_2$ concentrations could be detected. Co-incubation with apoptotic cells alone had no influence on PGE $_2$ release (Fig. 28B).

4.3.6.1 Kinetic of TNF α release

A kinetic of TNF α release after co-incubation with apoptotic S49.1 cells and stimulation with LPS was performed. TNF concentration increased exponentially during the observed period of 8 hours, whereas obvious lower concentrations were found in BMDM after treatment. No TNF α was detectable in control BMDM and BMDM co-incubated with apoptotic cells (Fig. 29).

RESULTS

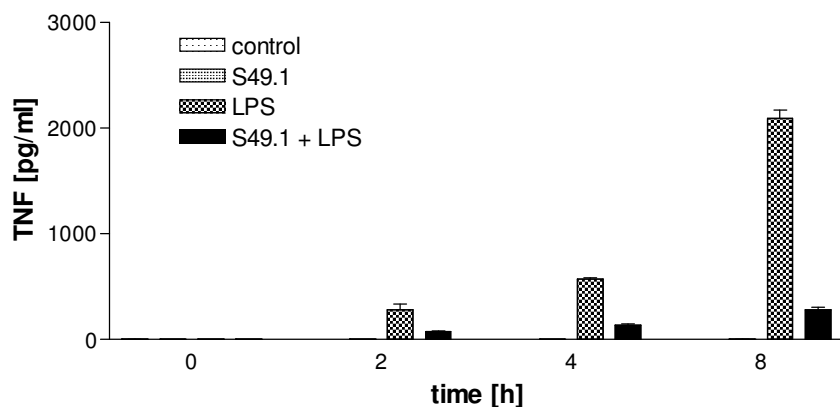


Figure 29: Immunomodulation by apoptotic S49.1 cells after co-incubation with BMDM followed by stimulation with LPS

BMDM were seeded on 96-well microtiter plates and co-incubated with apoptotic S49.1 for 2 h. After this period BMDM were washed several times with PBS, fresh medium was added and cells were challenged with LPS [100 ng/ml]. Supernatants were recovered after indicated times. TNF α concentrations were determined by ELISA. Data shown are means + SD of duplicate determinations.

4.3.6.2 Induction of apoptosis with staurosporine

In S49.1 cells ActD/CD95L was used to induce apoptosis. As an alternative stimulus staurosporine ([1 μ M], 24 h) was used. This treatment resulted in a population with some vital cells (around 20 %, fig. 26, p.51). STS treated S49.1 were incubated with BMDM followed by stimulation with LPS [100 nM]. These experiments were performed to see if comparable immunomodulating effects could be seen by the use of an alternative apoptosis inducing agent. Furthermore the presence of vital cells and their influence on immunomodulation could be investigated.

Interestingly, the STS treated S49.1 had the same effects on BMDM response as the apoptotic S49.1 cells after stimulation with ActD / CD95L. Apoptotic cells alone had no effect on the release of TNF α or IL-10. That was also interesting with respect to the vital cells during the co-incubation period. As seen before, TNF α concentration was always lower in the samples from BMDM pre-incubated with apoptotic cells. Concentration of IL-10 was just higher in these samples after eight hours of stimulation with LPS. This also served as control for the viability of the BMDM after co-incubation with apoptotic cells (Fig. 30).

RESULTS

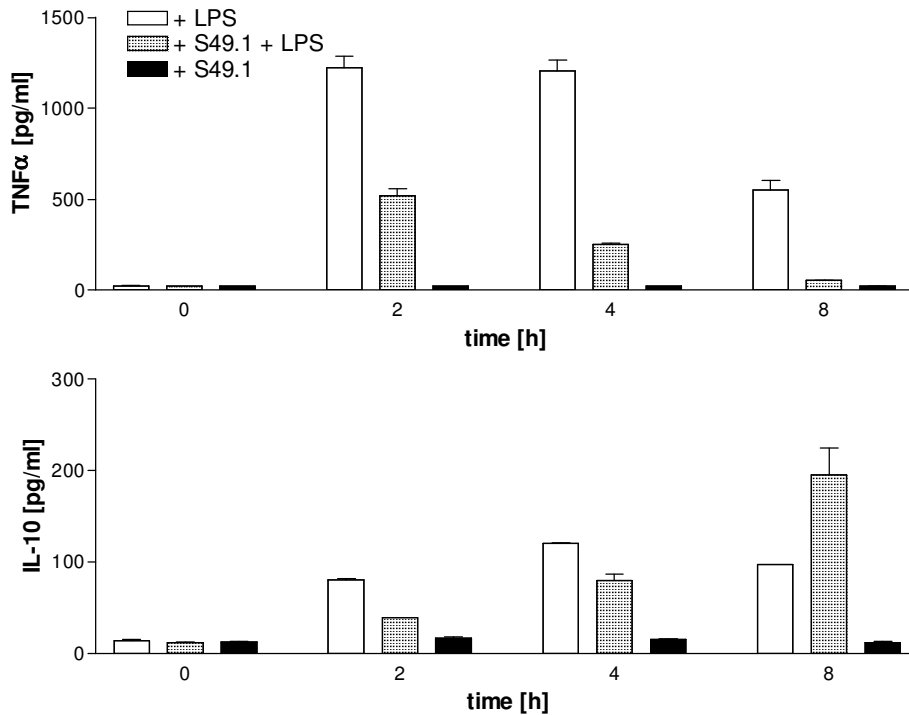


Figure 30: Immunomodulation by apoptotic S49.1 cells after co-incubation with BMDM followed by stimulation with LPS

BMDM were seeded on 96-well microtiter plates and co-incubated with STS ([1 μ M], 24 h) treated apoptotic S49.1 for 2 h. After this period BMDM were washed several times with PBS, fresh medium was added and cells were challenged with LPS [100 ng/ml]. Supernatants were recovered after indicated times. TNF α and IL-10 concentrations were determined by ELISA. Data shown are means + SD of duplicate determinations.

4.3.6.3 Freeze-thaw and hyperthermia as models of inflammatory cell lysis

Cell lysis is often correlated with inflammation. This mode of cell death is observed after stimulation with high concentrations of harmful compounds, for example after induction of liver necrosis by the analgesic acetaminophen. Freeze-thaw and hyperthermia were used to lyse the membrane of S49.1 cells. Both treatments resulted in a high number of necrotic cells as tested with the SYTOX/Hoechst33342 assay.

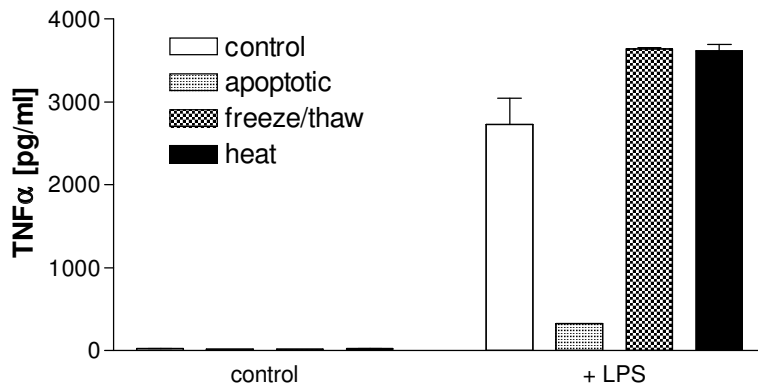


Figure 31: Influence of necrotic cells on macrophage response after stimulation with LPS

Necrosis was induced as described before by hyperthermia (56 $^{\circ}$ C, 15 min) or freeze-thaw. Necrotic cells were incubated with BMDM for 2 h. Samples for TNF determination were taken six hours after stimulation with LPS [100 ng/ml]. Co-incubation with apoptotic cells was taken as control. TNF α concentrations were determined by ELISA. Data shown are means + SD of duplicate determinations.

RESULTS

This necrotic typed cells were used to test their ability to induce inflammatory effects in combination with the BMDM. The effect of necrotic cells was compared with the one apoptotic cells had after co-incubation with BMDM for two hours. In control samples the effect of the necrotic cells without subsequent stimulation with LPS was observed.

Interestingly, the co-incubation of BMDM with dying cells had no effect on TNF α release. This result was seen previously with apoptotic cells, but also true for necrotic cells in the system here. After co-incubation with dying cells and activation of BMDM by LPS a release of TNF α was observed. As seen before, TNF α levels were decreased in BMDM after pre-incubation with apoptotic cells, whereas a co-incubation with necrotic cells increased TNF α concentration compared to BMDM stimulated with LPS alone (Fig. 31).

In conclusion the presented system was not able to resemble the inflammatory potential necrotic cells have *in vivo*. But it could be speculated that other endogenous stimuli could activate macrophages in a way like LPS. This stimulation could even occur after the insult and then result in an increase of inflammatory mediators and induction of inflammation.

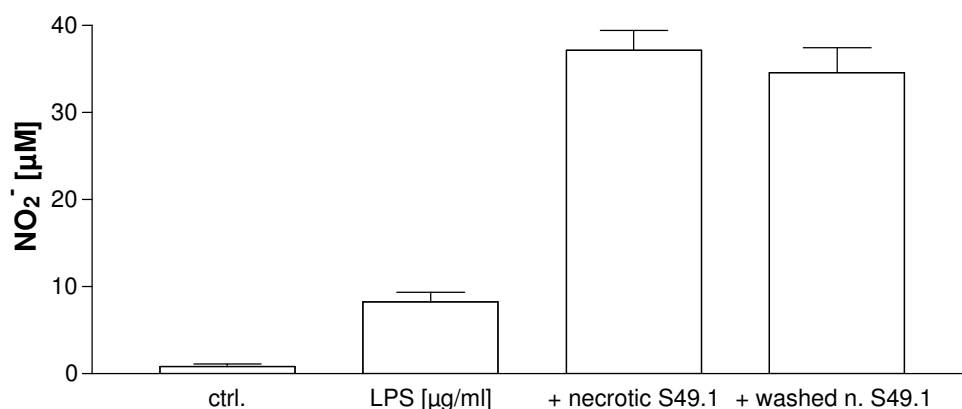


Figure 32: Effect of freeze/thawed S49.1 cells on NO release of BMDM

Necrosis was induced as described before by freeze-thaw. Necrotic cells were incubated with BMDM for 2 h. Samples for NO determination were taken 24 hours after stimulation with LPS [100 ng/ml]. NO was measured as nitrite with the Griess assay. Data shown are means + SD of triplicate determinations.

Necrosis is correlated with the loss of membrane integrity and the spillage of intracellular compounds. The latter is considered to be a reason for the induction of inflammation in surrounding tissue. To test the influence of intracellular components on the response of BMDM, freeze-thawed cells were washed twice to remove intracellular compounds of the lysed cells. These cells were compared with lysed cells co-incubated with BMDM directly after treatment.

Whether the release of intracellular components could activate BMDM was tested by the NO release after stimulation with LPS. The results presented in fig. 32 indicated that a pre-incubation with lysed cells led to an obvious increase of NO release. This observation could imply that BMDM are sensitised by necrotic cells. Interestingly, the washed necrotic cells had the same effect on activation of iNOS. For that reason it could be speculated that the sensibilisation of BMDM by necrotic cells was not induced by a spillage of intracellular components.

4.3.6.4 Impact of serum on immunomodulation

Immunomodulation of macrophages by apoptotic cells has been described before (Fadok *et al.*, 1998b). In these publications the authors also asked the question by which receptor the macrophages could recognise apoptotic cells. In some experiments they tested the influence of complement- and Fc-receptor mediated recognition and uptake. Their results indicated that the immunomodulation by apoptotic cells could only be observed in the absence of serum proteins. Recognition by these receptors seemed to be dominant compared to the effects of apoptotic cells on macrophages in the absence of serum proteins.

To see if these results could also be ascertained in the BMDM system with the apoptotic S49.1 cells, experiments in the presence and absence of FCS were performed (data not shown). In these experiments no influence of FCS (heat inactivated or not) could be seen on macrophage response after incubation with apoptotic or necrotic cells.

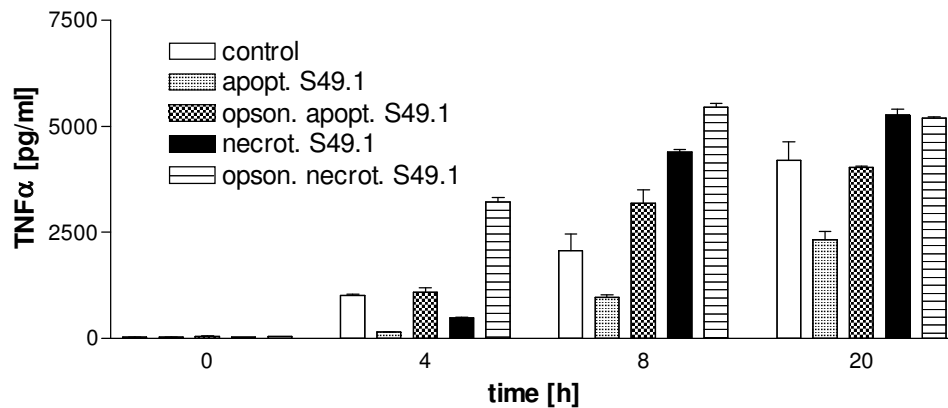


Figure 33: Influence of opsonisation of dying cells with mouse serum on macrophages response after stimulation with LPS

Necrosis was induced as described before by freeze-thaw. Apoptosis was induced by stimulation with ActD/CD95L. Opsonised cells were incubated with 10 % mouse serum at 37 °C for 30 min. Necrotic and apoptotic cells were incubated with BMDM for 2 h. Samples for TNF determination were taken after stimulation with LPS [100 ng/ml] at indicated times. Control samples were just stimulated with LPS. TNF α concentrations were determined by ELISA. Data shown are means + SD of duplicate determinations.

In a second approach mouse serum was used. The results in fig. 33 indicate that mouse serum had an impact on the recognition of apoptotic cells as described before. In addition, the presence of serum had an effect on the recognition of necrotic cells. An opsonisation of apoptotic and necrotic cells for 30 min at 37 °C, before these cells were co-incubated with the macrophages, increased the detected TNF α concentrations. Opsonisation of apoptotic cells increased TNF α concentration to levels seen after stimulation with LPS alone. Opsonisation of necrotic cells further increased TNF α release by BMDM, while also pre-incubation with necrotic cells in the absence of serum resulted in higher TNF α levels compared to control.

4.3.7 Modulation of macrophage response by dying Jurkat T cells

The Jurkat T cells were used to establish cell death models where necrosis could be induced by ATP depletion and stimulation with STS. These cells show no activation of caspases and lose cell membrane integrity after 5 hours (Hirt *et al.*, 2000).

Treatment with staurosporine alone resulted in an apoptotic phenotype, characterised by DEVD-afc cleavage and chromatin condensation. After incubation with the ionophore ionomycin Jurkat T cells show PS exposure, but no activation of caspases and no chromatin condensation. These cells were used to see if PS exposure alone was a sufficient trigger to induce immunomodulation compared to apoptotic cells.

Experiments with HMDM indicated that apoptotic Jurkat cells had the same effect as apoptotic S49.1 cells on BMDM. Surprisingly, the co-incubation with necrotic Jurkat cells resulted also in lower TNF α concentration and co-incubation with ionomycin treated cells increased TNF α levels compared to control (personal communication with U. Hirt).

To test if these effects were conserved within cells of different mammalian species, Jurkat cells were co-incubated with BMDM. Remarkably the same effects were seen in these experiments as before with the human macrophages. This was judged as an indication that different recognition mechanisms exist for dying cells, even necrotic cells, and that these mechanisms are conserved within mammals.

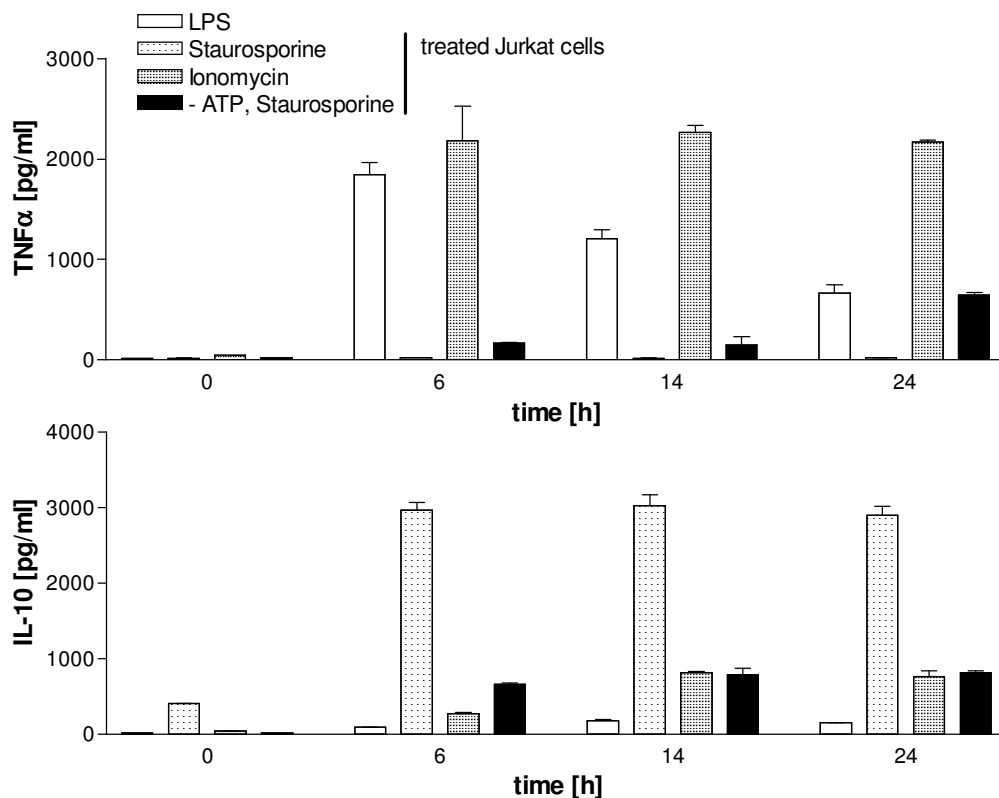


Figure 34: Immunomodulation by apoptotic S49.1 cells after co-incubation with BMDM followed by stimulation with LPS

BMDM were seeded on 96-well microtiter plates and co-incubated with apoptotic (STS, 1 μ M, 2 h), necrotic (ATP depleted, + STS, 1 μ M) or ionomycin treated Jurkat T cells for 2 h. After this period BMDM were washed several times with PBS, fresh medium was added and cells were challenged with LPS [100 ng/ml]. Supernatants were recovered after indicated times. TNF α and IL-10 concentrations were determined by ELISA. Data shown are means + SD of duplicate determinations.

As a second parameter to characterise the effects of these cells on BMDM IL-10 release was measured. As seen before, IL-10 release increases after pre-incubation with apoptotic cells and this was true for the apoptotic Jurkat cells as well. Interestingly, the necrotic Jurkat cells had only a weak effect on IL-10 release comparable to the one ionomycin treated cells (Fig. 34). This was remarkable because an increase of TNF α release was always correlated to a decrease of IL-10 and vice versa. Therefore, the ATP depleted Jurkat cells triggered with STS to induce a necrotic phenotype represent an unknown principle in this system. These results suggested an unknown diversity for the recognition of dying especially necrotic cells.

4.3.8 Influence of apoptotic cells on *E.coli* phagocytosis

One task of macrophages is the phagocytosis of pathogens like *E.coli*. In preliminary experiments the role of apoptotic cells as efficient modulators of macrophages response was shown. In this experiment the capacity of macrophages to engulf fluorescence labelled *E.coli* particles after pre-incubation with apoptotic cells was tested.

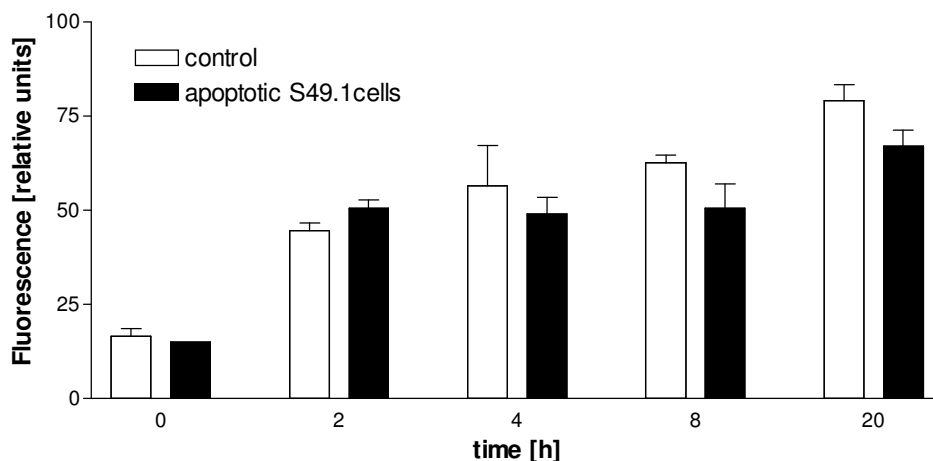


Figure 35: *E.coli* phagocytosis of BMDM after pre-incubation with apoptotic S49.1 cells

BMDM were pre-incubated with apoptotic S49.1 cells (ActD / CD95L, 16 h) for 2 h. Then *E.coli* particles [10 μ g/ml] were added and phagocytosis was measured after wash and lysis of the BMDM. Shown are means + SD from duplicate determinations.

Pre-incubation with apoptotic cells had an influence on macrophage response to LPS, but *E.coli* phagocytosis itself was unaffected as shown fig. 35. In this way the macrophages can still remove *E.coli* particles and just inform their surrounding neighbours about the new context, for example the recognition of apoptotic or necrotic cells.

4.3.9 Macrophage response is not induced by a soluble factor

Immunomodulation by apoptotic cells was shown to recruit the PS receptor on macrophages for signalling (Fadok *et al.*, 2000). Therefore PS exposure on apoptotic cells seems to be an important regulator of macrophage response. Interestingly, the ionomycin treated Jurkat cells could not mimic an apoptotic cells although they were PS positive on

RESULTS

the surface. This was an indication that further ligands could exist that influence macrophage response. This experiment was performed to see if soluble ligands could induce immunomodulation in the BMDM model. For this purpose cell inserts were used for the next co-culture experiments. These small cages allow a diffusion of soluble factor, but prevent contact between the different cell types. As endpoints NO and TNF release were measured after activation of the BMDM with LPS.

Results in fig. 36 indicated that not a soluble ligand induced immunomodulation, but rather a ligand connected to the surface of the apoptotic cell. The release of TNF α as well as NO indicates that immunomodulation was observed when a direct contact between apoptotic cell and macrophage was possible, but not when this contact was avoided by the cell insert.

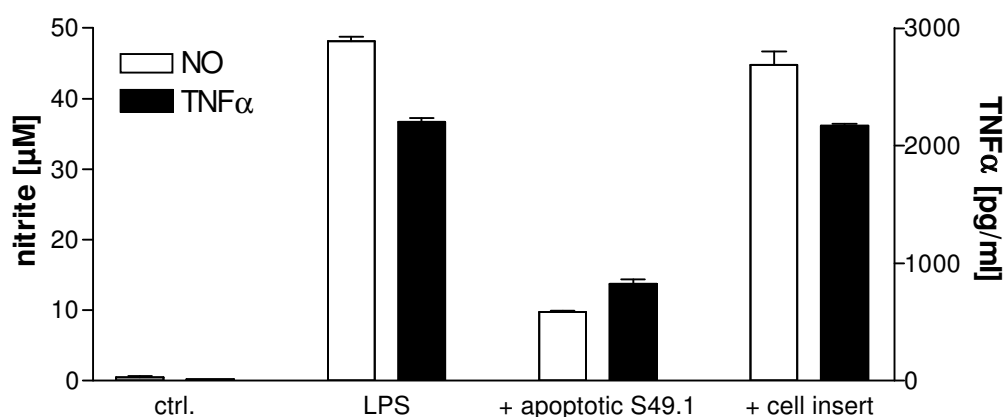


Figure 36: NO and TNF α release after co-incubation with apoptotic S49.1 and stimulation with LPS

BMDM were seeded on 24-well microtiter plates and co-incubated with apoptotic S49.1 cells (ActD/CD95L) for 2 h. After this period BMDM were washed several times with PBS, fresh medium was added and cells were challenged with LPS [$1 \mu\text{g/ml}$]. Supernatants were collected after 8 h for TNF measurement and after 24 h for NO determination by the Griess assay. Data shown are means + SD of duplicate determinations.

4.4 Effects of extracellular HSP 70 on macrophages

The primary function of HSP70 is the one of an intracellular molecular chaperon. HSC70 is the constitutively expressed form, HSP70 is the form induced by a broad range of stress factors. Despite this known function, new reports ascribe different extracellular roles for HSP70. One of these is the elicitation of inflammatory mediators by human macrophages.

4.4.1 Induction of inflammatory mediators in different macrophages

Initial experiments were performed to reproduce the finding that HSP70 can act as a chaperokine (a chaperone and a cytokine) in human monocyte derived macrophages (HMDM). In further experiments the question was asked if HSP70 was also able to stimulate the release of other cytokines in mouse macrophages like Kupffer cells, microglia or BMDM.

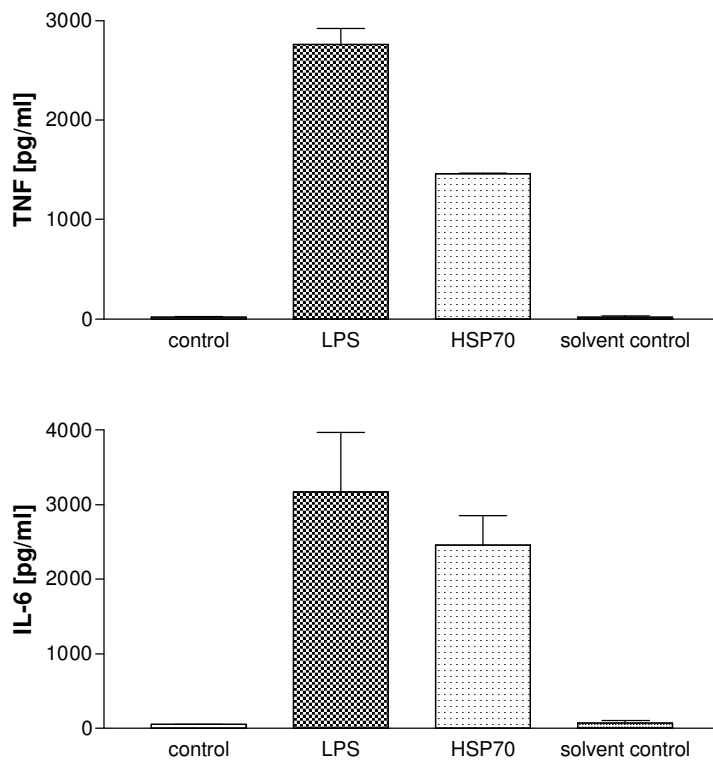


Figure 37: Cytokine release of HMDM (DIV7) 24 hours after stimulation with HSP70

HMDM were cultured in 96-well microtiter plates and stimulated with LPS [100 ng/ml] or HSP70 [10 ng/ml] for 24 h. Supernatants were collected and stored at -20 °C until measurement of cytokine concentrations. Data shown are means + SD of duplicate determinations.

As shown in fig. 37 the reports on the pro-inflammatory action of HSP70 could be reproduced. A stimulation of HMDM with HSP70 resulted in an increase of the pro-inflammatory cytokines TNF α and IL-6 after 24 h stimulation.

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The same experiments were repeated with human monocytes (DIV 0). In these experiments the monocytes showed stimulated control samples. This could be explained with the much higher stimulation of the cells due to the preparation (data not shown).

As another representative macrophage population Kupffer cells, the resident macrophages of the liver were tested. Kupffer cells were obtained from mouse liver preparations. The macrophages are enriched in the non-parenchymal cells (NPC) up to 50 %. The stimulation with HSP70 was stopped after five and twenty-four hours, then cytokine concentrations were measured. From preliminary experiments it was known that a maximal TNF α level is reached in Kupffer cells after five hours. In a second experiment the concentration / response relation of HSP70 on the TNF α induction in NPC was tested.

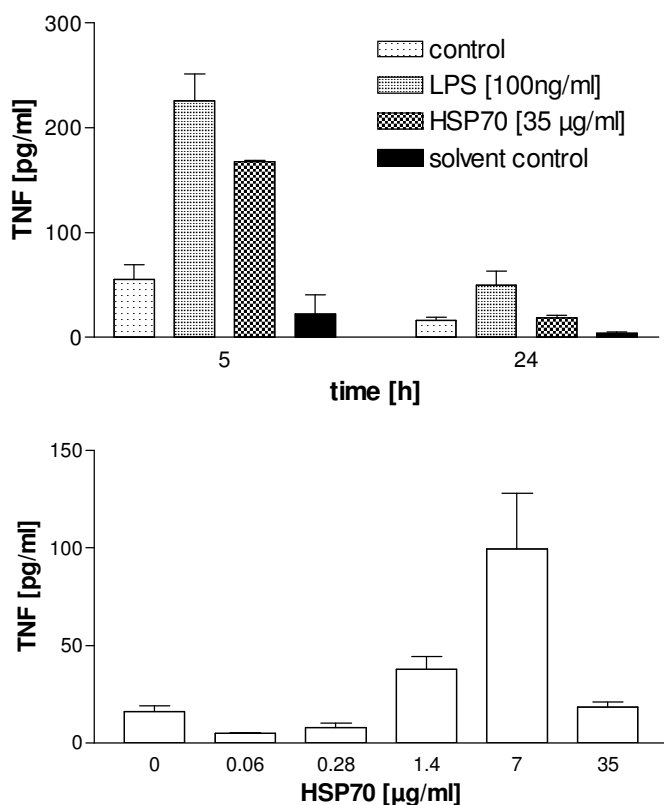


Figure 38: TNF α release of NPC after stimulation with HSP70

NPC were stimulated with HSP70. Samples for the TNF α determination were taken at the time points indicated. For the concentration / response relation supernatants were taken after 24 h. TNF α concentrations were measured by ELISA. Data shown are means + SD of duplicate determinations.

As can be seen in fig. 38 the detectable concentrations of TNF α after five hours were much higher compared to the release after 24 h. The concentration response curve showed the highest TNF α concentration after stimulation with a HSP70 concentration of 7 μ g/ml. This was a five fold lower concentration as used in the first experiment. It was unlikely that this effect was the result of a toxic compound in the solvent, because the HSP solvent was tested negative for cytotoxic effects in a MTT assay (data not shown).

The potential of HSP70 to induce the release of inflammatory mediators was also shown in BMDM and microglia cells (Fig. 39). The induction failed in incubation with mouse whole blood (data not shown). This result was explained by unspecific protein interactions of Hsp70 with serum proteins.

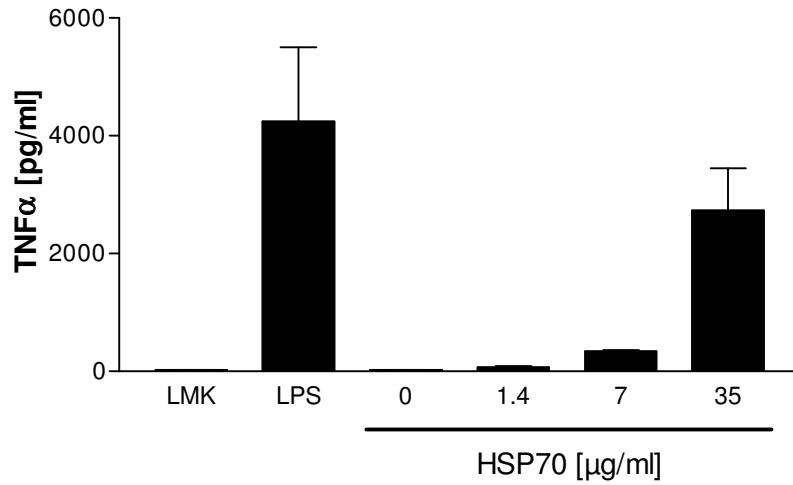


Figure 39: Hsp70 concentration dependent TNF α release after stimulation of microglia cells

Microglia cells were stimulated with Hsp70. Samples for the TNF α determination were taken after 24 h. LPS [100 ng/ml] was used as positive control. TNF α was measured by ELISA. Data shown are means + SD of duplicate determinations.

4.4.2 Induction of NO release and sensibilisation by IFN γ

NO is produced by activated macrophages by the inducible NO synthase (iNOS), which uses L-arginine as substrate. The induction of iNOS can be detected in macrophages after stimulation with LPS. In addition to the induction of cytokines it was of interest to know if Hsp70 could also induce the iNOS.

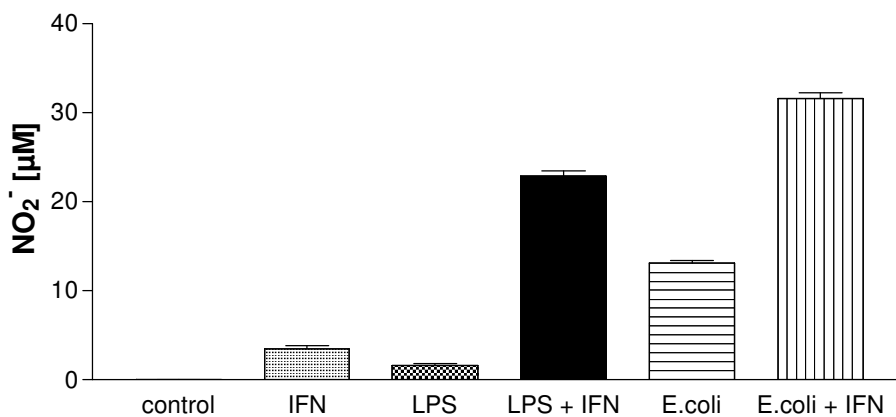


Figure 40: NO release by BMDM after stimulation with LPS or *E.coli* ± IFN γ

BMDM were exposed to IFN γ [100 U/ml] 2 h before cells were stimulated with LPS [1 µg/ml] or *E.coli* [1 µg/ml]. Supernatants were recovered after 24 h and NO production was measured by the concentration of nitrite by the Griess assay. Data are shown as means + SD of triplicate determinations.

The generation of NO via the iNOS was analysed as NO₂⁻ by the Griess assay (Ding *et al.*, 1988; Green *et al.*, 1982). BMDM cells were stimulated with LPS and *E.coli* in combination with interferon γ (IFN γ). IFN γ is an inducer of NO and a cytokine that has a synergy effect with stimuli like LPS and *E.coli* in this function.

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Results presented in fig. 40 showed that iNOS was induced by LPS and *E.coli* particles. The pre-incubation with IFN γ increased the release of NO. The release of NO after stimulation with 1 $\mu\text{g}/\text{ml}$ LPS was much lower compared with the release with the same concentration of *E.coli* particles.

In additional experiments the NO release of BMDM after stimulation with different LPS concentrations was tested. Concentrations of 10 ng/ml LPS were found to result in a detectable concentration of nitrite. Responsiveness and sensitivity of BMDM to LPS measured as NO release was found to be constant from 4 to 9 DIV (data not shown).

After these results were gained BMDM were tested for their ability to generate NO after stimulation with Hsp70. The synergistic effect of IFN γ on NO release in concert with different Hsp70 concentrations was examined in the same experiment. As a positive control LPS was used at a concentration range from 1 to 1000 ng/ml.

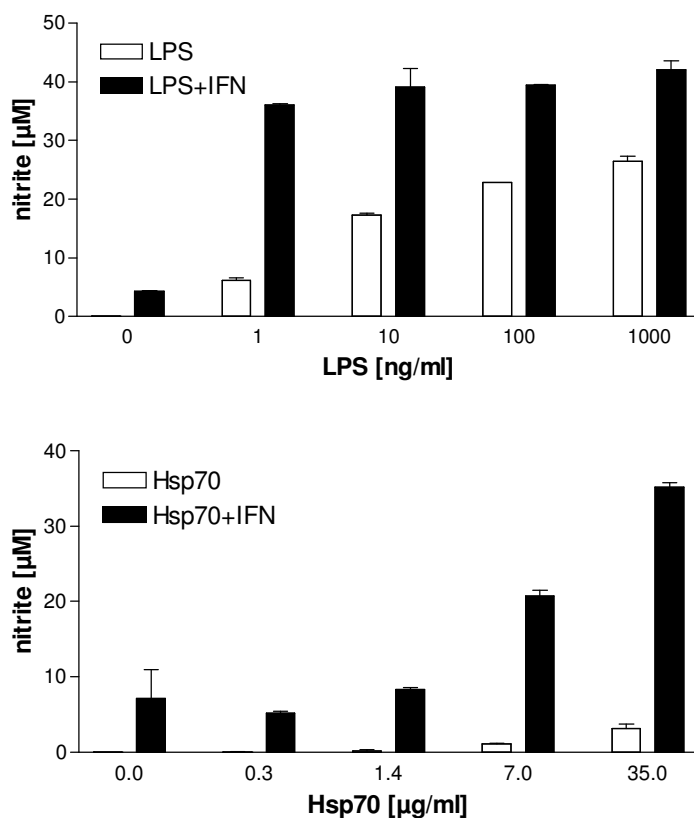


Figure 41: NO release of BMDM after stimulation with LPS and Hsp70 \pm IFN γ

BMDM were exposed to IFN γ [100 U/ml] 2 h before cells were stimulated with LPS or Hsp70. Supernatants were recovered after 24 h and NO production was measured by the concentration of nitrite by the Griess assay. Data represent means + SD of duplicate determinations.

The concentration dependent ability of LPS to induce NO release in BMDM is shown in fig. 41. This response was increased by pre-incubation [2 h] of BMDM with IFN. As seen with LPS, a Hsp70 sensitive and concentration dependent NO release of BMDM could be shown. The co-treatment with IFN γ resulted in a synergistic increase of NO. Compared to stimulation with LPS very high concentrations of Hsp70 were necessary to induce a significant release of NO. A concentration of 35 $\mu\text{g}/\text{ml}$ Hsp70 was as potent as a LPS concentration of 1 ng/ml, compared to the induction of NO release.

4.4.3 Effects of low endotoxin Hsp70 and the recombinant protein expressed in an eucaryotic system

The Hsp70 used was the recombinant human protein expressed in *E.coli*. The usage of this expression system has the drawback of a potential contamination with LPS. Especially in experiments that measure an inflammatory response of cells after stimulation with a recombinant protein one has to be careful not to detect LPS effects.

With the cDNA in hands, the bacculus virus system was used to express the protein in an eucaryotic system. The protein was combined with a his-tag to facilitate the purification.

In an initial experiment the FCS dependent activity of Hsp70 was tested. Serum contains a LPS binding protein (LBP) that can facilitate the binding at the TLR receptor and by this way the release of cytokines. A FCS independent Hsp70 activity would serve as a further argument for a LPS independent protein activity.

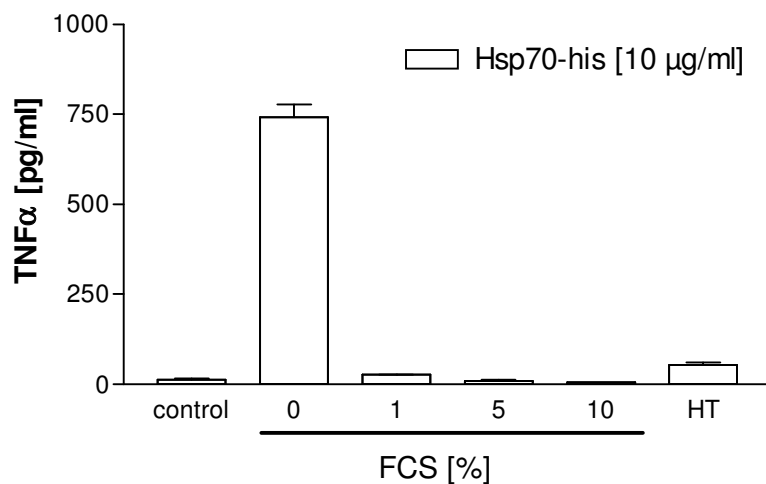


Figure 42: BMDM were stimulated with Hsp70 and different FCS concentrations

BMDM cells were stimulated with the recombinant human Hsp70-his protein expressed in an eucaryotic system. Stimulation with Hsp70 protein was tested with different FCS concentrations. The protein was heat denatured (HT) by incubation at 95° C for 30 min. Samples for the TNFα determination were taken after 7 h. Values are means + SD of duplicate determinations.

The co-incubation of Hsp70-his with increasing concentrations of FCS showed a dramatic decrease in the release of TNFα (fig. 42). The effect of FCS was explained by the binding of Hsp70 to denatured proteins of the heat inactivated FCS. The his-tag could be another explanation for these observations. The activity of Hsp70-his could be decreased by heat inactivation of the protein and no FCS was present. This result was an additional argument for the extracellular activity of Hsp70.

The increase in TNFα response after stimulations of BMDM in the absence of FCS was described for Hsp70-his. In experiments with the recombinant hu-Hsp70 expressed in *E.coli* 10 % FCS was used. The observations made before gave rise to the possibility to increase the signal intensity of this protein by lower FCS concentrations.

RESULTS

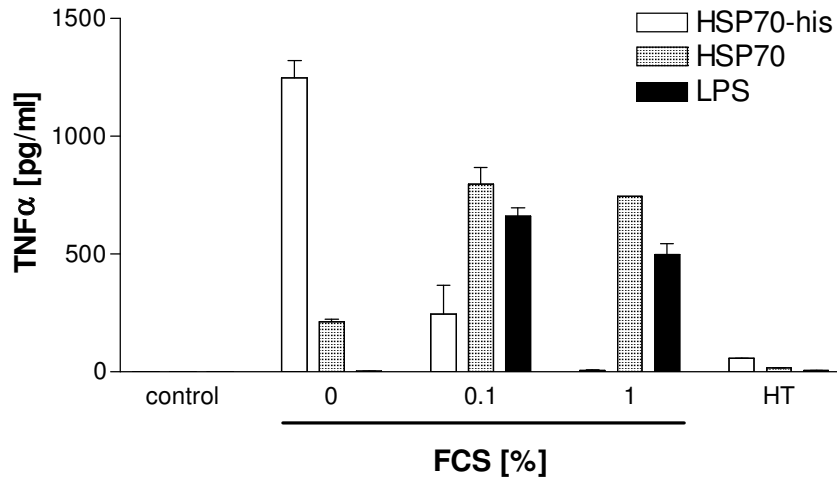


Figure 43: TNF α release of BMDM after stimulation with Hsp70

BMDM cells were stimulated with the recombinant human Hsp70-his protein expressed in an eucaryotic system (Hsp70-his [10 μ g/ml]) or the same protein expressed in *E.coli* (HSP70 [10 μ g/ml]). Stimulation with HSP70 protein was tested with different FCS concentrations. The protein was heat denatured (HT) by incubation at 95 $^{\circ}$ C for 30 min. LPS [1 ng/ml] was used as positive control for TNF α induction. Samples for the TNF α determination were taken after 4 h. TNF α concentrations were determined by ELISA. Data shown are means + SD of duplicate determinations.

The incubation with the recombinant protein from *E.coli* and FCS resulted in an increase of TNF α release in BMDM. This observation was in contradiction to the one with the Hsp70-his. The effects of FCS on the TNF α induction by Hsp70-his could be explained by the his-tag or by an influence of the protein purification protocol (Fig. 43).

The interaction of Hsp70 and Hsp70-his with FCS and their potency to induce TNF α release were inconsistent. The possibility of a contamination of the protein solution was tested indirect by comparison with a low endotoxin hu-Hsp70 (Stressgene). BMDM were incubated with a concentration range from 0.1 to 100 μ g/ml low endotoxin (i.e.) Hsp70 and the release of TNF α and NO were determined.

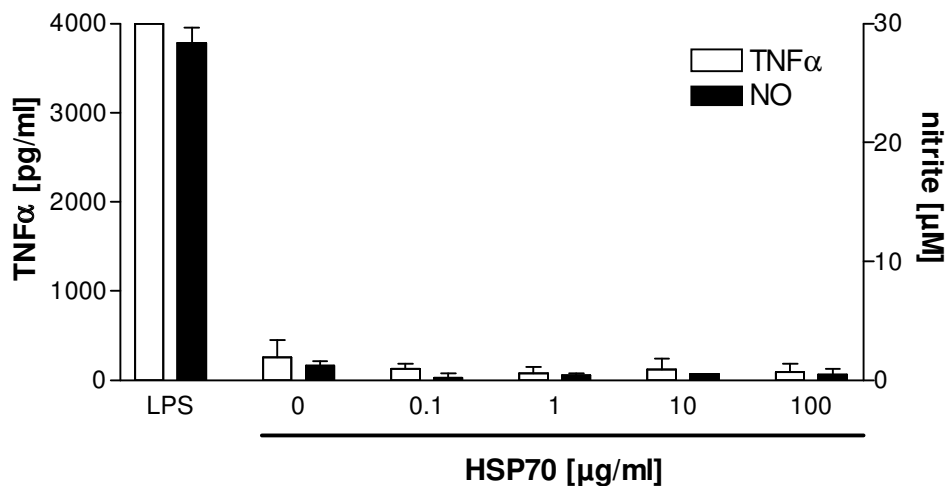


Figure 44: TNF α and NO release of BMDM after stimulation with low endotoxin Hsp70

BMDM cells were stimulated with the low endotoxin recombinant human Hsp70 protein expressed in *E.coli*. LPS [100 ng/ml] was used as positive control for TNF α induction. Supernatants were recovered after 6 h for TNF α release and after 24 h for NO production. Nitrite was measured by the Griess assay. TNF α concentrations were determined by ELISA. Data are means + SD of duplicate determinations.

RESULTS

As shown in fig. 44 the low endotoxin Hsp70 showed no activity in both assays but stimulation with LPS [100 ng/ml] resulted in a release of NO and TNF α . These results were confirmed in a further experiment with the microglia cell line BV-2 in combination with different FCS concentrations (Fig. 45).

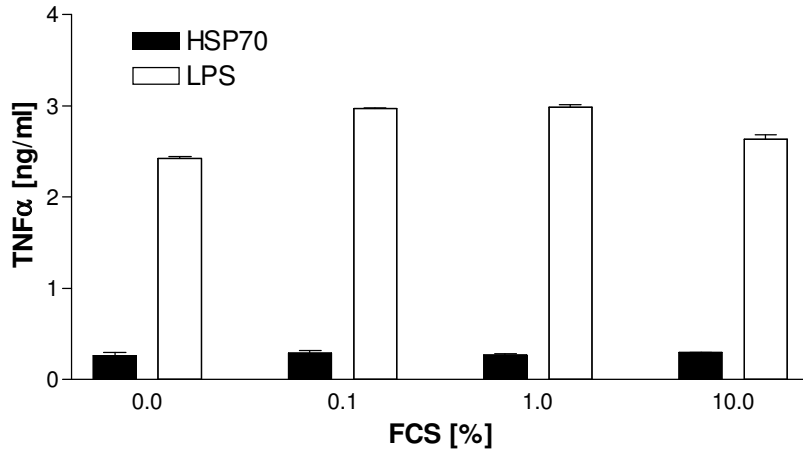


Figure 45: TNF α release of the microglia cell line BV-2 after stimulation with low endotoxin Hsp70 in combination with different serum concentration

BV-2 cells were stimulated with the low endotoxin recombinant human Hsp70 [10 μ g/ml] protein expressed in *E.coli*. LPS [100 ng/ml] was used as positive control for TNF α induction. Supernatants were recovered after 6 h for TNF α release and concentrations were determined by ELISA. Data are means + SD of duplicate determinations.

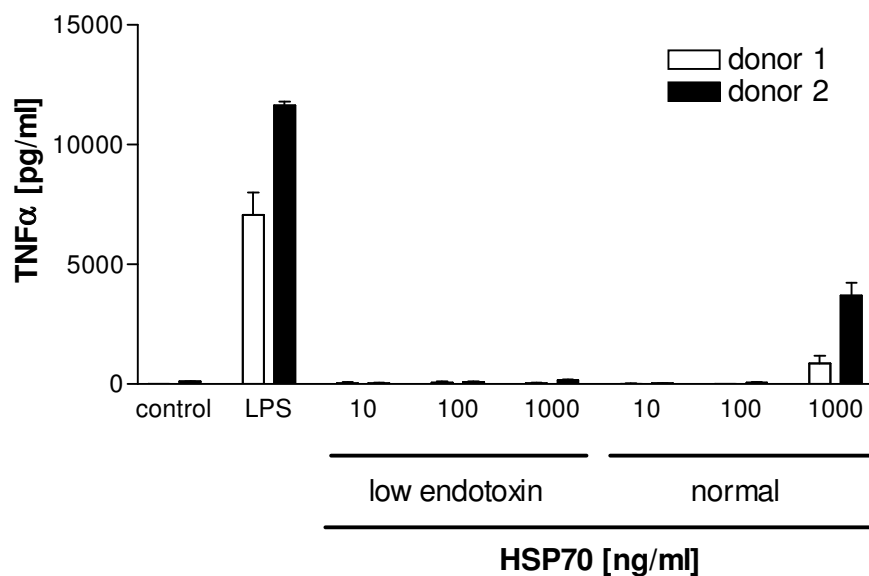


Figure 46: TNF α release of HMDM after stimulation with low endotoxin and standard Hsp70 preparations

HMDMs (DIV10) from two donors were stimulated with the low endotoxin recombinant human Hsp70 protein and the normal HSP70 protein both expressed in *E.coli*. LPS [10 ng/ml] was used as positive control for TNF α induction. Supernatants were collected after 6 h for TNF α release and concentrations were determined by ELISA. Data are means + SD of duplicate determinations.

No stimulation of TNF α release was seen in BV-2 cells after incubation with i.e. Hsp70. The presence of FCS had no influence on the induction. Interestingly, the absence of FCS in BV-2 cells only had weak effects on TNF α induction by LPS concentrations of 100 ng/ml. TNF α level was decreased, but not to the same degree as seen in BMDM (Fig. 43, p. 66). This could be explained by hundred fold lower LPS concentration used to stimulate BMDM in the corresponding experiment.

The induction of TNF α release in HMDM was possible with much lower concentrations of LPS or Hsp70. HMDM prepared from two donors were stimulated with the low endotoxin Hsp70 and the normal Hsp70. The preparation with the low endotoxin concentration resulted in no activity, whereas a concentration of 1 μ g/ml of the normal Hsp70 led to a significant increase in TNF α release (Fig. 46).

Next, the potential LPS binding of Hsp70 was tested in BMDM. Macrophages were stimulated with a concentration range from 1 to 1000 ng/ml LPS and a constant concentration of low endotoxin Hsp70 [10 μ g/ml]. In the same experiment the impact of FCS on TNF α release was tested.

Exposing BMDM to LPS in the absence of serum did not enhance TNF α release significantly, compared to the cells incubated with 1 % FCS. The Hsp70 protein did not have a strong effect on the induction of TNF α by LPS. The protein showed the ability to decrease TNF α induction in the presence of FCS and LPS concentrations of 10 and 100 ng/ml. But this effect was not very pronounced (Fig. 47).

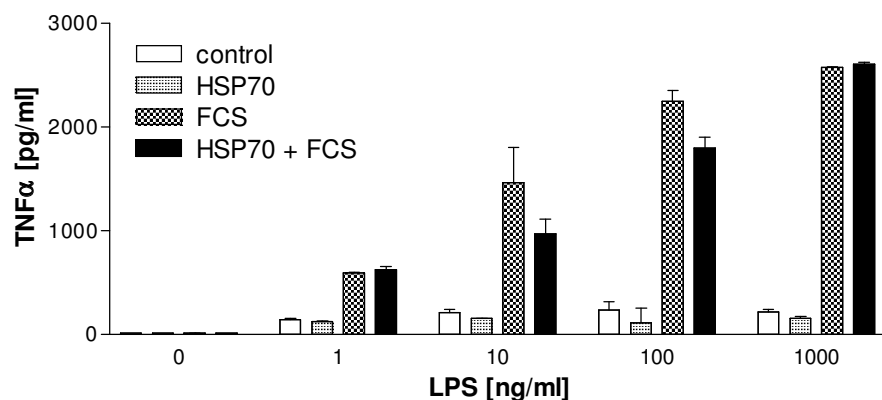


Figure 47: TNF α release of BMDM after co-stimulation with LPS and low endotoxin Hsp70

BMDMs were stimulated with the low endotoxin recombinant human Hsp70 [10 μ g/ml] protein. The incubation was performed in the absence or presence of FCS [1 %]. Supernatants were collected after 6 h for TNF α release and concentrations were determined by ELISA. Data are means + SD of duplicate determinations.

4.4.4 Hsp70 and the toll like receptors

Hsp70 was shown to be the endogenous ligand of the toll-like receptor 4 (TLR4). This receptor is also responsible for signalling of Hsp60 and other exogenous ligands, for example LPS. The experiments presented before in this work indicated that in low endotoxin preparations of Hsp70 no induction of inflammatory mediators could be seen. Nevertheless both preparations were examined in TLR4 deficient mice. The

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C57BL/10ScCr mice lack the receptor completely, the C3H/HeJ mice have a point mutation in TLR4 resulting in an inhibition of signal transduction.

As a control for the deficient TLR4 receptor signalling, LPS was used. As can be seen in fig. 48 the wildtype of both mice strains can transduce the LPS signal, whereas a TNF α release in the mutated forms was not detectable.

LTA was used as positive control to show that the wildtype and mutated mice response to an alternative stimuli with comparable intensity. LTA was shown to be of very high purity and is an exogenous ligand of toll-like receptor 2. TNF α release detected after stimulation with LTA indicated that both types of the respective strains response to an alternative stimulus with the same intensity. These results were taken as confirmation that the mutated forms had no general defect in response to inflammatory stimuli. These results would confirm the older publications on Hsp70 signalling through TLR4 (Asea *et al.*, 2002; Dybdahl *et al.*, 2002; Vabulas *et al.*, 2002).

The low endotoxin Hsp70 preparations were tested in BMDM of the C3H/HeN and HeJ mice. BMDM cells prepared from both mice showed no release of TNF α (Fig. 49). Stimulation with LPS resulted in a TNF α signal in C3H/HeN mice, but not in C3H/HeJ. As seen before, stimulation with LTA activated BMDM from both types. Taken together these results were judged as an indication for a TLR4 independent action of Hsp70 and a further hint for a LPS contamination of Hsp70 in data published before.

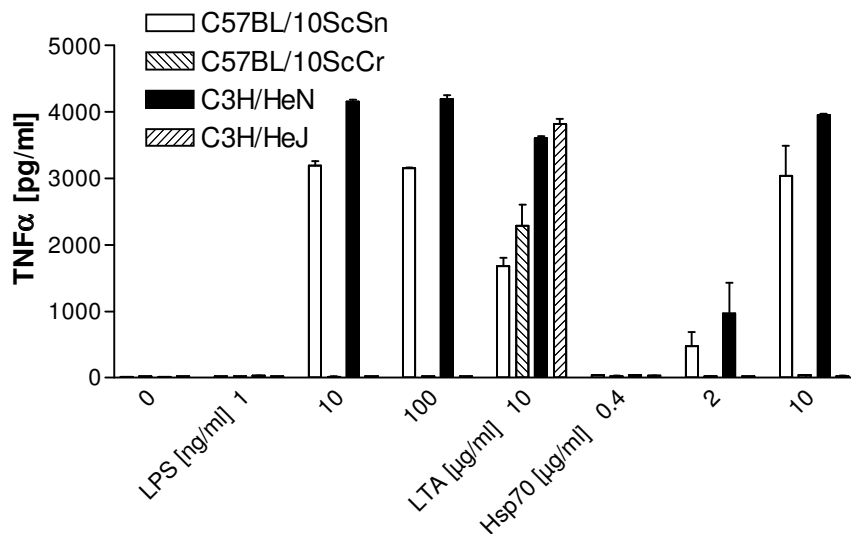


Figure 48: TNF α release in BMDM from two TLR4 deficient mice strain after stimulation with Hsp70

BMDM were plated and stimulated with increasing Hsp70 (Stressgene) concentrations. LPS and LTA were used as controls. The incubation was performed in the absence of FCS. Supernatants were recovered after 6 h for TNF α release and concentrations were determined by ELISA. Data are means + SD of duplicate determinations.

RESULTS

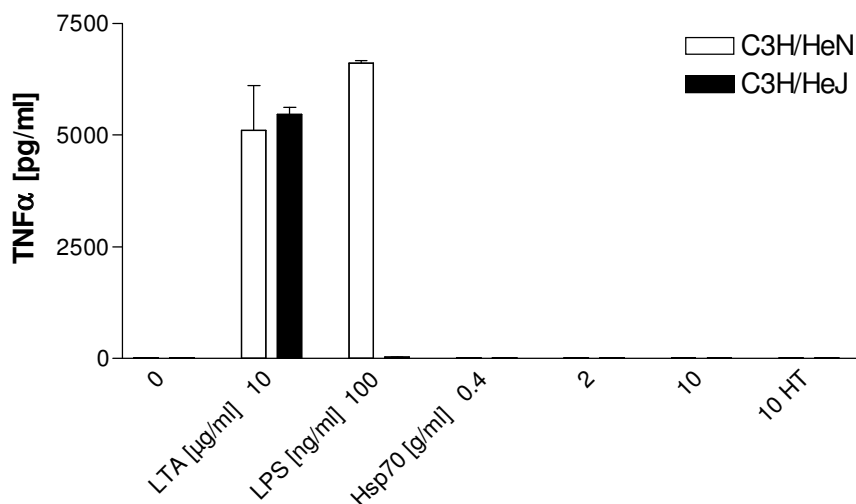


Figure 49: TNF α release of BMDM from TLR4 deficient mice after stimulation with low endotoxin Hsp70.

BMDM were plated and stimulated with different low endotoxin Hsp70 (from the University of Konstanz) concentrations. LPS and LTA were used as controls. The incubation was performed in the absence of FCS. Supernatants were recovered after 6 h for TNF α release and concentrations were determined by the TNF α ELISA. Data are means + SD of duplicate determinations.

4.4.5 HSP70 preparations in the LAL-test

In parallel with the different Hsp70 preparations LAL-tests (Limulus amoebocyte lysate, Endosafe[®] Endochrome-K[™], Charles River) were performed to get further information about the potential contamination of the protein solutions with LPS. In these assays Hsp70 protein concentrations of 10 μ g/ml were used.

Provider	Expression system	Purification	Contamination [EU/10 μ g protein]	Standard LPS[μ g/m]
University of Konstanz	insect cells		2 EU	200
University of Konstanz	insect cells	low endotoxin	< 0.05 EU	< 5
Stressgene	bacteria	low endotoxin	< 0.05 EU	< 5
Stressgene	bacteria		0.6 EU	60

Table 2: Detection of LPS in different Hsp70 preparations with the LAL-assay.

The different Hsp70 preparations were diluted to a concentration of 10 μ g/ml. The LAL-test was performed as described by the instructions of the supplier. *E.coli* strain 055:B5 was used as source of standard LPS. Data are means of duplicate determination.

As can be seen from the table 2 only the low endotoxin preparations had endotoxin concentrations below the detection level. Interestingly, the normal Stressgene preparation

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showed a low concentration of endotoxin. This concentration was not detectable with the assay used before (Limulus amoebocyte lysate test kit, QCL-1000, Bio Whittaker), explained by the lower detection limit. Remarkably the detected concentrations of LPS were too low to generate a TNF α signal in our tests with BMDM cells. This observation could be explained by a direct effect of Hsp70.

The low endotoxin preparations were also tested for their potential to induce TNF α release and compared with LPS and the normal preparation from Stressgene. As shown in fig. 50 both low endotoxin preparations had no effect on TNF α release in BMDM.

With respect to their higher sensitivity to LPS HMDM were challenged with normal and low endotoxin preparations from Stressgene (Fig. 46, p.67). These results together with the data presented in chapter 4.4.3 indicate that low endotoxin Hsp70 could not induce a TNF α release in contrast to the stimulation with Hsp70 preparations containing low concentrations of LPS.

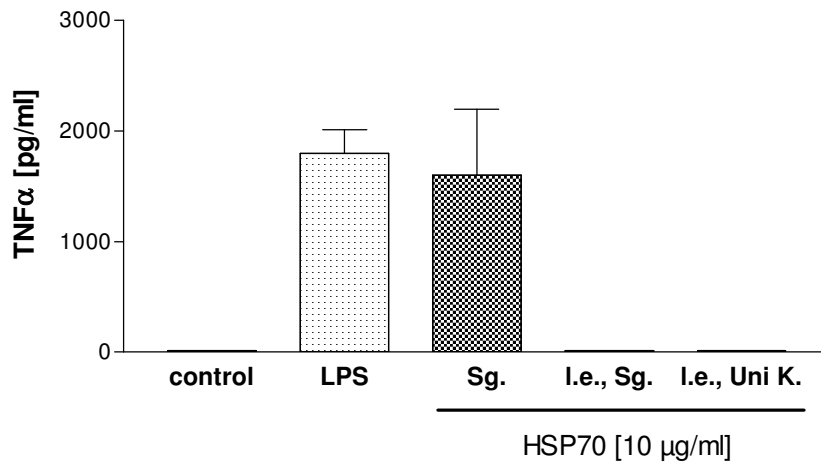


Figure 50: TNF α release of BMDM after stimulation with different Hsp70 preparations

BMDM were stimulated with different preparations of recombinant human Hsp70 [10 μ g/ml] protein. As positive control LPS [100 ng/ml] was used. The incubation was performed in the presence of FCS [1 %] with two low endotoxin (i.e.) preparations and a not further tested Hsp70 (Sg.: normal purification from Stressgene (*E.coli*), i.e. Sg.: low endotoxin preparation from Stressgene (*E.coli*), i.e. Uni K.: low endotoxin preparation from the University of Konstanz (insect cells)). Supernatants were recovered after 6 h for TNF α release and concentrations were determined by ELISA. Data are means + SD of duplicate determinations.

Protein activity is often demonstrated by the fact that observed properties are no longer present after protein denaturation by heat. Especially with regard to inflammatory studies this fact is often used to show activation of cells by proteins and to distinguish their effect from the one of LPS. This can be done because LPS is known to be heat stable.

Nevertheless low LPS concentration can be masked, when proteins are present during heat inactivation (personal communication: Dr. Dr. T. Hartung). Therefore, heat inactivation is not the appropriate test to show the absence of LPS in a protein preparation and often results in false negative results.

RESULTS

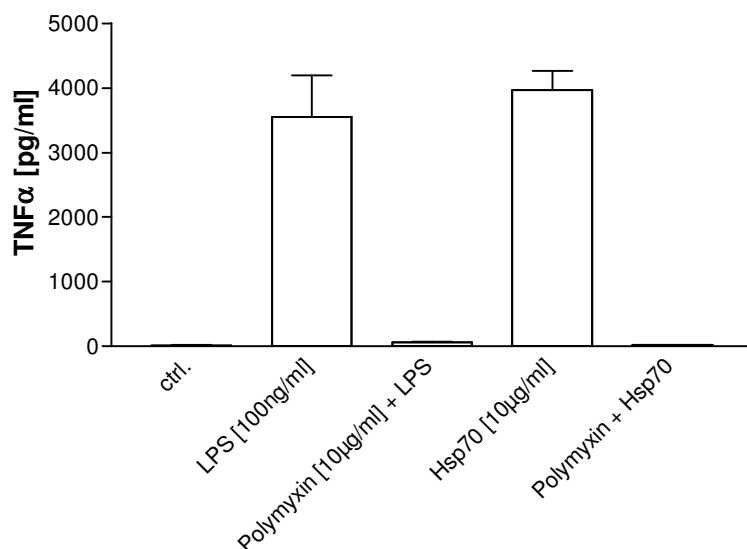


Figure 51: TNF α release from BMDM after co-incubation with LPS or Hsp70 and polymyxin

BMDM were treated with recombinant human Hsp70 [10 μ g/ml] and LPS [100 ng/ml]. Supernatants were recovered after 6 h for TNF α release and concentrations were determined by ELISA. Data are means + SD of duplicate determinations.

An alternative way to test LPS contaminations is by the use of polymyxin. This is a substance showing high binding affinity to LPS and low cellular toxicity. BMDM cells were incubated with LPS or Hsp70 in the presence or absence of polymyxin [10 μ g/ml]. This concentration was tested to be non-toxic previously. Stimulation of BMDM with LPS [100 ng/ml] and Hsp70 [10 μ g/ml] activated the macrophages resulting in the release of equal amounts of TNF α .

The co-incubation with polymyxin [10 μ g/ml] was sufficient to bind LPS [100 ng/ml] in a way that no TNF α signal was detectable. The same experimental outcome was observed in experiments with Hsp70 (Fig. 51). These results were taken as a further indication for LPS contamination in Hsp70 preparations that showed activity in a TLR4 dependent manner.

4.5 Extracellular caspase activity released from apoptotic cells

Caspases play a major role in the apoptotic death program. They represent a family of cystein proteases, which are homologues for the *C. elegans* cell-death gene product CED-3. Under physiological conditions the last step of the apoptotic cell death program is the phagocytosis of the cell. In this case the release of caspases is prevented by the rapid uptake of the dying cell.

Nevertheless apoptotic oligonucleosomal DNA fragments can be detected in plasma of cancer patients (Jahr *et al.*, 2001), or in mice after fulminant hepatic damage (Leist *et al.*, 1995; Leist *et al.*, 1996). In these situations the capacity for phagocytes to engulf apoptotic cells can be saturated. Under these conditions of impaired phagocytosis it could be possible that apoptotic cells loose their membrane integrity and active caspases are released.

Therefore the potential role of caspase-3 to propagate damage and to act as an inflammatory mediator was investigated. Further experiments were designed to describe extracellular stability of active caspase-3 and the release of this protease in different cell lines. We also had the possibility to test liquor samples from patients with traumatic brain injury as example for a massive tissue injury *in vivo*.

4.5.1 Stability of caspase-3 in extracellular environments *in vitro*

For initial experiments recombinant human caspase-3 was used to test the stability in different biological fluids and together with cells. The stability was tested by the activity of the enzyme after 2 h at 37 °C and expressed as recovery in percent of control.

As shown in fig. 52A the enzyme activity decreased after incubation in solution with low protein concentrations (RPMI 1640 medium) or in protein-free buffers (assay buffer, PBS). Incubations with intact or activated BMDM had no effect on activity. Freeze-thaw of Wehi or Jurkat T cells may result in a release of proteases that could decrease the activity of caspases by proteolysis. Nevertheless the activity was not changed.

Even when no serum was present, the activity was stabilized by coating the wells prior to use with 10 % FCS, indicating that lower activity was caused by adsorption of enzyme at the surface of the plates (Fig. 52A) or could be prevented by low amounts of stabilizing proteins. As shown for BMDM and Wehi cells the activity of the recombinant caspase-3 was maintained even after 24 hours (Fig. 52B).

Although 10 % FCS or human serum had no inhibitory effect (Fig. 52A) the influence of higher concentrations and the influence of heat inactivation (56 °C, 30 min) was tested. Neither untreated nor heat inactivated FCS or human serum had an effect on the activity of DEVD-afc hydrolysis (data not shown).

In further experiments the concentration of human serum was increased up to 98 %. As can be seen in fig. 53 the activity decreased dose dependently with increasing serum concentration. A comparable decrease was not seen when caspase-3 was mixed with 98 % mouse serum (data not shown). Interestingly the cleavage activity at high serum

RESULTS

concentration could be restored upon dilution with serum-free assay buffer or after increasing the substrate concentrations (data not shown).

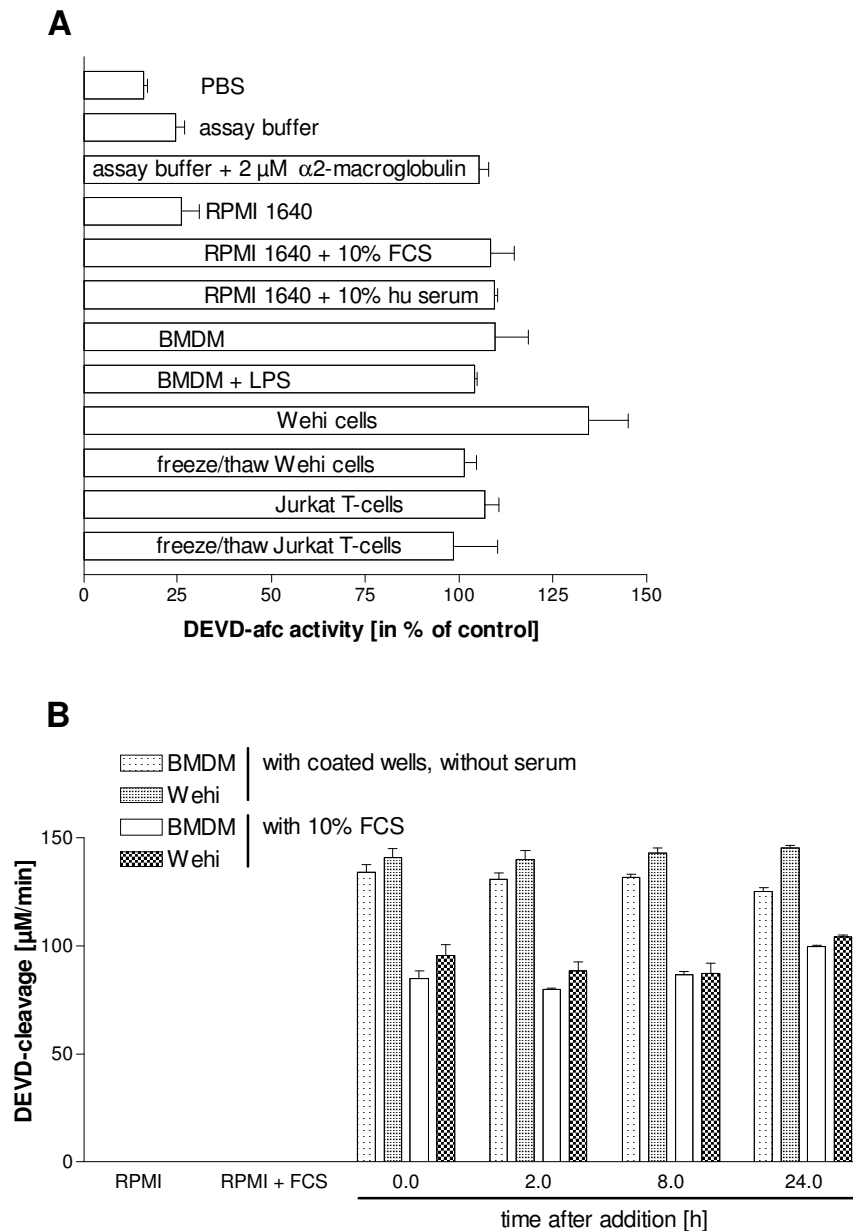


Figure 52: Stability of DEVDase activity

(A) Recombinant caspase-3 was incubated for 2 h at 37 °C in different media or incubated together with cultured cells, with dead cells (freeze-thaw), or with stimulated cells (BMDM, 1 μ g/ml LPS). Data are depicted as means + SD of duplicate determinations. Assay and measurement were performed in 96-well microtiter plates.

(B) The influence of FCS and unspecific surface binding of the enzyme was examined. Coating of 96-well microtiter plates was performed with 10 % FCS in PBS for 1 h at 37 °C before cells were added. Recombinant caspase-3 was incubated for the indicated time with BMDM and Wehi cells. Caspase activity was measured by the DEVD-afc cleavage assay (A+B). Data are depicted as means + SD of duplicate determinations.

RESULTS

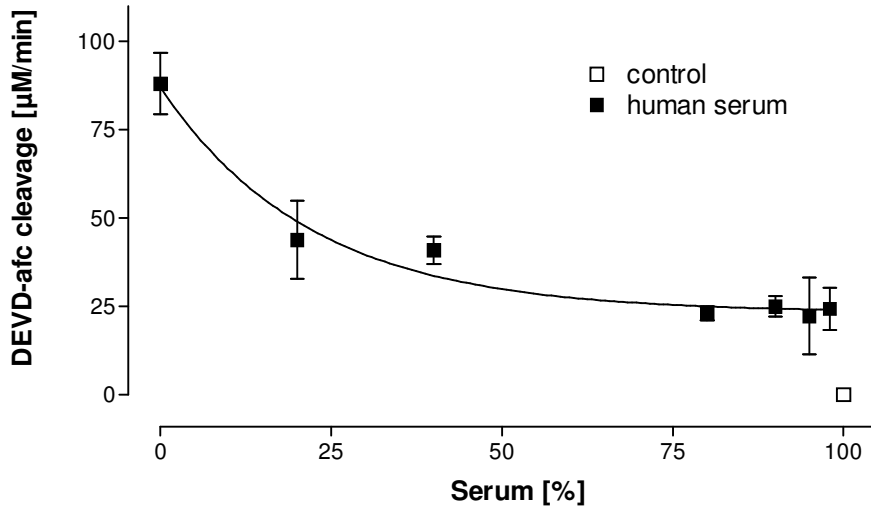


Figure 53: Stability of DEVDase activity

Caspase-3 was incubated with increasing concentrations of human AB-serum for 10 min before DEVDase was measured. Shown are means \pm SD of duplicate determinations.

Therefore an irreversible inhibition of caspase-3 is unlikely. The decrease in cleavage activity is rather an effect of unspecific binding or sequestration by serum proteins during the assay.

4.5.2 Release of active caspases from Jurkat cells

Next, the DEVDase activity from apoptotic Jurkat cells was examined. Jurkat cells were exposed to 1 μ M staurosporine. After two hours they showed a significant increase in nuclear condensation and DEVDase cleavage activity in the cell pellet.

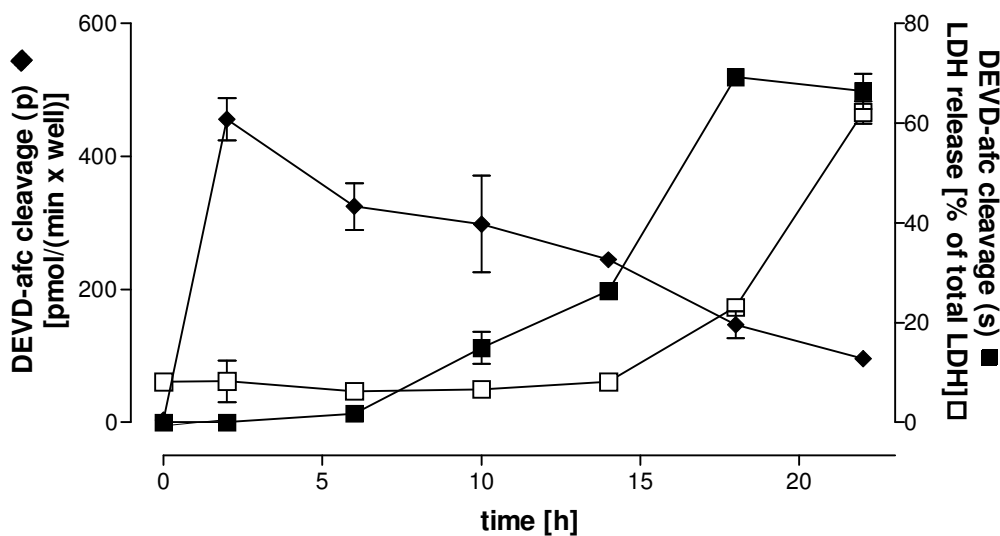


Figure 54: Release of DEVDase activity from Jurkat cells

Jurkat cells were exposed to 1 μ M staurosporine. After the indicated times samples were taken for the determination of DEVDase and LDH activity in the supernatants (s) and in the cell pellets (p). Data are means \pm SD of duplicate determinations.

In further experiments the release of caspase-3 like activity and LDH as a marker for cell lysis was determined. Compared to LDH a decline in the extracellular DEVDase activity was already detected after 6 hours compared with LDH at 14 h. Still, the kinetics of the DEVDase appearance paralleled LDH-release. The extracellular caspase activity reached a maximum after 18 hours, this activity represented around 40 % of the total activity at this time and only 20 % of the intracellular peak value after 2 hours (Fig. 54).

The measurement of extracellular caspase activity can be used as a sensitive method to detect cell lysis in this apoptotic model. Despite an overall decrease of caspase like activity in apoptotic Jurkat cells an extracellular activity was detectable and increased with cell lysis.

4.5.3 Elevated DEVDase activity in liquor of patients with traumatic brain injury

In initial experiments the stability and presence of caspase activity could be shown *in vivo* (Hentze *et al.*, 2001). The question was asked if DEVDase activity could be detected also in human extracellular fluids.

Samples from patients with traumatic brain injury (TBI) and controls were tested. In two out of 42 samples DEVDase activity was found. These samples were tested again with the caspase-3 specific inhibitor DEVD-CHO [100 nM]. In one of the two samples the DEVDase activity was inhibitable, the other sample lost activity (Fig. 55). No control sample showed a detectable DEVDase activity ($\leq 0.1 \mu\text{M}/\text{min}$).

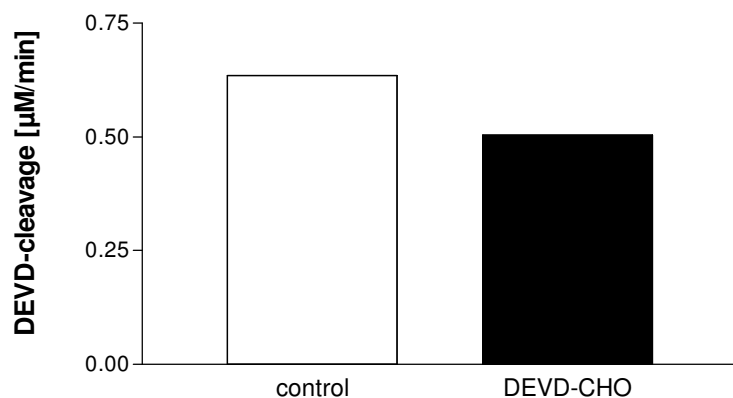


Figure 55: Caspase activity in human cerebrospinal liquor.

DEVDase activity was measured in liquor samples from traumatic brain injury patients (n=42) and in controls (n=7). TBI samples positive for DEVDase activity were measured in the presence of the caspase-3 inhibitor DEVD-CHO [100 nM]. Data shown are single values.

5 DISCUSSION

5.1 Immunomodulation by dying cells

Apoptotic cell clearance is efficient and non-inflammatory and is *in vivo* often mediated by neighbouring cells. The special interest on apoptotic cell removal is explained by its active anti-inflammatory and suggested anti-immunogenic effect.

Initially it was found that uptake of apoptotic cells does not induce the release of inflammatory mediators, but soon it was apparent that inflammatory stimuli can be actively suppressed *in vitro* (Fadok *et al.*, 1998b) and *in vivo* (Huynh *et al.*, 2002). Interestingly, the phagocytosis of apoptotic cells by macrophages, without further stimulation, seems to induce cytokine-independent survival and inhibition of proliferation by activation of Akt and inhibition of extracellular signal-regulated kinase 1 and 2 (Reddy *et al.*, 2002).

The focus of this study was on the anti-inflammatory effect of dying cells. The data presented here confirm the previous findings that a co-incubation with apoptotic cells, followed by stimulation with LPS, *E.coli*, LTA or zymosan results in a shift of the response of macrophages from pro- to anti-inflammatory mediators, when compared with the stimuli alone. For these effects the term immunomodulation was used. Results in this study further confirm that the co-incubation with apoptotic cells has no effect on *E.coli* phagocytosis. The immunomodulation by apoptotic cells has no obvious effect on the capability of macrophages to remove pathogens like *E.coli*.

Necrotic cells on the other hand are expected to trigger an inflammatory response by their lysis. This was shown for neutrophils (Fadok *et al.*, 2001a), but was not found for thymocytes and S49.1 cells in this study. Interestingly, in one report it was shown that necrotic cells in the interdigital space of developing mice, were not correlated with inflammation and fingers developed normally (Chautan *et al.*, 1999). These findings therefore suggest, that there are different ways and mechanisms for the uptake of non-apoptotic cells.

Even the immunomodulating effect of apoptotic cells on macrophages was abolished when the dying cells were opsonised with mouse serum, before they were added to the macrophages, suggesting that another recognition pathway was used involving Fc- and complement receptors. Interestingly, the data presented here indicate that this effect was not present when fetal calf serum in combination with mouse BMDM was used.

The differences in recognition and uptake may also explain the controversial results described for the presentation of antigens from apoptotic or necrotic cells (Rosen and Casciola-Rosen, 1999; Sauter *et al.*, 2000). Another drawback of these studies may be the way to induce necrosis by freeze-thaw. This method and also hyperthermia, neglect the complexity of necrotic cell death. Therefore in some experiments Jurkat T cells were used. Here necrosis was induced by staurosporine in combination with ATP-depletion. The data presented here can demonstrate that co-incubation of these necrotic cells with mouse BMDM resulted in immunomodulating effects, like the ones seen before with apoptotic cells. These effects are an increase in IL-10 and a decrease in TNF α production.

A phosphatidylserine (PS) receptor (Fadok *et al.*, 2000) on macrophages and PS on the apoptotic cells are implicated to be important for the immunomodulation described above (Fadok *et al.*, 1998b). PS exposure is very prominent on apoptotic cells and was shown to be involved in the release of TGF β and PGE $_2$. Data presented here with ionomycin treated Jurkat T cells indicated that also other receptors are involved. These cells expose PS on their surface, but lack further characteristics of apoptotic cells like chromatin condensation or caspase activation. This study demonstrates that these cells have no immunomodulating effect on macrophages.

These data indicate that the differences in dying cells have an individual influence on the macrophages, which are recognising these cells. Therefore, the form of cell death probably alters inflammatory and immunologic reactions. The identification of other receptors involved in these processes will shed more light on the mechanisms that are regulated by dying cells during phagocytosis.

One example of this expanding field was the identification of HMG-1 on necrotic cells (Scaffidi *et al.*, 2002). This protein is present on necrotic cells and could be shown to induce an inflammatory response in macrophages after co-incubation. Co-incubation of macrophages with necrotic HMG-1^{-/-} cells resulted in a much lower increase in TNF α production.

5.2 Hsp70 and the toll-like receptor 4

The classical role of Hsp70 is the one of an intracellular chaperone. First indications that inducible Hsp70 (Genome Data Base designation, HSPA) may also have an extracellular function came from reports indicating increased levels of antibodies against this protein in patients with autoimmune diseases (Minota *et al.*, 1988). Still the question is open, if the presence of antibodies is a result of cell lysis or of a regulated release. Studies supporting the second explanation showed an Hsp70 expression of the cell surface of cancer (Multhoff *et al.*, 1997; Multhoff *et al.*, 1995; Multhoff and Hightower, 1996), apoptotic (Sapozhnikov *et al.*, 2002), or stressed glia cells (Guzhova *et al.*, 2001) and one could speculate that once at the surface the protein can be released easily.

With regard to the function this expression on the surface or release of Hsp70 has, different explanations are discussed dependent on the different systems. The expression of Hsp70 on the surface of glia cells was suggested to be neuroprotective, as addition of Hsp70 could enhance neuronal stress tolerance (Guzhova *et al.*, 2001) and activate microglia and amyloid- β clearance (Kakimura *et al.*, 2002). On apoptotic and cancer cells Hsp70 could serve as a 'danger' or 'eat me' signal (Sapozhnikov *et al.*, 2002) of stressed cells.

A further and better-known function of Hsp70 is the eliciting of specific immune response against various tumour-derived and viral antigens (Srivastava, 2002). Another more general immunostimulatory effect of extracellular Hsp70 is the stimulation of proinflammatory cytokines and nitric oxide by monocytes/macrophages (Asea *et al.*, 2000b; Platt *et al.*, 1999). This cellular response was suggested to be dependent on CD14, toll-like receptor 4 (Asea *et al.*, 2000b; Dybdahl *et al.*, 2002) and toll-like receptor 2 (Asea *et al.*, 2002; Vabulas *et al.*, 2002). Nevertheless, neither an antibody against CD14 nor a natural ligand of CD14 (LPS) were able to inhibit cell surface binding of Hsp70 to monocytes and macrophages (Sondermann *et al.*, 2000). Suggesting the CD14-dependent activation of antigen presenting cells occur downstream of specific receptor binding of Hsp70.

Additionally, in CD8-enriched T cells stimulated with extracellular Hsp70 induced a concentration-dependent increase of β -chemokines like RANTES, macrophage inflammatory protein (MIP)-1 α or MIP-1 β (Lehner *et al.*, 2000).

The initial idea for the Hsp70 experiments performed in this report came from a publication demonstrating that Hsp70 can deliver a partial maturation signal to dendritic cells (Basu *et al.*, 2000). As Hsp70 is conserved from bacteria to mammals, their ability to activate antigen-presenting cells could be a mechanism for the response to internal and external stimuli. Additionally the release of Hsp70, that is often upregulated in stressed cells, could be a hint to explain inflammatory reactions when cell lysis occurs *in vivo*.

This study demonstrates on the one hand that extracellular Hsp70 can act as a cytokine in different macrophages populations like HMDM, mouse BMDM and Kupffer cells. Furthermore it was demonstrated that Hsp70 facilitates the toll-like receptor 4 (TLR4) for signaling. This was shown with the C3H/HeJ mice, characterised by a point mutation in the TLR4, and the C57BL/10ScCr mice lacking the TLR4.

The activation of macrophages was measured by detection of inflammatory cytokines and nitric oxide. For these experiments recombinant human Hsp70 (Nsp-555, Stressgene) expressed in *E.coli* was used. This protein solution was tested negative for LPS contamination in the Limulus amoebocyte lysate (LAL) assay.

Contamination with endotoxin is a major concern in these experiments; therefore human Hsp70 was expressed in insect cells to avoid the presence of any bacterial compound. To our surprise this protein preparation was positive in the LAL assay unless purified with polymyxin, a substance known to bind LPS with high affinity. Interestingly, this purified protein solution had no activity anymore. A recombinant low endotoxin human Hsp70 (Esp-555, Stressgene) was used in further experiments with the same result. These observations indicate that the release of cytokines after incubation of macrophages with Hsp70 was perhaps due to a contamination with LPS.

Consequently, the human Hsp70 (Nsp-555, Stressgene) used for the initial experiments was tested again. In a LAL assay with increased sensitivity a low contamination was found, whereas the low endotoxin preparation (Esp-555, Stressgene) was still negative. In an additional experiment the TNF release induced with the active Hsp70, could be completely inhibited by addition of polymyxin.

However, the contamination indicated by the sensitive LAL assay was too low to induce a detectable TNF release in our systems. To investigate if Hsp70 could exhibit synergistic effect on LPS, macrophages were stimulated with low endotoxin Hsp70 and increasing concentrations of LPS. This co-incubation resulted in a weak decrease in the LPS signal.

Therefore, the Hsp70 data presented in this report suggest that it is still open whether Hsp70 can act as a cytokine and if TLR4 is one of the endogenous receptor of this chaperone. The different observations made in this topic could also be influenced by the way the proteins were purified. One often used method is the purification of Hsp70 out of organs like the liver or kidney. The other one is the overexpression of the recombinant protein in *E.coli* or insect cells. One can only speculate about the possibility of contamination with other proteins or bacterial contaminations.

Nonetheless, the loss of activity observed with the recombinant human Hsp70 expressed in insect cells and with the recombinant human low endotoxin Hsp70 purchased from Stressgene, suggests that the cytokine function of Hsp70 is still doubtful. Both protein solutions were tested in a LAL with improved sensitivity and had endotoxin concentrations below the detection limit.

The similarity between the response to Hsp70 and lipopolysaccharide were discussed recently by Wallin (Wallin *et al.*, 2002). The author of this review emphasised that care must be taken when working with preparations of heat-shock proteins (Hsp). The different concentrations of Hsp required for activation, dependent on the origin (recombinant bacterial or tissue-derived) were one point of criticism, the number and quality of controls was another. In experiments with dendritic cells the mentioned author was not able to see activation with concentration as high as 300 µg/ml.

It was shown that Hsc70 can associate with LPS as well as surface receptors by fluorescence resonance energy transfer (Triantafyllou *et al.*, 2001). If this result is also true for Hsp70 remains open. Interaction assays between Hsp70 and fluorescence labelled LPS could be a further method to answer this question.

Finally, the controls performed to exclude contamination with LPS are discussed.

Limulus amoebocyte assay:

LPS-binding proteins in serum and other compounds are known to interfere with the assay. Serum was never present in tests performed in this study. A further control to exclude false negative results with the LAL-assay are samples spiked with LPS to control how much LPS could be recovered in the sample. The recovery of LPS in Hsp70 samples was always higher than 85 %.

The experiments with Hsp70 and increasing concentrations in BMDM gave some indications that Hsp70 could bind LPS, nevertheless it only had a very weak neutralising effect on the LPS response. A synergistic effect of Hsp70 on LPS is doubtful.

One should further keep in mind that the LAL-assay only detects endotoxin. But there are also other bacterial contaminations that could provoke an activation of macrophages.

Polymyxin B:

This substance can bind and neutralise LPS. Interestingly it does not neutralise all species of LPS and the ability to neutralise LPS bound to Hsp70 is unknown. One could further speculate that polymyxin B could have a denaturing or inactivating effect on the protein preparation. It is indicated to test if Hsp70 after preparation is still active. This could be done by testing the ATPase function (tested by Stressgene products) or the chaperone function.

In *in vitro* experiments polymyxin B is often added directly to the cells to exclude contamination. Controls are necessary in these experiments to exclude false negative (cytotoxicity) or false positive results (stimulation by polymyxin).

Heat inactivation:

The heat inactivation of a protein is a useful control when dealing with many models except of inflammatory ones, especially to exclude LPS contamination. To our point of view heat inactivation destroys the protein, but low concentrations of LPS can easily bind to denatured proteins and therefore result in false negative results.

Mice defective for the recognition of LPS:

The use of macrophages or other cells derived from these mice represent an excellent method to test LPS contamination. Nevertheless, the power of the results from these experiments rises and falls with the other controls performed to exclude LPS contamination.

5.3 Extracellular caspase-3

The observations presented here suggest the possibility of extracellular caspase activity. The implications of this result remain unknown until an extracellular substrate can be described. The first indication is the release of a caspase-like activity from dying neurons that can activate glia cells to produce cytokines (Viviani *et al.*, 2000).

In previous studies investigating the *in vitro* stability of different caspases in protein-free condition the activity was dependent on the presence of the detergent Chaps (Stennicke and Salvesen, 1997). Under these conditions the activity was maintained for two hours at 37 °C. The data presented here show that the addition of proteins has a comparable effect on the stability of the caspase activity. Interestingly, the DEVDase activity can also be maintained in biological fluids without requiring further additives. When added to cell lysates the recombinant caspase exhibited a remarkable resistance against intracellular proteases.

In the staurosporine treated Jurkat T cells the increase in DEVDase cleavage precedes and was paralleled by the detected LDH-release, indicating that apoptotic Jurkat cells can release active DEVDase. This could be shown in three further models *in vitro* and does also occur *in vivo* in pathological situations that are based on classical apoptotic cell death (Hentze *et al.*, 2001).

Detection of DEVDase cleavage in the liquor of traumatic brain injury patients was only possible in 2 out of 42 samples. In another work (Hentze *et al.*, 2001) 20 of 60 samples were positive and the activity of these samples could be reduced by addition of DEVD-CHO [100 nM].

These results suggest that measuring of DEVDase activity could be of a diagnostic value as a marker for exceeding apoptotic organ damage or in monitoring the success of tumour chemotherapy or transplant survival.

5.4 Quantitative phagocytosis assay using fluorescence labelled bioparticles

Metschnikoff first observed phagocytosis in 1887 (Metschnikoff, 1887). Various assays have been developed to quantify this process using all kinds of particles like latex beads and coloured lipid droplets and also bioparticles such as bacteria or yeast. However, quantification was performed by microscopic counting, radioisotopic detection, or counting of bacteria colonies and was therefore time consuming, with low specificity and sensitivity.

A new method to quantify phagocytosis was introduced by fluorescent labelling of particles (Stendahl *et al.*, 1977). This method was used and improved by other authors, and criteria were defined for quantitative phagocytotic assays (Stossel, 1977). These criteria are saturability of particle in question, complete inhibition of ingestion at 0° C or in the presence of a metabolic inhibitor and the lack of apparent ingestion at zero time.

One of the key problems of phagocytosis measurement was to distinguish between uptake and adhesion of a particle. The digestion of attached bacteria with lysozyme after fixation of the phagocytes was shown to be a useful method (Vray *et al.*, 1980). Other authors used trypan blue or crystal violet to quench extracellular fluorescence. These dyes were not able to cross the cell membrane, leaving intracellular fluorescence unaffected (Wan *et al.*, 1993).

Another step ahead was the use of 96well-microtiter plates. In this work the whole experiment including the measurement was performed on the same microtiter plate (Wan *et al.*, 1993). This test system facilitated the screening of large number of various biological and pharmacological substances and their effects on phagocytosis.

The standard fluorescent dye used for these assays was fluorescein isothiocyanate (FITC), with its disadvantage of pH sensitivity. When phagocytes were lysed in these assays, it was always necessary to use buffered solutions. With new dyes like tetramethylrhodamin (TMR) or BODIPY the use of such buffers is no longer required.

We decided to use these well-established methods to test the influence of apoptotic cells on phagocytes and their capacity to ingest *E.coli* particles. During the process of establishing these test systems for our purposes several unexpected results were observed.

Initially we used the simple and rapid *in vitro* phagocytosis assay (Wan *et al.*, 1993) using TMR or BODIPY-labelled bioparticles and trypan blue solution for quenching extracellular fluorescence (Hed *et al.*, 1987; Sahlin *et al.*, 1983). As demonstrated, all criteria for the quantitative phagocytosis could be fulfilled. Interestingly, an additional control failed (competition with unlabelled particles). In competition experiments with unlabelled *E.coli* particles an increase in the fluorescent signal by the use of high concentrations of unlabelled particles was observed. This result was surprising, but reproducible.

Therefore, a different phagocytosis assay was used, to examine if identical results could be obtained with another method. This new assay was based on a technique with a lysis step at the end of the experiment (Oda and Maeda, 1986; Uff *et al.*, 1993). We compared both assays and found that after washing away the not attached particles was quite effective and additional quenching had no strong effect on the measured signal.

Interestingly, the signal after lysis increased two up to five fold, when compared with the quenching technique and dependent on the fluorescent dye that was used. These results could be explained by the fact that the labelled particles are distributed in a bigger volume and therefore can be excited much better compared with the volume they had in the endosomes of the phagocytes.

Another explanation for this unexpected finding aroused after the measurement of the relation between fluorescence and particle concentration. This relation can be used to correlate the fluorescence after phagocytosis with the number of ingested particles. Surprisingly, the concentrations for the standard-curve exceeded the concentrations used in the phagocytosis assay, but measured signal intensity was much lower. As the increase in fluorescence was always compared with time zero, it was not noticed that the overall fluorescence increased with the ongoing of the experiment. As shown here, the total fluorescence of the lysed phagocytes and the extracellular fluorescence already increased after two hours compared with the signal at time zero. The increase is due to the digestion of the labelled particles and the liberation of the fixed fluorescent molecules on the surface of the bioparticles. It was suggested here that the fluorescence measured at the beginning of the assay is the result of 'self-quenching' of the fluorophores. On the surface of the particles these molecules are located so close to each other, that fluorescent quenching dominates over emission. This hypothesis could also explain the results seen in the competition experiments with the unlabelled *E.coli*. The signal measured after four hours phagocytosis was increased with high concentrations of unlabelled *E.coli*, because the 'self-quenching' effects in the endosomes was reduced by unlabelled particles. The immense increase in signal : noise ratio of the lysing assay was also explained partially by this point of view.

Also the lysing assay fulfilled all criteria outlined by Stossel (Stossel, 1977), but compared with the quenching assay the signal : noise ratio was superior even after short phagocytosing periods. With respect to the increasing fluorescence during the phagocytosis assay, it was suggested to limit phagocytosis to maximal two hours. In cases when longer incubation times are indicated the use of a mixture of labelled and unlabelled particles could resolve some problems.

Data presented here indicate to limit phagocytosis assay in time, because with increasing time not only the uptake of particles is examined. The increase in fluorescence is not only the result of passive diffusion of liberated fluorescent molecules. As well, it is dependent on acidification of the endosome, endosome trafficking, re-uptake by pinocytosis or viability of the phagocyte. One should keep in mind that these activities have a time dependent influence on the measured signal and their influence is rising with the ongoing of the assay.

If phagocytosis is viewed not only as simple uptake, but more as a multistep process, longer incubation times are no problem. In this case the phagocytosis assay gets the character of a viability test.

The data presented here therefore suggest some further criteria for quantitative phagocytosing assays with fluorescence labelled bioparticles. If possible, the particles should not be labelled to a maximum to avoid 'self-quenching' or it has to be tested that the total fluorescence is not increasing during the assay.

DISCUSSION

This modified lysing assay was used to characterise the influence of apoptotic cells on phagocytosis of bioparticles in this work and also to characterise MAPKAP2 null mice (Lehner *et al.*, 2002).

6 SUMMARY

Phagocytosis of apoptotic cells is a fast and efficient process. This is documented by the very rare observation of apoptotic cells *in vivo*. In fact the uptake of apoptotic cells by professional phagocytes like macrophages is not only a non-inflammatory but also an active anti-inflammatory process.

- To examine these immunomodulating effects of dying cells an *in vitro* co-culture system using primary murine cells was established. The influence of apoptotic cells was described by the measurement of factors like TNF α , IL-10, PGE₂ and NO.
- Co-incubation of macrophages with apoptotic cells followed by stimulation with LPS resulted in a decrease of inflammatory TNF α and an increase of anti-inflammatory IL-10 release and thereby changed the response of macrophages, when compared with LPS stimulation alone. Similar results were obtained with as different target cells as apoptotic isogenic mouse thymoma S49.1 cells, Jurkat T cells or primary thymocytes. Interestingly, some types of necrotic cells such as ATP-depleted Jurkat T cells had also anti-inflammatory effects in the co-culture system.
- The effect apoptotic cells have on other macrophage functions, i.e. phagocytosis, was examined with a sensitive assay, based on fluorescence labelled *E.coli* particles. Pre-incubation with apoptotic cells had no effect on *E.coli* phagocytosis.

In cases of massive apoptosis the phagocytic capacity can be limited and secondary lysis occurs. Caspase-3 release should be detected under these circumstances.

- Stable activity of recombinant caspase-3 was demonstrated in several biological fluids by DEVD-afc cleavage.
- Caspase-3 activity could be detected *in vitro* in the supernatant of apoptotic cells.

Hsp70 can be released from necrotic cells and is also expressed on the surface of stressed cell as a 'danger-signal'. Hsp70 was postulated to take the function of an inflammatory cytokine.

- Addition of some Hsp70 preparations was found to induce macrophages to produce inflammatory mediators.
- The use of two low endotoxin Hsp70 preparations reproducibly failed to induce this response.



7 ZUSAMMENFASSUNG

Die Phagozytose von apoptotischen Zellen ist ein schneller und effizienter Vorgang. Verdeutlicht wird dies durch das sehr seltene Auffinden apoptotischer Zellen *in vivo*. Die Aufnahme von apoptotischen Zellen durch professionelle Phagozyten, wie zum Beispiel Makrophagen, ist nicht nur ein nicht entzündlicher, sondern vielmehr ein antiinflammatorischer Vorgang.

- Um diese immunmodulatorischen Effekte sterbender Zellen zu untersuchen wurde ein *in vitro* Cokultursystem mit primären Mauszellen aufgebaut. Der Einfluß der apoptotischen Zellen auf die Makrophagen wurde durch die Messung der TNF α -, IL-10-, PGE $_2$ - und NO-Freisetzung beschrieben.
- Im Vergleich zur Stimulation mit LPS wurden nach Coinkubation von Makrophagen mit apoptotischen Zellen und anschließender Stimulation mit LPS erhöhte anti-inflammatorische IL-10 und reduzierte inflammatorische TNF α Konzentrationen gemessen. Vergleichbare Ergebnisse wurden erzielt, wenn statt der apoptotischen primären Thymozyten apoptotische Zellen der isogenetischen Mausthymomazelllinie S49.1 oder der humanen Jurkat T Zelllinie verwendet wurden. Interessant war, daß gewisse Typen nekrotische Jurkatzellen die gleichen Effekte hervorriefen wie apoptotische Zellen.
- Der Einfluß apoptotischer Zellen auf die Phagozytoseaktivität von Makrophagen wurde mit einem sensitiven Assay untersucht, der auf der Verwendung von fluoreszenzmarkierten *E.coli*-Partikeln beruht. Apoptotische Zellen haben keinen Einfluß auf diese Makrophagenfunktion.

In Fällen massiver Apoptose kann die Phagozytosekapazität begrenzt sein. In diesen Situationen könnte durch sekundäre Lyse aktive Caspase-3 freigesetzt werden.

- In dieser Arbeit konnte die Stabilität einer rekombinanten Caspase-3 anhand ihrer Aktivität in unterschiedlichen biologischen Lösungen nachgewiesen werden.
- Des weiteren konnte Caspase-3 Aktivität im Überstand apoptotischer Zellen festgestellt werden.

Vergleichbar zur Caspase-3 könnte auch Hsp70 unter ähnlichen Umständen von apoptotischen Zellen freigesetzt werden und ein ‚Gefahrensignal‘ lysierter Zellen darstellen. Es wurde postuliert, daß Hsp70 die Funktion eines inflammatorischen Zytokins hat.

- Stimulation von Makrophagen mit einigen Hsp70 Präparationen führt zur Freisetzung inflammatorischer Mediatoren über den TLR4.
- Bei Verwendung zweier gering Endotoxin-kontaminierter Hsp70-Präparationen konnte dieser Effekt nicht beobachtet werden.

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