universität wien

# MASTERARBEIT

Titel der Masterarbeit

„Delineation of the cellular responses to MDA-modified proteins in macrophages“

1 von 1

Verfasser

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# 1. Introduction

The function of the immune system is to protect the host against any diseases. To accomplish this goal, the immune system has to perform four major tasks [1]. First, it has to recognize potentially harmful disease-causing agents by detecting and binding antigens of invading pathogens. This task is fulfilled by both the innate and the adaptive immune system. The second task is to stop the disease and destroy all dangerous substances, which is carried out by immune effector functions, such as antibodies and the complement system. A further task the immune system has to perform is to regulate immune responses to avoid an overreaction (e.g. allergies) and a subsequent damage of its host. Finally, the immune system should also protect against re-emerging pathogens. Therefore, the adaptive immune system provides an immunological memory, which allows immune reactions with the same pathogen to be much stronger and faster at a second contact.

Plenty of pathogens are already repelled by physical barriers of the human body, such as the skin and ciliated epithelia. In addition also chemical barriers, such as acids and enzymes, prevent infectious agents to enter the body. Only if pathogens can pass these two barriers, innate and adaptive immune responses are activated.

## 1.1 Innate Immunity

The innate immune system reacts immediately and eliminates most of the pathogens. In contrast, the adaptive immune system reacts more slowly (days not hours), but clears the infection much more efficiently.

### 1.1.1 Cells of the innate immunity

Innate immunity contains a wide variety of cells of hematopoietic origin (Fig. 1). All cells of the innate immune system derive from pluripotent hematopoietic stem cells in the bone marrow. These pluripotent stem cells give rise to common myeloid progenitor cells, which then differentiate into either granulocyte/macrophage progenitors or megakaryocyte/erythrocyte progenitors. Moreover, granulocyte/macrophage progenitors then give rise to polymorphonuclear leukocytes, such as neutrophils, eosinophils, basophils and monocytes. These ‘moving cells’ reside in the

blood until an inflammation occurs. If inflammation occurs, the cells are guided by cytokines and chemokines to the site of inflammation. Furthermore, monocytes continually migrate to the tissue without inflammation and differentiate into macrophages. Macrophages, dendritic cells and mast cells (the precursor of mast cell is still not known) belong to tissue-residing cells.

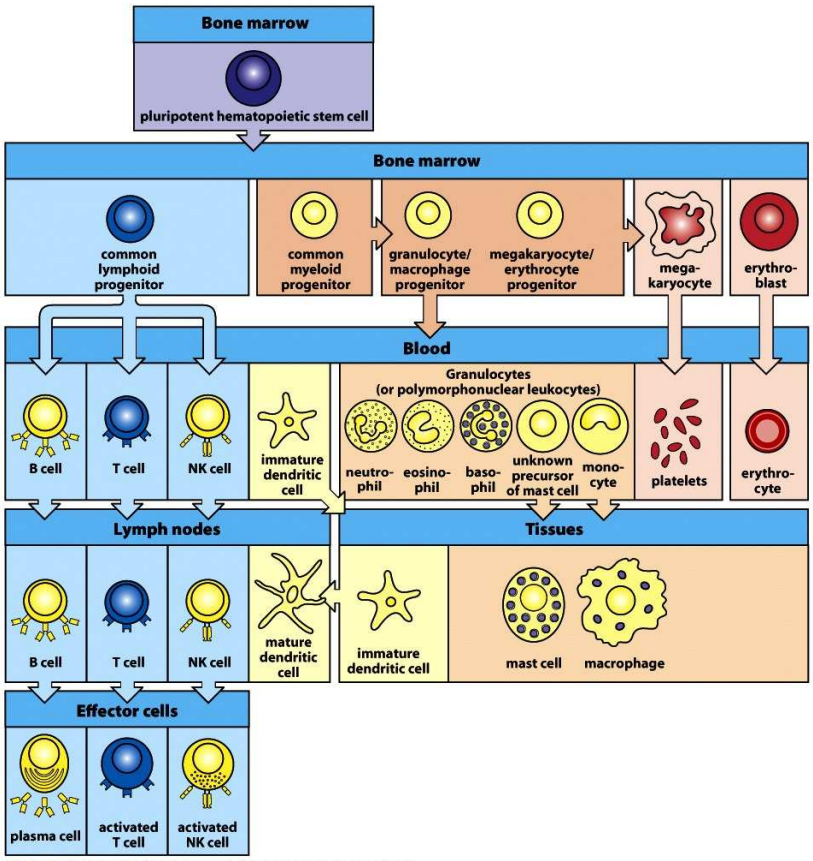


Figure 1-3 Immunobiology, 7ed. (© Garland Science 2008)

**Fig. 1** Cells from both the adaptive and the innate immunity derive from a pluripotent hematopoietic stem cell in the bone marrow. (Murphy et al., Janeway's Immunobiology 2008)

*Eosinophils and Basophils*

The exact function of eosinophils and basophils is still in question. There is increasing evidence, that their function in innate immunity is to eliminate parasites, which are too big to be ingested by macrophages or neutrophils. When they are activated, they start to release their granules, which contain toxic proteins and enzymes. In addition, they have been attributed a role in allergic inflammatory reactions as well.

*Mast Cells*

As already mentioned above, the precursor of mast cells is still unknown. Mast cells contain granules, which are released upon contact with e.g. parasitic worms. Furthermore, mast cells release tumour necrosis factor (TNF) α in response to bacteria. Secreted TNFα then induces an influx of neutrophils to the site of inflammation [2].

*Dendritic Cells*

Dendritic Cells are, like macrophages and neutrophils phagocytic cells, which participate in the innate immune response by clearing pathogens through phagocytosis and macropinocytosis. In addition, they play an important role in the adaptive immune response. After encountering invading pathogens, dendritic cells have the ability to activate T-lymphocytes of the adaptive immunity.

*Neutrophils*

Neutrophils are one out of three types of phagocytic cells and are the most abundant cells in the innate immune system. After encountering an infection, released cytokines and chemokines (e.g. from macrophages) guide neutrophils to the site of inflammation. They migrate into the tissue, ingest pathogens by phagocytosis and efficiently destroy them [3].

*Macrophages*

Macrophages reside in all tissues. They arise from circulating monocytes. Similar to neutrophils, their major task is phagocytosis and thereby clearance of pathogens and cellular debris. Additionally, macrophages also interact with the adaptive immune system by presenting antigens on their surface. Like other cells, macrophages express specific markers on their surface (CD14, CD11b and F4/80) and can therefore be easily identified with specific antibodies [4].

Formerly, activated macrophages could be divided into M1 and M2 macrophages. M1 macrophages are described as classically activated macrophages, whereas M2 macrophages are considered alternatively activated [5]. M1 polarization is induced through ligation of certain pattern-recognition-receptors (PRRs) and interferon-γ (INF γ). Moreover, it was recently suggested [6] that macrophages should be divided into three different groups, because M2 macrophages are a heterogeneous group, which differ dramatically in their biochemical and physiological properties. Therefore, it was suggested to divide M2 macrophages into a) wound-healing (triggered by interleukin-4 (IL4) and/or IL13) and b) regulatory macrophages (triggered by immune complexes and TLR ligands).

In my research project, we mainly used macrophages derived from bone marrow cells stimulated with M-CSF , and they have been shown to exhibit characteristics of M2 macrophages.

## 1.2 Pattern-Recognition-Receptors (PRRs)

Cells of the innate immune system, especially macrophages, recognize pathogenic invaders by germline encoded pattern-recognition-receptors. Those PRRs bind to, amongst microbial species highly conserved structures called pathogen-associated-molecular-patterns (PAMPs). The group of PRRs contains actually four different classes of receptors [7]. One class is the RIG-I-like receptor (RLRs) family, which recognize dsRNA as for example the genomic RNA of certain viruses [8]. Another class is the NOD-like receptor (NLRs) family. These receptors are located in the cytoplasm and detect structures of bacterial peptidoglycans [9]. C-type lectin receptors (CLRs) are a third family of PRRs, which recognizes β-glycans of fungi [10] and carbohydrates of microorganism [11]. The last, and for this diploma thesis most important class of PRRs, is the family of Toll-like receptors that will be described in more detail below. Additionally scavenger receptors, such as scavenger receptor A (SR-A) and CD36, belong to the PRRs [12]. In contrast to the four classes of PRRs, no signalling pathway is described for SR-A. However, there is evidence that SR-A ligands are signalling through TLRs [13].

*Toll-like receptors*

Toll was first discovered in the development of drosophila, in which it triggers anti-fungal responses [14]. Subsequently ten similar receptors (all of them involved in pathogen detection) were identified in the human body [15]. TLR1,2,4,5 and 6 are expressed on the cell surface, whereas TLR3,7,8,9 and 11 are localized intracellular. Most TLRs dimerize upon stimulation. TLR 3,4 and 8 act as homodimers, whereas TLR2 acts as a heterodimer together with TLR1 or TLR6.

When stimulated, TLR4 homodimerizes on the plasma membrane and associates with CD14 and MD2 to form the Lipopolysaccharide (LPS)-receptor [1]. Intracellularly, TLR4 is linked to TIRAP or TRIF and could signal through either the MyD88-dependent or the TRIF-depending (also known as MyD88-independent) pathway, respectively. Both pathways finally activate transcription factors and trigger the upregulation of inflammatory genes.

The most prominent ligand for TLR4 has been first described by Poltorak et al. in 1998 as the bacterial endotoxin Lipopolysaccharide (LPS) [16]. LPS first binds to CD14 and subsequently to the MD2/TLR4 complex.

*TLR2*

TLR2 forms heterodimers with TLR1 or 6. Moreover, pathogen recognition by TLR2 is strongly enhanced by the co-receptor CD14 [17]. Intracellularly, it is linked to TIRAP and hence signals only through the MyD88-dependent pathway.

TLR2 is involved in the recognition of a wide array of microbial molecules. LTA (a major constituent of the gram positive cell wall) is probably the best known ligand for TLR2 [18]. Furthermore, TLR2 participates in the recognition of some types of LPS [19].

## 1.3 Oxidation Specific Epitopes

Oxidative reactions are crucial for survival. During oxidative reactions, reactive oxygen species (ROS) are formed, which have important roles in homeostasis and cell signalling. Moreover, they also have the ability to damage proteins and lipids. Under normal circumstances, antioxidant mechanisms like vitamin C, vitamin E and superoxide dismutases (SOD) buffer the effect of ROS and therefore maintain a balance. Enhanced formation of ROS, as it occurs for example during inflammation, can result in a state referred to as “oxidative stress”. If lipids are targeted by ROS, lipid peroxidation can occur. Lipid peroxidation mainly affects unsaturated lipids, as they offer highly reactive ethylene-bonds. Many unsaturated lipids are to be found in the cell membrane. When cells undergo apoptosis, unsaturated lipids (polyunsaturated fatty acids of phospholipids) become oxidized and give rise to highly reactive products; amongst them, different highly reactive aldehydes, including malondialdehyde (MDA) and 4-hydroxynonenal, which can form covalent adducts with the ε-aminogroups of lysines or aminogroups of phospholipids, such as phosphatidylethanolamine (Fig. 2). These newly generated “oxidation specific epitopes” are found on apoptotic and necrotic cells, but not on viable cells. Furthermore microparticles (apoptotic blebs) and oxidized LDL, but not LDL, carry them as well [20, 21]. These epitopes have been shown to be recognized by the innate immune system via natural antibodies, scavenger receptors and innate effector proteins. Additionally, some oxidation specific epitopes are mediate effects via TLRs (eg. OxPL stimulates TLR4 [22]). Due to the fact that oxidation spe-

cific epitopes are recognized by the same receptors as PAMPs, they are similarly classified as damage (danger) associated molecular patterns [23].

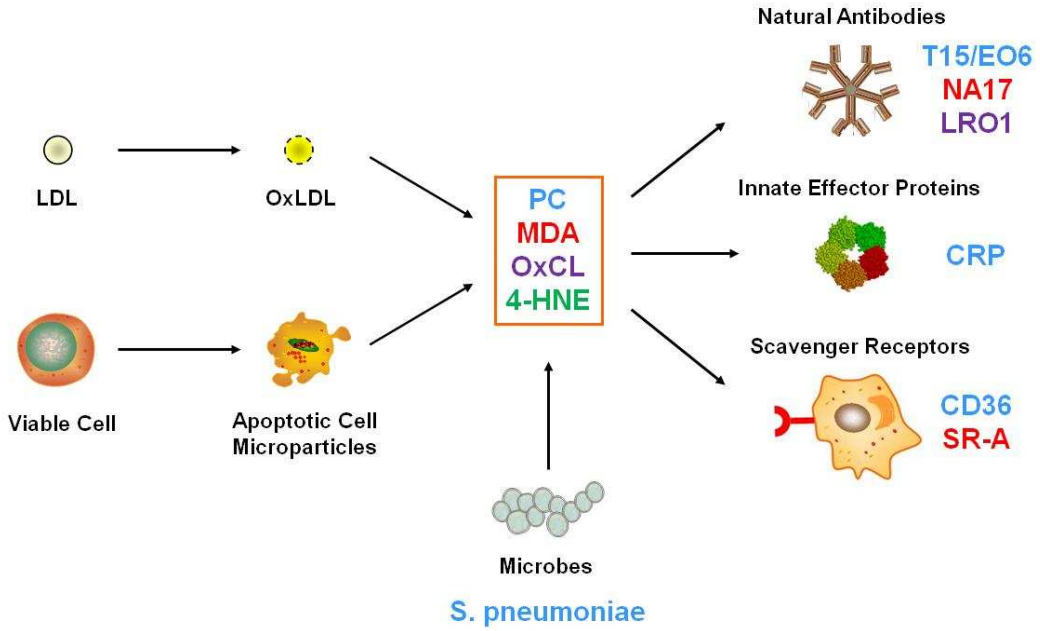



**Fig. 2** Oxidation specific epitopes are major targets of innate immunity (modified from Binder CJ et al., JLR 2005).

### 1.3.1 Malondialdehyde

Malondialdehyde (MDA) is one major secondary product of lipid peroxidation [24]. Similarly, phosphocholine (PC) containing oxidized phospholipids, oxidized cardiolipin (OxCL) and 4-hydroxynonenal have been shown to form adducts as well. Aldehydes, generated during lipid peroxidation are highly reactive and attack targets intra- or extracellularly. Together with acetaldehyde (AA), MDA forms the malondialdehyde-acetaldehyde-adduct (MAA) that are believed to be the biologically most active MDA-epitopes. MDA-epitopes are found in various pathological settings like atherosclerosis, acute lung injury, Alzheimer's disease, rheumatoid arthritis and apoptotic cells (Fig 3).

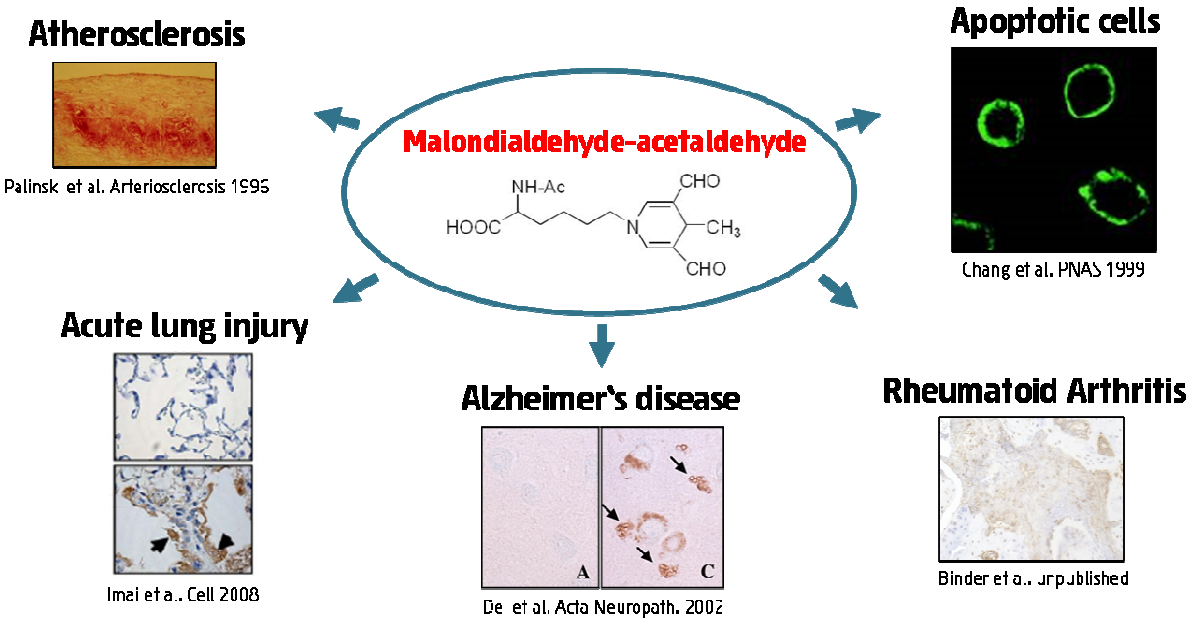



**Fig. 3** Malondialdehyde-acetaldehyde is found in various pathological settings.

*In vitro*, mainly four different MDA-adducts are generated upon reaction of MDA with a carrier protein (Fig 5), depending on the pH and the supply of acetaldehyde (AA). If MDA reacts with an aminogroup on lipids or proteins at a neutral pH of 7.4, mainly simple (A) and cross-linking (B) adducts are generated. Both adducts are non-fluorescent. Bovine serum albumin modified this way is referred to as MDA-BSA in this thesis. If MDA reacts with aminogroups under acidic conditions, 3,5-diformyl-1,4-dihydropyridine-4-yl-pyridinium (FHP) -adducts (D) are generated. FHP is a fluorescent, cross-linking adduct. Modification of primary aminogroups with AA and MDA in acidic conditions, generates the malondialdehyde-acetaldehyde adduct (MAA), which is a ring structure also referred to as 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (MDHDC)[25] (C).

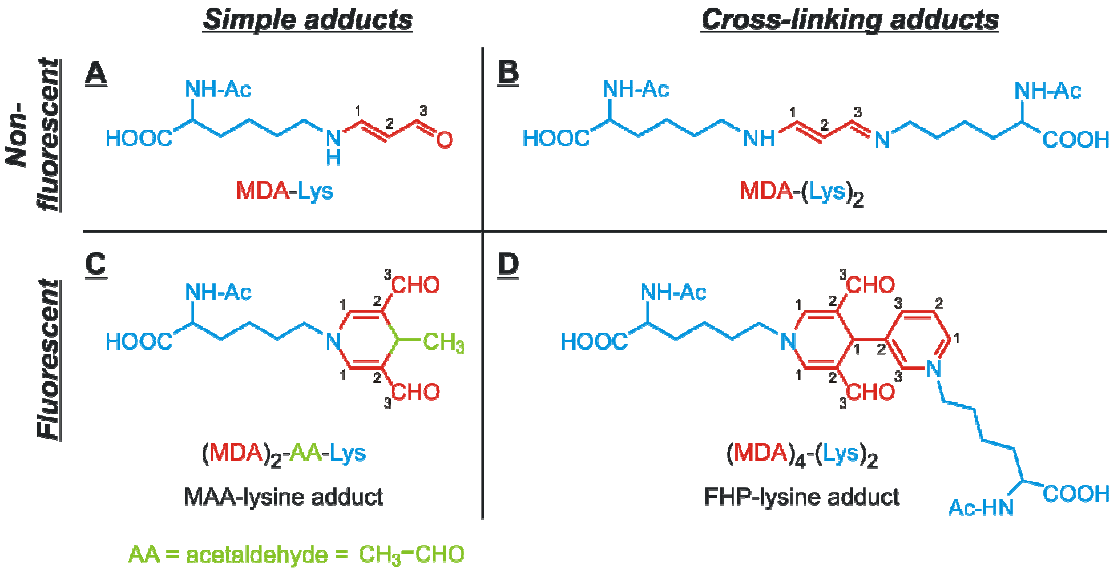



**Fig. 4** MDA is a highly reactive aldehyde, which reacts immediately and therefore generates different adducts (A,B: MDA-BSA, C: MAA-BSA, D: FHP-BSA)

MDHDC is fluorescent but in contrast to FHP involves only one aminogroup. BSA modified with acetaldehyde and MDA as just described will be named MAA-BSA in this thesis.

In this Master Thesis, I aim to define malondialdehyde-acetaldehyde-adduct (MAA) as a novel danger-associated-molecular-pattern (DAMP) and delineate the cellular responses of macrophages to it. DAMPs are endogenous danger signals that induce inflammatory responses in absence of microorganisms. For this reason, the induced response is called sterile inflammation. I will provide evidence that MAA acts as a danger signal, hence is recognized by various PRRs and has to be cleared effectively by the immune system to avoid chronic inflammation. The obtained insights suggest a crucial role of TLR4 and TLR2 for the responses towards MAA. Furthermore, CD14 is necessary for MAA signalling. Additionally I will present data that suggest that MAA-BSA binds to TLR2.

# 2. Material & Methods

## 2.1 Material

Mice

TLR4$^{-/-}$, C57BL/6, SR-A$^{-/-}$ and CD36$^{-/-}$ were purchased from The Jackson Laboratory. TLR2$^{-/-}$ and CD14$^{-/-}$ mice were a generous gift from Sylvia Knapp. Mice were housed under conventional conditions and fed regular rodent chow. All animal experiments were performed in accordance with relevant guidelines and regulations.

Cells, chemicals, plates and buffers

The human monocytic cell line THP1 and the murine macrophage cell line RAW267.1 (RAW) were grown according to ATCC protocols. Mouse bone marrow derived macrophages (BMDM), mouse peritoneal macrophages were isolated as described below. Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 + Glutamax (contains a stabilized form dipeptide from L-glutamine, L-alanyl-L-glutamine, that prevents degradation and ammonia build-up even during long-term cultures) (RPMI) and Hank's Buffered Salt Solution (HBSS) media were purchased from Invitrogen and supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Peprotech), 1% Penicillin/Streptomycin (P/S) (Sigma), 1% HEPES (Sigma) and 0.1% Gentamycin (Invitrogen). Macrophage colony-stimulating factor (MCSF) was purchased from PeproTech. BCA protein assay kit was provided by Pierce.
ELISA plates (Immuno Maxi Sorp) were obtained from Nunc and microfluor plates from Thermo Scientific. PTS cartridges for endotoxin test (LAL test) were purchased from Charles River. Stripping buffer was obtained from Thermo Scientific.

SOC medium (1000ml)

20g Bacto
5g Bacto Yeast Extract
2ml 5M HCl
10ml 1M $MgCl_2$
10ml 1M $MgSO_4$
2,5ml 1M KCl
20ml 1M Glucose
filled up with $H_2O$ to 1000ml

*Protein Lysis Buffer*
50mM HEPES
150mM NaCl
5mM EDTA
1% NP-40
50mM NaF
1mM $Na_3VO_4$
1mM PMSF
1:25 Complete
$H_2O$

*Protein Wash Buffer I*
50mM HEPES
500mM NaCl
5mM EDTA
1% NP-40
50mM NaF
1mM $Na_3VO_4$
1mM PMSF
1:25 Complete
$H_2O$

*Protein Wash Buffer II*

50mM HEPES

500mM NaCl

5mM EDTA

$H_2O$

*10x Running Buffer (1l)*

144g Glycin

33g TRIS

10g SDS

pH 8,3 (HCl)

*Transfer Buffer*

5,89g TRIS

9,93g Glycine

0,375g SDS

*HEBS Buffer*

400ml $H_2O$

8g NaCl

0,2g $Na_2HPO_4$

6,5g HEPES (Sigma)

*1x TAE buffer*

40 mM Tris acetate

1 mM EDTA

<u>Protein Gel</u>

*4x Lower Buffer*

1,5M TRIS

0,4% SDS

pH 8,8 (HCl)

*4x Upper Buffer*
0,5M Tris.HCl
0,4% SDS
pH 6,8 (HCl)

*Lower Gel 1,5mm*
8,2ml 30% acrylamid
7ml $H_2O$
5ml 4x Lower Buffer
200µl APS
10µl Temed

500µl isopropanol was added on the lower gel until it became solid, then discarded before addition of upper gel.

*Upper Gel 1,5 mm*
2,4ml 30% acrylamid
7,2ml $H_2O$
3,2ml Upper Buffer
128µl APS
10µl Temed

## 2.2 Methods

Modification of bovine serum albumin (BSA)

*MAA-BSA*
To generate MAA-BSA, a 0.5M Malondialdehyde (MDA) solution was prepared by adding HCl (0.01M) and $H_2O$ to MDA and incubated 10 min at 37°C until the colour turned slightly yellow. After turning yellow, the pH of the MDA solution was adjusted to 4.8. Meanwhile, to facilitate pipetting, the highly volatile acetaldehyde (AA) was diluted 1:4 in phosphate buffered saline (PBS) at pH4.8. MDA (0.1M), BSA or L-polylysine (20mg) and AA (0.015M) were mixed and

the pH was adjusted to 4.8. Then the reaction mix was incubated at 37°C for 4h and dialyzed against PBS pH7.4 at 4°C o/n.

*MDA-BSA*

The modification was done as described for MAA-BSA, but without addition of AA and the pH was adjusted to 7.4 instead of 4.8. Subsequently, the reaction mix was incubated for 45min at 37°C.

*FHP-BSA*

The modification was done as described for MAA-BSA, but without AA and the pH was adjusted to 5 instead of 4.8. Subsequently, the reaction mix was incubated for 4h at 37°C.

*AA-BSA*

The modification was done as described for MAA-BSA, but without adding MDA, the pH adjusted to 4.8 and the reaction mix incubated 4h at 37°C.

Modification of LDL

Copper oxidation and MDA modification of LDL were carried out by Maria Ozsvar-Kosma as described in detail previously [26].

Bicinchoninic acid assay (BCA)

The BCA assay is commonly used to quantify protein in solutions. The protein bonds from each protein reduce $Cu^{2+}$ to $Cu^{1+}$. Thereby, the amount of $Cu^{1+}$ ions is proportional to the amount of protein. In the next step, $Cu^{1+}$ ions are bound by each two molecules of bicinchoninic acid, forming a violet complex.

The working reagent was prepared by mixing solution A to solution B in a 1:50 ratio. 25µl of each sample and standard (both diluted 1:5 and 1:25 in $H_2O$) were mixed with 200µl of the working reagent. After this, the samples were incubated for 30min at 37°C. Subsequently the absorbance was measured at a wavelength of 562nm using a Biotek Synergy 2 plate reader.

Measurement of fluorescence

2µg of modified BSA/polylysine were resuspended in 200µl of $H_2O$ and fluorescence was measured at the wavelengths 355(excitation)/460nm (emission) using a Biotek Synergy 2 plate reader.

Trinitrobenzene sulphonic acid (TNBS) Assay

This assay measures the availability of free amino groups.

First, 50µg of protein were diluted in 250µl $H_2O$ and 500µl of 4% bicarbonate buffer (pH8.5) were added. Subsequently, 50µl of picrylsulfonic acid were added, mixed and incubated for 60 minutes at 37°C. Following the incubation, 200µl of 1N HCl and 100µl of 10% SDS were added and well mixed. After 15 minutes the absorbance of the reaction mixture was measured at a wavelength of 340nm using a Biotek Synergy 2 plate reader.

Generation of polylysine and MAA-polylysine beads

First, 5ml of bead suspension (N-hydroxysuccinimide (NHS) activated Sepharose 4 fast flow in isopropanol) were activated by washing with 45 ml 1mM HCl. The suspension was centrifuged for 5 min at 800rpm and beads were resuspended in 8ml polylysine/MAA-polylysine (2mg/ml in PBS, pH 7.4). Following an incubation for 4h at room temperature (RT), beads were sedimented as before, the supernatant (SN) was aspirated and the remaining reactive NHS-groups were blocked by incubation for 2h at RT in 40ml of 0.1M Tris-HCl pH 8.5. Subsequently, beads were sedimented by centrifugation and washed in 16ml of 0,1M acetate buffer, 0.5M NaCl, pH 4.5. The suspension was distributed to 2x 15ml tubes and centrifuged again for 1min at 1000 rpm. Then SN were aspirated and 8ml of 0.1M Tris-HCl, pH 8.5 were added. The acetate-tris-wash cycle was repeated 5x (6 in total), beads were combined and stored as a 1:1 suspension in PBS, pH 7.4.

Antibody depletion with MAA-PL-beads

Microfluor round bottom 96-well ELISA plates (white) were coated with 50µl/well of CuOx-LDL, MDA-LDL or MAA-BSA (1µg/ml) o/n at 4°C. On the following day coated plates were washed 4x with PBS and blocked 30min at RT with 100µl/well of PBS 1% BSA. Subsequently plates were washed 4x with PBS. The beads (1:1 suspension in PBS) were mixed with 2x stock

solutions of antibodies (EO6 [2,34mg/ml], EO14 [0,2mg/ml] and LRO4 [0,75mg/mL]) (2µg/ml). Following incubation for1h, the suspension was sedimented by centrifugation (1min at 230g) and 50µl of the supernatant were added to the plate o/n. On the next day, the plate was washed 4x with PBS and incubated 1h with a secondary antibodyagainst mouse IgM coupled to alkaline phosphatase (αIgM-AP diluted 1:30,000 in PBS 1% BSA, Sigma-Aldrich). The plate was washed 4x with TBS to remove phosphates and finally LumiPhos (diluted 1:3 in $H_2O$) was added for 90min at RT in darkness. Afterwards chemiluminescence was measured using a Biotek Synergy 2 plate reader. All washing steps were performed on a Biotek microplate washer model ELx405.

ELISA based assay for the binding of Fc-TLR2 to MAA

Microfluor round bottom 96-well ELISA plates (white) were coated o/n at 4°C with 50µl/well (1µg/ml) of MAA-BSA, BSA or corresponding controls (diluted in PBS). On the next day, coated plates were washed with PBS and blocked for 60min with 100µl of PBS containing 1% BSA. After washing with PBS, the plates were incubated for 120min at RT with 50µl of the TLR2 Fc chimera (20µg/ml in PBS), washed again with PBS, and then 50µl of monoclonal α Fc IgG-biotin (Sigma; 1:50000 in PBS) were added for 60min at RT. Subsequently, the plates were washed with TBS and incubated with 25µl of Lumiphos for 90min in the dark. Finally the chemiluminescence was measured at a wavelength of 530nm. All washing steps were performed on a Biotek microplate washer model ELx405.

Competition of MAA-BSA with TLR2 Fc Chimera

Microfluor round bottom 96-well ELISA plate (white) were coated with 50µl/well of MAA-BSA, BSA or $Pam_3CSK$ at a concentration of 1µg/ml (in PBS) for 1h at 37°C. Subsequently, plates were washed 4x with PBS and thereafter blocked for 1h at RT with PBS 1%BSA. TLR2 Fc chimera (1µg/ml in PBS 1% BSA) was pre-incubated with BSA or MAA-BSA to minimize binding capacities for 1h. After blocking, the plate was washed again 4x with PBS, 50µl of the pre-incubated Fc chimera were added and incubated over night (o/n) at 4°C. On the following day plates were washed 4x with PBS, and monoclonal α Fc IgG-biotin (Sigma; 1:50,000 in PBS) was added and incubated for 1h at RT. Thereafter plates were washed 4x with PBS-EDTA and 1x with $H_2O$, and chemiluminescence was measured using LumiPhos as described above. All washing steps were performed on a Biotek microplate washer model ELx405.

IL-8 ELISA

For the detection of IL-8 in cell culture supernatants, I used the human IL-8 ELISA set from Becton, Dickinson and Company (BD). Maxisorp 96-well flat bottom plates (Nunc, transparent) were coated with 100µl IL-8 capture AB (in $NaHCO_3$ buffer; (1:250) o/n at 4°C. On the next day paltes were washed 3x in PBS 0.05% Tween 20 and blocked for 1h at RT with PBS 10% FCS. Then plates were washed again 3x with PBS 0.05% Tween 20, and 100µl of samples and standards (both diluted in PBS 10% FCS; 1:1 dilution series for standard starting with 200pg/ml) were added and incubated for 2h at RT. Subsequently, plates were washed 6x with PBS 0.05% Tween 20, and 100µl of biotinylated anti-human IL-8 Ab (1:250) + streptavidin-HRP in PBS 10% FCS) were added. Plates were incubated for 1h at RT, followed by 6 washing steps with PBS 0.05% Tween 20 and addition of 100µl of TMB solution (Peroxidase substrate; TMB A and TMB B (Lumigen, Inc) were mixed 1:1). After 20 min the reaction was stopped with 100µl 1N $H_2SO_4$ and the OD (450/570nm) was measured using a Biotek Synergy 2 plate reader. All washing steps were performed on a Biotek microplate washer model ELx405.

KC/MIP-2 ELISA

For the detection of KC/MIP-2 in cell culture supernatants, I used the DuoSET ELISA development kit from R&D. Nunc Maxisorp plates were coated with 100µl of capture Abs [500µg/ml] (in PBS) o/n at 4°C. On the following day, plates were washed 3x in PBS 0.05% Tween 20 and blocked for 1h at RT with PBS 1% BSA. Afterwards plates were washed again 3x, 100µl of samples and standards (both diluted in PBS 1% BSA; starting dilution of standards at 1000pg/ml) were added and incubated for 2h at RT. Plates were washed 3x and subsequently 100µl of biotinylated detecting Abs [50µg/ml] in PBS 1% BSA) were added. Following another incubation of 2h at RT, the plate was washed 3x, 100µl streptavidin-HRP solution (R&D; 1:200 in PBS 1% BSA) were added and incubated for 20min. Subsequently plates were washed 3x, 100µl of TMB solution were added, incubated for 20min and finally the reaction was stopped by adding 100µl 1N $H_2SO_4$. Then the OD (450/570nm) was measured using a Biotek Synergy 2 plate reader. All washing steps were performed on a Biotek microplate washer model ELx405.

Limulus amebocyte lysate (LAL) -Test

The assay is based on limulus amebocyte lysate, which is extracted from blood cells from *Limulus polyphemus* (horseshoe crab), and is used to detect bacterial endotoxin or lipopolysaccharide

(LPS). This lysate contains enzymes that are activated by endotoxin and subsequent split the chromophore paranitro aniline (pNA) from the chromogenic substrate, producing a yellow colour.

4x 25µl of the sample (diluted to its working concentration in PBS) were tested using PTS™ cartridges (Charles River; detection limit >0.05EU/ml). The assay was performed and evaluated on Endosafe®-PTS™ from Charles River. If the value is below 0.05 EU/ml, the sample is considered endotoxin free.

Stimulation of THP1 cells

THP1 cells were diuled to a concentration of $5x10^5$ cells/ml in RPMI. Subsequently, 100µl of this suspension was added into a 96-well v-bottom plate. The suspension was centrifuged for 5min at 300g and the supernatant (SN) discarded. Finally the pellet was resuspended in 100µl stimulation medium (RPMI + BSA or MAA-BSA) and transferred to a 96 u-bottom well tissue culture plate. After incubation for the indicated time at 37°C / 5%$CO_2$, cell suspension was transferred to 96well v-bottom plate, cells were pelleted by centrifugation (5min 300g) and the SNs were harvested for analysis by ELISA or stored at -80°C.

Apoptotic Assays

*Staurosporine*

1µg/ml of staurosporine was added toTHP1 cells and incubated o/n at 37°C / 5% $CO_2$.

*Etoposide*

1µg/ml of etoposide was added to THP1 cells and incubated o/n at 37°C / 5% $CO_2$.

*UVA/UVB*

$30x10^6$ cells (THP1 and RAW) were resuspended (adherent cells were scraped before counting) in 5ml PBS and transferred to a 10cm dish. Following irradiation (UVA: Sellamed 3000; UVB: Waldmann Medizin Technik) without lid for the desired time, cells were centrifuged for 5min at 300g and resuspended in 10ml RPMI without FCS. Subsequently, the cells were allowed to undergo apoptosis by o/n incubation at 37°C.

Flow cytometric analysis of apoptotic cells

Apoptotic cells were resuspended à $1x10^6$ cells/ml. 1ml of cells were centrifuged for 5min at 500g and resuspended in 99µl PBS 1% BSA. Then 1µl of α-CD16/CD32 (1:200; BioLegend) was added and incubated for 15min at RT to block Fc-receptors. Subsequently, 700µl of PBS 1% BSA were added and the suspension was distributed to micronic tubes (100µl per tube). Following a 5min centrifugation at 500g, the SN was aspirated and 100µl of LRO4 [0,75mg/ml] (1:438; MDA-specific mouse monoclonal IgM) or α-KLH [0,5mg/ml](1:100; BioLegend; isotype control) were added. After an incubation of 30min at 4°C 300µl of PBS 1% BSA were added, centrifuged for 5min at 500g and 100µl of α-IgM-APC (Allophycocyanin) [0,5mg/ml] (1:800; BD) were added. The suspension was incubated for 30min at 4°C, 300µl of PBS 1% BSA were added and again centrifuged for 5min at 500g. Afterwards cells were resuspended in 100µl of 7AAD+Annexin V solution (1µl of Annexin V (1:100; BD) + 1µl of 7AAD (1:100; BD) + 98µl of PBS 1% BSA) and incubated for 10min at RT. Finally 400µl of Annexin V buffer (PBS 1% BSA; 2mM $CaCl_2$) was added. Flow cytometry was performed on a FACSCalibur (BecktonDickinson). All antibodies were diluted in PBS 1% BSA.

Pulldown Assay

*Cell Lysate*

First, adherent RAW cells were washed 2x with ice-cold PBS. Then the dish was placed on ice and 600µl of protein lysis buffer (see material) were added. Following scraping of the cells in lysis buffer, the lysate was homogenized by repeated pipetting. To remove cellular debris, the solution was transferred to an eppendorf tube and centrifuged for 10min at 18000g at 4°C. The supernatant was transferred to a new eppendorf tube and used for analysis after protein quantification by BCA assay.

*Pulldown Assay*

Eppendorf tubes were prepared with either 80µl of MAA beads (1:1 suspension in PBS) or 80µl of polylysine beads (as negative control; 1:1 suspension in PBS). Then 2ml of cell lysates were added to each tube and incubated for 2h at 4°C on a roller mixer. After this, the suspension was transferred to spin columns (90µm filters, Mobicol) and centrifuged for 2min at 200g at 4°C. The flow through was discarded, 500µl of protein wash buffer I were added, followed by centrifugation for 2min at 200g at 4°C. Again the flow through was discarded and the washing cycles were

repeated 3 times. Then columns were washed 2x with protein wash buffer II (buffer II was applied to the walls of the columns from the top in a circular fashion, to reduce the risk of possible contamination with detergens from the lysis buffer that could interfere with the shotgun proteomic analysis). Afterwards beads were resuspended in 1ml washing buffer II, transferred to a new eppendorf tube and centrifuged for 1min at 100g. Subsequently, supernatants were aspirated and samples boiled in 80µl NuPage LDS sample buffer (Invitrogen) + β-Mercaptoethanol (28mM) for 5min at 95°C. Finally, supernatants were taken to new eppendorf tubes, boiled again for 5min at 95°C and samples were loaded an a 10% SDS gel.

Silverstain of a SDS gel

First the gel was fixed o/n in 50% methanol / 10% acetic acid. On the next day, the gel was washed 20min in 50% methanol and 2x 10min in $H_2O$. Then it was sensitized for 30sec using 0.02% sodiumthiosulfate ($Na_2S_2O_3 \cdot 5H_2O$). Subsequently, the gel was washed 2x shortly in $H_2O$ and incubated 2x 10min in freshly prepared $AgNO_3$ (0,1% $AgNO_3$ in 4°C cold $H_2O$). Afterwards it was rinsed 1x in $H_2O$ and developed in 3% $Na_2CO_3$ / 0,05% HCHO. The developing solution was exchanged if needed. The gel was developed until the background turned slightly brown. The reaction was stopped with 1% acetic acid and the gel was stored in 1% acetic acid at 4°C.

Generation of bone marrow derived macrophages (BMDMs)

Femurs and tibias from mice were harvested, cleaned, opened at..., and flushed with wash medium (RPMI + 10% Pen/Strep) using a 25G-needle attached to a 10ml syringe. The cells were mixed by pipetting and passed through a 100µm filter. Following centrifugation for 10min at 300g and resuspension in 2ml wash medium, 8ml Erythrocyte-Lysis-buffer (Morphisto) were added and incubated for 5min. Addition of 10ml culture medium (RPMI+FCS) was followed by mixing and centrifugation for 10min at 300g. Then cells were washed 1x in 20ml culture medium, centrifuged, resuspended in 10ml culture medium and counted (54µl medium, 6µl trypan blue and 20µl cells). Finally, cells were resuspended in the calculated volume ($1x10^6$cells/ml for RNA isolation; $3x10^6$cells/ml for ELISA), macrophage-colony-stimulating-factor (MCSF; 1:1000) was added and subsequently cultured in cel culture dishes at 37C / 5% $CO_2$. After 3 days, 1 volume of medium + MCSF (1:500) was added. At day 6, the monolayer was washed twice with PBS and used for experiments. The differentiation was confirmed by staining cells for expression of CD11b by flow cytometry using an anti-CD11b-APC (BioLegend).

Isolation of RNA

RNA was isolated with Qiagen RNeasy Kits according to manufacturer's instructions.

cDNA generation (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems)

*Mastermix*

1µl RT buffer

0,4µl dNTPs

1µl random primer

0,5µl RT

2,1µl $H_2O$

*Reaction mix:*

5µl mastermix

5µl RNA

*Reverse transcription cycler programme (Eppendorf vapo.project thermal cycler)*

1) 25°C 25min

2) 37°C 120min

3) 85°C 5sec

4) 4° unlimited

cDNA was diluted 1:10 in $H_2O$ for RT-qPCR.

Real Time quantitative PCR (RT-qPCR)

*Mastermi*x

0,5µl Primer Mix (10µM forward primer, 10µM reverseprimer in H2O)

5µl SYBR Green (Peqlab)

*PCR reaction*

5,5µl mastermix

4,5µl cDNA

*PCR program*

1) 50°C 2min
2) 94°C 10min
3) 95°C 15sec
4) 60°C 15sec
5) 72°C 30sec
6) 60°C 5min

Steps 3-5 were repeated 39x. A melting curve was generated after the PCR to assess the quality of the product.

Peritoneal Lavage

To isolate resident peritoneal macrophages of mice, a peritoneal lavage was performed. Following euthanasia, the peritoneal cavity was flushed with 10ml HBSS 2% FCS. Cells were detached by gentle shaking before recovery of the lavage fluid. Subsequently, the cell suspension was centrifuged for 5min at 300 g and cells (macrophages) were resuspended in RPMI + FCS and seeded. In order to enrich for macrophages, cells were cultured in cell culture dishes over night and non-adherent cells were removed by washing with PBS on the next day.

Intraperitoneal injections

25µg/g body weight of MAA-BSA or $10x10^6$ apoptotic cells/mouse were injected into the peritoneal cavity. After 2 or 5h, mice were sacrificed and a peritoneal lavage was peformed with 10ml of PBS 1%BSA. After centrifugation for 10min at 300g, SN were analyzed by ELISA or stored at -80°C. Peritoneal cells were stained with anti-mouse CD11b-APC [0,5mg/ml] (1:800; Biolegend) and anti-mouse Ly6C-FITC [0,5mg/ml] (BioLegend; 1:200) for FACS analysis to elucidate specific cell influx into the peritoneal cavity. Monocytes are $Cd11b^+Ly6C^-$ and macrophages are $Cd11b^{high}$ $Ly6C^-$. Moreover, neutrophils are $CD11b^+$ $Ly6C^+$ and inflammatory monocytes are $CD11b^+$ $Ly6C^{high}$. Additionally, cells were stained with anti-mouse Ly6G-PE [0,5mg/ml] (1:2000; BioLegend), to better distinguish between neutrophils ($Ly6G^+$) and inflammatory monocytes ($Ly6G^-$).

Western Blot

*Cell lysis*

Cell monolayers (12well) were washed 1x with 1ml ice cold PBS. Then 100µl of protein lysis buffer were added and lysates were homogenized by repeated passing through a 25G needle. Subsequently, lysates were placed on ice for 30min, centrifuged for 10min at 4°C and supernatants were used for further analysis.

*Blotting, detecting and stripping*

A polyacrylamide gel was prepared as described before. The samples (30µl/slot) and the molecular weight marker (2µl/slot, Fermentas) were loaded and the gel was run at 120V for 1,5h. Afterwards, proteins were transferred from the gel to a nitrocellulose membrane (Whatman) using the BioRad TransBlot SD (semi-dry) system and the transfer buffer (described above). The blot was run for 30min at 15V.

After blotting, the membrane was first blocked for 60min in TBS 0.05%Tween 5%milk. Subsequently the 1st AB (1:1000) in TBS 0.05%Tween 5% milk was added and incubated o/n at 4°C. On the following day the membrane was washed 3x 5min in TBS 0,05%Tween and then the 2nd AB (1:2000) in TBS 0,05%Tween 5%milk was added for 60min at RT. After washing 3x 10min in TBS 0.05%Tween, ECLplus (GE healthcare) was prepared according to manufacturer's instructions (1ml reagent A + 25µl reagent B), the membrane was covered with 1ml of ECLplus and incubated 5min in the dark. Finally, the membrane was exposed and pictures were taken (Bio Rad Universal Hood II).

To remove all antibodies that could interfere with further detections, membranes were stripped. First, membranes were washed in TBS 0,05%Tween 5% milk for 10min. Subsequently, the membranes were incubated for 20min in stripping solution (Thermo Scientific Prod #21059). After stripping, membranes were washed 2x for 5min in TBS 0,05%Tween and further treated as described above (blocking, 1st antibody, 2nd antibody, detection).

*Antibodies for Western Blot*

1st AB:

P-p44/42 MAPK Mouse mAB [100µg/ml] (Cell Signalling)

p44/42 MAPK Mouse mAB [100µg/ml] (Cell Signalling)

2nd AB:

αMouse-HRP [1mg/ml] (abcam)

Cloning

*Design of primers*

First, the coding sequence (CDS) of the gene of interest was identified on Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/). Following, the CDS was copied into the primer3 source sequence window (http://frodo.wi.mit.edu/primer3/). Primers flanking a splicing site were designed and blasted on the genome of interest. The E-value describes the random background noise. The lower the value, the more significant the hit is. Therefore the E-value should be around 0.015.

*PCR from original vector*

Primers were designed for the coding region including start and stop codons and restrictions sites were added. The annealing temperature of forward and reverse primer should differ max. 2°C.

*Primer*

MD2 attB1 primer fw.

gggg aca agt ttg tac aaa aaa gca ggc tcc atg tta cca ttt ctg ttt ttt tcc

MD2 attB1 primer re:

gggg ac cac ttt gta caa gaa agc tgg gt cta att tga att agg ttg gtg

*40µl PCR reaction to amplify coding insert from template plasmid*

8µl 5x GoTaq green buffer

0,4µl 25mM dNTP

2µl 10µM primer mix

17,2µl $H_2O$

0,4µl GoTaq-Polymerase

1µl template plasmid (40ng DNA)

*PCR program*

1) 94°C 3min
2) 94°C 30sec
3) 65°C 30sec
4) 72°C 1min
5) 72°C 5min
6) 4°C unlimited

Steps 2-4 were repeated for 35x.

*Run Agarose Gel*

A gel containing 1% agarose in 1x TAE buffer  Gelstar (1:30,000) was prepared. Samples were loaded and the gel was run at 80V. Bands were visualized on Bio Rad Universal Hood II. Ineeded some cases, bands were excised under UV light.

*Extract DNA from Agarose Gel*

For the extraction of DNA from an agarose gel the QIAEX II kit from Qiagen was used. First, the DNA band from the agarose gel was excised weighed and 3 volumes of Buffer QX1 were added. QIAEX II beads were vortexed for 30 s, and 10µl were added to the sample followed by incubation for 10min at 50°C. During this incubation, the sample was vortexed every 2 minutes. Then the solution was centrifuged for 30 s at full speed. The pellet was washed with 500µl of Buffer QX1 and centrifuged again for 30 s. Subsequently, the pellet was washed twice with 500µl Buffer PE, centrifuged for 30 s and air dried for 15min. Finally, DNA was eluted by vortexing the beads in 20µl $H_2O$.

*EcoRI/SalI restriction digest*

1µl EcoRI (Roche)
1µl SalI (Roche)
5µl buffer H
23µl $H_2O$
20µl  PCR product or vector

Samples were incubated for 2h at 37°C.

*Ligation*

The insert and vector were used in a volume ratio 3:1

*20µl reaction*

4µl 5x dilution buffer

1µl digested vector

3µl digested insert

10µl 2x ligation buffer

1µl T4 ligase

The samples were incubated for 30min at RT

*Heat shock transformation into chemically competent XL1-blue bacteria*

15µl XL1-blue bacteria (thawed on ice for 15min) and 10µl ligation mix were combined on ice. The samples were incubated first for 30min on ice and subsequently 40sec at 45°C and 300rpm. Afterwards 250µl of SOC medium (preheated to 37°C) were added and incubated for 1h at 37°C, shaking at 200rpm. Finally 200µl of the bacteria were plated on amp-agar plates (50ng/ml ampicillin) at 37°C o/n. On the next day the colonies were picked and inoculated o/n.

Transfection of HEK293T cells

On the day before transfection, HEK293T cells were washed in HBSS, trypsinized (Trypsin obtained from Sigma) for 2 min and resuspended in 10ml of culture medium (DMEM+FCS). 1.5ml of culture medium containing 500,000 HEK293T cells were added to a 6 well plate. On the following day, the transfection mix was prepared:

2µg DNA

9µl $CaCl_2$ (2M)

61µl $H_2O$

The transfection mix was added drop wise into 71µl of HEBS buffer and incubated for 5min at RT. Afterwards the whole mix was added drop wise to the seeded cells.

DNA/RNA quantification

DNA/RNA concentrations were quantified using the Nanodrop spectrometer 2000C (Peglab) according to the manufacturer's instructions .

DNA isolation Mini Prep

The 5prime FastPlasmid Mini-Prep Kit (5prime) served for isolation of the plasmids amplified in *E. coli*. A single colony grown on a LB-Ampicillin plate was picked off and inoculated in a 2 ml liquid LB-Ampicillin culture. It was grown over night at 37 °C at 12g.The isolation was done according to the manufacturer's instructions. The DNA was resuspended in 50µl H2O.

DNA isolation Midi Prep

For high-yield plasmid purification, PureLink Midi 50 kit from Invitrogen was used. A single colony grown on a LB-Ampicillin plate was picked off and inoculated in a 50 ml liquid LB-Ampicillin culture. It was grown over night at 37 °C at 12g. The culture was centrifuged for 10min at 4,000g. It was proceeded according to the manual. The purified DNA was dissolved in 200µl TE buffer and stored at -20°C.

# 3. Results

## 3.1 MDA-modifications exhibit differences in biological activity

Malondialdehyde is a highly reactive aldhehyde and hardly ever found in free form in the human body, as it promptly forms stable adducts on proteins, lipids and DNA. For our experiments we modified a carrier protein, namely BSA, with MDA. Based on increasing published evidence, we postulated that certain MDA-epitopes are specifically recognized by the innate immune system. To prove this, I incubated BSA with MDA under various conditions, thereby generating different types of MDA adducts, including MDA-BSA, FHP-BSA, MAA-BSA and AA-BSA. The percentage of modified amino groups in each preparation was assessed by TNBS assay, demonstrating a similar extent of modification for MAA-, FHP-, and MDA-BSA (Table 1). AA-modifications appeared not to be successful.

| | | |
|---|---|---|
| **BSA** | **0,0** | **%** |
| **MAA** | **84,6** | **%** |
| **FHP** | **88,9** | **%** |
| **MDA** | **79,0** | **%** |
| **AA** | **-3,7** | **%** |

**Table 1** Percentage of modified amino groups measured by TNBS test.

| | | |
|---|---|---|
| **BSA** | **0** | **0** |
| **MAA** | **62** | **59** |
| **FHP** | **12** | **11** |
| **AA** | **1** | **1** |
| **MDA** | **2** | **3** |
| **$H_2O$** | **2** | **3** |

**Table 2** BSA modifications show different fluorescence ability.

Subsequently, I also measured the fluorescence in each preparation (Table 2), as described in Material & Methods. The negative controls, $H_2O$, BSA (and AA-BSA), did not show any fluorescence. Similarly, MDA-BSA showed no fluorescence. In contrast, FHP-BSA showed low,

and MAA-BSA high fluorescence. The fluorescence of the MAA-BSA is thought to be due to the enrichment of the MDHCS structure and the low fluorescence of FHP-BSA is likely due to some MDHDC epitopes in this modification.

To assess and compare the biological activity of the differently modified protein preps, THP1 cells were stimulated for 16h with the differentially modified proteins. After the treatment, supernatants were assayed for IL-8 secretion by ELISA (Fig. 6A). THP1 cells showed a small, but significant increase in IL-8 secretion upon the stimulation with MDA-BSA. However, when stimulated with MAA-BSA, THP1 cells showed much higher IL-8 secretion (up to ~400pg/ml). As expected, stimulation with FHP-BSA also showed significant IL-8 secretion.

Because the biological activity is highest in MAA-BSA, all following experiments were conducted using this specific adduct.

### 3.2. Excluding endotoxin contamination

To rule out experimental artefacts as a result of endotoxin contamination, I performed LAL assays of our BSA preparations. Every single preparation had an endotoxin content of less than 0,050EU/ml. THP1 cells lack CD14 and in our hands only respond to high doses of LPS (>1µg/ml) [27]. Because THP1 cells respond well to MAA-BSA stimuli, endotoxin-contamination would require high amounts and should thus be easily detectable by LAL test. To further exclude endotoxin contamination, I performed a stimulation experiment in the presence of polymyxin B (Fig. 5), which has the ability to bind and neutralize endotoxin [28].

For this experiment RAW macrophages were used. Cells were stimulated with BSA or MAA-BSA in the presence or absence of keto-deoxyoctulosonate (KDO, the active component of LPS) and polymyxin B. KDO caused a general increase in MIP-2 secretion. Moreover, a synergistic effect of MAA-BSA and KDO could be observed. In the presence of polymyxin B, the effect of

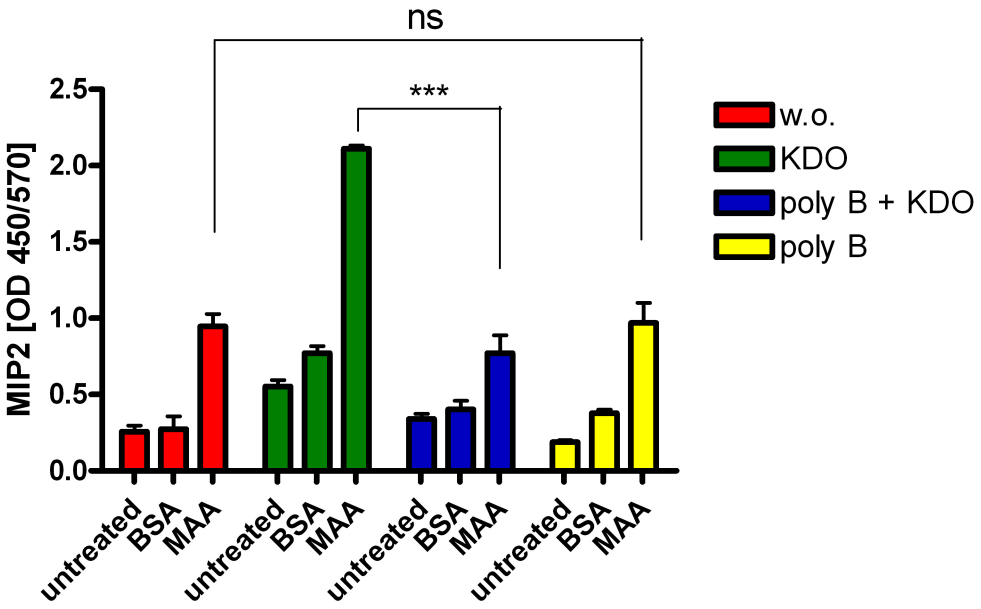



**Fig. 5** Polymyxin B neutralizes KDO but not MAA-BSA. ***<0,001, ns = non significant

KDO was lost, whereas the MAA-BSA effect remained almost entirely unaffected. Finally, co-stimulation with polymyxin B alone did not show any effect of the MIP-2 secretion induced by MAA-BSA.

## 3.2 MAA-BSA induces a complex immune response

Our research group as well as others have shown that MDA-adducts induce a chemokine response in monocytes. To confirm this, THP1 cells (a human monocyte cell line) were stimulated with different concentrations of either MAA-BSA or BSA as a negative control for 16h (Fig. 6B). Subsequently, the supernatants were taken and IL-8 levels were measured by ELISA.
In this experiment, a dose-dependent effect of MAA-BSA on THP1 cells can be appreciated. Furthermore, the negative control BSA did not trigger any IL-8 secretion. However, a basal IL-8 secretion can be observed. For all further experiments using THP1 cells, 50µg/ml of MAA-BSA was used, if not described otherwise.

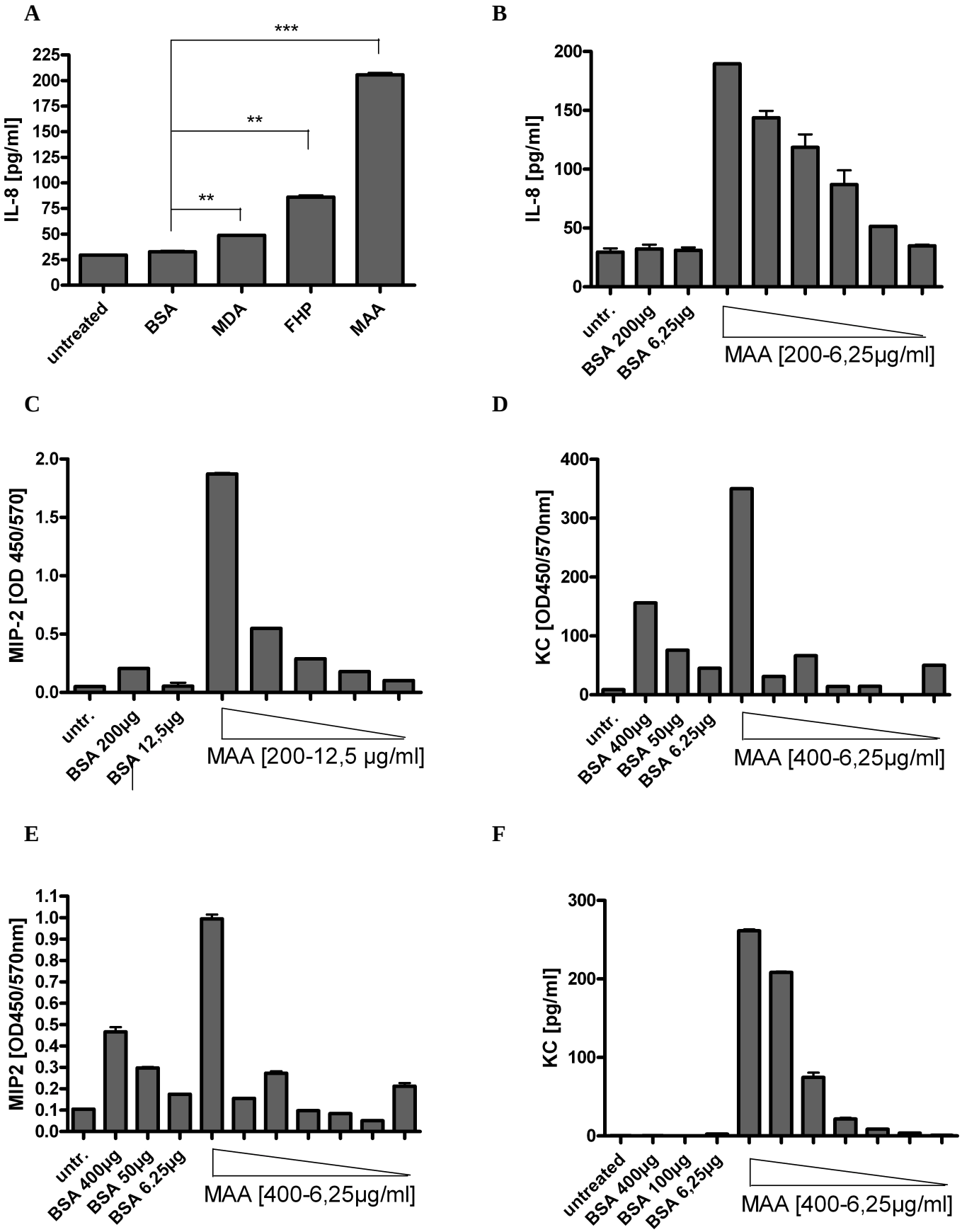

A
IL-8 [pg/ml]
225
200
175
150
125
100
75
50
25
0
***
**
**
untreated
BSA
MDA
FHP
MAA
B
IL-8 [pg/ml]
200
150
100
50
0
untr.
BSA 200µg
BSA 6,25µg
MAA [200-6,25µg/ml]
C
MIP-2 [OD 450/570]
2.0
1.5
1.0
0.5
0.0
untr.
BSA 200µg
BSA 12,5µg
MAA [200-12,5 µg/ml]
D
KC [OD450/570nm]
400
300
200
100
0
untr.
BSA 400µg
BSA 50µg
BSA 6.25µg
MAA [400-6,25µg/ml]
E
MIP2 [OD450/570nm]
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
untr.
BSA 400µg
BSA 50µg
BSA 6.25µg
MAA [400-6,25µg/ml]
F
KC [pg/ml]
300
200
100
0
untreated
BSA 400µg
BSA 100µg
BSA 6,25µg
MAA [400-6,25µg/ml]

**Fig. 6** MAA-BSA acts as a danger signal. (A) Biological activity of different BSA modifications (100µg/ml each) (SN1:2). MAA-BSA triggers (B) THP1 cells (SN1:2), (C) RAW cells (SN1:3), (D),(E) peritoneal macrophages and (F) BMDMs to secrete various chemokines. untr. = untreated. Bars represent mean +/- SD of biological triplicates. These data are representative examples of 2 (A, D) or 3 (B, C, E, F) independent experiments. **p<0,01, ***<0,001

As we planned to investigate the role of MAA-BSA also in mice, it was important to confirm these results in a murine cell system. Therefore, I stimulated the immortalized peritoneal macrophage cell line RAW267.1 with different concentrations of MAA-BSA, and BSA as a negative control. 16h after stimulation, I measured the concentration of the chemokine KC (CxCl1) in the supernatant by ELISA. KC is the murine equivalent of human IL-8. Surprisingly, there was no KC secretion detectable.

However, in the same experiment, a dose-dependent effect on MIP-2/Cxcl2 secretion was observed (Fig. 6C). The fact that RAW macrophages responded to MAA-BSA by secreting MIP-2, was suggested that chemokines other than IL-8/KC may be affected as well.

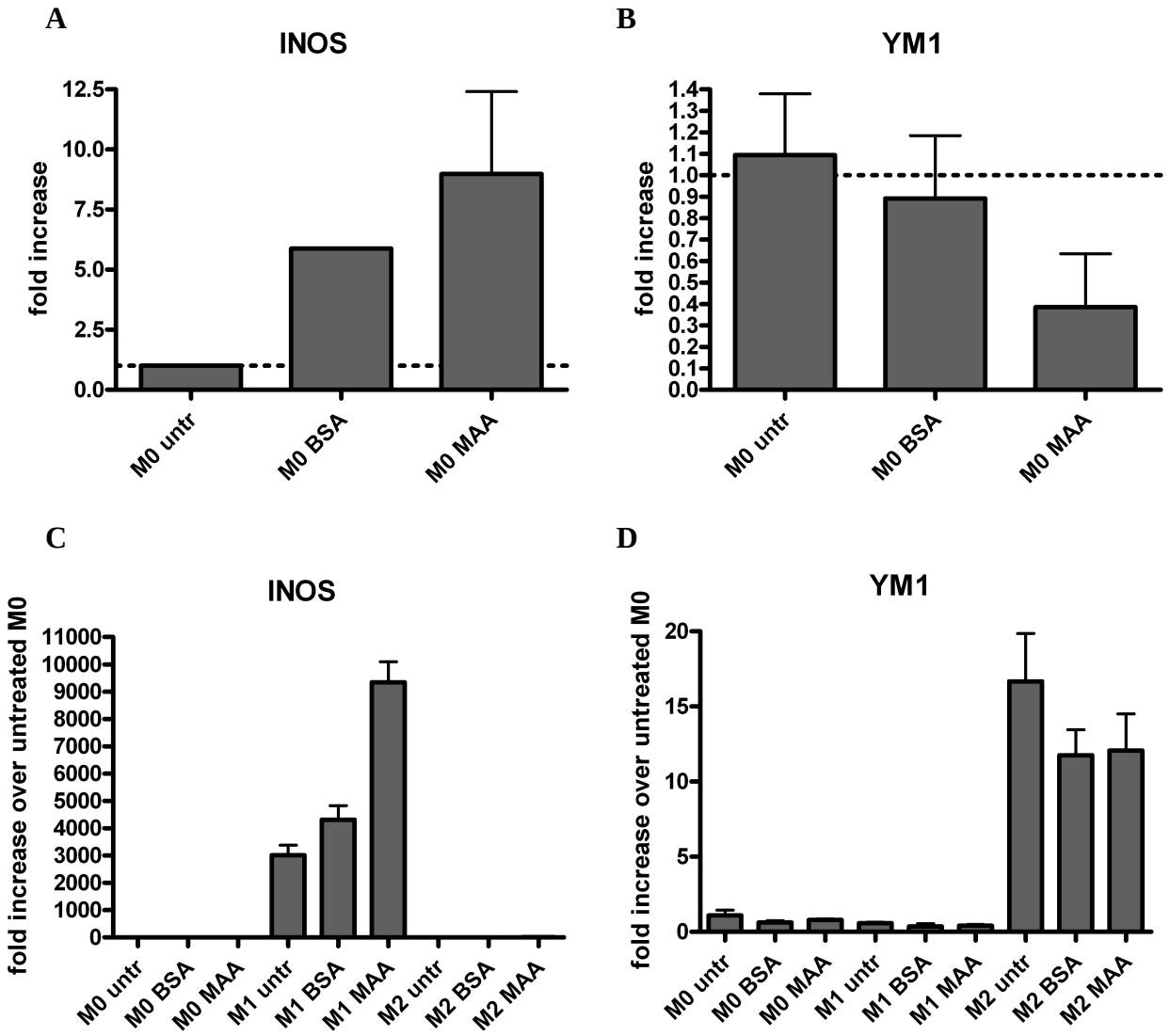
So far, all experiments had been carried out with immortalized cell lines. Therefore, we decided to test the response in primary cells. Therefore, I performed peritoneal lavages of wild type (WT) mice to isolate resident peritoneal macrophages. After seeding the cells over night, they were stimulated with different amounts of MAA-BSA for 16h. Upon MAA-BSA stimulation, peritoneal macrophages responded by secreting KC (Fig. 6D) and MIP-2 (Fig. 6E) in a concentration dependent manner. However, only high concentrations of MAA-BSA were sufficient to induce a response in resident peritoneal macrophages.

For this reason and also because of the limited number of peritoneal macrophages per mouse, we decided to obtain macrophages from a different source, namely the bone marrow. I isolated bone marrow of WT mice and differentiated macrophages (BMDMs) by addition of M-CSF. The differentiated cells were treated with different concentrations of MAA-BSA. In contrast to peritoneal macrophages, BMDMs were very sensitive to the MAA-BSA-treatment and showed a dose dependent KC secretion (Fig. 6F). Given that BMDMs responded very well to MAA-BSA, and the yield of cells following isolation from the bone marrow was much higher than from the peritoneum, all following experiments in primary cells were done with BMDMs.

Macrophages can display different phenotypes [6]. Thus, we first addressed the question, whether MAA-stimulation induces a phenotypic change in macrophages. Freshly isolated bone marrow cells were seeded and differentiated into macrophages with M-CSF. Following complete

differentiation, cells were stimulated with MAA-BSA or BSA for 3.5h and RNA was isolated. Subsequently, I quantified the expression of the M1-specific gene iNOS (Fig. 7A) or the M2-specific gene YM1 (Fig. 7B) by RT-qPCR. As seen in figure 7A, iNOS is upregulated upon MAA-BSA stimulation. In contrast, YM1, a M2 marker is downregulated upon MAA-BSA treatment. This suggested that stimulation of BMDM with MAA induces a shift towards an M1 phenotype.

Secondly, I assessed the MAA-specific response in M1 versus M2 macrophages. To elucidate this, BMDMs were isolated and subsequently polarized towards M1 with interferon-γ (IFN- γ) or M2 with interleukin-13 (IL-13). Successful polarization was confirmed by assessing the expression of iNOS (Fig. 7C) and YM1 (Fig. 7D) by RT-qPCR , respectively. The results given in Fig. 7 demonstrate the successful polarization into M1 and M2 macrophages, allowing me to use the polarized macrophages to investigate their response towards MAA-BSA (Fig. 7E, 7F). Both CxCl1 and CxCl10 were found to be upregulated upon MAA-treatment.



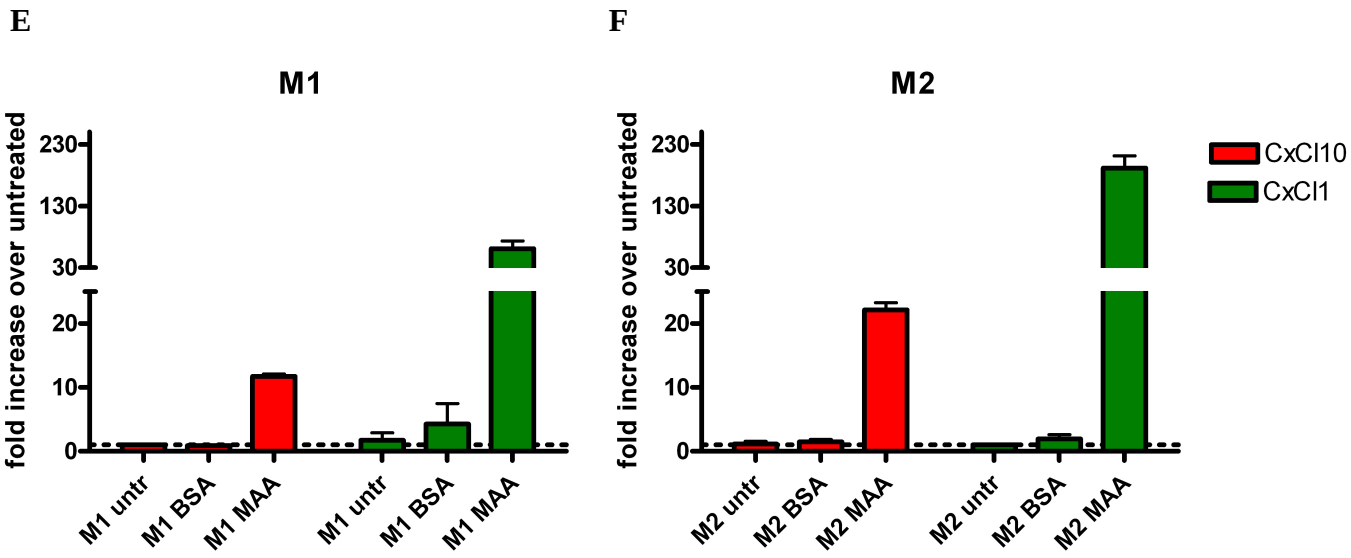
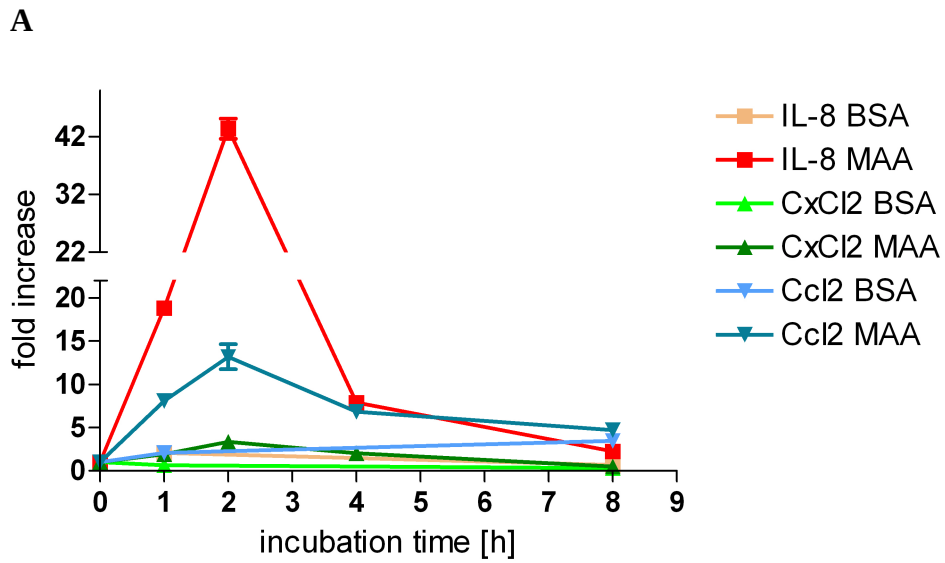
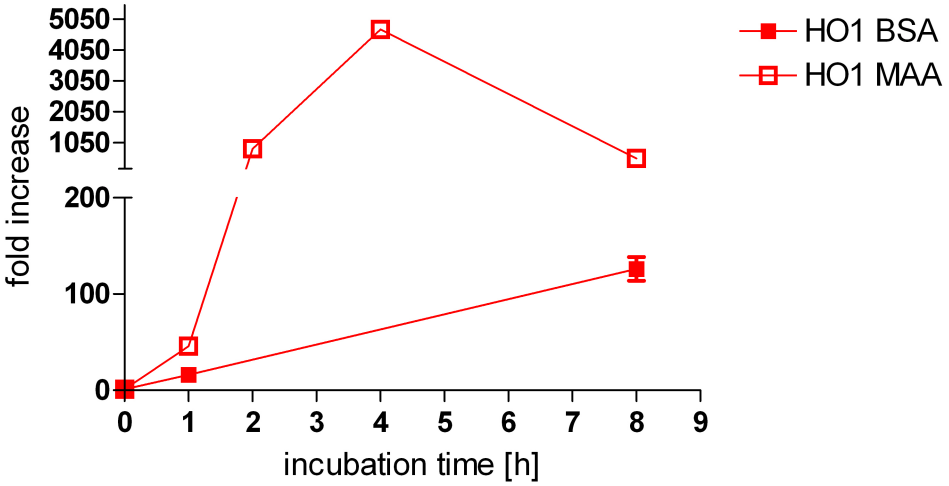



**Fig. 7** Macrophage phenotypes. Upregulation of (A) iNOS and (B) YM1 after treatment of M0 with MAA-BSA. Fold increase of (C) iNOS and (D) YM1 over untreated M0 to verify subclass polarization. MAA-BSA triggers upregulation of inflammatory genes in (E) M1 as well as in (F) M2. Bars represent mean +/- SD of biological triplicates. These data are representative examples of 2 independent experiments.



**B**




**C**

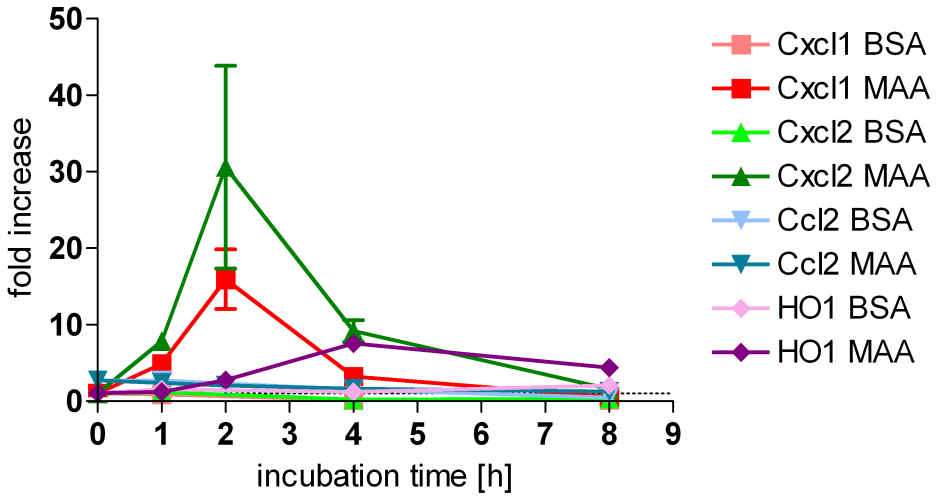



**Fig. 8** Upregulation of inflammatory genes upon MAA-BSA stimulation. (A) experiment of stimulated THP1 cells. (B) HO-1 is highly induced after MAA-BSA stimulation. (C) Time course experiment on BMDMs. Data points represent mean +/- SD of biological triplicates.

We have shown that different inflammatory chemokines are upregulated, when cells of the innate immune system are stimulated with MAA-BSA. To better understand the response to MAA-BSA, I also screened for the induction of other inflammatory genes and the time points of their upregulation. Hence, I performed a time course experiment using THP1 cells. Cells were stimulated for stimulation 0,1,2,4 and 8 hours and RNA was isolated to monitor the upregulation of different genes at each time point (Fig. 8A).

We first assessed the expression of IL-8 (CxCl8), MIP-2 (CxCl2), and MCP-1 (Ccl2). Upon the stimulation with MAA-BSA, all 3 genes were upregulated. CxCl2 was slightly induced (3x), whereas IL-8 (43x) and Ccl2 (14x) were highly upregulated. When stimulated with BSA, none of the genes showed an induction. The peak of upregulation was reached at 2h. Therefore, I chose this time point to screen for MAA-regulated genes on a genome wide scale using expression microarrays. Table 3 shows a list of the genes that were most strongly induced by MAA-BSA in THP-1 cells.

| **Fold increase** | **Gene Title** | **Gene Symbol** |
|---|---|---|
| 36.20 | chemokine (C-C motif) ligand 4 | CCL4 |
| 30.38 | interleukin 1 receptor antagonist | IL1RN |
| 10.92 | ELOVL family member 7, elongation of long chain fatty acids (yeast) | ELOVL7 |
| 10.02 | early growth response 2 | EGR2 |
| 9.65 | interleukin 1 receptor antagonist | IL1RN |
| 8.88 | chemokine (C-C motif) ligand 3 | CCL3 |
| 8.83 | chemokine (C-C motif) ligand 24 | CCL24 |
| 7.11 | UL16 binding protein 2 | ULBP2 |
| 6.23 | heme oxygenase (decycling) 1 | HMOX1 |
| 6.11 | PR domain containing 1, with ZNF domain | PRDM1 |
| 5.82 | 5'-nucleotidase, ecto (CD73) | NT5E |
| 5.76 | pleckstrin homology-like domain, family A, member 1 | PHLDA1 |
| 5.66 | platelet-derived growth factor alpha polypeptide | PDGFA |
| 5.58 | interleukin 1 receptor antagonist | IL1RN |
| 5.51 | perilipin 2 | PLIN2 |
| 5.44 | carbonyl reductase 3 | CBR3 |
| 5.35 | v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian) | MAFB |
| 5.31 | CDK5 regulatory subunit associated protein 2 | CDK5RAP2 |
| 5.15 | dual specificity phosphatase 5 | DUSP5 |
| 5.04 | human immunodeficiency virus type I enhancer binding protein 3 | HIVEP3 |
| 5.02 | CD83 molecule | CD83 |

**Table 3** List of upregulated genes after stimulation of THP1 cells with MAA-BSA (only genes with at least 5x induction are shown).

Surprisingly, none of the hitherto monitored cytokines were found in the list of high (>5x) upregulated genes. However, Ccl3, Ccl4 and Ccl24 which are closely related to Ccl2 were induced. Interestingly, HO-1 (HMOX1), which is known to play a protective role against oxidative stress [29] was also induced. To verify this upregulation, I assessed the expression of HO-1 at various time points following MAA-stimulation in THP1 cells by RT-qPCR (Fig. 8B). Confirming the results of the microarray, HO1 was highly expressed upon MAA-BSA stimulation, but not after BSA stimulation. Interestingly, IL1RN was induced as well. This antagonist, modulates immune reactions by inhibiting IL1 alpha and beta (IL1b).
To show whether the kinetics of gene expression following MDA-administration show a similar pattern in murine primary cells, I performed a time course experiment in BMDMs (Fig. 8C). Again RNA was isolated at different time points, following MAA-BSA stimulation, and the expression of inflammatory genes CxCl1 (KC), CxCl2, Ccl2 and the protective gene HO1 was assessed. BMDMs stimulated with the negative control BSA showed no upregulation of the genes at any time. In contrast, MAA-BSA stimulated BMDMs express CxCl1 and CxCl2. Contrary to THP1 cells, BMDMs did not express Ccl2 in response to MAA-BSA. Corresponding with the THP1 time course, the peak of gene induction was around 2h. HO1 reached its peak at 4h and slightly decreased until 8h.

In summary, MAA-BSA induces a complex response that is mainly characterized by the induction of genes known to be involved in the early inflammatory response. This demonstrates its capacity to act as an endogenous danger signal.

## 3.3 Biological Relevance of MAA epitopes

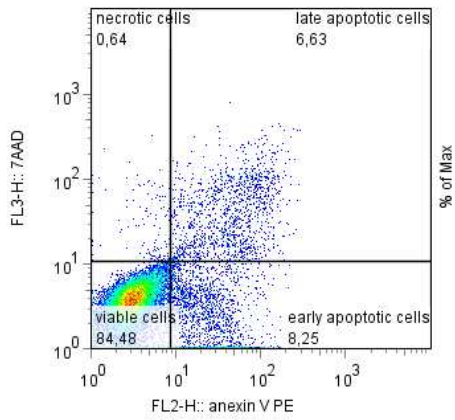
An important carrier of MDA-epitopes *in vivo* is represented by dying cells. In 2009, Chou *et al.* [30] generated apoptotic thymocytes by treating them with either dexamethasone or PMA, and found that late apoptotic cells carry MDA epitopes using an MDA-specific Ab.
Apoptosis can be induced by various chemical agents and physical treatments. Therefore I compared different ways of inducing apoptosis. Generating apoptotic THP1 cells by adding staurosporine or dexamethasone was successful as assessed by 7-AAD and Annexin-V staining. Vi-

able cells are 7AAD and Annexin V double negative. Early apoptotic cells are Annexin V positive, but 7AAD negative. Most important, late apoptotic cells are 7AAD and Annexin V double positive. After gating the different markers, each subpopulation was stained with the MDA-specific IgM Ab LRO4 for MDA epitopes. Surprisingly, under the experimental conditions used, I did not observe the generation of MDA-epitopes on late apoptotic THP1 cells. However, treatment with the DNA-damage inducing agent etoposide induced late apoptotic cells with MDA-epitopes on their surface (Fig. 9A, 9B).

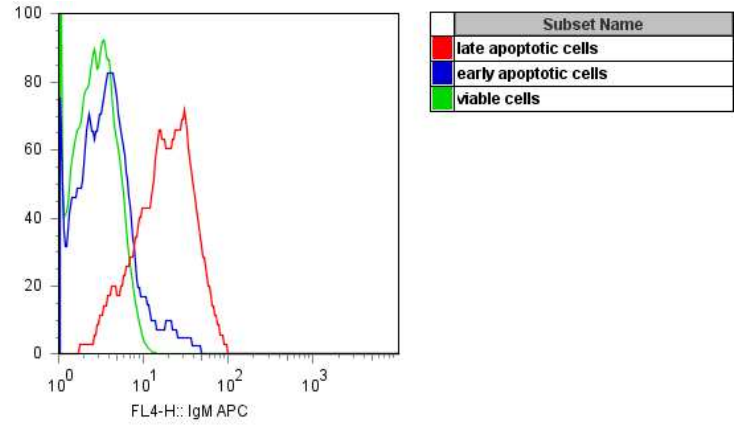
Because, the yield of late apoptotic cells after etoposide treatment was not satisfying, and higher concentrations did not increase the amount of late apoptotic cells, I switched to an alternative treatment. This time I decided to treat THP1 cells with UVA/UVB light. UVA light worked well in different concentrations, but again the amount of late apoptotic cells was unsatisfying. The use of UVB light induced high amounts of late apoptotic cells and high numbers of MDA-carrying apoptotic cells in different concentrations. I decided to irradiate THP1 cells with 40mJ UVB light (Fig. 9C, 9D) in the following experiments, as the amount of apoptotic cells generated was sufficient at a moderate dose.

After identifying a reliable way to generate late apoptotic cells that carry MDA epitopes, I also tried the same treatment with RAW cells. Like THP1 cells, RAW macrophages were irradiated with different concentrations of UVA and UVB light, but the amount of apoptotic cells was difficult to assess (Fig. 9E, 9F). Unlike THP1 cells, RAW macrophages showed a high autofluorescence which made it impossible to gate individual sub-populations. However, an overall increase of LRO4-binding was observed following UVB irradiation, indicating a higher amount of MAA-epitopes.

A




B



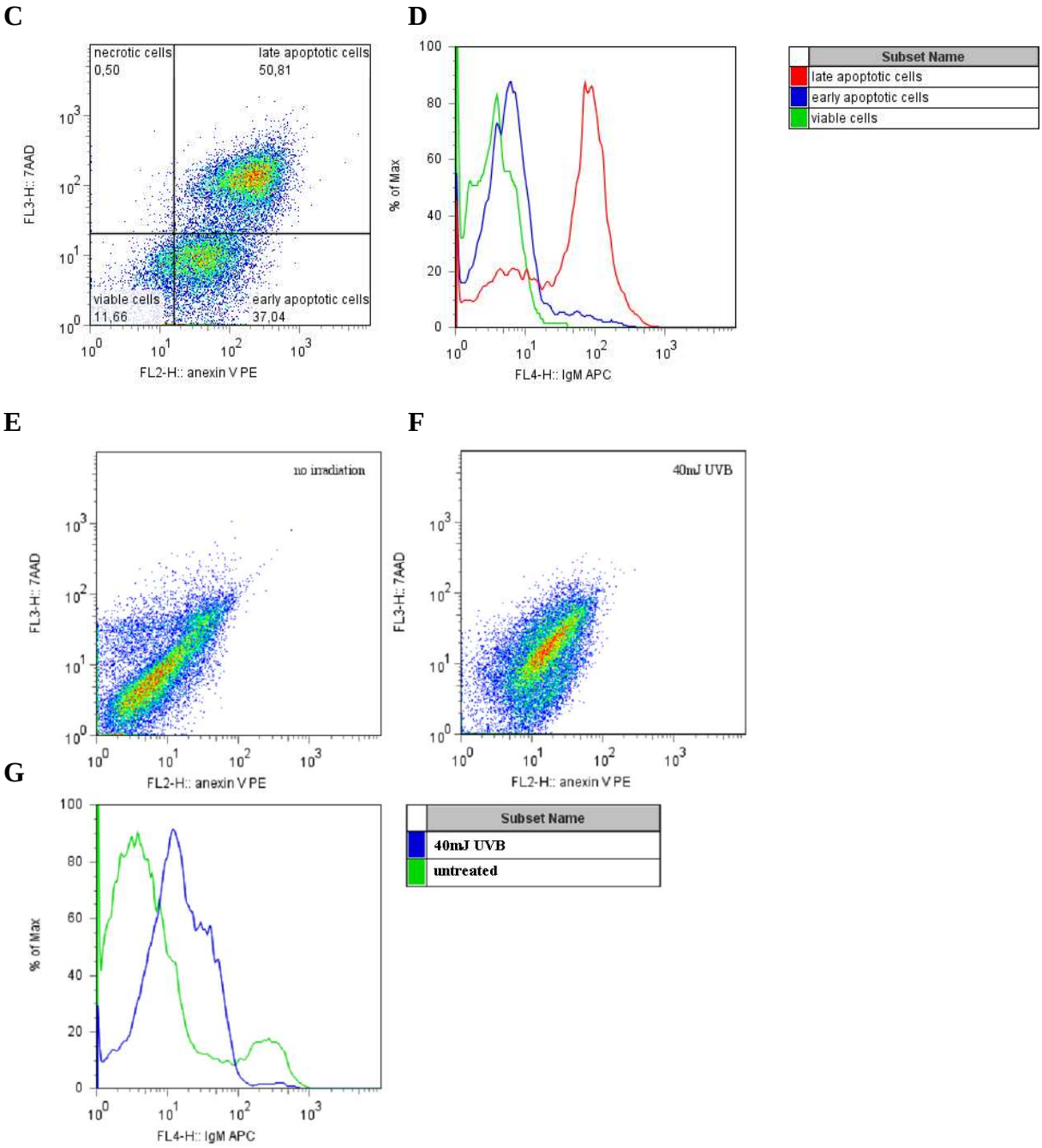



**Fig. 9** Generation of apoptotic cells, carrying MDA epitopes. (A) Etoposide treated THP1 cells. Stained with 7AAD and Annexin V. (B) Gated cell-populations stained with LRO4. (C) THP1 cells irradiated with 40mJ UVB and stained with 7AAD and Annexin V. (D) Gated cell-populations stained with LRO4. (E) viable/untreated RAW macrophages were irradiated with (F) 40mJ UVB and stained with LRO4 for MDA epitopes (G). Different conditions were tested with similar results. Shown blots are representative.

Subsequently, I wanted to investigate if MAA epitopes on late apoptotic cells have the same biological 'danger' effect as MAA-BSA. Indeed, when RAW cells were stimulated with apoptotic RAW cells (AC), an increase in MIP-2 secretion was observed similar to that observed with

MAA-BSA(Fig. 10). However, it needs to be demonstrated that this effect is indeed due to the presence of MAA-epitopes on late apoptotic cells.

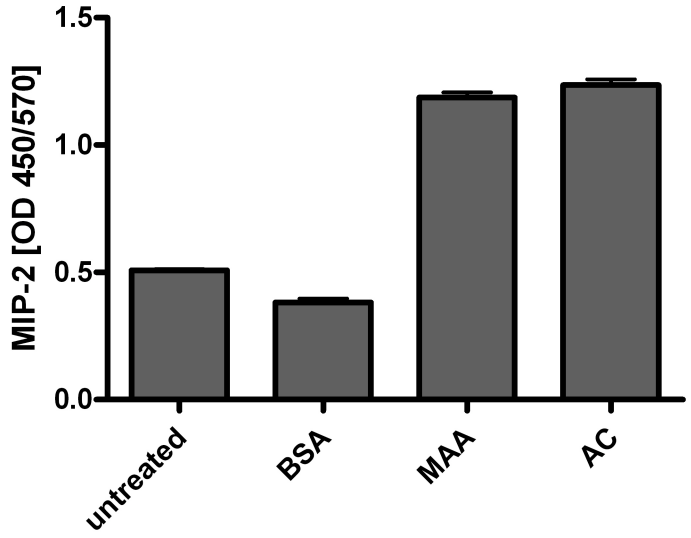


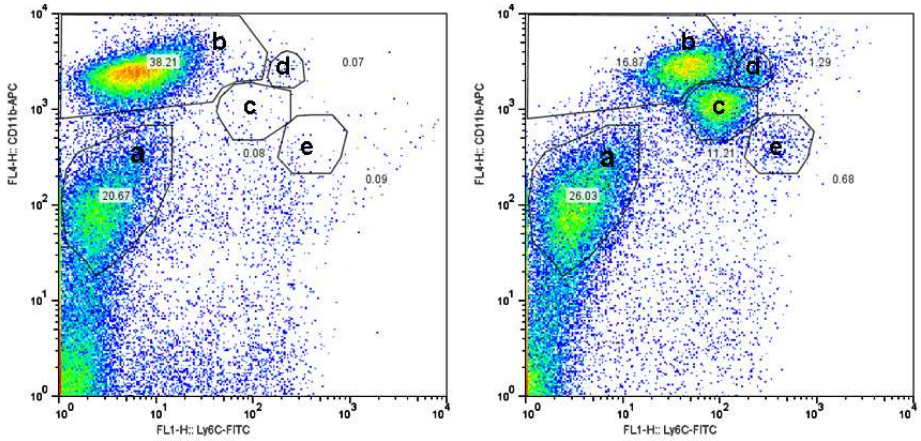

**Fig. 10**. Apoptotic cells (AC) induce MIP-2 secretion. Data is representative of 2 independent experiments. Bars represent mean +/- SD of biological triplicates. This graph is a representative example of 3 independent experiments.

### 3.3.1 In vivo experiments

In the previous chapters, I provided evidence that MAA-BSA acts as a danger signal and is found on apoptotic cells. As all these experiments were only done *in vitro*, we also wanted to test the ability of MAA-BSA to induce immune responses *in vivo*. The peritoneal cavity is an easy accessible, relatively closed system, and high concentrations of a given stimulus can be reached by local administration. Furthermore, most immunologically relevant cell types are present there. To assess the pro-inflammatory activity of MAA-BSA *in vivo* in a pilot experiment, I injected 25µg/g mouse of BSA or MAA-BSA into the peritoneal cavity. After 2h, mice were sacrificed and the peritoneal cavity was lavaged. The obtained peritoneal cells were then stained for CD11b, Ly6C and Ly6G and subsequently analysed by FACS (Fig. 11A, 11B). Resident monocytes were defined as $Cd11b^{+}Ly6C^{-}$ and macrophages as $Cd11b^{high}$ $Ly6C^{-}$. Moreover, neutrophils are $CD11b^{+}$ $Ly6C^{+}$ and inflammatory monocytes are $CD11b^{+}$ $Ly6C^{high}$. Additionally, the cells were stained for Ly6G, to distinguish between neutrophils ($Ly6G^{+}$) and inflammatory monocytes ($Ly6G^{-}$) (data not shown). Interestingly, the whole population of macrophages (a) is shifted upon MAA-BSA injection. This can be explained by the uptake of the fluorescent MAA-BSA which

interferes with the detection. Most striking, there was a significant influx of neutrophils (c) as well as of inflammatory monocytes (e). Furthermore, in this experiment, an increase of a cellular population (d) could be observed, which may represent other granulocytes, such as mast cells. In addition, we compared the percentages of granulocyte influx at 2h and 5h post injectionem (Fig. 11C). The neutrophil influx peaked at 2h, while after 5h, the percentage of granulocytes started to normalize again. Neutrophil influx usually depends on secreted chemokines like IL-8. Therefore, I also assayed the peritoneal lavage fluid for possible secretion of KC. I screened for KC secretion 2h and 5h after BSA or MAA-BSA injection (Fig. 11D), respectively. At the 2h time point, high KC secretion upon MAA-BSA injection was detected. After 5h, no KC was detectable. In contrast, injected BSA did not cause any KC secretion at both time points. Subsequently, BSA (n=7) or MAA (n=8) were injected into mice at the 2h time point (Fig. 11E), strengthening the data from the pilot experiment.

**A** **B**

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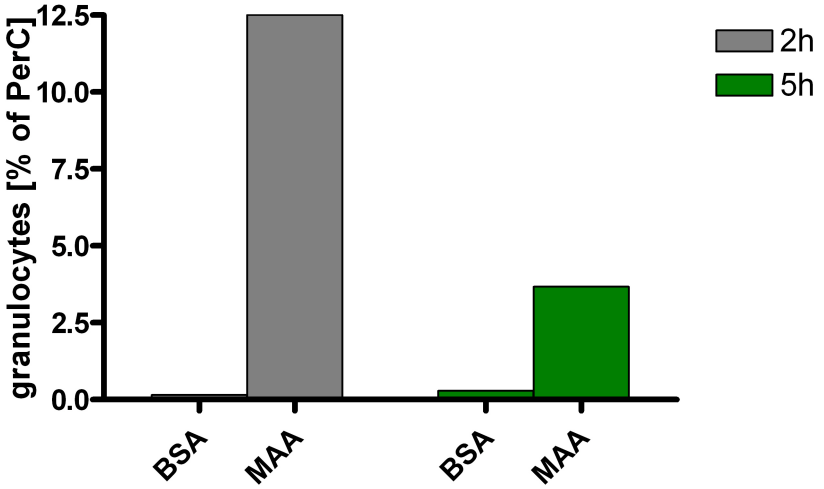

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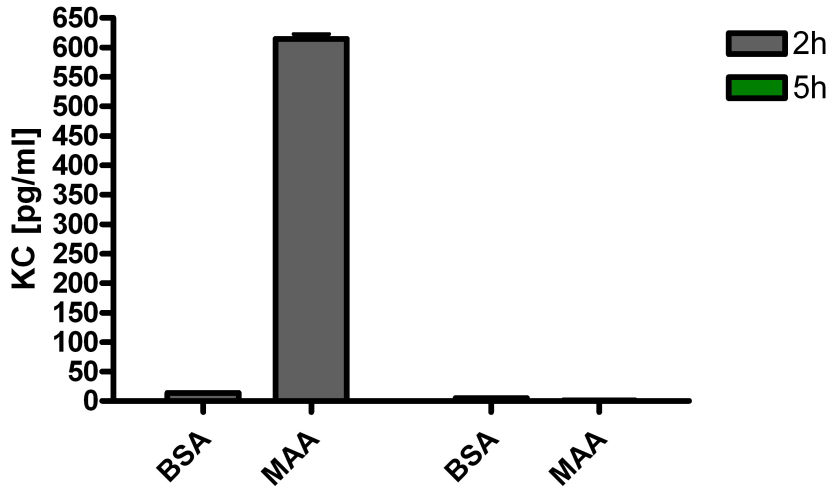



**E**

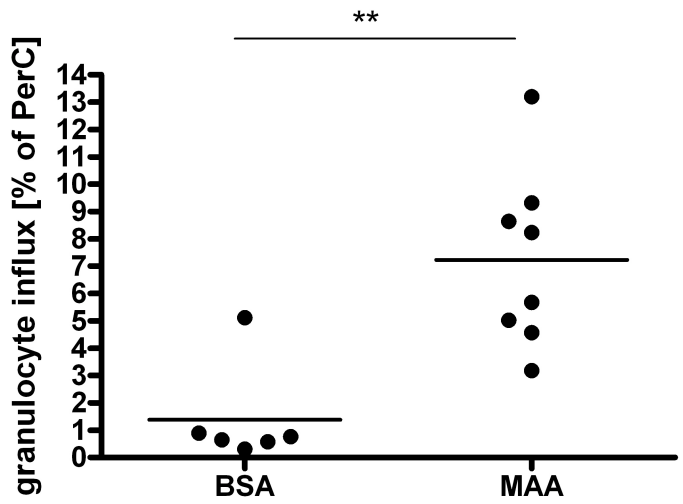
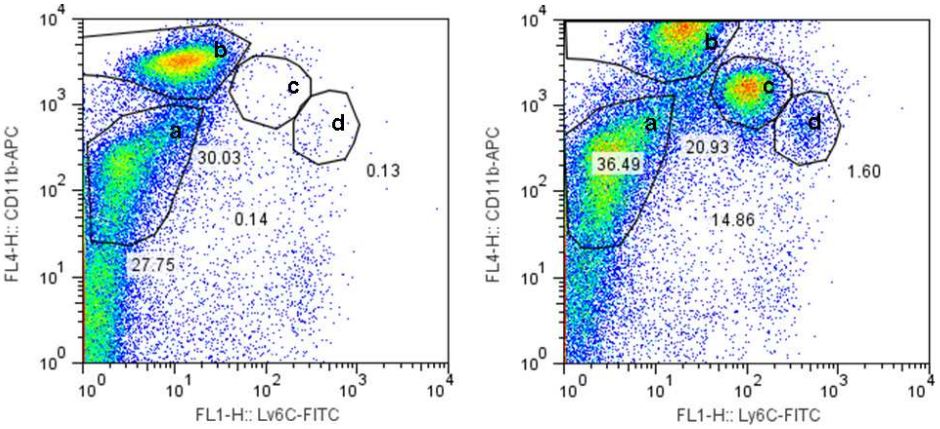



**Fig. 11** Intraperitoneal injections of MAA-BSA**.** (A), (B), (C) and (D) are graphs of one conducted experiment. Following BSA (A) or MAA-BSA (B) injection, isolated peritoneal cells were stained for CD11b and Ly6C. (a) Monocytes, (b) macrophages, (c) neutrophils, (d) unknown and (e) inflammatory monocytes. (C) Granulocyte influx out of total peritoneal cells after 2h and 5h. (D) KC secretion upon BSA or MAA-BSA injection after 2 and 5h. (E) Peritoneal influx after IP injection (2h) of BSA (n=7) or MAA (n=8). **p<0,01

Taken together, injections of MAA-BSA, but not BSA, cause secretion of inflammatory chemokines and influx of inflammatory cells, demonstrating that MAA-BSA can induce a sterile peritonitis, as both neutrophil influx and KC secretion are markers thereof [31].

To test, whether apoptotic cells carrying MDA on their surface can elicit a similar response in the peritoneum, we performed a pilot experiment, in which apoptotic RAW cells were generated by UVB-irradiation and injected into the peritoneal cavity (n=2). Injections of PBS and viable RAW cells were used as negative controls (n=2 each). After 2h, the peritoneal cavity was washed and cells stained for Cd11b and Ly6C (Fig. 12A, 12B). In addition, cells were stained for Ly6G to better discriminate between neutrophils and inflammatory monocytes. Similar to the MAA-BSA injection, injected apoptotic cells caused an increased influx of neutrophils and a modest influx of inflammatory monocytes in contrast to injected viable cells. Furthermore, the granulocyte influx was quantified, showing that apoptotic RAW cells mimic MAA-BSA (Fig. 12C). Injection of PBS and viable RAW cells did not cause any granulocyte influx, whereas injected apoptotic RAW cells caused a highly significant influx. Thus, apoptotic cells mimic to some extent the effects of MAA-BSA *in vitro* as well as *in vivo*. However, the experiments

**A** **B**

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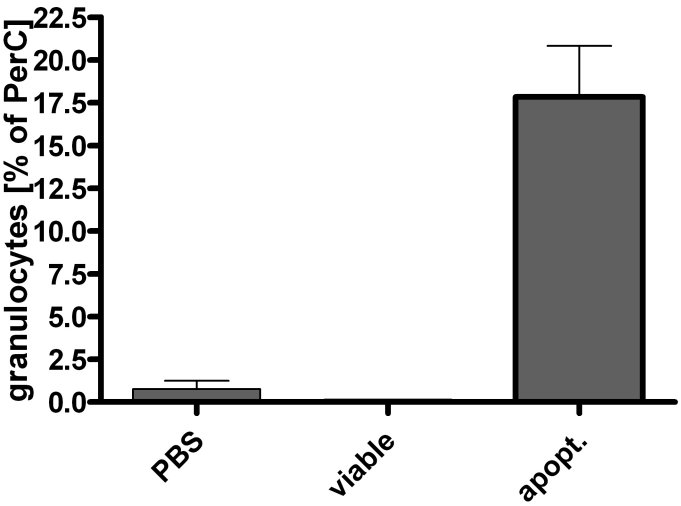


**Fig. 12** After intraperitoneal injection of (A) viable or (B) apoptotic RAW cells, isolated peritoneal cells were stained for CD11b and Ly6C. (a) Monocytes, (b) macrophages, (c) neutrophils and (d) inflammatory monocytes. (C) Quantification of inflammatory cells out of total peritoneal cells. (n=2)

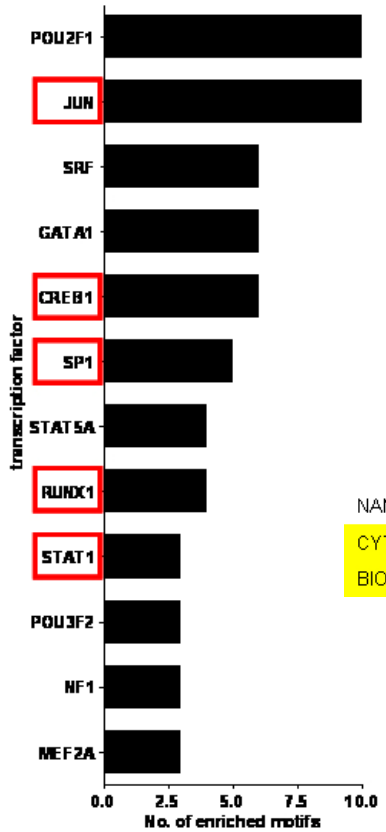
have to be repeated with larger numbers of mice and it has to be demonstrated that MDA epitopes on the surface of apoptotic cells induce the neutrophil influx.

Taken together, I have shown that MDA epitopes on apoptotic cells can act as an endogenous danger signal *in vitro*. Moreover, I elucidated that this effect is also present *in vivo*. Therefore I continued to investigate the response on a molecular level.

## 3.4 Intracellular signalling

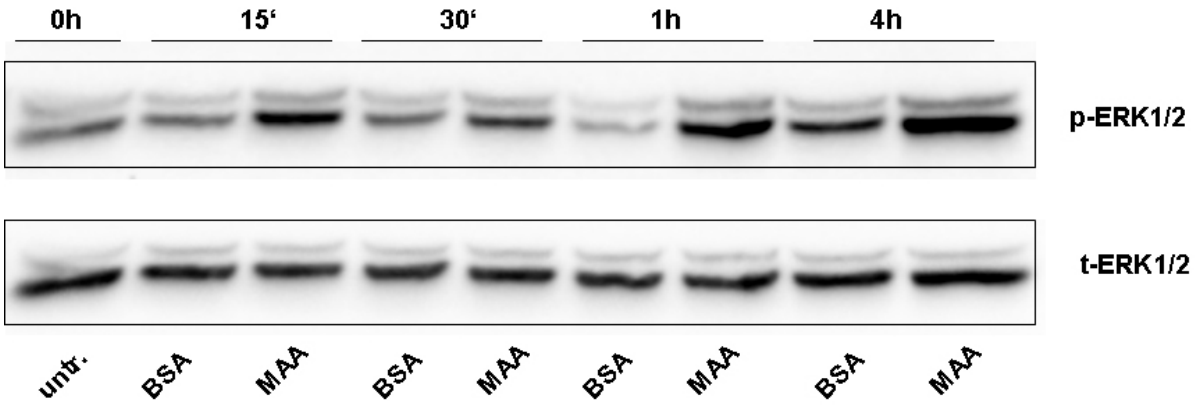
The microarray expression data set I had generated from MAA-BSA stimulated THP1 cells was analyzed for enriched DNA motifs and enriched pathways as annotated in the KEGG-database (Kyoto Encyclopedia of Genes and Genomes) (Fig 13A). This analysis was conducted with GSEA (gene set enrichment analysis provided by the Broad Institute). Interestingly, most of the

**A**

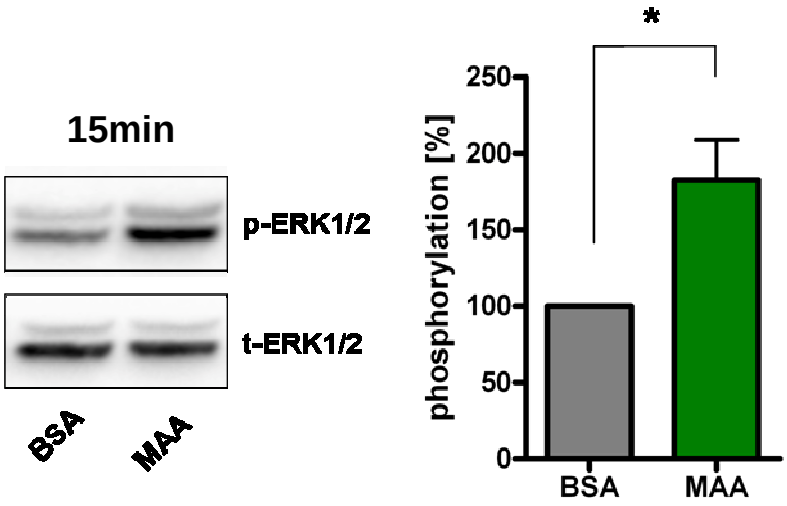



| NAME | SIZE | ES | NES | FDR | FWER |
|---|---|---|---|---|---|
| CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION | 45 | 0.69 | 2.10 | 0.00 | 0.00 |
| BIOSYNTHESIS_OF_STEROIDS | 23 | 0.69 | 1.86 | 0.01 | 0.02 |

**B**


0h
15‘
30‘
1h
4h
p-ERK1/2
t-ERK1/2
untr.
BSA
MAA
BSA
MAA
BSA
MAA
BSA
MAA

**C**


15min
p-ERK1/2
t-ERK1/2
BSA
MAA
*
phosphorylation [%]
250
200
150
100
50
0
BSA
MAA

**D**

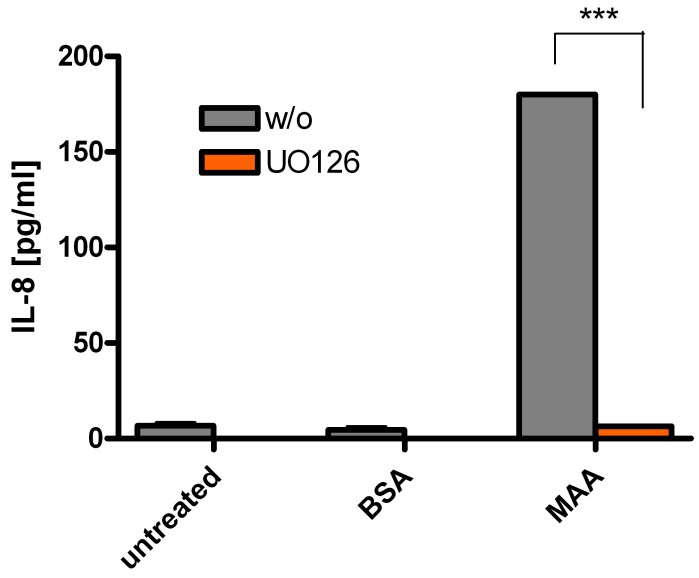

***
IL-8 [pg/ml]
200
150
100
50
0
w/o
UO126
untreated
BSA
MAA

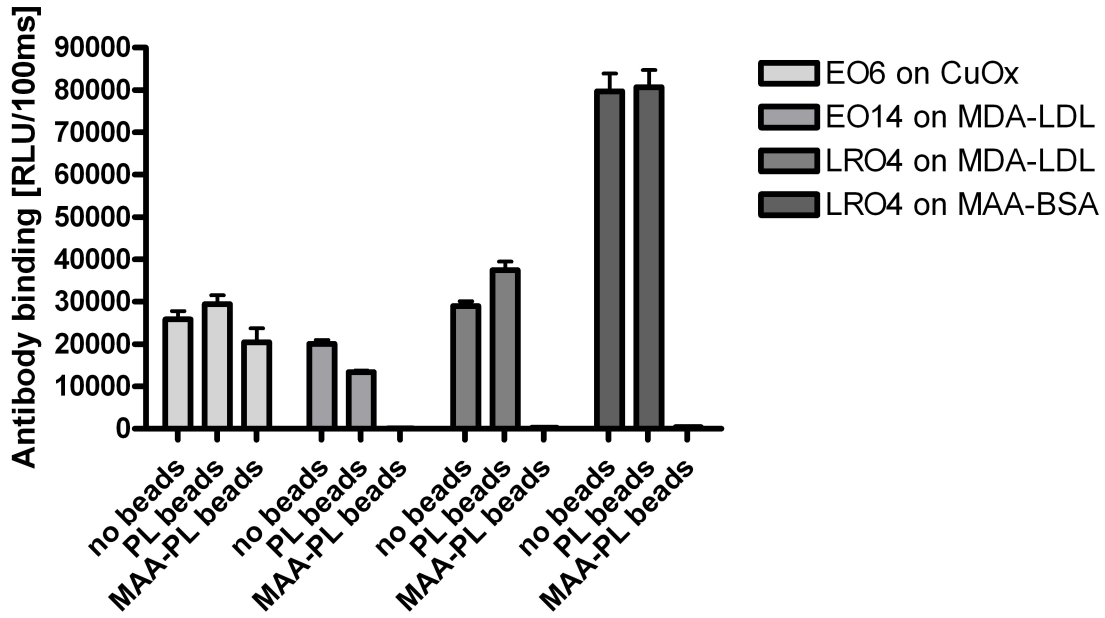
**Fig. 13** The intracellular response to MAA-BSA. (A) Analysis of microarray data for enriched pathways and DNA motifs. (B) MAA-BSA stimulation leads to increased phosphorylation of ERK. (C) Quantification of ERK-phosphorylation after 15min of stimulation (3 pooled experiments). (D) The ERK inhibitor UO126 completely depletes MAA induced IL-8 secretion. *p<0,05, ***p<0,001

genes upregulated by MAA-BSA treatment carried DNA motifs of MAPK regulated transcription factors (13A, red circled), therefore indicating the involvement of the MAPK-pathway in MAA signalling. The pathway analysis revealed an enrichment of genes annotated as part of 1) the cytokine-cytokine receptor interaction pathway and 2) the biosynthesis of steroids pathway.
The enrichment of the former can be explained by the fact that many cytokines and chemokines were induced upon MAA-BSA stimulation. Enrichment of the latter may be the result of a biased annotation, as many enzymes involved in antioxidant response also play a role in steroid biosynthesis.
Extracellular signal regulated kinases (ERK) belong to the mitogen activated protein kinases (MAPK) and regulate a plethora of important cellular functions including proliferation, secretion, migration and apoptosis [32, 33]. To prove this hypothesis, THP1 cells were stimulated for different time points with either MAA-BSA or BSA. Cell lysates were analyzed for possible phosphorylation of ERK by Western Blotting (Fig 13B). ERK was found to be highly phosphorylated upon MAA-BSA treatment at each time point. In contrast, stimulation with BSA did not cause an increase in ERK-phosphorylation. Levels of pERK were assessed after 15min and densitometrically quantified (Fig 13C). Phosphorylation of ERK (p-ERK) was first normalized to total ERK (t-ERK), BSA controls were set as 100%, and the increase of phosphorylation was calculated. There was a statistical significance ($p<0.05$) in phosphorylation between BSA and MAA-BSA stimulation. To address the question if phosphorylation of ERK is crucial for MAA signalling, I then tested the effect of the ERK inhibitor UO126 on MAA induced IL-8 secretion in THP1 cells (Fig. 13D).
As reported above, THP1 cells secreted IL-8 upon MAA-BSA stimulation. When UO126 was added, IL-8 secretion was completely blunted. This data suggest that MAA induced IL-8 secretion is ERK dependent.

### 3.5 MAA binds to TLRs

After delineation of the most important intracellular mediators, we decided to focus on identifying the receptors involved in MAA signalling. To identify possible binding partners of MAA, we

**A**

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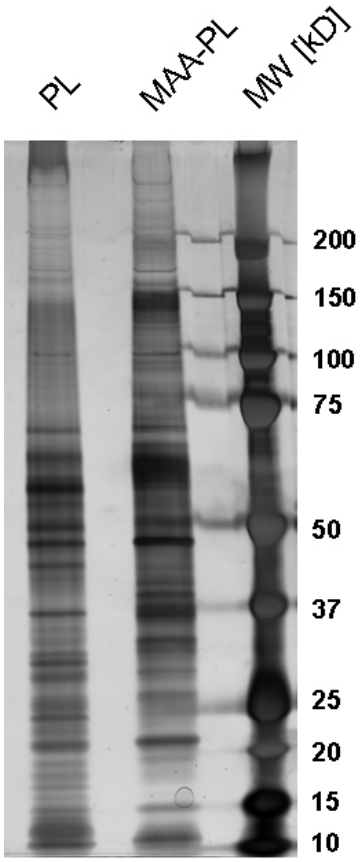


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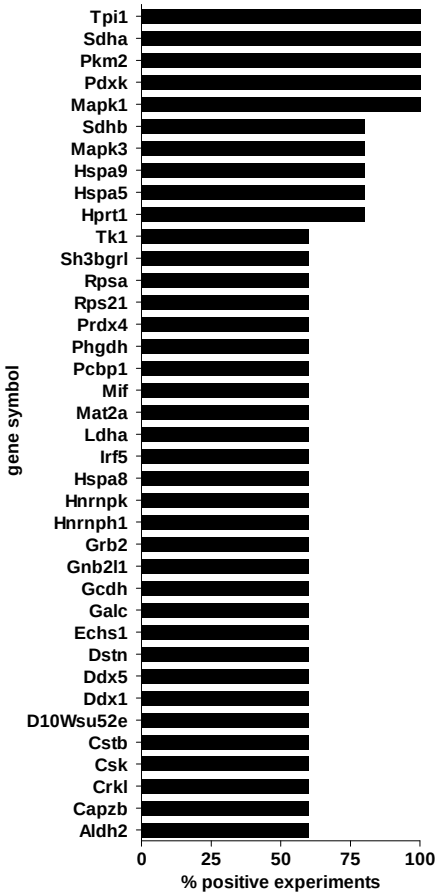


**Fig. 14** MAA pulldown in RAW lysate. (A) MAA-PL beads compete with MAA-BSA or MDA-LDL for binding to MDA-specific natural antibodies. (B) Silverstain of one representative pulldown with MAA-PL or PL beads in RAW lysate. (C) Samples were handed over to the mass spectrometry facility and analyzed. The figure shows MAA-binding partners which were found in at least 50% of the conducted experiments.

used a proteomic approach. We coupled sepharose beads to either MAA-polylysine or polylysine as a negative control and used them to pull down MDA-binding proteins from cell lysate obtained from RAW-cells. To verify the biological activity of our newly generated MAA-PL beads an immunocompetition experiment was conducted (Fig. 14A). MAA-PL beads completely compete with MAA-BSA. Therefore we could proceed with the pulldown experiment. Aliquots of the eluted samples were run on a gel and visualized by a silver stain (Fig. 14B).
When the pulldowns of MAA-PL beads and PL- beads were compared (Fig. 14B), many additional bands were identified in the MAA-PL pulldown compared to the PL pulldown (e.g. bands ~60kDA and ~150kDA). Eluted samples were subsequently analyzed by Keiryn Bennett (CeMM), using tandem mass spectrometry [34]. Following the subtraction of PL binding partners, we show in Fig. 14C only the MAA binding partners which appeared in at least 66% of the experiments. This list of binding partners reveals that MAA interacts with various proteins of the MAPK pathway, for instance MAPK1 and MAPK2. These results are congruent with the already shown data that MAPK pathways are crucial for signalling. However, receptors on the cell surface could not be detected as MAA binding partners with this method.

As the uptake of MDA-epitopes is mediated by SR-A [35], a possible role is conceivable. Therefore we treated THP1 cells with Fucoidan, a SR-A agonist (Fig. 15). If SR-A plays a role in MAA signalling, Fucoidan should compete with MAA and therefore decrease IL-8 secretion. THP1 cells were pre-incubated 1h together with Fucoidan and then stimulated with MAA or LPS. As expected, Fucoidan had no effect on LPS induced effects. In contrast, Fucoidan treatment diminished MAA-BSA induced IL-8 secretion. This indicates a possible role for SR-A in MAA signalling. Fucoidan treatment diminished but did not ablate IL-8 secretion. Hence, other receptors may play a role. However, no membrane proteins or receptors were found in the pulldown experiment, which may be due to technical limitations [36] of our approach. SR-A-ligands synergize with TLR4 ligands in the context of macrophage cell death [13]. Moreover, another paper showed that oxidized phospholipids, which are CD36 ligands, acts through a heterodimer of TLR4 and TLR6 [37]. To assess the role of TLR4 in MAA induced inflammation, I isolated BMDMs from WT and $TLR4^{-/-}$ mice and treated them with MAA-BSA.

**A**

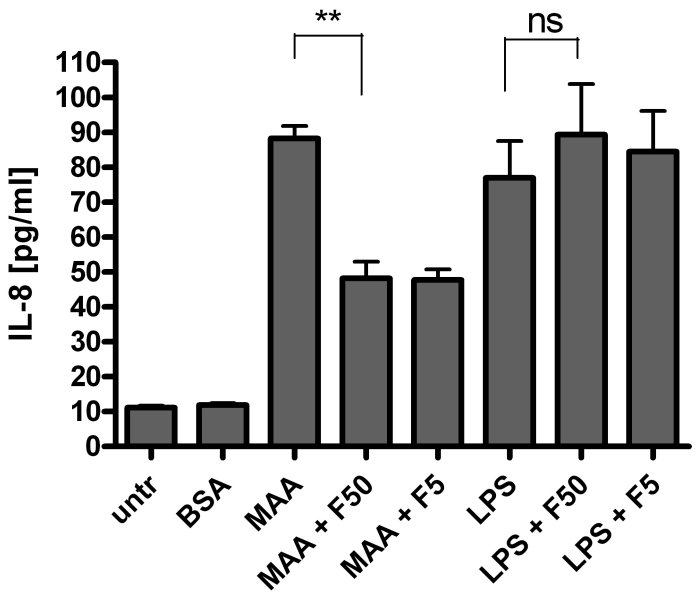
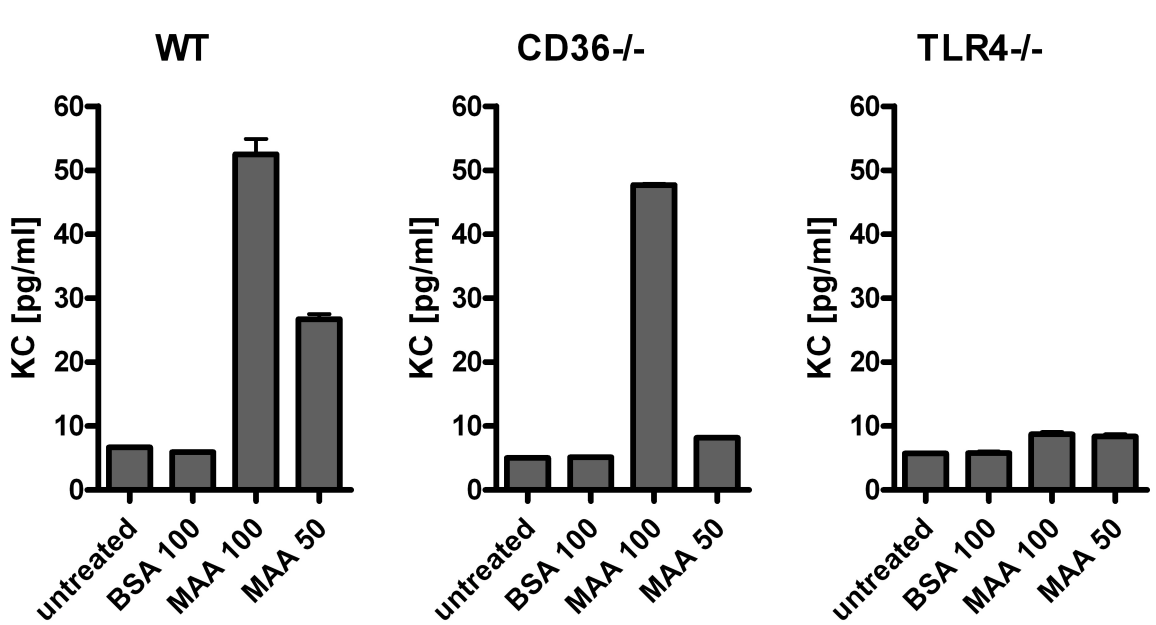


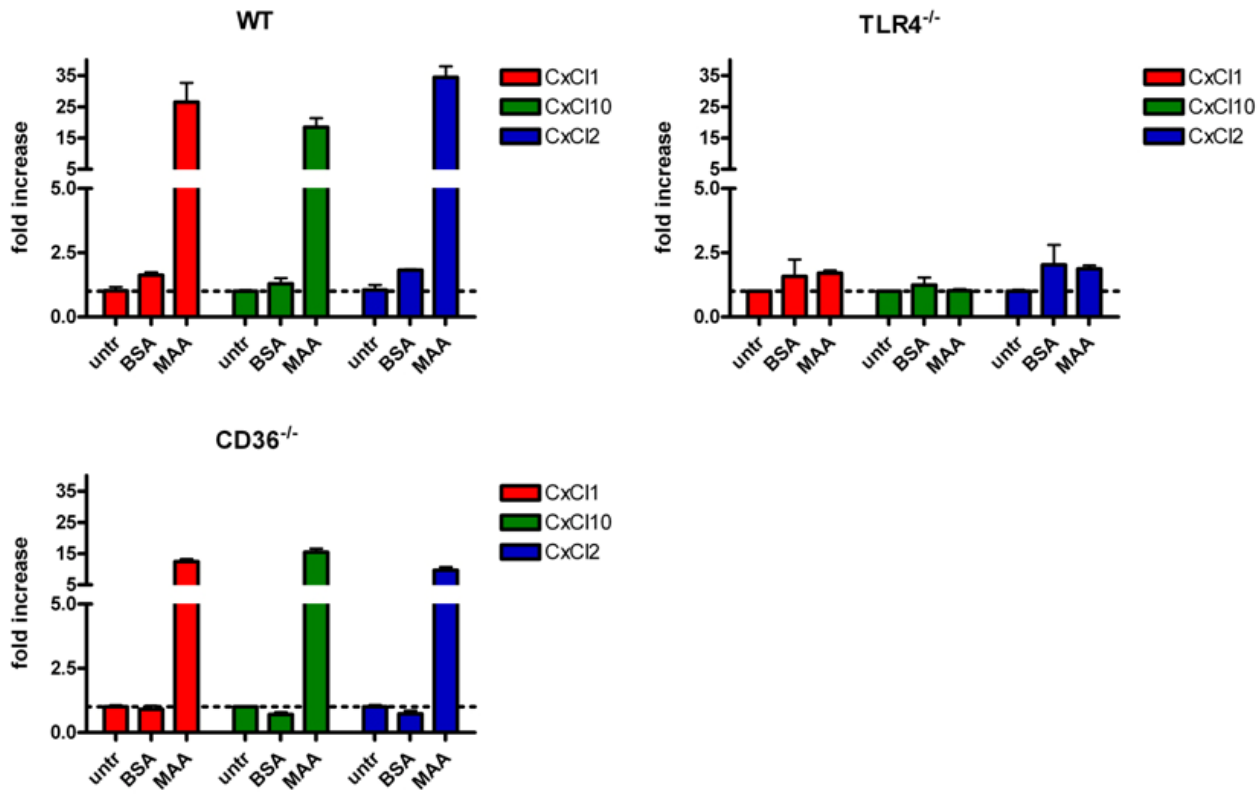

**Fig. 15** Impact of Fucoidan (50 and 5 µg/ml) on IL-8 secretion (SN 1:2). Data is representative of 2 similar experiments. **p<0,01, ns = not significant

Macrophages obtained from mice lacking the scavenger receptor CD36, which is responsible for the uptake of phosphocholine-decorated molecules [38] , were used as an additional control. WT and $CD36^{-/-}$ BMDMs responded to the MAA-BSA stimulation by secreting KC (Fig. 16A). In contrast, $TLR4^{-/-}$ BMDMs did not secrete KC in response to the MAA-BSA stimuli. BMDMs from the same knockout mice were stimulated with BSA or MAA-BSA for 3h, RNA was isolated and expression of inflammatory genes was quantified by RT-qPCR (Fig. 16B). Upon stimulation with MAA-BSA, BMDMs from WT and $CD36^{-/-}$ induce expression

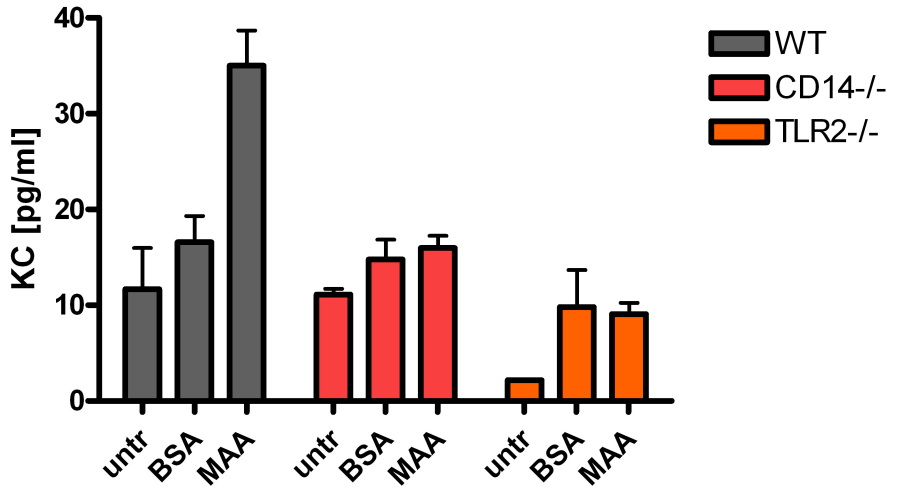
**A**



**B**


WT
TLR4-/-
CD36-/-
fold increase
35
25
15
5
5.0
2.5
0.0
CxCl1
CxCl10
CxCl2
untr
BSA
MAA

**C**


KC [pg/ml]
40
30
20
10
0
WT
CD14-/-
TLR2-/-
untr
BSA
MAA

**D**

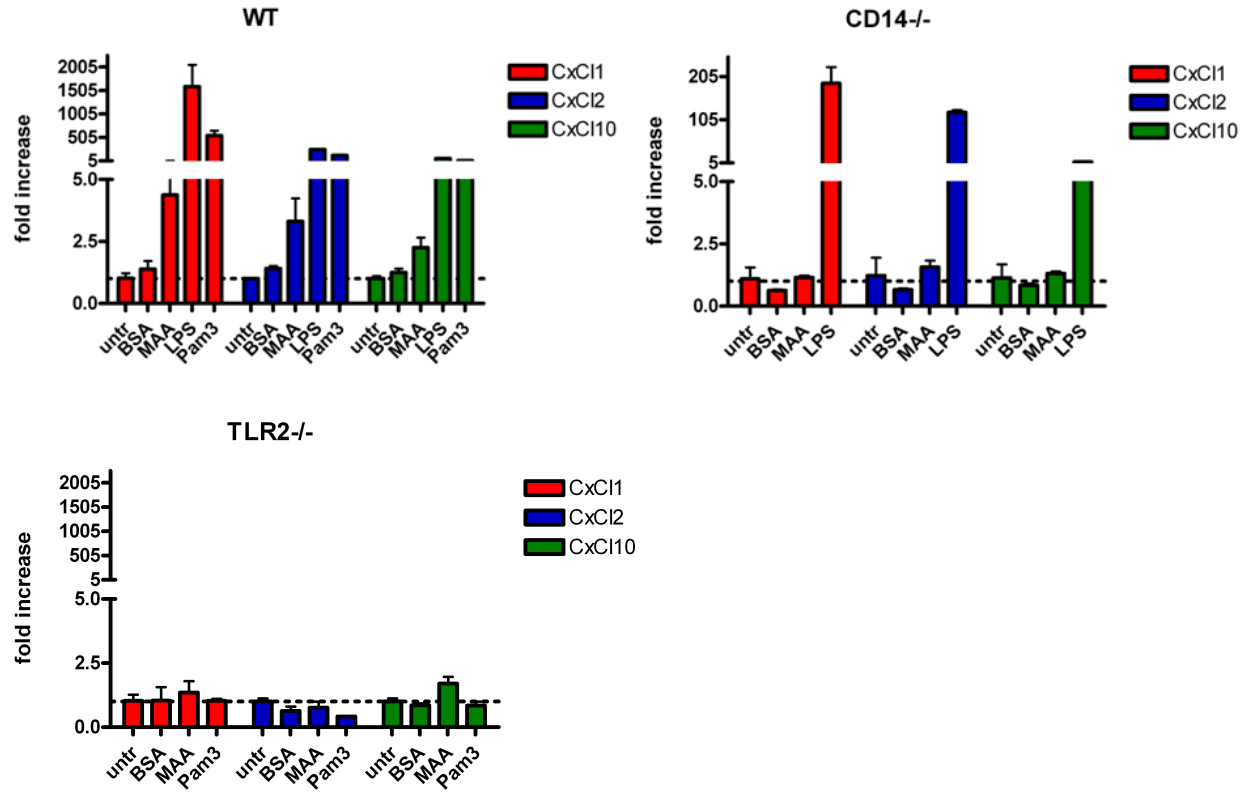



**Fig. 16** Screen for different pattern recognition receptors. BMDMs from WT, TLR4$^{-/-}$ and CD36$^{-/-}$ mice were stimulated with MAA-BSA and checked by ELISA for KC secretion (A) or by qPCR for upregulation of CxCl1, CxCl2 and CxCL10 (B). BMDMs from WT, TLR2$^{-/-}$ and CD14$^{-/-}$ mice were stimulated with MAA-BSA and checked for KC secretion (C) or upregulation of CxCl1, CxCl2 and CxCl10 (D). Bars represent mean +/- SD of biological triplicates. Data are representative of 2 independent experiments.

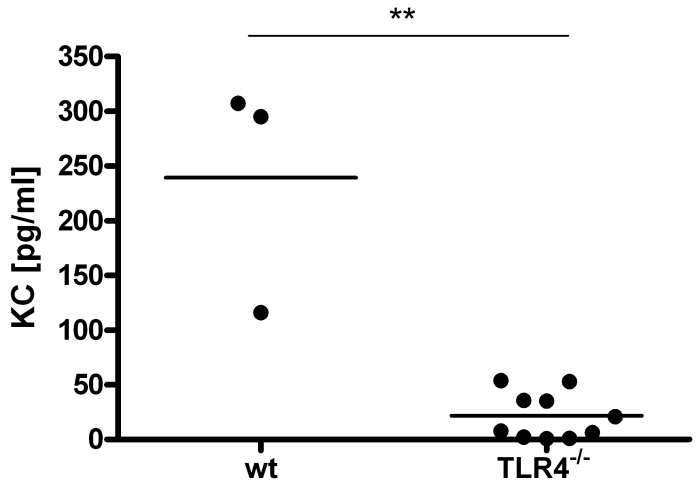
of CxCl1, CxCl2 and CxCl10. According to the previous ELISA results, none of these genes were upregulated in TLR4$^{-/-}$. Therefore, TLR4 seems to play a crucial role in MAA signalling.

The TLR4 complex consists, as previously described, of TLR4 itself as a dimer, MD2, and the co-receptor CD14. To test, whether CD14 is necessary for MDA-induced chemokine-secretion, BMDMs from CD14$^{-/-}$ mice were isolated and stimulated. In a first experiment, levels of secreted KC following MAA-stimulation were assessed by ELISA (Fig.16C). In contrast to wt cells, CD14$^{-/-}$ BMDMs did not induce KC secretion upon MAA-BSA stimulation. In line with this, expression of CxCl1, CxCl2 and CxCl10 was not induced in CD14$^{-/-}$ BMDM 2h after stimulation with MAA-BSA. As control, cells were stimulated with LPS, which induces a residual, but significantly reduced response in CD14$^{-/-}$ macrophages. Combining the ELISA and qPCR results, CD14 seems to be necessary for MAA-signalling. Therefore the results indicate that several components of the TLR4 complex are needed for the response to MAA-BSA.

We confirmed the involvement of TLR4 in the model of MAA-induced sterile peritonitis that we introduced in chapter 3.3.1, using TLR4$^{-/-}$ mice. After injection of MAA-BSA into TLR4$^{-/-}$ mice (n=10), levels of KC in the peritoneal lavage fluid remained low as measured by ELISA (Fig. 17A). On the other hand, injection of MAA-BSA into WT mice (n=3) caused significant KC secretion. These results indicate a crucial role of TLR4 in MAA-induced sterile inflammation *in vivo*.

According to our data, blocking of TLR4 should diminish MAA signalling. Therefore, I stimulated THP1 cells with MAA-BSA and LPS and added Eritoran to some of the samples (Fig. 17B). The chemical structure of Eritoran is similar to LPS and thus it acts as a TLR4 antagonist

**A**




**B**

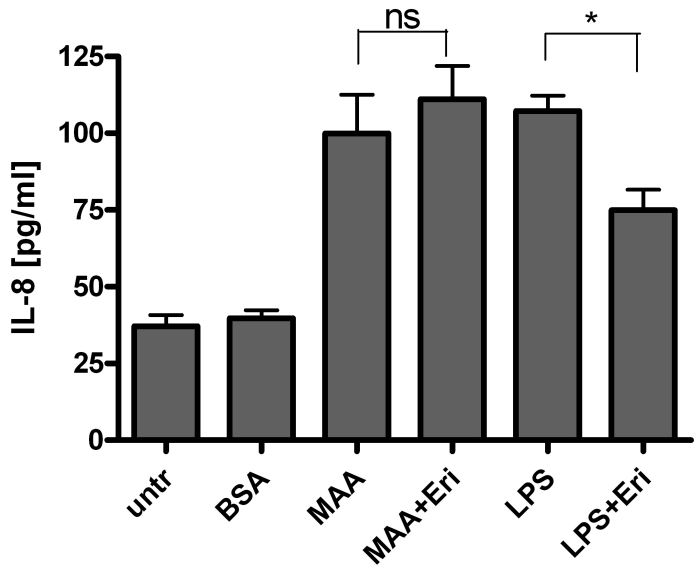



**Fig. 17** The role of TLR4$^{-/-}$ in MAA signalling. (A) Amount of KC in the peritoneal lavage after 2h of intraperitoneal injections of MAA-BSA into WT (n=3) or TLR4$^{-/-}$ (n=10) mice. (B) Eritoran interferes

with LPS but not with MAA-BSA. These data are representative of 2 experiments. *p<0,05, **p<0,01, ns = not significant

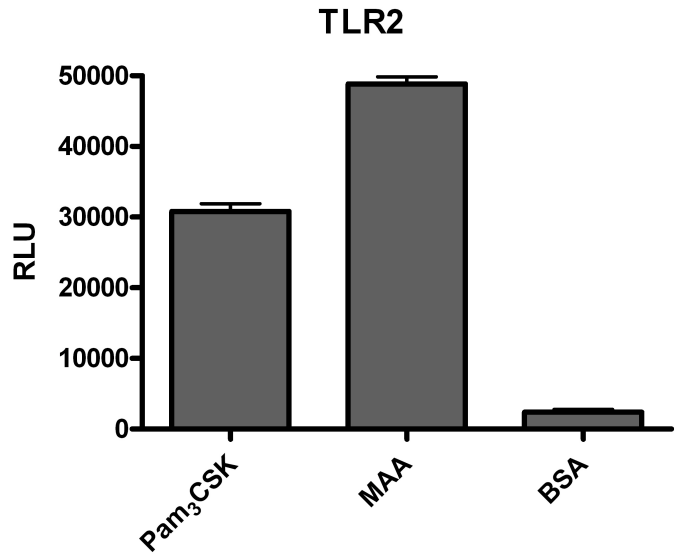
and blocks TLR4 induced immune responses [39]. Consistent with the literature, Eritoran competed with LPS also in our setting, thereby decreasing LPS induced IL-8 secretion. Importantly, MAA-BSA stimulation was not affected by Eritoran pretreatment. This data suggest that MAA-BSA does not bind in the same way as LPS. Therefore, it either binds at a different position or other receptors are involved.

Because v-(2-carboxyethyl) pyrrole (CEP), another oxidation specific epitope, was shown to signal through TLR2 [40], I stimulated $TLR2^{-/-}$ BMDMs with MAA-BSA (Fig. 16C). Similar to $CD14^{-/-}$ and $TLR4^{-/-}$ mice, BMDMs from $TLR2^{-/-}$ showed no response to the MAA-BSA stimuli, suggesting also an important role for this receptor in MAA signalling. Again, we confirmed this data by qPCR (Fig. 16D). As expected, $TLR2^{-/-}$ did also not show any response to Pam3CSK, a common TLR2 ligand.

Importantly, the expression analysis confirmed the results described in Fig. 16C: MAA-BSA failed to induce the expression of chemokines in TLR2-deficient macrophages. None of the tested inflammatory genes were upregulated in the $TLR2^{-/-}$ mice. ELISA and qPCR results together suggest a crucial role for TLR2 in MAA specific signalling.

After elucidating various receptors which are involved in MAA signalling, I wanted to explore the possibility whether MAA-BSA physically interacts with them. Therefore, I used an ELISA

**A**



**B**

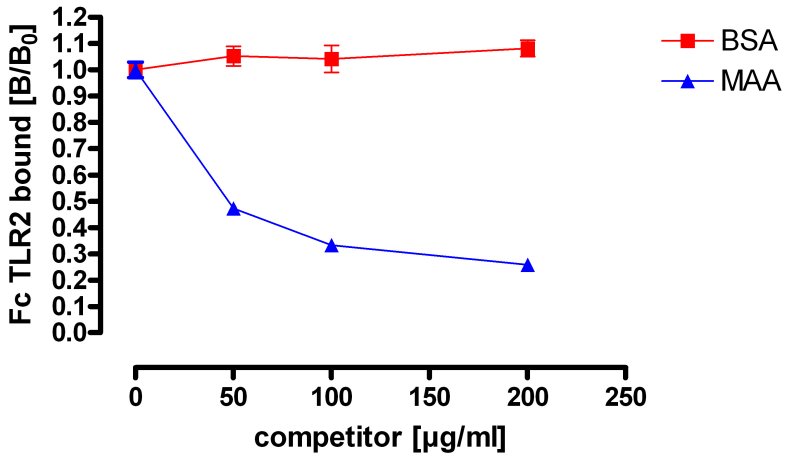



**Fig. 18** MAA-BSA binds to TLR2. (A) TLR2 Fc chimera binds to $Pam_3CSK$ and MAA-BSA. Data are representative of 3 independent experiments. (B) MAA-BSA competes with the TLR2 Fc chimera.

based binding assay based on Fc-receptor chimeras of TLR2, as described in the materials and methods section. Fc chimeras are constructed by genetic fusion between the extracellular domain of the receptors and the Fc portions from IgG antibodies and can be easily detected by labelled Fc specific 2$^{nd}$ ABs. Fc-TLR 2 bound to coated MAA-BSA as well as to coated $Pam_3CSK$, which was used as a positive control (Fig. 18A). Furthermore specific binding of MAA-BSA to TLR2 was confirmed in a competition ELISA experiment (Fig. 18B). MAA-BSA, but not BSA, competes with the TLR2 Fc chimera for binding to coated MAA-BSA.

# 4. Discussion

MDA is a highly reactive aldehyde, which immediately reacts with aminogroups on proteins and lipids, generating a variety of different adducts, including MAA and FHP. For our experiments, I modified BSA with MDA. By adjusting the pH and incubation time, and by adding acetaldehyde, I was able to enrich for MAA-type adducts. Here I show that the generated MAA-BSA modification causes increased IL-8 secretion, when THP1 cells are stimulated with it, identifying MAA-adducts as biologically active MDA-modifications. In contrast, other types of aldehyde-modifications, such as simple MDA-BSA, caused only very few IL-8 secretion. FHP induced intermediate IL-8 secretion, probably due to residual generation of the MAA-adduct. These data suggest that MDHDC modifications, which are enriched in MAA-BSA, are responsible for the observed inflammatory response. This inflammatory potential of MAA supports the notion that also other oxidation epitopes than CEP and minimally modified low-density lipoprotein (mmLDL), induce inflammation [40, 41] Furthermore, I demonstrate that the response is not to due to any endotoxin contamination of our MAA-BSA modifications. All preparations used in this manuscript were tested for endotoxin by the LAL-assay. Moreover, the LPS-binding molecule polymyxin B failed to inhibit MAA-BSA induced chemokine-secretion. In addition, THP1 cells, which are expressing very low amounts of the LPS-co-receptor CD14 [27] and therefore would not respond to low doses of endotoxin, are very sensitive to MAA-BSA treatment.

The hypothesis, that MAA-BSA acts as a danger signal, is further supported as both human as well as mouse cell lines (THP1 and RAW), and also primary mouse cells (peritoneal macrophages and BMDMs) respond to MAA-BSA treatment by the secretion of a number of proinflammatory chemokines, including IL-8/KC and MIP-2. Additionally, I show that protective, antioxidant genes like HO-1 are also induced. Furthermore, different macrophage subtypes (M1 and M2) respond similarly to this stimulus. Taken together, I have shown that MAA-BSA induces a complex inflammatory response. As MAA is an oxidation specific epitope and is generated endogenously, it is suggested that MAA is a major inducer of sterile inflammation. The fact that natural antibodies (like LRO4), which are found in plasma without any previous infections, and complement factor H recognize and bind MAA, further strengthens our hypothesis that MAA is a major inducer of sterile inflammation.

I further demonstrate that UVB treated monocytes and macrophages that undergo apoptosis and carry MDA/MAA epitopes. This is in line with previously reported findings [42]. When THP1 cells were stimulated with apoptotic THP1 cells, no inflammatory response was detectable. Possible explanations could be, that the detected epitopes on apoptotic THP1 cells generated by

UVB-irradiation are mainly simple MDA adducts or the density of MAA epitopes is too low to elicit a response. Additionally, I show that apoptotic RAW cells carry MDA/MAA epitopes and cause an inflammatory response. Given that MAA epitopes are present on apoptotic cells, MAA qualifies as endogenous DAMP (damage associated molecular pattern).

UVB irradiated, apoptotic RAW cells were found to mimic the MAA-BSA effect, inducing a robust inflammatory response, probably due to an enrichment of MAA epitopes on their surfaces. However, a neutralization experiment with LRO4 (or other MDA-specific Abs) still needs to be done to prove, that the effect of apoptotic RAW cells is mediated by MDA epitopes.

I further found that MDA elicits a sterile peritonitis *in vivo*. Following MAA-BSA injection into the peritoneal cavity of mice, I observed an influx of granulocytes and high KC secretion in the lavage fluid. These hallmarks are consistent with the already well established model of LPS induced sterile peritonitis [43]. It is interesting that the MAA-BSA induced inflammation was cleared rapidly in 5 hours, as no KC is anymore detectable and the amount of granulocytes were significantly decreased in contrast to the 2 hours time point. Therefore it can be suggested that accumulation of MAA causes a fast inflammatory response, which is cleared quickly and effectively to prevent further damage. Furthermore, I provide evidence, that apoptotic RAW cells mimic the effects of MAA-BSA also *in vivo*. Like MAA-BSA, injected apoptotic cells cause the influx of a considerable amount of granulocytes.

Genomic analysis of the gene expression changes caused by MAA stimulation suggested an involvement of MAPK pathways. Indeed, western blot analysis indicated higher ERK1/2 phosphorylation after MAA-BSA treatment. We further show that ERK activity is necessary for MAA-induced chemokine-secretion, as ERK inhibition by UO126 completely abolished IL-8 secretion. Based on this data, it is believed that intracellular signalling through activation of ERK1/2 and further activation of the JNK pathway, which activates transcription factors.

Interestingly, our proteomic approach to identify cellular MAA-binding proteins in RAW lysate revealed many potential MAA specific binding partners. However, as all of them were intracellular proteins, this assay did not help in delineating involved extracellular receptors. Together with the already shown interaction of SR-A and MAA [35], it is an indication for a possible role of phagocytosis in MAA signalling and has to be investigated in future experiments. Independent of phagocytosis, SR-A could still be necessary for MAA signalling. To elucidate this possibility, the SR-A antagonist Fucoidan was used. The stimulation together with Fucoidan revealed, that this antagonist interferes with the MAA stimuli but not with LPS pointing towards a possible role of SR-A in MAA-induced chemokine secretion. Because SR-A has no known intracellular signalling cascade [44], other receptors are likely to be involved.

TLR4 has been shown to mediate the inflammatory response to other oxidation epitopes [37]. Furthermore, SR-A ligands have been reported to act synergistically with TLR4-ligands in inducing macrophage cell death [13, 37]. Using TLR4 deficient macrophages, I was able to show a crucial role for TLR4 in MAA-induced inflammation both *in vitro* as well as *in vivo*. In BMDMs the co-receptor CD14 is also necessary for MAA-signalling, as all the knockout mice did not respond anymore to the stimuli. In contrast, THP1 cells, which lack CD14 [27], respond normally to MAA-BSA, suggesting a need for CD14 only in primary cells. According to the data obtained, blocking of TLR4 should deplete IL-8 secretion. By using the TLR4 antagonist Eritoran, I was able to show that unlike with LPS, MAA-induced IL-8 secretion was not diminished.
A recent publication [40] reported that CEP, another oxidation epitope, is TLR2 dependent. TLR2 deficiency caused an ablation of the macrophage response to MAA-BSA.
Using an in vitro binding assay based on Fc-receptor-chimeras, I was able to show that MAA directly interacts with TLR2 and specific binding was proved in a competition experiment. However, further binding studies with TLR4 have to be conducted.

A working model for MAA-induced signalling based on the data presented here would be following: MAA-BSA binds directly to TLR2, which subsequently forms a dimer with TLR4. Moreover the whole dimer complex is stabilized by co-receptor CD14. Following, dimerization, the complex signals through phosphorylation of ERK1/2 and subsequent activation of transcription factors. However, the possible role of SR-A is yet not fully understood and will require further investigation.
One approach would be to express all receptors in all possible combinations in HEK293T cells and check the transcriptional response to MAA in a luciferase reporter assay. A possible promoter to identify transcriptional response would be AP1, as the microarray data suggests an upregulation of the JNK pathway. Furthermore, dimerization of TLR2 and TLR4 should be proved by fluorescence microscopy and the binding to TLR2 has to be confirmed by colocalization with fluorescent MAA-BSA. However, achieving a pure MAA-solution is impossible. Therefore, FHP will always be a part of the MAA-BSA modification and *vice versa.* Fur further studies, it will be advisable to use one defined adduct, as with the protocols applied in this thesis, only mixtures can be generated.

# 5. References


1. Murphy, K., *Janeway's Immunobiology* 7th ed. 2008.
2. Malaviya, R., et al., *Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha.* Nature, 1996. **381**(6577): p. 77-80.
3. Borregaard, N., *Neutrophils, from marrow to microbes.* Immunity. **33**(5): p. 657-70.
4. Khazen, W., et al., *Expression of macrophage-selective markers in human and rodent adipocytes.* FEBS Lett, 2005. **579**(25): p. 5631-4.
5. Gordon, S., *Alternative activation of macrophages.* Nat Rev Immunol, 2003. **3**(1): p. 23-35.
6. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.
7. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell. **140**(6): p. 805-20.
8. Takeuchi, O. and S. Akira, *Innate immunity to virus infection.* Immunol Rev, 2009. **227**(1): p. 75-86.
9. Inohara, et al., *NOD-LRR proteins: role in host-microbial interactions and inflammatory disease.* Annu Rev Biochem, 2005. **74**: p. 355-83.
10. Robinson, M.J., et al., *Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection.* J Exp Med, 2009. **206**(9): p. 2037-51.
11. Geijtenbeek, T.B., J. den Dunnen, and S.I. Gringhuis, *Pathogen recognition by DC-SIGN shapes adaptive immunity.* Future Microbiol, 2009. **4**(7): p. 879-90.
12. Greaves, D.R. and S. Gordon, *Thematic review series: the immune system and atherogenesis. Recent insights into the biology of macrophage scavenger receptors.* J Lipid Res, 2005. **46**(1): p. 11-20.
13. Seimon, T.A., et al., *Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages.* Proc Natl Acad Sci U S A, 2006. **103**(52): p. 19794-9.
14. Lemaitre, B., et al., *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults.* Cell, 1996. **86**(6): p. 973-83.
15. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity.* Nature, 1997. **388**(6640): p. 394-7.

16. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.
17. Lotz, S., et al., *Highly purified lipoteichoic acid activates neutrophil granulocytes and delays their spontaneous apoptosis via CD14 and TLR2.* J Leukoc Biol, 2004. **75**(3): p. 467-77.
18. Schwandner, R., et al., *Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2.* J Biol Chem, 1999. **274**(25): p. 17406-9.
19. Ozinsky, A., et al., *The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors.* Proc Natl Acad Sci U S A, 2000. **97**(25): p. 13766-71.
20. Shaw, P.X., et al., *Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity.* J Clin Invest, 2000. **105**(12): p. 1731-40.
21. Huber, J., et al., *Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions.* Arterioscler Thromb Vasc Biol, 2002. **22**(1): p. 101-7.
22. Imai, Y., et al., *Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury.* Cell, 2008. **133**(2): p. 235-49.
23. Matzinger, P., *The danger model: a renewed sense of self.* Science, 2002. **296**(5566): p. 301-5.
24. Esterbauer, H., *Chemistry and Biochemistry of 4-hydroxynonenal, Malondialdehyde and Related Aldehydes.* Free Radical Biology & Medicine, 1991.
25. Tuma, D.J., et al., *Elucidation of reaction scheme describing malondialdehyde-acetaldehyde-protein adduct formation.* Chem Res Toxicol, 2001. **14**(7): p. 822-32.
26. Palinski, W., et al., *Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein.* Arteriosclerosis, 1990. **10**(3): p. 325-35.
27. Wang, J., *Induction of bacterial lipoprotein tolerance is associated with suppression of toll-like receptor 2 expression.* J Biol Chem., 2002.
28. Cardoso, L.S., et al., *Polymyxin B as inhibitor of LPS contamination of Schistosoma mansoni recombinant proteins in human cytokine analysis.* Microb Cell Fact, 2007. **6**: p. 1.
29. Nath, K.A., et al., *Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat.* J Clin Invest, 1992. **90**(1): p. 267-70.

30. Chou, M.Y., et al., *Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans.* J Clin Invest, 2009. **119**(5): p. 1335-49.
31. Gokal, R., *Icodextrin-associated sterile peritonitis.* Perit Dial Int, 2002. **22**(4): p. 445-8.
32. Steelman, L.S., et al., *Dominant roles of the Raf/MEK/ERK pathway in cell cycle progression, prevention of apoptosis and sensitivity to chemotherapeutic drugs.* Cell Cycle. **9**(8): p. 1629-38.
33. Xia, Z., et al., *Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis.* Science, 1995. **270**(5240): p. 1326-31.
34. Haura, E.B., et al., *Using iTRAQ combined with tandem affinity purification to enhance low-abundance proteins associated with somatically mutated EGFR core complexes in lung cancer.* J Proteome Res. **10**(1): p. 182-90.
35. Shechter, I., et al., *The metabolism of native and malondialdehyde-altered low density lipoproteins by human monocyte-macrophages.* J Lipid Res, 1981. **22**(1): p. 63-71.
36. Wu, C.C. and J.R. Yates, 3rd, *The application of mass spectrometry to membrane proteomics.* Nat Biotechnol, 2003. **21**(3): p. 262-7.
37. Stewart, C.R., et al., *CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer.* Nat Immunol. **11**(2): p. 155-61.
38. Boullier, A., et al., *Phosphocholine as a pattern recognition ligand for CD36.* J Lipid Res, 2005. **46**(5): p. 969-76.
39. Baumgarten, G., et al., *Toll-like receptor 4, nitric oxide, and myocardial depression in endotoxemia.* Shock, 2006. **25**(1): p. 43-9.
40. West, X.Z., et al., *Oxidative stress induces angiogenesis by activating TLR2 with novel endogenous ligands.* Nature. **467**(7318): p. 972-6.
41. Bae, Y.S., et al., *Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2.* Circ Res, 2009. **104**(2): p. 210-8, 21p following 218.
42. Chang, M.K., et al., *Apoptotic cells with oxidation-specific epitopes are immunogenic and proinflammatory.* J Exp Med, 2004. **200**(11): p. 1359-70.
43. Ajuebor, M.N., et al., *Role of resident peritoneal macrophages and mast cells in chemokine production and neutrophil migration in acute inflammation: evidence for an inhibitory loop involving endogenous IL-10.* J Immunol, 1999. **162**(3): p. 1685-91.

44. Nikolic, D.M., et al., *Class A scavenger receptor-mediated cell adhesion requires the sequential activation of Lyn and PI3-kinase.* Am J Physiol Cell Physiol, 2007. **292**(4): p. C1450-8.

**Figures**

Fig. 1 taken from Murphy et al., Janeway's Immunobiology 2008

Fig. 2 modified from Binder CJ et al., JLR 2005

Fig. 3 taken from Palinski et al., Arteriosclerosis 1996, Imai et al., Cell 2008, Dei et al., Acta Neuropath 2002, Binder et al., unpublished, Chang et al., Proc Natl Acad Sci U S A 1999

# 6. Appendix

## 6.1 Abstract (English)

Malondialdehyde (MDA) *per se* is a highly reactive aldehyde generated during lipid breakdown that forms adducts on amino groups. One of the main generated adducts is the Malondialdehyde-acetaldehyde-adduct, further named MAA. MAA mainly consists of the ring structure 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (MDHDC). These epitopes are found on the surface of apoptotic and necrotic cells and on the surface of apoptotic blebs (microparticles) and accumulate in many diseases, such as atherosclerosis.

We show that MAA causes a fast and complex inflammatory response in vitro and in vivo. Macrophages and monocytes immediately respond to this epitope by secreting various pro-inflammatory chemokines. The recognition of MAA by macrophages involves the pattern-recognition-receptors (PRRs) TLR4 and TLR2. Additionally, CD14 is crucial for eliciting a response to MAA. Conducted binding assays prove a direct binding of MAA to TLR2 as well as to TREM1. Moreover, competition assays suggest a role for scavenger receptor A (SR-A) in MAA-signalling.

We show that stimulation with MAA-BSA leads to an upregulation of MAPK pathways on the genomic level. Specifically, ERK1/2 is phosphorylated in response to MAA-BSA treatment. Using an ERK-specific inhibitor, we show that the response to MAA-BSA is ERK-dependent.

Taken together we present evidence that MAA represents a novel damage-associated-molecular-pattern (DAMP) which is abundant in inflammatory conditions and diseases associated with it. A better understanding of MAA signalling could identify novel therapeutical targets and thereby lead to a better treatment of these diseases.

## 6.2 Abstract (German)

Malondialdehyd (MDA) ist ein hochreaktives Aldehyd, das während der Peroxidation von Lipiden (Fettsäuren) produziert wird. Es reagiert sofort mit Acetaldehyd und freien Aminogruppen und bildet dabei eine Reihe verschiedener chemischer Strukturen (Epitope). Ein sehr häufig gebildetes Epitop ist das Malondialdehyd-Acetaldehyd-Addukt (MAA). Dieses bezeichnet vorrangig eine Ring-Struktur namens 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde (MDHDC). MAA Epitope findet man an der Oberfläche von apoptotischen und nekrotischen Zellen, sowie an der Oberfläche von Mikropartikeln.

In dieser Master Thesis zeigen wir, dass eine Stimulation mit MAA schon nach kurzer Zeit Makrophagen und Monozyten zur Sekretion von inflammatorischen Chemokinen veranlasst. Unsere Resultate weisen darauf hin, dass Macrophagen MAA mittels TLR2 und TLR4 erkennen. CD14 scheint dabei ebenso eine wichtige Funktion zu erfüllen. Weiters zeigen wir mittels *in vitro* Bindungsstudien, dass MAA an TLR2 bindet. Überraschenderweise fanden wir auch heraus, dass MAA an TREM1 bindet. Bisher ist kein TREM1 Ligand bekannt, daher ist MAA der erste nachgewiesene, direkte Interaktionspartner.

Wir zeigen, dass nach Bindung an diese Pattern-Recognition-Rezeptoren (PRRs) MAPK-pathways hochreguliert werden. Eine Stimulation mit MAA führte zu einer erhöhten Phosphorylierung von ERK1/2. Inhibition von ERK mit einem künstlichen Inhibitor führte zu einer reduzierten Sekretion von Chemokinen nach MAA-BSA Stimulation.

Zusammenfassend, beschreiben wir ein neues damage-associated-molecular-pattern (DAMP), welches in vielen Krankheitsbildern vorkommt. Ein besseres Verständnis, von den von MAA ausgelösten Immunreaktionen, könnte in Zukunft zu einer besseren Behandlung von Krankheiten führen, in denen MAA eine wichtige Rolle spielt.

## 6.2 Curriculum Vitae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Postiglione MP, Juschke C, Haas GA, Caralambous C, and Knoblich JA
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