MEDICAL UNIVERSITY OF VIENNA

Role of Malondialdehyde Epitopes in Sterile Inflammation

Doctoral thesis at the Medical University of Vienna

for obtaining the academic degree

Doctor of Philosophy

Submitted by
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Vienna, April 2017

Declaration

I hereby declare that I, Clara Jana Lui Busch, have carried out the PhD project entitled "Role of Malondialdehyde Epitopes in Sterile Inflammation" under the supervision of Prof. Dr. Christoph Binder, PhD, at the Medical University of Vienna, Department of Laboratory Medicine, and at the Research Center for Molecular Medicine (CeMM) of the Austrian Academy of Sciences. I designed and performed most experiments, analyzed the majority of data presented, interpreted all results and wrote a manuscript for publication of this work. Part of the experimental work was conducted at the University of Maastricht and at the Universitätsmedizin Mainz. The results of my work are described and discussed in this thesis, written and compiled in full by me using no other than the indicated sources.

The PhD project was funded by the Austrian Science Fund FWF (SFB F30, Lipotox), Boehringer Ingelheim Fonds (PhD fellowship), and supported by the doctoral programme of CeMM. The contributions of the co-authors to the manuscript are as follows: the co-first author Tim Hendrikx performed crucial experiments, analyzed and interpreted part of the data and contributed to the manuscript. He was a postdoc at that time and will not use the article for graduation. David Weismann, Sven Jäckle, Sofie Walenbergh, André Rendeiro, Juliane Weißer, Florian Puhm, Anastasiya Hladik, Laura Göderle, Nikolina Papac-Milicevic, Gerald Haas, Vincent Millischer and Saravanan Subramanian performed experiments or contributed to data analysis and interpretation. Sylvia Knapp, Keiryn Bennett and Christoph Bock contributed to experimental design or analysis and edited the manuscript. Christoph Reinhardt and Ronit Shiri-Sverdlov provided technical and material support and edited the manuscript. Christoph Binder conceived the project, supervised the work, provided funding and wrote the manuscript.

This PhD thesis is to be submitted to the Medical University of Vienna for obtaining the degree "Doctor of Philosophy" and has neither been published nor submitted to any other university before.

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English Abstract

Increased oxidative stress during inflammatory responses leads to the generation of damaged lipids and proteins with oxidation-specific epitopes such as malondialdehyde (MDA) epitopes. These lipid peroxidation-derived moieties have been associated with many inflammatory diseases including Alzheimer's disease, acute lung injury, multiple sclerosis and age-related macular degeneration. Importantly, malondialdehyde-epitope production is also observed in diet-induced inflammatory diseases such as nonalcoholic fatty liver disease and cardiovascular disorders. There have been many hypotheses trying to link the diet-induced changes such as hyperlipidemia with the subsequent inflammatory events but a clear insight into the underlying mechanism has remained elusive. Recently, natural IgM antibodies as well as complement factor H have been identified as major malondialdehyde-binding proteins in plasma. Additionally, malondialdehyde epitopes were demonstrated to be pro-inflammatory in vitro but the functional responses they induce in cells of innate immunity are not well understood. In my thesis project, I wanted to characterize the malondialdehyde-induced pro-inflammatory responses in more detail, identify signalling pathways and associated receptors on immune cells that sense the presence of malondialdehyde epitopes, and elucidate the functional role of malondialdehyde epitopes as danger-associated molecular pattern with the capacity to elicit sterile inflammation. Together, I could identify innate immunity and oxidative stress processes as major response pathways in livers of $Ldlr^{-/-}$ mice that were fed a Western-type diet using RNA sequencing and in silico functional analyses of transcriptome data. Furthermore, I showed that malondialdehyde epitopes are detectable in hepatic inflammation predominantly on dying cells and stimulate cytokine secretion as well as leukocyte recruitment in vitro and in vivo. Malondialdehyde-induced cytokine secretion in vitro was dependent on the presence of the scavenger receptors CD36 and MSR1. Moreover, in vivo neutralization of endogenously generated malondialdehyde epitopes by intravenous injection of a specific malondialdehyde antibody resulted in decreased hepatic inflammation in $Ldlr^{-/-}$ mice on a Western-type diet. In conclusion, I have provided evidence for a better understanding of the link between a lipid-rich diet and liver inflammation and suggested a putative novel point for therapeutic intervention.

Deutschsprachige Zusammenfassung

Im Kontext einer entzündlichen Immunantwort tritt auch erhöhter oxidativer Stress auf, der zur Fragmentierung von Lipiden sowie zur Bildung von sogenannten oxidationsspezifischen Epitopen wie Malondialdehyd-(MDA)-Epitopen führen kann. Diese Epitope wurden nicht nur in zahlreichen entzündlichen Erkrankungen wie bei der Alzheimer-Krankheit, akuter Lungenschädigung, Multipler Sklerose und altersabhängiger Makuladegeneration festgestellt, sondern auch in ernährungsbedingten Krankheiten wie nicht-alkoholischen Fettleber- und Herz-Kreislauf-Erkrankungen. Obwohl mehrere Hypothesen im Hinblick auf den Zusammenhang zwischen ernährungsbedingten Veränderungen wie beispielsweise Hyperlipidämie und den darauffolgenden entzündlichen Reaktionen existieren, herrscht bis heute keine Klarheit über die zugrunde liegenden Mechanismen. Während in vitro Untersuchungen bereits zeigen konnten, dass MDA-Epitope eine pro-inflammatorische Wirkung auf Zellen des angeborenen Immunsystems haben und dass wichtige Plasmaproteine wie natürliche Antikörper vom Isotyp IgM oder Komplementfaktor H eine bedeutende Rolle bei der Neutralisierung von MDA-Epitopen spielen, sind die funktionalen Konsequenzen einer solchen Interaktion weiterhin unbekannt. In meinem Dissertationsprojekt möchte ich MDA-vermittelte Entzündungsantworten im Detail charakterisieren, deren Signalwege und Rezeptoren auf Immunzellen identifizieren, und die Rolle von MDA-Epitopen als Gefahr-assoziiertes molekulares Muster, besser bekannt unter dem Begriff “damage-associated molecular pattern”, im Rahmen von Entzündungen untersuchen. Zusammenfassend konnte ich anhand von RNA-Sequenzierungsanalysen zahlreiche Prozesse identifizieren, die sowohl charakteristisch für Signalwege des angeborenen Immunsystems als auch für oxidativen Stress sind, und die im Lebergewebe von *Ldlr*$^{-/-}$-Mäusen bei cholesterinhaltiger Diät verstärkt aktiviert werden. Des Weiteren konnte ich zeigen, dass MDA-Epitope in entzündetem Lebergewebe insbesondere auf apoptotischen Zellen auftreten und sowohl Zytokinsekretion als auch Infiltration von Leukozyten stimulieren. Für eine MDA-induzierte Zytokinsekretion in vitro waren die Scavenger-Rezeptoren CD36 und MSR-1 erforderlich. Außerdem konnten endogen gebildete MDA-Epitope in vivo durch intravenöse Gabe eines spezifischen MDA-Antikörpers neutralisiert werden, was eine verringerte Leberentzündung in *Ldlr*$^{-/-}$-Mäusen bei cholesterinhaltiger Diät zur Folge hatte. Abschließend habe ich experimentelle Daten für ein besseres Verständnis des Zusammenhangs zwischen einer cholesterinhaltigen Diät und der darauffolgenden Entzündung in der Leber geliefert, was einen potentiellen, neuen Anknüpfungspunkt für Therapien darstellt könnte.

Publications Arising From This Thesis

Busch CJ*, Hendrikx T*, Weismann D, Jäckel S, Walenbergh SM, Rendeiro AF, Weißer J, Puhm F, Hladik A, Göderle L, Papac-Milicevic N, Haas G, Millischer V, Subramaniam S, Knapp S, Bennett KL, Bock C, Reinhardt C, Shiri-Sverdlov R, Binder CJ. Malondialdehyde epitopes are sterile mediators of hepatic inflammation in hypercholesterolemic mice. Hepatology. 2016 Dec 16. doi: 10.1002/hep.28970. [Epub ahead of print] PubMed PMID: 27981604. *equal contr.

Abbreviations

4-HNE	4-Hydroxynonenal
7-AAD	7-Aminoactinomycin D
ALE	Advanced lipoxidation end-product
AP	Alkaline phosphatase
AP1	Activator protein 1
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
Atgl	Adipose triglyceride lipase
BMDM	Bone-marrow-derived macrophage
bMGL	Bacterial monoacylglycerol lipase
C4BP	C4b-binding protein
CEP	2-(ω-Carboxyethyl)pyrrole
CFH	Complement factor H
CLR	C-type lectin receptor
CONV-R	Conventionally-raised
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
GF	Germ-free
FH	Familial hypercholesterolemia
HFD	High-fat diet
HMG-CoA	Hydroxymethylglutaryl-CoA synthase
HMGB1	High mobility group box 1
HRP	Horse-radish peroxidase
IgM	Immunoglobulin M
IL-8	Interleukin-8
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
KC	Kupffer cell
LDL	Low-density lipoprotein
Ldlr	LDL receptor
LPS	Lipopolysaccharide

MAL	Myelin and lymphocyte protein
MAPK	Mitogen-activated protein kinase
MCD	Methionine-choline-deficient
MDA	Malondialdehyde
MDHDC	4-Methyl-1,4-dihydropyridine-3,5-dicarbaldehyde
mmLDL	Minimally-modified LDL
Mpo	Myeloperoxidase
MSR1	Macrophage scavenger receptor 1
mtDNA	Mitochondrial DNA
MyD88	Myeloid differentiation primary response gene 88
NAFLD	Non-alcoholic fatty-liver disease
NASH	Non-alcoholic steatohepatitis
Ncf1	Neutrophil cytosolic factor 1
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
OxLDL	Oxidized LDL
PRR	Pathogen-recognition receptor
qPCR	Quantitative polymerase-chain reaction
RLU	Relative light unit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SR	Scavenger receptor
SR-A1	Scavenger receptor A1
TBS	Tris-buffered saline
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TRAF	TNF-receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
UV	Ultraviolet
WD	Western-type diet

Acknowledgements

First, I would like to thank my doctoral supervisor Prof. Christoph J. Binder for having offered me a position in his research group, for his continuous support in developing this project, and for the opportunity to present its results at numerous conferences. His suggestion to become a BIF Fellow and his help with my application have allowed me to extend my scientific network early on by a group of young scientists of remarkable diversity and interests, and I am grateful for this experience. He was also very supportive of my post-doctoral aims, especially when the transition to the next stage was complicated by the then still unpublished manuscript and I would like to express my gratitude for his understanding of my situation.

Next, I would like to thank my PhD committee members Prof. Giulio Superti-Furga and Prof. Rudolf Zechner for their critical comments and insights during the yearly meetings about the progress of my project.

Thanks also go to David and Vincent, from whom I inherited the "MDA story", for introducing me to this topic and its techniques (though not the TBARS assay!), to Tim for his fantastic support in a critical phase of this project, and all the members of the Binder lab for an exceptionally friendly and helpful working atmosphere; especially Maria for being the gentle heart of the lab; Nina, Barbara and Máté for our memorable, fun times in the "MDA bay"; and Dimitris for sharing his diverse and intriguing thoughts on aspects of inflammation, immunology and life.

I am very grateful to have been part of CeMM and the PhD program, as the institute's collaborative and youthful character teaches aspiring scientists a lot about what is important in science, and also, what is not. My time at CeMM would have been less joyful without my fellow "group students 2011", of whom I have to thank most Nina for becoming a dear companion during my time at CeMM, being always there for strategic decisions, counsel or simply a cup of coffee.

Last, thanks to Hermann and LaTeX for helping me make this thesis a plain text.

1 Introduction

This chapter serves the purpose of delineating the conceptual framework of the thesis, with the aim of putting the main findings of my PhD project into perspective. Although I will show in this thesis that a specific molecule derived from the chemical compound malondialdehyde plays a critical role in one inflammatory condition, namely steatohepatitis, I speculate that the conclusions of the herein described findings can be extended to other inflammatory diseases where malondialdehyde or similar structures are also formed and have been detected in earlier studies by us and others. More specifically, I propose that our data support the existence of a common mechanism linking environmentally induced oxidative stress with subsequent immune responses that promote tissue damage and inflammation. Of course, the validity of any such extrapolations remains to be shown.

1.1 The Importance of Being Homeostatic

Resilience is the capacity of live and inanimate systems to withstand stress signals, be they of endogenous or exogenous origin. The key feature of resilient systems is their ability to allow only temporary prevalence of a disturbed condition before safe return to a balanced state. Living systems are obviously strongly affected by perturbances of various kinds due to their integration in and interaction with the surrounding world. Thus, it comes as no surprise that the survival of an organism is critically dependent on its capability to deal with stressors. Indeed, homeostatic mechanisms have evolved in multi-cellular organisms that operate as buffers against the action of damage-inducing agents by absorbing their consequences. One such mechanism is inflammation. Though it appears to cause harm and damage at first sight, inflammation is actually a physiological process with fundamental importance for maintaining tissue homeostasis and promoting wound repair. Certainly, the initial, classical symptoms of inflammation, i.e. heat, redness, swelling, pain and loss of function (calor, rubor, tumor, dolor, functio laesa) do not augur well, and one thus easily overlooks that physiological inflammation not only combats e.g. invading pathogens to prevent further damage but also, importantly, resolves self-reliantly after the danger has waned. Figure 1.1 illustrates the five pillars or hallmarks of both inflammation and resolution.

Of note, both initiation and resolution of inflammation are actively orchestrated processes that require tight regulation on different levels to ensure that their potentially

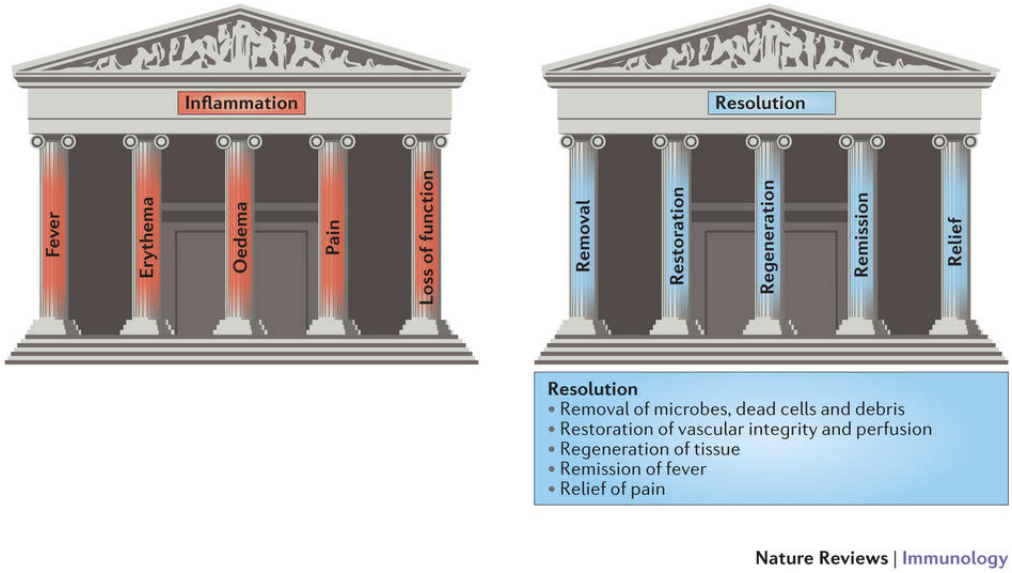

Figure 1.1: Hallmarks of inflammation and resolution. The cardinal signs of inflammation, originally only four (redness, swelling, heat and pain described by Cornelius Celsus, see also Medzhitov (2010)), were expanded to five by Rudolph Virchow in 1858 to include "loss of function". On the other hand, the five pillars of resolution consist of removal of the inciting stimulus, restoration of nutrient/blood supply, tissue regeneration, remission of fever and relief of pain. Image source: Basil and Levy (2016). Reprinted with permission.

damaging consequences are kept under control. While the initiation of inflammation is regarded as a protective reaction to homeostasis-disturbing stressors, it simultaneously modulates certain parameters of the existing homeostasis, thus making space for temporary dysbalance to deal with stress signals, and then awaits its resolution and the system's return to homeostasis (Kotas and Medzhitov, 2015). The flexibility of tolerating such temporary disturbances characterizes resilient systems. However, as inflammatory responses have to interfere with the homeostatic mechanisms in place, faulty regulation of inflammation can result in a failure to resolve, and in the persistence of inflammation-caused chronic diseases. From a therapeutic point of view, novel treatment strategies could therefore aim at tipping the balance back from chronic inflammatory responses towards promotion of resolving mechanisms.

1.2 Sterile or Infectious: That is not the Question

Medzhitov's analytical view on inflammation ascribes the following four components to it: inducers, sensors, mediators and target tissues (Figure 1.2). Whereas all inducers initiate inflammatory responses, both the sensors engaged and mediators released depend on the inducer type. For example, inducers derived from viral infections differ structurally from those of bacterial infections and thus activate virus-specific sensors resulting in the

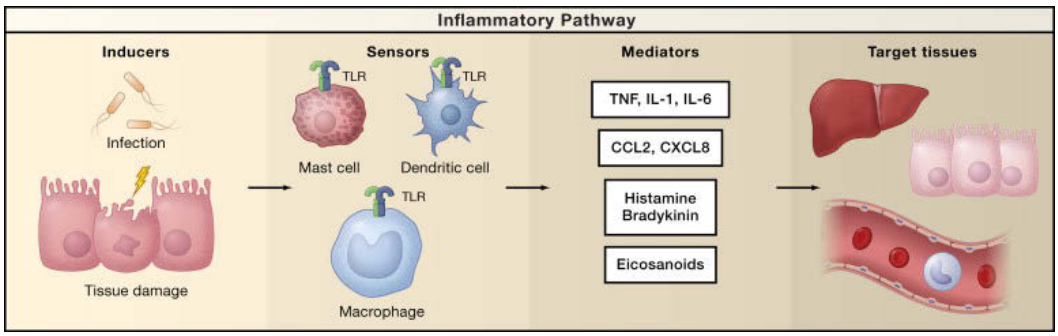

Figure 1.2: The four components of inflammation. Inducers, sensors, mediators and target tissues make up the inflammatory response. The interaction of inducers derived from e.g. infections with sensors instructs the carrier of the sensor to produce mediators, which stimulate target tissues to react to the harmful effects of the inducer. Sensors are molecules expressed by immune cells to scan the surrounding space for danger signals and upon detection alert the immune system to commence clearance of undesired or harmful agents. Image source: Medzhitov (2010). Reprinted with permission.

production of type-I interferons, while bacterial triggers are recognized by different sensors and elicit a distinct response characterized by secretion of inflammatory cytokines and chemokines.

Infections were regarded as the major cause of inflammation in the past, however, more recently, the growing incidence of chronic inflammatory disorders strongly associated with unhealthy dieting and increased body weight such as non-alcoholic fatty liver diseases (NAFLDs) and atherosclerosis has raised the awareness for a role of non-infectious, sterile triggers as causes of inflammation. The term "sterile trigger" or "sterile stimulus" has been established to describe a heterogeneous mixture of inflammation-inducing compounds that may include toxins, chemical substances such as minerals but also biomolecules that are under normal conditions enclosed within cells but exposed during stress, as well as endogenous structures that become modified and immunogenic due to cellular damage. For example, sterile inflammation can be caused by trauma, ischaemia reperfusion injury or exposure to chemicals without any contribution of microbial origin (Chen and Nuñez, 2010; Rock et al., 2010). Independent of their origin or structure, these sterile triggers act as danger signals similar to microbial agents and their presence engages the same host receptors for recognition and signal transduction. Such host receptors are predominantly expressed on the surface of several immune cells including macrophages, which are responsible for scanning tissues for unwanted or damaged material to be removed.

1.3 Of Monocytes and Macrophages

Elie Metchnikoff (1845-1916) was the first to describe the phagocytic activity of macrophages and delineate their important role for host defense and tissue homeostasis in a

time of controversy between humoralists and cellular immunologists over the dominance of their respective fields. His insights into the contribution of macrophages to innate immunity represented a major mile stone in the development of the then rather novel field of cellular immunity, which had been overshadowed by powerful evidence that unequivocally appears to demonstrate the superiority of humoral responses: the discovery of antibodies, complement factors and the success of immunotherapy by use of antitoxins (Medzhitov, 2010; Cavaillon, 2011).

Today, macrophages are recognized as members of the mononuclear phagocyte system of multicellular organisms together with monocytes and dendritic cells and they possess important functions in immune defense, homeostasis, development and regeneration upon tissue injury (Davies et al., 2013; Wynn and Vannella, 2016). Depending on their anatomical location, they may have additional specialized functions. For example, microglia residing in the brain are involved in synaptic remodelling, while osteoclasts are important for bone resorption and Kupffer cells in the hepatic sinusoids of the liver scan the circulating blood from the gut and clear aged erythrocytes; all of these cells are by ontogeny and function macrophages with distinct adaptations to their respective host tissue (Davies et al., 2013). During inflammation, neutrophils are the first cells in place through chemoattraction by activated tissue-resident macrophages and parenchymal cells. Soon after removal of pathogens or unwanted debris they undergo apoptosis and their corpses are phagocytosed by monocytes recruited to the site of inflammation. Figure 1.3 shows the sequence of events after initiation of an inflammatory response. Understandably, for the maintenance of homeostasis it is of critical importance to tightly regulate every single step in this program, from the recruitment of neutrophils to the clearance of apoptotic debris and the cessation of inflammation.

1.4 Aims of This Thesis

Based on previous observations in my thesis laboratory, I hypothesized that MDA epitopes are generated during the course of an inflammatory response induced by sterile triggers due to increased oxidative stress. These MDA epitopes could act as mediators of inflammation and sustain the existing inflammatory response as long as MDA-epitope-carrying structures such as dying cells or oxidized proteins remain uncleared by innate immune cells, e.g. owing to impaired resolution mechanisms or overwhelming MDA production. In this thesis, I show and discuss data relating to the following three aims:

1. Investigate the role of MDA epitopes as mediators of sterile inflammation
2. Identify the receptor(s) mediating MDA-induced responses
3. Characterize MDA-epitope generation during programmed cell death (apoptosis)

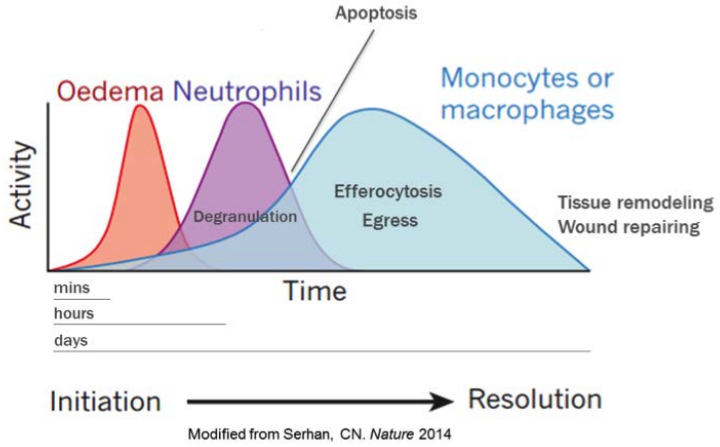

Figure 1.3: Representative time-course of an inflammatory response from initiation to resolution. Tissue swelling (oedema) is followed by neutrophil influx, their apoptosis and phagocytosis (efferocytosis) by monocytes. Courtesy of Máté Kiss, adapted from Serhan (2014).

2 Background

This chapter reviews the current knowledge on important themes of the thesis project. There are three parts. In the first part, I have included a book chapter published by Papac-Milicevic, Busch et Binder, which reviews relevant literature on major topics around MDA: biochemistry and generation, its mechanism of adduction to biomolecules resulting in epitope formation and in vivo targets of MDA adduction (section 2.1). The primary focus of this bookachapter, however, is the role of MDA epitopes as targets of innate immune responses. Hence, we review the many innate immune mechanisms that have evolved to recognize also MDA epitopes, including both soluble and membrane-bound structures such as natural IgM antibodies, complement and pattern-recognition receptors (PRRs). Addressing the role of adaptive immunity in this context, the immunogenicity of MDA epitopes is discussed. From a clinical perspective, the contribution of MDA epitopes to the development of atherosclerosis is best-studied and reviewed in detail.

In the second part, I included a review written with Christoph Binder, focussing specifically on carriers of MDA epitopes and in vivo models where MDA epitopes have been investigated (see Section 2.2). In the third part, I review the background literature complementary to parts I and II by describing certain concepts relevant to the thesis work that are not included in the published review article and book chapter, such as the disease model used to investigate the in vivo role of MDA epitopes (Sections 2.3 to 2.7).

2.1 Book Chapter: Malondialdehyde Epitopes as Targets of Immunity and the Implications for Atherosclerosis

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Adv Immunol. 2016;131:1-59. doi: 10.1016/bs.ai.2016.02.001. PubMed PMID: 27235680.

CHAPTER ONE

Malondialdehyde Epitopes as Targets of Immunity and the Implications for Atherosclerosis

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Contents

Abstract

Accumulating evidence suggests that oxidation-specific epitopes (OSEs) constitute a novel class of damage-associated molecular patterns (DAMPs) generated during high oxidative stress but also in the physiological process of apoptosis. To deal with the potentially

[1] Contributed equally.

Advances in Immunology, Volume 131
ISSN 0065-2776
http://dx.doi.org/10.1016/bs.ai.2016.02.001

harmful consequences of such epitopes, the immune system has developed several mechanisms to protect from OSEs and to orchestrate their clearance, including IgM natural antibodies and both cellular- and membrane-bound receptors. Here, we focus on malondialdehyde (MDA) epitopes as prominent examples of OSEs that trigger both innate and adaptive immune responses. First, we review the mechanisms of MDA generation, the different types of adducts on various biomolecules and provide relevant examples for physiological carriers of MDA such as apoptotic cells, microvesicles, or oxidized low-density lipoproteins. Based on recent insights, we argue that MDA epitopes contribute to the maintenance of homeostatic functions by acting as markers of elevated oxidative stress and tissue damage. We discuss multiple lines of evidence that MDA epitopes are proinflammatory and thus important targets of innate and adaptive immune responses. Finally, we illustrate the relevance of MDA epitopes in human pathologies by describing their capacity to drive inflammatory processes in atherosclerosis and highlighting protective mechanisms of immunity that could be exploited for therapeutic purposes.

1. OVERVIEW

Cellular stress, senescence, and cell death are tightly associated with oxidative stress. A major consequence of increased oxidative stress is the peroxidation of membrane lipids resulting in the generation of various oxidation-specific epitopes (OSEs). OSEs and the immune responses targeting them have been implicated in many acute and chronic inflammatory diseases, most prominently atherosclerosis. Studies of the biological activities of oxidized LDL (OxLDL), which is a key pathogenic driver of atherosclerosis, have helped identify OSE as a novel class of damage-associated molecular patterns (DAMPs). In this chapter, we will particularly focus on a certain group of OSEs, namely malondialdehyde (MDA) epitopes. MDA epitopes have been documented on the surface of dying cells and in damaged tissues. Recent studies have identified them as major targets of various immune responses that modulate homeostatic processes, eg, the clearance of apoptotic cells. In atherosclerosis, which is characterized by impaired resolution and chronic inflammation, MDA epitopes have been identified as mediators of inflammation and therefore serve as interesting potential targets for immunological therapeutic interventions in cardiovascular diseases (CVDs).

2. BIOCHEMISTRY AND GENERATION OF MDA IN VITRO AND IN VIVO

Oxygen is a fundamental prerequisite for energy production by cellular respiration in aerobic organisms. However, this also results in the constant

generation of reactive oxygen species (ROS) as potentially damaging by-products, which are produced endogenously in mitochondria, peroxisomes, the endoplasmic reticulum, and even in the plasma membrane of cells, but can also be induced exogenously by UV light, heat, bacterial, and environmental agents, such as tobacco smoke and ionizing radiation (Bae, Oh, Rhee, & Yoo, 2011; Nathan & Cunningham-Bussel, 2013). Newly generated ROS can attack membrane lipids containing carbon–carbon double bonds (eg, polyunsaturated fatty acids (PUFAs) of phospholipids), and damage them by a process called lipid peroxidation. Lipid peroxidation of free fatty acids occurs through both enzymatic and nonenzymatic mechanisms. If not efficiently controlled this emanates in the perturbed integrity of different cellular structures potentially leading to cellular death (Nathan & Cunningham-Bussel, 2013). Enzymatic mechanisms involve the activation of lipoxygenases, myeloperoxidases, cyclooxygenases, and cytochrome P450 (Niki, 2009). After the enzymatic removal of hydrogen from the double allylic-activated CH_2 group of PUFAs, oxygen is added, generating a peroxydienyl radical. This is then transformed into an anion and the reaction is terminated by back-transfer of the proton generated in the first reaction step, resulting in the formation of a lipid-hydroperoxide molecule (LOOH). Nonenzymatic mechanisms are mediated by free radicals, which can be indirectly generated by nicotinamide adenine dinucleotide phosphate oxidases and nitric oxide synthases (Niki, 2009). In turn, free radicals are able to remove hydrogen from a CH_2 group of PUFAs, resulting in the generation of LOOH and new dienyl radicals, which propagate this chain reaction. LOOHs that are generated by both reactions then decompose and during their degradation a great variety of secondary products such as MDA, 4-hydroxynonenal (4-HNE), and the remaining core aldehyde of oxidized phospholipids, such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine, are produced (Yin, Xu, & Porter, 2011). These end products of lipid peroxidation are highly reactive aldehydes, which can further propagate oxidative damage and are therefore considered to be downstream mediators of oxidative stress. Under normal conditions, lipid peroxidation is inhibited enzymatically by the activities of glutathione peroxidases, superoxide dismutases and catalases, or nonenzymatically by antioxidants, such as vitamin C and vitamin E (Niki, 2009). It has been extensively shown that an imbalance between oxidative stress and antioxidant mechanisms results in pathological oxidation (Finkel & Holbrook, 2000; Nathan & Cunningham-Bussel, 2013). Therefore, total levels of end products of lipid peroxidation, especially of free and adducted MDA, are widely used as indicators of oxidative stress in higher

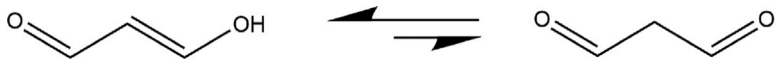

Fig. 1 Chemical structure of malondialdehyde. MDA is an aldehyde with the chemical formula $C_3H_4O_2$. At physiological pH, it exists mainly in its enol form (*left structure*). MDA has a molar mass of 72.06 g/mol, a density of 0.991 g/mL, and its melting point and boiling point are at 72°C and 108°C, respectively. It is produced by acid hydrolysis of 1,1,3,3-tetramethoxypropane at room temperature in vitro (Nair et al., 2001), and due to its high reactivity usually not observed in a pure form.

eukaryotic organisms and have been shown to correlate with the extent of tissue damage in acute injury and chronic diseases (Imai et al., 2008; Pamplona et al., 2005; Weismann et al., 2011; Yla-Herttuala et al., 1989).

Here, we will mainly focus on the biology of one of these reactive terminal degradation products: MDA. MDA (also known as Malonic aldehyde; Propanedial; 1,3-Propanedial) is an aldehyde with the chemical formula $C_3H_4O_2$ that at physiological pH mainly exists in its enol form (Nair, O'Neil, & Wang, 2001). The chemical structure of MDA is given in Fig. 1.

Under physiological conditions, newly generated MDA can modify free amino acids, proteins, nucleotides, and phospholipids creating stable covalent epitopes. Major in vivo carriers of MDA epitopes identified so far are apoptotic/necrotic cells (Amir et al., 2012; Chang et al., 1999, 2004; Chou et al., 2009; Weismann et al., 2011), microvesicles (MV) (Huber et al., 2002; Liu, Scalia, Mehta, & Williams, 2012; Tsiantoulas et al., 2015), and oxidized lipoprotein particles (Palinski et al., 1989; Shao, Tang, Heinecke, & Oram, 2010).

The main substrates for the generation of MDA in vivo are arachidonic acid (AA, 20:4) and docosahexaenoic acid (DHA, 22:6) present in membrane phospholipids. Already in the 1970s Pryor et al. (Pryor & Stanley, 1975) proposed possible mechanisms of MDA generation from these two PUFAs. He suggested the formation of intermediates that are bicycloendoperoxides, which are then broken down by thermal- or acid-catalyzed reactions giving rise to free MDA. Additionally, it was observed that certain amounts of MDA can be generated as a side product of thromboxane A2 synthesis. The enzymes cyclooxygenase 1 (COX-1) or COX-2 metabolize AA to prostaglandin H_2 (PGH_2), and newly formed PGH_2 is then metabolized by the thromboxane synthase, to thromboxane A2, 12-hydroxyheptadecatrienoic acid, and MDA (Hecker & Ullrich, 1989). Several reports suggested that MDA can also formed by oxidation of spermine (Quash et al., 1987), UV-irradiation of the skin surface lipid squalene (Dennis & Shibamoto, 1989), or by gamma irradiation of carbohydrates (Cheeseman, Beavis, & Esterbauer, 1988).

3. GENERATION OF MDA EPITOPES

Free MDA exists as a bifunctional electrophile and its reactivity is pH dependent. At physiological pH MDA is present in the enolate ion form. MDA reactivity can be increased by lowering the pH, which favors the formation of beta-hydroxyacrolein, thereby increasing its affinity toward nucleophilic molecules in the vicinity (Esterbauer, Schaur, & Zollner, 1991). In addition, it has been demonstrated that at high concentrations of MDA in an acidic milieu (pH range 4–7) long oligomers of MDA are formed, which results in hydrolytic cleavage of newly formed MDA oligomers, generating MDA and acetaldehyde (AcA) (Gomez-Sanchez, Hermosín, & Maya, 1990). This observation is very important, as AcA and MDA can react together and create a more complex form of immunogenic epitopes, malondialdehyde-acetaldehyde (MAA). Due to their chemical nature free AcA and MDA possess the ability to create epitopes on major cellular macromolecules in different tissues serving as mediators of oxidative stress (Fig. 2). Forming stable epitopes with biomolecules has been suggested to increase the half-life of MDA in vivo (Siu & Draper, 1982). Furthermore, MDA modifications of lipids, nucleotides, free amino acids, and proteins may result in their loss of function, loss of structural integrity, and may lead to altered cellular responses (Fogelman et al., 1980; Hyvarinen, Uchida, Varjosalo, Jokela, & Jokiranta, 2014; Wallberg, Bergquist, Achour, Breij, & Harris, 2007). Modifications of each type of macromolecules will be discussed separately.

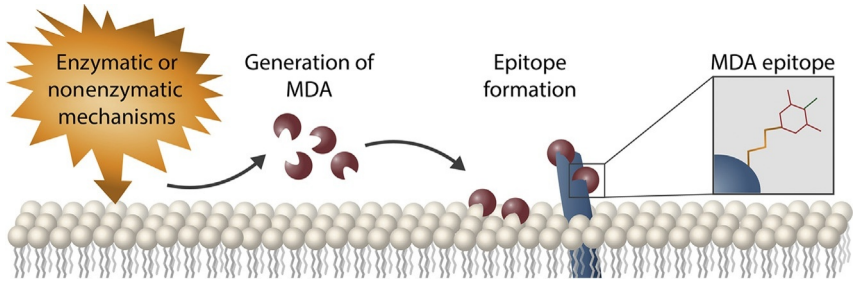

Fig. 2 Mechanism of MDA epitope formation. Lipid peroxidation of polyunsaturated fatty acids by enzymatic or nonenzymatic mechanisms results in the generation of highly reactive aldehydes such as MDA, acting as mediators of oxidative stress. Covalent attachment of MDA to amino groups present in, eg, Lys of biomolecules nearby results in the formation of MDA epitopes.

3.1 MDA Modification of Amino Acids and Proteins

MDA-modified biomolecules such as free amino acids or proteins have been identified in different tissues and body fluids in humans and animals in vivo (Akalin, Baltacioglu, Alver, & Karabulut, 2007; Gonenc, Ozkan, Torun, & Simsek, 2001; Imai et al., 2008; Pamplona et al., 2005; Weismann et al., 2011). Indeed, at neutral pH in the presence of free lysine (Lys) and arginine (Arg), MDA can hydrolyze and form epitopes on their side chains. Additionally, at lower pH MDA has also been shown to form epitopes on another group of amino acids, including glycine (Gly), leucine (Leu), valine (Val), histidine (His), arginine (Arg), tryptophan (Trp), tyrosine (Tyr), serine (Ser), and cysteine (Cys) (Esterbauer et al., 1991; Hadley & Draper, 1988; Nair et al., 2001). Proteins are typically modified by MDA on the ε-amino groups of their side chains forming various epitopes by Schiff base reaction (Slatter, Bolton, & Bailey, 2000; Watson, Preedy, & Zibadi, 2012). These include preferentially side chains of Lys, but modifications of His, Tyr, Arg, ethionine (Met), glutamine (Gln), and Cys have also been observed (Esterbauer et al., 1991; Gurbuz & Heinonen, 2015; Watson et al., 2012). Moreover, it has been demonstrated that both glycosylation and the acetylation of proteins render them more susceptible for MDA modification, possibly as a result of slight alterations in the tertiary structure of the protein that facilitate access for MDA (Mooradian, Lung, & Pinnas, 1996; Tuma, Thiele, Xu, Klassen, & Sorrell, 1996).

In human and animal models of disease MDA epitopes have been detected on proteins found in serum and urine, as well as on proteins of the extracellular matrix, and many intracellular ones (Akalin et al., 2007; Draper, Csallany, & Hadley, 2000; Gonenc et al., 2001; Imai et al., 2008; Weismann et al., 2011; Yla-Herttuala et al., 1989). Based on available literature we have generated an extensive list of endogenous proteins that had been reported to be modified by MDA, and these are presented in Table 2. In four different species, MDA modifications of a total of 107 proteins have been reported, of which five were found in at least two different organisms. These five proteins are ATP synthase (subunit β), NADH dehydrogenase Fe–S protein 2, cytochrome *b*–*c*1 complex (subunit 1), albumin, and actin. Interestingly, the majority of the proteins have mitochondrial origin, strengthening the notion that mitochondria, being major reservoirs of ROS, could be one site within the cell where MDA epitopes are preferentially generated.

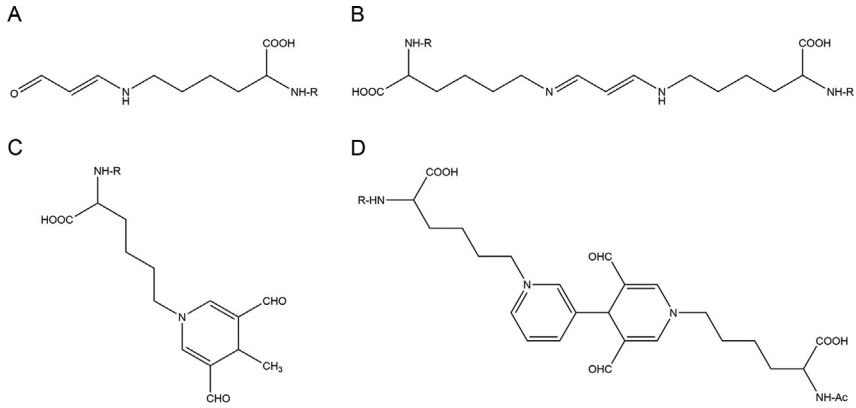

Fig. 3 Chemical structures of MDA epitopes found on Lys side chains of protein (Gonen et al., 2014; Uchida, 2000). *R*, protein backbone. (A) *N*-ε-(2-propenal)Lys. (B) 1-Amino-3-iminopropene. (C) 4-Methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative. (D) Pyridium–dihydropyridine.

Structurally different types of epitopes between MDA and primary amines have been reported, including fluorescent and nonfluorescent ones as well as cross-linking or noncross-linking ones. Important examples are presented in Fig. 3.

The most common type of adduct is *N*-ε-(2-propenal)lysine (Fig. 3A), which is a linear nonfluorescent adduct without the ability to generate cross-links and forms in the absence of AcA at pH 7 (Uchida, Sakai, Itakura, Osawa, & Toyokuni, 1997). It has been identified as the major form of endogenous MDA excreted in rat and human urine using ion exchange and high-performance liquid chromatography (Draper, McGirr, & Hadley, 1986; Piche, Cole, Hadley, van den Bergh, & Draper, 1988) and has been detected on in vitro MDA-modified keyhole limpet hemocyanin (KLH), MDA-modified LDL, and MDA-modified Apoprotein A-I (ApoAI), as well as copper-oxidized LDL (CuOx-LDL) (Shao et al., 2010). More complex MAA epitopes on ε-amino groups of Lys are formed by MDA and AcA in a 2:1 ratio (Tuma et al., 1996). The two major MAA epitopes are a 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative (MDHDC adduct) and a 2-formyl-3-(alkylamino) butanal derivative (FAAB adduct). In contrast to FAAB, MDHDC is a fluorescent adduct with $\lambda_{ex} = 395$ nm and $\lambda_{em} = 485$ nm. It requires the close proximity of two intermediates, including an FAAB adduct that is generated when one molecule of MDA and one of AcA react with free amino groups of a protein side

chain, and an MDA Schiff base (MDA-enamine) generated by the reaction of MDA with another free amino group. After the generation of these two intermediates, the FAAB moiety is transferred to the nitrogen of the MDA-enamine followed by ring closure yielding the MDHDC adduct (Fig. 3C) (Tuma et al., 2001). The formation of MDHDC epitopes has been demonstrated to be favored by acidic conditions (pH 4). Using NMR spectroscopy (Kearley, Patel, Chien, & Tuma, 1999) and performing chemical analysis (Xu et al., 1997) it has been shown that MAA epitopes are different from those epitopes formed when both aldehydes are allowed to react alone. Several lines of evidence suggest that this type of MAA epitopes possesses potent biological activity (Tuma et al., 1996; Uchida et al., 1997), although *N*-*ε*-(2-propenal) lysine epitopes have also been shown to be highly immunogenic (Gonen et al., 2014). Moreover, epitopes with the ability to form cross-links between different Lys residues in vitro have been found as well. These epitopes form cross-links within the same domain of a protein or domains in close proximity. In these reactions, one lysine residue forms a Schiff base with MDA, which is stabilized by equilibration to an enolate, and reacts then with a second Lys residue to form a diimine cross-link (Requena et al., 1996). Both simple MDA cross-links, such as 1-amino-3-iminopropenes (Fig. 3B), and more complex ones that are also fluorescent, such as pyridium–dihydropyridine epitopes have been described (Fig. 3D) (Itakura, Uchida, & Osawa, 1996). Stable cross-linking MDA epitopes were first demonstrated under semi-physiological conditions using NMR and EI-MS (Slatter, Murray, & Bailey, 1998). Such epitopes have been proposed to play a role by affecting structural proteins, such as collagen in the vessel wall where this could contribute to vascular stiffening and development of vasculopathies. In addition, in vitro MDA cross-links distant Lys residues of ApoAI (Shao et al., 2010) as well as purified ApoE3 and ApoE4 molecules (Montine et al., 1996), and cross-links apoprotein(a) to Apolipoprotein B100 (ApoB100) (Haberland, Fogelman, & Edwards, 1982). Finally, endogenous cross-linking MDA epitopes of proteins are formed on platelet proteins upon platelet activation and have been found to be increased in diseases associated with platelet activation, such as metabolic syndrome and sickle cell anemia (Zagol-Ikapite et al., 2015).

3.2 MDA Modification of Nucleotides and Nucleic Acids

Free MDA has been shown to form covalent epitopes with nucleotides, such as deoxy-guanosine, deoxy-adenosine, and deoxy-cytidine, in a

reaction that is enhanced in the acidic milieu. The major epitopes that can be formed by these reactions are pyrimido-[1,2-α]purin-10(3*H*)-one deoxyribose (M_1G), N6-(3-oxopropenyl)deoxyadenosine (M_1A), and N4-(3-oxopropenyl)deoxycytidine (M_1C) (Marnett, 1999). Of these, M_1G are five times more abundant than M_1A adducts in the liver, and the amounts of M_1C are generally very low (Chaudhary, Reddy, Blair, & Marnett, 1996). MDA is considered to be a potent mutagen (Esterbauer et al., 1991), and MDA modifications of nucleotide bases of DNA have been shown to induce genotoxicity by various mechanisms. MDA epitopes on nucleotides can cause template changes (Maddukuri et al., 2010; VanderVeen, Hashim, Shyr, & Marnett, 2003), result in the formation of interstrand cross-links (Niedernhofer, Daniels, Rouzer, Greene, & Marnett, 2003), or create DNA–protein cross-links (Voitkun & Zhitkovich, 1999). Interestingly, it has been shown that DNA isolated from human and rat liver exposed to enhanced lipid peroxidation contains increased amounts of MDA epitopes on DNA (Chaudhary et al., 1994). Nucleotide excision repair has been suggested to be the main mechanism of repair for M_1G-caused damage in DNA (Marnett, 1999). Moreover, mitochondrial DNA has been found to have a higher abundance of MDA-DNA epitopes compared to nuclear DNA. This increased amount of MDA epitopes in mitochondrial DNA is thought to be due to the lack of nucleotide excision repair in mitochondria (Cline, Lodeiro, Marnett, Cameron, & Arnold, 2010). Interestingly, increased levels of MDA-adducted nucleotides are found in the serum and urine of patients suffering from cancer as well as diseases associated with increased ROS production (Gungor et al., 2010; Munnia et al., 2006; Peluso et al., 2011).

3.3 MDA Modification of Phospholipids

In contrast to MDA modifications of proteins or nucleotides, only little data are available on MDA modification of phospholipids, which can be modified as well. Indeed, aminophospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), have been found to form epitopes with MDA by enaminal derivatization. MDA-modified PS and PE have been identified in aging erythrocytes possibly as a consequence of peroxidative lipid damage (Gil, Farinas, Casado, & Lopez-Fernandez, 2002; Jain, 1988) and as metabolic waste products excreted in urine (Draper et al., 2000; Uchida et al., 1997).

3.4 Metabolism of MDA Epitopes In Vivo

Not much is known about metabolism of MDA epitopes in vivo. Marnett et al. injected intraperitoneally ^{14}C-labeled MDA into male and female Swiss Webster mice and found rapid and uniform distribution of labeled MDA throughout the body (Marnett, Buck, Tuttle, Basu, & Bull, 1985). Moreover, it was observed that conversion of MDA to CO_2 is completed in 4 h. In another study, urine samples obtained from rats challenged with oxidative stress by treatment with iron nitrilotriacetate or carbon tetrachloride or vitamin E deficiency were found to have MDA present in proteins, nucleic acid bases, and phospholipids as measured by HPLC (Draper et al., 2000). A similar excretion pattern, albeit in much smaller amounts, was observed in urine samples obtained from humans (Draper et al., 2000). Additionally, it has been shown that MDA levels in human urine can be modulated by diet, physical exercise, and smoking (Hadley, Visser, & Vander Steen, 2009; Mellor, Hamer, Smyth, Atkin, & Courts, 2010).

4. CARRIERS OF MDA EPITOPES

Tissue stress and subsequent cellular damage result in the generation of dying cells, the release of MV, and the accumulation of cellular debris. A number of studies have documented the presence of MDA epitopes on apoptotic cells and MV. For example, we and others could show that MDA-specific antibodies bind to serum-deprived apoptotic endothelial cells, dexamethasone-treated or UV-irradiated apoptotic T cells, as well as to necrotic cells and apoptotic blebs from retinal pigment epithelial cells (Amir et al., 2012; Aredo et al., 2015; Chang et al., 1999; Chou et al., 2009; Weismann et al., 2011) (see Table 1 for an overview). Furthermore, MDA epitopes have been found on the membranes of oxidized MV released from endothelial cells (Huber et al., 2002), and human monocytic cells treated with unesterified cholesterol (Liu et al., 2012), as well as on in vitro generated platelet-derived MV and on circulating MV collected from human blood (Gil et al., 2002; Tsiantoulas et al., 2015). In addition, MDA has been detected in aging erythrocytes, suggesting the presence of MDA epitopes in their membranes.

MDA epitopes have been identified on plasma lipoproteins and proteins. Among the lipoproteins, oxidized high-density lipoprotein (HDL) and

Table 1 Cellular and Subcellular Carriers of MDA Epitopes

Carrier Type	Induction	Method of Detection	References
Microvesicles (MV)			
Circulating MV		Flow cytometry	Tsiantoulas et al. (2015)
Platelet MV	Ionomycin	Flow cytometry	Tsiantoulas et al. (2015)
Endothelial MV	*tert*-Butyl hydroperoxide, Fe^{2+}	Flow cytometry	Huber et al. (2002)
Monocytic MV	Cholesterol	Flow cytometry	Liu et al. (2012)
Activated or dying cells			
Retinal pigment epithelial cells	Heat	Immunofluorescence	Weismann et al. (2011)
Thymocytes	PMA	Flow cytometry, immunofluorescence	Chou et al. (2009)
Jurkat T cells	UV	Flow cytometry	Amir et al. (2012)
Endothelial cells	Serum deprivation	Flow cytometry, immunofluorescence	Chang et al. (1999)
Thymocytes	Dexamethasone	Flow cytometry, immunofluorescence	Chang et al. (1999)
Jurkat T cells	UV	Flow cytometry, immunofluorescence	Tuominen et al. (2006)
THP-1 cells	Cholesterol	Immunofluorescence	Liu et al. (2012)
Erythrocytes	Aging	HPLC	Gil et al. (2002)
Platelet	Metabolic syndrome, sickle cell anemia	Mass spectrometry	Zagol-Ikapite et al. (2015)

low-density lipoprotein (LDL) eluted from the lesional tissues have been shown to carry MDA epitopes (Haberland, Fong, & Cheng, 1988; Palinski et al., 1989; Shao et al., 2010; Yla-Herttuala et al., 1989). In addition, other proteins modified by MDA have been detected in body fluids and tissues of different organisms listed in Table 2.

Table 2 List of Identified MDA-Modified Proteins

Organism	Tissue	Protein	NCBI (GI)	Detection Method	References
Homo sapiens	Retina	Tubulin, beta chain	5174739	2D gel electrophoresis, immunoblot with anti-MDA antibody	Schutt, Bergmann, Holz, and Kopitz (2003)
		Tubulin, alpha-1 chain	135395		
		ATP synthase beta chain	1145449		
		Ubiquinol-cytochrome *c* reductase core protein I	4507841		
		Pyruvate kinase, muscle	14750405		
		ATP synthase, alpha subunit	4757810		
		Enolase 1, alpha	4503571		
		S-arrestin	14737493		
		NADH dehydrogenase Fe–S protein 2	4758786		
		Guanine nucleotide-binding protein, beta polypeptide 2	4885283		
		Pyruvate dehydrogenase	2144337		
		Annexin A2	16306978		
		Glyceraldehyde-3-phosphate dehydrogenase	31645		

		Porin 31HM (anion channel 1)	238427		
		Voltage-dependent anion channel 2	4507881		
		H119n carbonic anhydrase Ii	2554664		
		Annexin V (Lipocortin V)	999937		
		Prohibitin	4505773		
		ATP synthase, subunit d	5453559		
		Calmodulin 2 (phosphorylase kinase, delta)	14250065		
		Cytochrome *c* oxidase subunit Va precursor	4758038		
		Peroxiredoxin 2	13631440		
		Crystallin, alpha A	4503055		
		Crystallin, alpha B	4503057		
	Atherosclerotic lesion	ApoA-I	4557321	Detection with MDA2 antibody on lesional HDL	Shao et al. (2010)
	Atherosclerotic lesion	Apo B-100	105990532	Western blot on lesional LDL	Palinski et al. (1989)

Continued

Table 2 List of Identified MDA-Modified Proteins—Cont'd

Organism	Tissue	Protein	NCBI (GI)	Detection Method	References
	Frontal cortex	Superoxide dismutase 2	119568015	2D gel electrophoresis/MS	Dalfo et al. (2005)
		α-Synuclein	80475099		
	Brain cortex	Neurofilament light polypeptide	62511894	2D gel electrophoresis/MS	Pamplona et al. (2005)
		Vimentin	55977767		
		Tubulin beta-2	55977480		
		Tubulin alpha-1B chain	55977471		
		Tubulin alpha-4A	55977476		
		Tubulin alpha-1C chain	20455322		
		Actin, cytoplasmic 1	46397316		
		Actin, cytoplasmic 2	54036665		
		Glial fibrillary acidic protein	121135		
		Gamma-enolase	20981682		
		Alpha-enolase	119339		
		Cytochrome *b*–*c*1 complex subunit 1	92090651		
		ATP synthase subunit beta	114549		
		Creatine kinase B-type	125294		

		Glutamine synthetase	1169929		
		Glutamate dehydrogenase 1	118541		
		Guanine nucleotide-binding protein G(I)/G(S)/G (T) subunit beta-1	51317300		
		60 kDa heat shock protein	129379		
		Dihydropyrimidinase-related protein 2	3122051		
	Plasma	**Albumin**	4502027	LC/MS, Western blot	Odhiambo et al. (2007)
	Aortic tissue	Elastin		Fluorescence of elastin fraction	Yamamoto et al. (2002)
Mus musculus	Broncho-alveolar lavage	Surfactant protein D	6677921	Immunopreciptation and Western blot	McCaskill et al. (2011)
	Blood	Hemoglobin		Sodium borohydrate reduction of MDA adducts	Kautiainen, Tornqvist, Svensson, and Osterman-Golkar (1989)
	Heart and skeletal muscle mitochondria	Aconitase	18079339	SDS-PAGE/MS of MDA positive bands	Yarian, Rebrin, and Sohal (2005)
		Acyl-coenzyme A dehydrogenase	23956084		
		Albumin	163310765		
		ATP synthase	31980648		
		α-ketoglutarate dehydrogenase	21313536		

Continued

Table 2 List of Identified MDA-Modified Proteins—Cont'd

Organism	Tissue	Protein	NCBI (GI)	Detection Method	References
	Kidney mitochondria	NADH dehydrogenase (ubiquinone) Fe–S protein 1	148667767	2D gel electrophoresis/MS	Choksi, Nuss, Boylston, Rabek, and Papaconstantinou (2007)
		ATP synthase subunit alpha	6680748		
		ATP synthase subunit beta	31980648		
		NADH dehydrogenase (ubiquinone) Fe–S protein 2	16359270		
		NADH dehydrogenase [ubiquinone] iron–sulfur protein 4	281485615		
		NADH dehydrogenase [ubiquinone] 1 subunit C2	18859597		
		Succinate dehydrogenase [ubiquinone] flavoprotein subunit	54607098		
		Gamma-glutamyltranspeptidase 1	807201132		
		Isocitrate dehydrogenase 2 (NADP+)	37748684		
		Catalase	157951741		

		Cytochrome *b*–*c*1 complex subunit 1	46593021		
		Cytochrome *b*–*c*1 complex subunit 2	22267442		
		Cytochrome *b*–*c*1 complex subunit Rieske	13385168		
		Peroxisomal acyl-coenzyme A oxidase 1 isoform 1	66793429		
		Long-chain-specific acyl-CoA dehydrogenase	31982520		
		Cytochrome *c* oxidase subunit II	34538601		
Rattus norvegicus	Limb and heart muscles	Elongation factor 2	8393296	Immunoprecipitation with anti-MDA antibody, EF2 western	Arguelles, Cano, Machado, and Ayala (2011)
	Gastrocnemius	Beta-enolase	122065177		Marin-Corral et al. (2010)
		Creatine kinase M-type	124056470		
		Carbonic anhydrase 3	5921194		
		Actin, alpha skeletal muscle	61217738		
		Tropomyosin alpha-1 chain	92090646		
		ATP synthase subunit beta, mitochondrial	114562		
	Tibialis anterior	Beta-enolase	122065177		

Continued

Table 2 List of Identified MDA-Modified Proteins—Cont'd

Organism	Tissue	Protein	NCBI (GI)	Detection Method	References
		Fructose biphosphate aldolase A	113609		
		Creatine kinase M-type	124056470		
		Actin, alpha skeletal muscle	61217738		
		Tropomyosin alpha-1 chain	92090646		
	Extensor digitorum longus	Beta-enolase	122065177		
		Fructose biphosphate aldolase A	113609		
		Creatine kinase M-type	124056470		
		Actin, alpha skeletal muscle	61217738		
		ATP synthase subunit beta, mitochondrial	114562		
	Soleus	Beta-enolase	122065177		
		Fructose biphosphate aldolase A	113609		
		Creatine kinase M-type	124056470		
		Carbonic anhydrase 3	5921194		
		Actin, alpha skeletal muscle	61217738		

		Tropomyosin alpha-1 chain	92090646		
		ATP synthase subunit beta, mitochondrial	114562		
	Heart	Creatine kinase M-type	124056470		
		Actin, alpha cardiac muscle 1	54036667		
		Tropomyosin alpha-1 chain	92090646		
		Myosin-6	127741		
		Myosin light chain	127151		
		Vacuolar proton pump subunit C1	81882798		
		ATP synthase subunit beta, mitochondrial	114562		
		NADH-ubiquinone oxidoreductase 75 kDa subunit mitochondrial	53850628		
		Aldehyde dehydrogenase mitochondrial	14192933		
	Kidney	Type IV collagen	157821889	IHC, Western blot	Neale et al. (1994)
Oryctolagus cuniculus		Type II collagen	2190238	Western blot	Tiku, Allison, Naik, and Karry (2003)

5. TARGETS OF IMMUNE RESPONSE

As described earlier, MDA modifications of endogenous molecules are typically associated with structural changes and subsequently functional alterations of these macromolecules. Moreover, MDA modifications occur as a consequence of increased oxidative stress and therefore signify tissue damage. Thus, for the host to deal with potentially harmful effects of altered self-molecules it needs to recognize them and respond to their accumulation, eg, by mounting specific immune responses. Already in the 1980s Witztum and colleagues have shown that injection of guinea pigs with glucosylated autologous LDL or glucosylated autologous albumin resulted in the generation of antisera recognizing the glucosylation itself but not the proteins that were modified (Witztum, Steinbrecher, Fisher, & Kesaniemi, 1983).

In a follow-up study, specific antisera could also be generated with differently modified autologous LDL, including methylated, ethylated, acetylated, and carbamylated LDL (Steinbrecher, Fisher, Witztum, & Curtiss, 1984). These pioneering studies demonstrated that even the smallest modifications of autologous proteins can result in the haptenization of self-proteins leading to the formation of neo-self antigens that are recognized by the immune system. This concept has provided insights into the mechanisms by which oxidation of LDL renders it immunogenic, as this process generates several different OSEs that modify OxLDL particles and are recognized by the immune system in a hapten-specific manner. Many different OSEs have been characterized, including 4-HNE, phosphocholine (PC) of oxidized phospholipids, and MDA (Hartvigsen et al., 2009). There is now ample evidence that OSEs are recognized by both innate and adaptive immunity.

6. INNATE IMMUNE RESPONSES TOWARD OSEs

The innate immune system is instantaneously capable of recognizing a broad range of structures via pattern-recognition receptors (PRRs). Depending on whether the ligands originate from a foreign source (eg, microbes) or from within the host, they have been collectively named pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), respectively. Tissue-resident macrophages expressing PRRs on their surfaces are among the first cells of the innate

immune system to sense PAMPs or DAMPs and initiate an inflammatory response to ultimately remove pathogens or the triggering agent, respectively. Several classes of PAMPs, such as bacterial lipopolysaccharide (LPS), bacterial flagellin, lipoteichoic acid from Gram-positive bacteria, double-stranded RNA, unmethylated CpG motifs and DAMPs, such as high mobility group box 1 protein, heat shock proteins, ATP, and uric acid have been described (Chen & Nunez, 2010; Krysko et al., 2012). DAMPs are typically intracellular components that are secluded from the recognition by the immune system and can be released into extracellular environment upon cellular damage. Because DAMPs convey a message of danger to other cells employing specific cellular receptors and triggering innate immunity response, OSEs, including MDA, which fulfill these criteria can be considered a novel class of DAMPs (Fig. 4) (Miller et al., 2011).

Indeed, OSEs are recognized by PRRs present on macrophages, such as the heterogeneous family of scavenger receptors (SRs), which bind and internalize (scavenge) oxidized but not native LDL (Canton, Neculai, & Grinstein, 2013). Among SRs, CD36 and SR-A1 are the two most relevant receptors for the uptake of OxLDL, which leads to the formation of lipid-laden foam cells—a hallmark of atherosclerotic lesions. Importantly, some

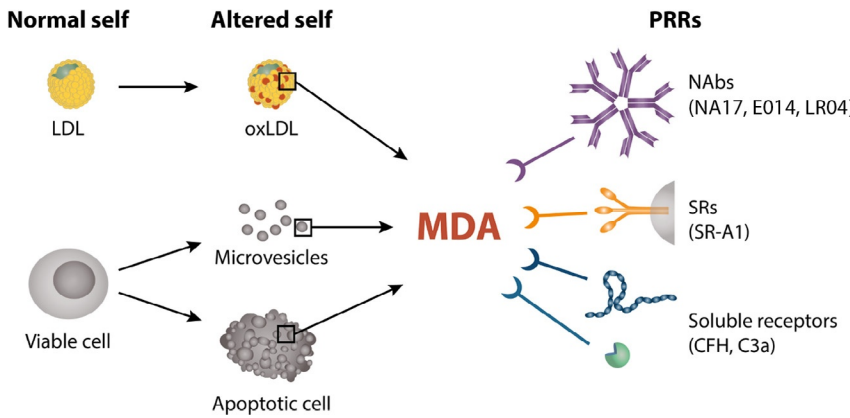

Fig. 4 Overview of MDA epitopes and their interaction with different arcs of innate immunity. MDA epitopes are DAMPs generated on biomolecules such as LDL in atherosclerotic lesions due to oxidative stress and on the surface of cells, cellular debris, or microvesicles during apoptosis and cell activation. Both soluble- and membrane-bound factors of the innate immune system bind to MDA epitopes presented on OxLDL or cell membranes including natural antibodies such as E014 and NA17, the scavenger receptor SR-A1 and two members of the complement system, CFH and C3a. Also other OSEs are recognized by innate immunity (not shown here).

OSEs are not merely taken up by SRs but can also serve as inflammatory mediators alarming the immune system about the damage inflicted by atherogenic lipoproteins. Another important PRR involved in clearing damaged lipoproteins are toll-like receptors (TLRs), which bind to a vast variety of different PAMPs and DAMPs, including OSEs. Certain TLRs have been shown to bind—as part of a multimeric complex—to oxidized phospholipids, OxLDL, and other OSEs, thereby mediating proinflammatory signals. For example, recognition of OxLDL by CD36 on macrophages enables the formation of a heterotrimeric complex consisting of CD36, TLR4, and TLR6, which mediates inflammatory responses resulting in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway activation and expression of chemokines including chemokine (C–X–C motif) ligand 1 (Cxcl1), Cxcl2, chemokine (C–C motif) ligand 5 (Ccl5), and Ccl9 (Stewart et al., 2010). Macrophages lacking any of the three receptors fail to respond to OxLDL stimulation, while TLR2 deficiency does not have any effect. TLR2, however, seems to play a role when macrophages subjected to endoplasmatic reticulum stress are stimulated with several oxidized phospholipids, saturated fatty acids, and OxLDL, which results in a TLR2/CD36-dependent induction of apoptosis (Seimon et al., 2010).

Thus, a number of PRRs recognize OSEs and act as sensors of oxidative stress. Upon ligation of OSEs with their corresponding PRRs, signal transduction events are initiated that culminate in sterile inflammatory responses characterized by chemokine secretion, leukocyte recruitment, or tissue repair. There is accumulating evidence that certain PRRs and pattern recognition proteins (PRPs) are also involved in sensing of MDA epitopes as DAMPs, and in the following section we will review published work both on the recognition by innate immunity and on the proinflammatory effects of MDA epitopes.

6.1 Cellular Receptors of MDA Epitopes

In line with initial studies showing that modified but not native LDL is recognized by macrophages via SRs (Goldstein, Ho, Basu, & Brown, 1979; Henriksen, Mahoney, & Steinberg, 1981), Shechter et al. demonstrated that both acetyl-LDL and fucoidin, a known SR-A1 ligand, inhibited the uptake of MDA-modified LDL (MDA-LDL) by human monocyte–macrophages (Shechter et al., 1981), indicating that MDA-LDL is recognized and degraded also via a SR-dependent mechanisms. Similarly, lipoprotein (a) (Lp(a)) is recognized by human monocyte–macrophages via SRs only

upon modification with MDA, as MDA-LDL, CuOx-LDL, and polyinosinic acid inhibited binding and degradation of MDA-Lp(a) (Haberland, Fless, Scanu, & Fogelman, 1992). In addition, binding of LDL isolated from rabbit atherosclerotic lesions to mouse peritoneal macrophages was competed for by MDA-LDL or polyinosinic acid, suggesting SR-A1-mediated uptake (Yla-Herttuala et al., 1989). In light of this, MDA epitopes present on altered self-molecules or on the surface of dying cells could be sensed and identified by SRs as waste markers and signals of disturbed homeostasis that are required to be scavenged, ie, removed from the system.

More recently, additional groups have confirmed that SR-A1 is involved in binding to MDA-modified proteins in a number of different cell types, including J774 cells (Willis, Klassen, Carlson, Brouse, & Thiele, 2004), bovine or human bronchial epithelial cells (Berger et al., 2014; Wyatt, Kharbanda, Tuma, Sisson, & Spurzem, 2005), murine bone marrow-derived macrophages (Wallberg et al., 2007), mouse peritoneal macrophages, and SR-A1-expressing human embryonic kidney (HEK) cells (Nikolic, Cholewa, Gass, Gong, & Post, 2007), corroborating prior data that SR-A1 is one but not necessarily the only receptor binding to MDA epitopes. Indeed, Zhu et al. accidentally found that CD16, an Fcγ receptor (FcγR) included in the study as a negative control, inhibited the binding of J774 cells to MDA-LDL-coated wells to a larger extent than soluble CD36, which was the original target of interest (Zhu et al., 2014). Similar to SRs, FcγRs have been shown to identify and eliminate microbial pathogens. Binding to the Fc portion of antibodies attached directly to pathogens or to the surface of infected cells allows enhanced uptake and clearance by cells equipped with an FcγR. Four groups of FcγRs exist in mice: FcγRI (CD64), FcγRIIB (CD32), FcγRIII (CD16), and FcγRIV (CD16-2) of which all but FcγRIIB are activating receptors containing an immunoreceptor tyrosine-based activation motif in their intracytoplasmic domain. In the study, the inhibition of MDA-LDL binding occurred in a dose-dependent manner and was competed for by immune complexes, known ligands of CD16. Of note, CD16 did not bind to OxLDL. The authors also observed decreased MDA-LDL and immune complex binding to macrophages after silencing or knocking out CD16, while OxLDL binding was unchanged between wild-type and $CD16^{-/-}$ macrophages. Lack of CD16 resulted also in reduced secretion of tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and CCL5 upon MDA-LDL stimulation, which was dependent on Syk phosphorylation. Together, the authors showed that CD16 might be

an additional receptor for MDA epitopes next to SR-A1, possibly implying that a redundancy of uptake and clearance mechanisms could be beneficial for an organism to protect from consequences of oxidative stress.

6.2 Proinflammatory Effects of MDA Epitopes

Inflammation is a physiological process caused by the body's own immune mechanisms that have evolved to sense and handle the undesirable presence of both altered endogenous and exogenous stimuli. Resolution of an inflammatory response can only occur when the inflammatory stimuli are cleared and no longer cause any harm. Impaired resolution and/or continuous generation of inflammatory agents might result in the establishment of a chronic inflammation accompanied by persistent tissue damage. Various studies have addressed the proinflammatory capacity of MDA-modified proteins in different cell types including primary cells and cell lines from different animal models. Stimulation of cells derived from rat heart or liver such as endothelial (Duryee et al., 2008; Hill et al., 1998; Thiele et al., 2004) and stellate cells (Kharbanda, Todero, Shubert, Sorrell, & Tuma, 2001) results in the release of TNF-α, MCP-1, macrophage inflammatory protein 2-alpha (MIP2α), and fibronectin or the expression of intercellular adhesion molecule 1 (ICAM-1), major histocompatibility complex (MHC) class II, and vascular cell adhesion molecule (VCAM-1), MCP-1, and MIP-2. In bovine bronchial epithelial cells, MDA epitopes induce interleukin (IL-8) and urokinase-type plasminogen activator (uPA) secretion via protein kinase C (PKC) activation (Kharbanda, Shubert, Wyatt, Sorrell, & Tuma, 2002; Wyatt, Kharbanda, Tuma, & Sisson, 2001). Indeed, several studies demonstrate that the proinflammatory effects induced by MDA epitopes involve not only the activation of PKC (Kharbanda et al., 2002; Wyatt et al., 2012, 2001) but also phosphoinositide 3-kinase (PI3-kinase), Src-kinase (Nikolic et al., 2007), PL-C/IP3 (Cai et al., 2009), and NF-κB (Raghavan, Subramaniyam, & Shanmugam, 2012; Shanmugam et al., 2008) have been implicated. Interestingly, in analogy to cholesterol crystals, MDA epitopes were shown to induce lysosomal rupture upon uptake (Willis et al., 2004) when stimulating J774 macrophages with MDA-modified hen egg lysozyme (MDA-HEL). The authors found that binding of MDA-HEL to the cells occurs in a SR-A1-dependent manner and results in lysosomal rupture followed by cell death. Other studies have shown that treatment of a human hepatic stellate cell line with MDA-modified horse-serum albumin induces activation of ERK1/2 and NF-κB (Kwon et al., 2014). The same

authors also demonstrated that murine Kupffer cells stimulated with MAA-modified human serum albumin secrete IL-6 after 2 h in the presence of LPS, building on an earlier observation that MAA-BSA-stimulated Kupffer cells exhibited a synergistically enhanced cytokine response in the presence of LPS (Duryee et al., 2004). In contrast, no synergistic effect of the TLR2 ligand Pam3CSK4 with MDA-BSA in macrophages could be observed (Saeed et al., 2014).

Additional validation of the proinflammatory capacity of MDA epitopes in vitro is provided in an unbiased approach, where Shanmugam et al. (2008) analyzed conditioned media of THP-1 cells treated with MDA-modified Lys using hybridization-based cytokine arrays. MDA-Lys was found to increase the levels of 20 cytokines, including CCL11, CCL18, CCL28, tumor necrosis factor superfamily member 14 (TNFSF14), MCP-1 and MCP-2. Further in silico analysis of the networks involved using a pathway analysis software revealed that treatment with MDA-Lys induces targets involved in biological processes such as immune response, cellular movement, and cell-to-cell signaling. Using reporter gene assays or chemical inhibitors, the authors showed that MDA-Lys induced activation of NF-κB and p38 MAPK pathways. Furthermore, they observed that incubation of monocytes with MDA-Lys increases their adhesion to smooth muscle or endothelial cells. Together, they demonstrated that MDA-Lys impacts inflammatory gene expression, activation, and motility of monocytes. This was also observed in a separate study using Jurkat T cells stimulated with free MDA (Raghavan et al., 2012).

Next to the expression of cytokines and adhesion molecules, several groups have also investigated motility, morphological changes, and effects on cell viability caused by MDA epitopes. For example, MDA-BSA-coated plates incubated with SR-A1-expressing HEK cells results a spread morphology within 10 min, accompanied by an increase in cell surface area and the formation of membrane extensions after 2 h in a PI3-kinase- and Src-kinase-dependent manner (Nikolic et al., 2007). Morphological changes were also observed in rat heart endothelial cells stimulated with MAA-BSA (Hill et al., 1998) or in J774 macrophages (Willis et al., 2004) and in some cases these morphological alterations are followed by detachment and cell death (Thiele et al., 2004; Willis et al., 2004; Willis, Klassen, Tuma, & Thiele, 2002). In contrast, MDA-LDL only weakly induced the expression of collagen I, collagen III, and fibronectin in human hepatic stellate cells (Schneiderhan et al., 2001), while the cell morphology was found to be unchanged. Also THP-1 cells stimulated with MDA-LDL

displayed even increased cell proliferation in one study (Suzuki, Sasaki, Kumagai, Sakaguchi, & Nagata, 2010), and other researchers found that free MDA induces migration in peripheral blood mononuclear cells and BMDMs after 1 h of incubation (Geiger-Maor et al., 2012), while rat neurons were damaged by free MDA via induction of Ca^{2+} influx (Cai et al., 2009) or via a MAPK pathway in a dose- and time-dependent manner (Cheng, Wang, Yu, Wu, & Chen, 2011). These apparently inconsistent results may be due to the different cell types used in these studies. Nevertheless, there is little doubt about the proinflammatory properties of MDA epitopes in vitro. However, there are only few studies investigating the in vivo effect of MDA epitopes. One study by us showed that MAA-BSA induces a proinflammatory response in the retinal pigment epithelium of mice upon intravitreal injection characterized by a robust increase in KC expression (Weismann et al., 2011). In another murine model of lung injury, MAA-BSA or MAA-modified surfactant D (SPD) was instilled into the lungs of wild-type mice upon which elevated levels of neutrophils and KC were detected in the lung lavage fluid (Wyatt et al., 2012). Even though the amount of in vivo data is scarce, it clearly corroborates the in vitro data demonstrating that MDA epitopes are DAMPs capable of alerting the immune system of high oxidative stress, inflammation, and potential tissue damage by inducing secretion of cytokines.

6.3 Innate Recognition by IgM Natural Antibodies and Complement

Natural antibodies (NAbs) are predominantly of the IgM class and constitute an important component of humoral innate immunity. In addition to their key role in the first line defense against microbial infections, NAbs also play a vital role in the removal of apoptotic cells and metabolic waste to maintain tissue homeostasis (Manson, Mauri, & Ehrenstein, 2005). They are naturally occurring antibodies that can bind microbial structures and stress-induced self-antigens through an evolutionary conserved repertoire of variable regions made out of nonmutated germline genes. In mice, IgM NAbs are secreted by B-1 cells, which constitute a subset of primordial B cells with different surface marker expression, activation requirements, and a distinct anatomical location (Baumgarth, 2011). NAbs are present at birth and detectable already at the fetal stage. They can be found in germ-free mice, indicating that their occurrence is independent of external antigenic stimuli. Nevertheless, their titers can be enhanced by positive antigenic stimulation (Baumgarth, 2011; Baumgarth, Tung, & Herzenberg, 2005).

First insights into the concept that IgM NAbs bind OSEs came from studies in which spleens of 9 months old Apolipoprotein E (Apoe)$^{-/-}$ mice fed a high fat high cholesterol diet for 7 months, which exhibit high titers of OSE-specific IgM, were used to generate hybridoma cell lines (Palinski et al., 1996). About 30% of the hybridoma cell lines producing IgM antibodies were recognizing epitopes of OxLDL. Interestingly, a large part of the clones identified were found to have specificity for MDA-LDL. Detailed characterization of one of the clones termed E06 that specifically binds to PC of oxidized phospholipids of OxLDL demonstrated its CDR3 region to be encoded by the canonical rearrangement of the identical V-D-J germ line genes that encode a previously characterized IgA NAb, called T15 (Shaw et al., 2000). This suggested that many more OSE-specific IgM could be NAbs. Indeed, we could subsequently show that plasma of mice that were kept under complete germ-free conditions contained IgM antibodies with specificity for several OSEs, including MDA-type epitopes (Chou et al., 2009), demonstrating their natural occurrence in the absence of exposure to foreign antigens. Notably, colonization of the gut of these mice with microbiota of conventionally housed mice resulted in the expansion of some, but not all, OSE-specific IgM. The latter is consistent with the fact that many IgM NAbs possess dual reactivity for microbial and self-antigens (Baumgarth, 2011; Racine & Winslow, 2009). Moreover, we could also demonstrate that IgM antibodies in human umbilical cord blood of newborns contained high titers of IgM with specificity for OxLDL and MDA-LDL. In contrast to IgG, IgM are not transported across the placenta. Therefore, IgM in umbilical cord blood are exclusively of fetal origin and therefore can be regarded as human NAbs. Interestingly, compared to the matched maternal plasma of the newborns, the IgM titers to OxLDL and MDA-LDL were significantly enriched in the umbilical cord plasma. Key evidence for the existence of OSE specific and in particular MDA-specific IgM NAbs came from characterization of the binding specificity of IgM derived from murine B-1 cells, isolated from the peritoneal cavities of wild-type mice. Both supernatants of purified B-1 cells stimulated with LPS or IL-5 as well as plasma of recombination-activating gene 1-deficient mice (Rag1$^{-/-}$) (that do not have any functional B or T cells) reconstituted with purified B-1 cells contained IgM with specificity for MDA epitopes. Importantly, ELISpot studies for the frequencies of IgM-secreting cells in the spleens and antigen absorption studies of the plasma IgM of B-1 cell reconstituted mice revealed that ca. 15% of all B-1 cell-derived IgM NAbs have

specificity for MDA epitopes, as well as more complex MAA epitopes, which constitute the majority of OSE-specific IgM NAbs that represent 30% of the NAb repertoire. Finally, sequence analyses of the variable region of a newly cloned anti-MDA IgM mAb, termed NA17, derived from the spleens of these mice, did not reveal nucleotide variation to germline genes in the V_H rearrangements, and only 1 nucleotide insertion between V_L and J_L germline gene segments (Chou et al., 2009). Similarly, previously cloned anti-MDA IgM NAbs (E014, LR04) have also been found to express unmutated germline variable genes (Lichtman, Binder, Tsimikas, & Witztum, 2013). Thus, several lines of evidence identify MDA epitopes as major targets of IgM NAbs.

The high prevalence of MDA-specific IgM NAbs is consistent with an important role in homeostatic housekeeping functions. Indeed, as described earlier, apoptotic cells as well as MV carry MDA epitopes and are recognized by MDA-specific IgM. NAbs have been shown to play a critical role in the clearance of apoptotic cells via complement-dependent mechanisms (Chen, Park, Patel, & Silverman, 2009; Ogden, Kowalewski, Peng, Montenegro, & Elkon, 2005). We could also demonstrate that the MDA-specific IgM NAb NA17 binds apoptotic thymocytes but not viable cells. Moreover, when injected together with apoptotic thymocytes into the peritoneal cavity of mice, NA17 enhances their uptake by peritoneal macrophages in vivo (Chou et al., 2009). It has been suggested that MDA epitopes exposed on apoptotic cells guide the immunosilent and antiinflammatory clearance of apoptotic cells by recruitment of MDA-specific IgMs and corecruitment of C1q (Chen et al., 2009). Thus, IgM NAbs with specificity for MDA have the capacity to engage certain functions of the complement system for host homeostasis.

The complement system represents another humoral component of innate immunity. It is a complex system consisting of about 40 different proteins organized in three major pathways: the classical, the lectin, and the alternative pathway (Ricklin, Hajishengallis, Yang, & Lambris, 2010). Major functions of complement involve the protection of the host from invading pathogens, the orchestration of innate and adaptive immune responses, and housekeeping functions in promoting clearance of apoptotic cells. Immune complexes deposited on pathogenic surfaces initiate the classical pathway, a range of different carbohydrates exposed on pathogens initiate the lectin pathway, and the alternative pathway has a low grade of constitutive activation. Although each complement pathway has a unique way of initiation, they all converge at the step of C3 cleavage, a central component of the

complement, by C3 convertases into a smaller C3a and a larger C3b fragment. Downstream of C3, all pathways are merged into one resulting in the generation of the terminal complement complex (TCC). Clearly, the proteolytic cascade of complement needs to be tightly controlled at several levels to prevent deleterious consequences for the host, which is achieved by a group of proteins called regulators of complement activity (RCA). RCA family members are divided into two major subgroups, soluble ones, such as complement factor H (CFH) and C4-binding protein (C4BP), and membrane-bound ones, including CD46, CD55, CR1, CR2, CR3, and CR4 (Zipfel & Skerka, 2009).

There is now increasing evidence that MDA epitopes also directly trigger specific aspects of complement. Using an unbiased approach to identify MDA-reactive plasma proteins in pull-down assays using MDA-modified polystyrene beads and plasma from $Rag^{-/-}Ldlr^{-/-}$ mice, we identified CFH as a major binding protein for MDA epitopes (Weismann et al., 2011). CFH is a glycoprotein in plasma with a concentration of 500 μg/mL and acts as major regulator of the alternative complement pathway. CFH plays an important complement inhibitory role both in solution and attached to host cell surfaces. CFH regulates complement in three different ways: it disables the formation of the C3 convertase, facilitates its decay, and enhances proteolytic cleavage of C3b to generate inactive iC3b. Employing different ELISAs, we showed that CFH binds to MDA epitopes directly and independently of their protein carrier, but not to PC or 4-HNE epitopes (Weismann et al., 2011). Furthermore, this binding seems to require the presence of advanced MAA epitopes such as MDHDC and is Ca^{2+}- and Mg^{2+}-independent. When CFH is bound to MAA-modified BSA, its complement regulatory activity in factor-I-mediated C3 cleavage is retained, as demonstrated by the fact that MAA-bound CFH had the capacity to cleave C3b into iC3b in the presence of factor I. This is important as opsonization of apoptotic cells with iC3b has been shown to mediate their antiinflammatory clearance by macrophages, and we have demonstrated that binding of CFH to dying cells and blebs is in part mediated by MDA epitopes. CFH is a multidomain molecule composed of 20 domains named short consensus repeats (SCRs) arranged in a "beads on the string" fashion. Using recombinant constructs of CFH, in which different numbers of SCRs had been deleted, we could demonstrate that only SCR7 and SCR19/20 were able to mediate binding of CFH to MAA epitopes. Notably, SCR7 and SCR20 have been described as clustering sites for mutations in CFH that are associated with many complement-related diseases, such as

age-related macular degeneration (AMD), atypical hemolytic uremic syndrome (aHUS), and C3 glomerulopathies (Ricklin & Lambris, 2013). In this regard, we could demonstrate that CFH of carriers of the common CFH SNP rs1061170 that results in an H to Y exchange on position 402 (H402Y variant) exhibits severely impaired binding to MAA in a gene-dosage-dependent manner with >60% decreased binding for homozygous carriers compared to homozygous controls. These findings provided important insights into the strong association of rs1061170 with the risk to develop AMD—a chronic inflammatory disease of the retina that is associated with increased oxidative stress and the most common cause of blindness in the elderly. One may speculate that binding of MAA on apoptotic and necrotic cells by CFH may allow it to inhibit proinflammatory effects of MAA and regulate complement activation on these surfaces. Indeed, we could show that CFH at physiological concentrations has the capacity to inhibit MAA-induced IL-8 secretion by the human monocytic THP-1 cell line. Moreover, the induction of KC expression in the retinal pigment epithelium of mice injected intravitreally with MAA-BSA was completely abolished when CFH was coinjected. Thus, CFH has been shown to scavenge proinflammatory properties of MAA in vitro and in vivo, and to mediate important cofactor activity on MAA-carrying surfaces. Both properties would favor an antiinflammatory removal of potentially harmful dying cells. Recently, Aredo et al. generated transgenic CFH mice carrying human CFH sequence for SCR6–8 (with either 402Y or 402H), flanked by the mouse sequence for SCR1–5 and SCR9–20 (Aredo et al., 2015). Aged mice transgenic for the human CFH H402 variant, in contrast to wild-type mice, had increased accumulation of MDA-modified proteins, increased microglial/macrophage activation, and induction of proinflammatory gene expression in the retina. Similar results were observed when 11 months old mice of both groups where subjected to another model of chronic oxidative stress using hydroquinone diet and exposure to white fluorescent light for 8 weeks (Aredo et al., 2015). These findings support the notion that CFH binding to MDA may play an important role in AMD pathogenesis by modulating the consequences of oxidative stress and inflammation in the retinal pigment epithelium.

Further insights into the potential importance of CFH binding to MDA were provided by studies investigating the effect of SNPs in SCR19–20 of CFH that predispose to the development of aHUS (Hyvarinen et al., 2014). This disease is characterized by the development of microangiopathic hemolytic anemia and thrombocytopenia leading to renal failure

(Nester et al., 2015). In this study, recombinant CFH SCR19–20 constructs harboring various SNPs were tested for their ability to bind MDA-BSA. Eight out of 12 SNPs were found to alter binding to MDA-BSA. The authors suggested that impaired MDA binding observed in these CFH variants may be involved in pathogenesis of aHUS. In addition, CFH SCR19–20 fragments were found to bind to MDHDC epitopes as it has been shown for full-length CFH and SCR7-containing fragments (Hyvarinen et al., 2014; Weismann et al., 2011). Furthermore, the authors suggested that binding of SCR6–8 to MDA-BSA has a different nature of interaction, in contrast to the binding of SCR19–20, as the SCR6–8 could not be inhibited with increasing concentrations of NaCl (Hyvarinen et al., 2014). Although CFH is a single chain molecule with SCRs organized in a "beads on the string" fashion, structural analyses suggest that when CFH is bound to, eg, C3b on cellular surface it is bent, and SCR4 and SCR9 are brought together in close proximity (Kajander et al., 2011; Wu et al., 2009). It is not known how the 3D structure of CFH looks when it is bound to MDA, but it can be speculated that both domains (SCR7 and SCR19/20) cooperate in mediating binding to MDA, although both SCRs may bind to MDA epitopes in a different manner. The binding affinity of CFH to MAA-BSA, which actually represents the avidity of the whole molecule, was found to be ~62 nM (Weismann et al., 2011).

In addition to SCR-containing complement proteins, it has also been shown that recombinant human C3a, a proinflammatory anaphylatoxin, binds specifically to MDA- and MAA-modified LDL or BSA (Veneskoski et al., 2011). The same authors also showed that MAA- and MDA-modified LDL comigrated with C3a in gel shift assays and facilitated scavenging of C3a by J744A1 macrophages. Using ELISA, they demonstrated that C3a was binding to MDA- and MAA-modified proteins (HDL and LDL) but not to PC-modified proteins, suggesting that MAA/MDA epitopes on OxLDL mediate binding to C3a. Additionally, we also tested the binding of plasma purified C3 to MAA-BSA by ELISA, but could not observe any binding. These data suggest that only upon cleavage of C3, the C3a fragment can bind to MDA (Weismann et al., 2011).

Thus, certain complement regulators and complement effectors bind to MDA and MAA epitopes in particular, which are present on OxLDL, apoptotic cells, and MV (Veneskoski et al., 2011; Weismann et al., 2011). While CFH has been shown to limit MAA-induced inflammation, future studies need to explore the effects of MAA-decorated surfaces on complement activation in general. Because MDA/MAA epitopes are major targets of IgM

NAbs as well as CFH, they may represent important hubs on dying cells and oxidized lipoproteins allowing complement to mediate critical housekeeping functions to maintain host homeostasis. The fact that MDA/MAA epitopes are recognized by membrane PRR, such as SR-A1, as well as soluble PRPs, such as CFH and IgM NAbs, identifies them as potent regulators of tissue homeostasis and as novel DAMPs of innate immunity (Fig. 4).

7. ADAPTIVE IMMUNITY RESPONSES ON MDA EPITOPES

In contrast to innate immunity, adaptive immunity is acquired throughout life following exposure to specific antigens. This results in the generation of a nearly unlimited repertoire of receptors with high antigen specificity and the generation of immune memory, which is carried out by various subsets of CD4+ Th and CD8+ Tc as well as B cells and the antibodies they secrete (Lichtman et al., 2013). Different subsets of Th cells, including IFNγ- and TNF-α-secreting Th1 cells; IL-4, IL-5, IL-10, and IL-13-secreting Th2 cells; and IL-17-secreting Th17 cells, have been described.

A multitude of studies exists demonstrating that active immunization with MDA-modified proteins or lipoproteins triggers adaptive immune responses. These characterizations were mainly done in atherosclerosis-prone rabbits and mice. Moreover, adaptive IgG antibodies with specificity for MDA have been documented in humans and several animal models of atherosclerosis as well as models of ethanol-induced chronic inflammatory tissue injury (Tuma et al., 1996; Xu et al., 1997). Based on this, Witztum and colleagues initiated studies to demonstrate the possibility to induce hapten-specific immune response against MDA epitopes. Both antisera (MAL-2) and monoclonal IgG antibodies (MDA2) could be generated in guinea pigs and mice immunized with MDA-modified homologous LDL (Palinski et al., 1989, 1990). These data suggested that the robust production of hapten-specific IgG antibodies occurs in a T cell-dependent manner. We could show that immunization of mice with homologous MDA-LDL induced the robust production of T cell-dependent IgG antibody titers with specificity for MDA epitopes. Moreover, this immunization in which MDA-LDL was emulsified in complete Freund's adjuvant for the primary and incomplete Freund's adjuvant for the boosting injections resulted in a preferential production of Th2-dependent IgG1, whereas Th1-dependent IgG2a/c antibodies were only moderately induced. Antigen stimulation assays of splenocytes of immunized mice led to a secretion of primarily

IL-5, IL-10, IL-13 and to a much lower degree IFNγ following stimulation with MDA-LDL, but not native LDL, consistent with a Th2-biased immune response (Binder et al., 2004; Gonen et al., 2014). The studies also demonstrated the induction of T cells specific for MDA epitopes, which likely are recognized by T cells in the context of a specific peptide sequence. Indeed, Wuttge et al. showed that subcutaneous immunization of nude mice that lack T cells with MDA-modified autologous albumin fail to mount specific IgG responses (Wuttge, Bruzelius, & Stemme, 1999). We have confirmed the requirement of T cells for IgG responses triggered by MDA-LDL, as immunization of MHC class $II^{-/-}$ or T-cell receptor $\beta^{-/-}$ mice with MDA-LDL failed to induce robust IgG antibody titers (Binder et al., 2004). In addition, immunization of $CD4^{-/-}$ $Apoe^{-/-}$ mice with MDA-LDL results in reduced IgG1, IgG2a/c, and IgM antibody titers to MDA-LDL (Zhou, Robertson, Rudling, Parini, & Hansson, 2005). Notably, the remarkable Th2 dominated immune response to MDA-LDL induced by immunization occurs independently of the carrier, and can be observed also when other proteins, eg, mouse serum albumin, modified with other adducts, such as MAA and propanal are used as antigens (Gonen et al., 2014). Nevertheless, MAA-modified proteins have been found to induce the strongest antibody responses. The strong immunogenicity of MAA epitopes in particular has been demonstrated before in several immunization studies of various organisms (bovine, human, rabbit, and mice), as immunization with MAA-modified homologous albumin induced specific antibodies even in the absence of any adjuvant (Thiele et al., 1998). Of considerable interest is the fact that immunization of SR-$A1^{-/-}$ mice with MAA-BSA resulted in a reduced generation of anti-MAA-BSA antibodies compared to control wild type, suggesting that SR-A1-mediated uptake of MDA-modified proteins by antigen-presenting cells is critical (Duryee et al., 2005). Thus, both endogenous and exogenous MDA/MAA haptens have the capacity to induce robust adaptive immune responses, which are characterized by antibody production and induction of specific Th cells.

8. MDA EPITOPES IN DISEASES

There is a growing list of diseases in which increased levels of MDA have been detected using various methods (Bhuyan, Bhuyan, & Podos, 1986; Brown & Kelly, 1994; Dei et al., 2002; Dexter et al., 1989; Gonenc et al., 2001; Grigolo, Roseti, Fiorini, & Facchini, 2003; Haider

et al., 2011; Imai et al., 2008; Jain, McVie, Duett, & Herbst, 1989; Odhiambo et al., 2007; Pemberton et al., 2004; Schoenberg et al., 1995; Shimizu et al., 2001; Sikar Akturk et al., 2012; Tuma et al., 1996; Valles, Aznar, Santos, & Fernandez, 1982; Wade, Jackson, Highton, & van Rij, 1987; Weismann et al., 2011; Yla-Herttuala et al., 1989). MDA has been documented in both chronic and acute diseases associated with high levels of oxidative stress, such as cardiovascular, neurodegenerative, metabolic, and communicable diseases (Fig. 5). However, the levels of MDA determined in the plasma of healthy individuals have shown great variability, in part due to the method of blood drawing and sample preparation (Del Rio, Stewart, & Pellegrini, 2005). Moreover, the detection methods for MDA still possess a number of limitations. For example, the most commonly used method for MDA detection, the thiobarbituric acid reactive substances assay, has long been known to be nonspecific (Esterbauer et al., 1991). Although more reliable methods for detecting MDA levels with high specificity exist including MDA-specific antibodies or assays based on mass spectrometry (Zagol-Ikapite et al., 2015), it still remains unclear if the detected changes of MDA are reliable biomarkers for all these diseases.

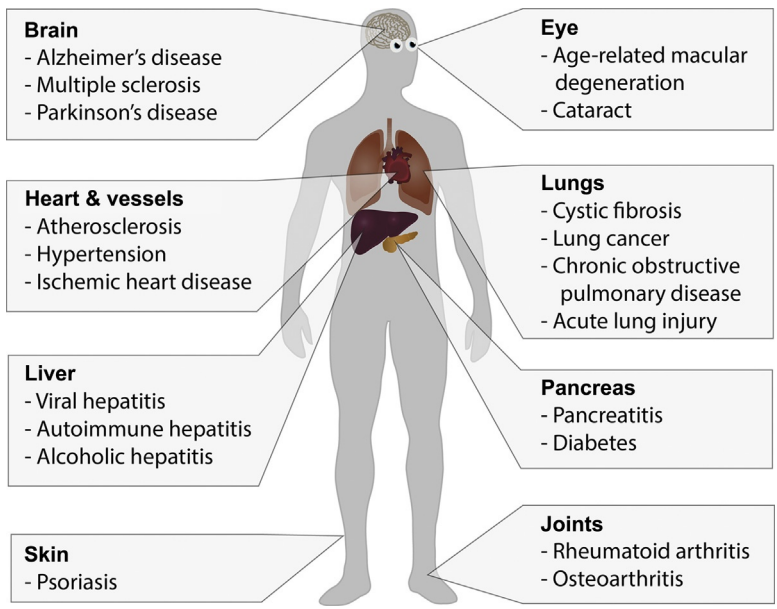

Fig. 5 MDA epitopes in diseases. MDA epitopes can be found in many pathological settings affecting multiple organs including neurodegenerative diseases, metabolic diseases, cancers, and also infectious diseases.

9. RELEVANCE OF OSEs IN CARDIOVASCULAR DISEASE

CVDs are among the most-studied diseases with respect to a role for MDA epitopes. CVDs constitute the major cause of mortality and disability worldwide (Mendis et al., 2011). Generally, CVDs can be divided into two major subgroups, CVDs that arise due to atherosclerosis (stroke, heart attack, hypertension, and peripheral vascular disease) or CVDs that are independent of atherosclerotic process (congenital and rheumatic heart diseases, cardiomyopathies, and arrhythmias). According to the WHO, CVDs with atherogenic origin are responsible for 80% of CVD deaths.

Atherosclerosis is a chronic inflammatory disease of large- and medium-sized arteries and the underlying cause for heart attacks and strokes. Several nonmodifiable and modifiable risk factors, such as hypercholesterolemia, hypertension, diabetes, smoking, gender, and age have been identified. Atherosclerotic lesion formation is sustained by high levels of plasma LDL cholesterol that are deposited in the artery wall, where an inflammatory reaction is triggered. These initial fatty streak lesions can progress to more complex plaques with many inflammatory infiltrates and large acellular necrotic areas. Once these advanced plaques become unstable they are prone to rupture, which triggers a local thrombotic event that can ultimately lead to a heart attack or stroke.

LDL belongs to a group of lipoprotein particles ranging in size from 18 to 25 nm with the major purpose to transport cholesterol in the circulation. It contains a single ApoB100 molecule (with 4536 amino acids, of which 356 are Lys) and a lipid part consisting of phospholipid and cholesterol molecules. Atherogenesis is initiated by endothelial cell dysfunction and intimal retention of LDL. Once LDL is trapped in the subendothelial space of the arteries it undergoes several types of modifications, most prominently enzymatic and nonenzymatic oxidation leading to the generation of OxLDL. OxLDL itself triggers proinflammatory responses in endothelial cells and enhanced recruitment of monocytes into the intima of the artery wall (Gerrity, 1981; Quinn, Parthasarathy, Fong, & Steinberg, 1987). Recruited monocytes then differentiate into macrophages that further propagate the oxidation of LDL through 12/15-lipoxygenase (12/15-LO) and myeloperoxidase (MPO) pathway, thus enhancing the proatherogenic inflammatory cascade. OxLDL is then taken up by macrophages using an array of SRs expressed on their surface, such as CD36 and SR-A1 (Greaves & Gordon, 2009; Moore, Sheedy, & Fisher, 2013). An impaired

balance between cellular uptake and efflux of cholesterol results in the transformation of lesional macrophages into lipid-laden foam cells—hallmark cells of atherosclerosis. Consequently, intracellular accumulation of OxLDL results in the formation of cholesterol crystals that trigger lysosomal rupture and activation of the inflammasome. In addition, intracellular accumulation of free cholesterol can enhance ER stress and promote an unfolded protein response that triggers apoptosis foam cell macrophages (Feng et al., 2003). Usually, lesional apoptotic cells are silently cleared by professional phagocytes, but once the efferocytotic mechanism becomes overloaded either with a multitude of apoptotic cells or excess of OxLDL this emanates in impaired clearance of apoptotic cells. Uncleared apoptotic cells then undergo secondary necrosis, lose membrane integrity, and release cellular debris and DAMPs, further propagating the inflammatory response. Thus, OxLDL, apoptotic cells, and cellular debris accumulate in atherosclerotic lesions and sustain the inflammatory process (Glass & Witztum, 2001; Hartvigsen et al., 2009; Tabas, 2010; Tsiantoulas, Diehl, Witztum, & Binder, 2014). Moreover, in addition to innate immune responses adaptive immunity also plays a role in atherogenesis (Lichtman et al., 2013). T cells—most prominently IFNγ-secreting Th1 cells—have been found in lesions and were demonstrated to promote atherogenesis. Although B cells are rarely found in lesions, their modulatory roles on atherogenic process have recently gained much attention.

10. MDA EPITOPES IN ATHEROSCLEROSIS

During the oxidative modification of LDL, the phospholipid and cholesterol moiety of LDL undergoes lipid peroxidation, which leads to the generation of many different OSEs, including MDA epitopes. OxLDL has been shown to be proinflammatory and immunogenic, suggesting that it is a major driver of the inflammatory process during atherogenesis. Indeed, the characterization of OSEs as novel class of DAMPs has provided important insights into the sterile inflammatory process of atherosclerosis. Accumulating evidence suggests that MDA epitopes represent critical mediators of inflammation in atherosclerosis.

The fact that atherosclerotic lesions contain MDA epitopes has been known for more than 30 years. Using a monoclonal antibody raised against MDA-Lys residues, the presence of MDA epitopes was demonstrated in atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits (Haberland et al., 1988), and later Yla-Herttuala et al. (1989) have shown that LDL in

rabbit and human lesions is in fact oxidized and a major carrier of MDA epitopes. MDA-modified LDL—in contrast to native LDL—possesses a strong chemotactic potential and can be engulfed more readily by macrophages (Yla-Herttuala et al., 1989). Consistent with findings in rabbits and humans, lesions from Apoe$^{-/-}$ mice were shown to contain MDA epitopes in regions rich in macrophages as well as in the necrotic core, which is an area replete with apoptotic cells (Palinski et al., 1994). Possibly, accumulating apoptotic cells prominently contribute to MDA epitopes in atherosclerotic lesions. Indeed, we have recently shown that the levels of plasma MV isolated from the culprit lesion site of patients suffering a myocardial infarction are increased and enriched in MDA epitopes compared to plasma MV of the same patients obtained from the periphery (Tsiantoulas et al., 2015). Additionally, it has been shown that HDL isolated from human atherosclerotic tissues is enriched in MDA epitopes compared to HDL from plasma of healthy donors. Modification of ApoAI, the major protein moiety of HDL, with MDA in vitro results in dysfunctional HDL. MDA epitopes on ApoAI cause structural and conformational changes, thereby impairing its ability to interact with ABCA1 and promoting cholesterol efflux onto HDL, which is a homeostatic mechanism to remove access cholesterol from macrophages. Thus, oxidative modification of ApoAI by MDA impairs an important cardioprotective function of HDL, which may also contribute to atherosclerosis development (Shao et al., 2010).

Inflammation in vascular lesions is considered a key driver of atherogenesis. Major carriers of MDA epitopes, such as OxLDL, MV, and dying cells have been shown to possess robust proinflammatory properties, and there is accumulating evidence that MDA epitopes themselves are mediators of these effects. For example, the cytotoxic effect of OxLDL on endothelial cells can be blocked by a polyclonal antibody against MDA but not by a polyclonal antibody against ApoB100. Moreover, it was shown that MDA epitope-mediated OxLDL cytotoxicity was partially dependent on Akt pathway activity (Yang et al., 2014). Additionally, the direct proinflammatory nature of MDA epitopes has been demonstrated by several groups. As discussed earlier, treatment of THP1 cells with MDA-Lys resulted in strong activation of NF-κB activity as well as the activation of signaling pathways related to inflammation, cellular motility, and cell-to-cell signaling. Furthermore, MDA-Lys also increased monocyte binding to vascular smooth muscle and endothelial cells (Shanmugam et al., 2008). Additionally, MAA-BSA induced expression of proinflammatory cytokines IL-8, IL-1β, TNF-α, and IL-12β in THP-1 cells (Weismann et al., 2011).

All these cytokines have been shown to play an important role in atherosclerotic lesion formation (Ait-Oufella, Taleb, Mallat, & Tedgui, 2011). For example, IL-8 or CXCL1 promotes leukocyte recruitment to the vascular wall and atherosclerosis-prone mice deficient in either Cxcl1 or its receptor Cxcr1 develop significantly reduced atherosclerosis. Interestingly, also MDA-carrying MV were able to induce IL-8 secretion in human monocytes, and this effect could be neutralized in the presence of the MAA-specific IgM NAb LR04, suggesting that MAA epitopes are in part responsible for the proinflammatory effect of MV (Tsiantoulas et al., 2015). All these data strongly support the fact that MDA epitopes act as DAMPs that modulate lesional inflammation and have a fundamentally important role in the process of atherogenesis.

10.1 MDA Immunization Protects from Atherosclerosis

The importance of MDA in atherogenesis has been demonstrated in a large number of immunization studies, which showed induction of atheroprotective immunity by immunization with MDA antigens. These studies indicate that endogenously generated MDA is relevant for atherogenesis, as the immune response against MDA specifically mediates protection. For example, immunization of WHHL rabbits with MDA-modified homologous LDL resulted in increased antibody titers to MDA epitopes and atheroprotection, in contrast to immunization with KLH (Palinski, Miller, & Witztum, 1995). Similar atheroprotective effects as well as increased antibody levels were observed in immunization studies of atherosclerosis-prone Apoe$^{-/-}$ and Ldlr$^{-/-}$ mice (Binder et al., 2004; Fredrikson et al., 2003; Freigang, Horkko, Miller, Witztum, & Palinski, 1998; George et al., 1998; Gonen et al., 2014; Zhou, Caligiuri, Hamsten, Lefvert, & Hansson, 2001; Zhou et al., 2005). Apoe$^{-/-}$ mice immunized with homologous MDA-LDL develop high titers of anti-MDA-LDL IgG antibodies and had 50% less lesional area at the aortic sinus compared to control mice injected with PBS (George et al., 1998). Additionally, Ldlr$^{-/-}$ mice immunized with homologous MDA-LDL or native LDL developed high IgM and IgG titers to MDA-LDL and had attenuated atherosclerosis. Interestingly, immunization with homologous native LDL also protected from atherosclerosis without inducing high titer of antibodies, suggesting a different mechanism for atheroprotection with native LDL (Freigang et al., 1998). Subsequently, seven peptides within human ApoB100 were shown to be immunogenic and were recognized by IgM and IgG autoantibodies in human serum (Fredrikson et al., 2003). In a follow-up study,

immunization of Apoe$^{-/-}$ mice with a mixture of two peptides of human ApoB100 (peptides 142 and 210 with 85–90% similarity to the mouse ApoB100 sequence) resulted in a reduction of plaque area by 60%, in contrast to control immunized mice. Immunization with ApoB100 peptides increased IgG titers against MDA-modified peptides, suggesting that the peptides used as antigens became modified with MDA in vivo (Fredrikson et al., 2003). The importance of MDA and MAA as a key antigen has been demonstrated by the fact that immunization of Apoe$^{-/-}$ mice with plaque homogenates from Apoe$^{-/-}$ mice triggers MDA-specific antibodies and reduces lesion formation. More precisely, immunization with MDA-LDL or plaque homogenates led to increased T cell-dependent IgG antibodies recognizing MDA-LDL, the titers of which were found to negatively correlate with plaque size or serum cholesterol levels (Zhou et al., 2001). In an attempt to identify the exact requirements for the atheroprotective effect of immunization with MDA-LDL, extensive immunization studies with different types of related antigens have been performed. In these studies, Ldlr$^{-/-}$ mice were immunized with MDA-LDL (MDA-LDL), MDA-modified murine serum albumin (MDA-MSA), MAA-MSA, propanal-modified MSA, native MSA, and PBS, and then fed an atherogenic diet for 28 weeks. The most robust reduction in aortic lesion area (40%) compared to PBS control was observed when MAA-MSA was used as an immunogen. Despite similar induction of hapten-specific immune responses with the other antigens, only mice immunized with MDA-LDL and MDA-MSA had a significant reduction of lesion area, albeit to a lesser extent than immunization with MAA-MSA. Notably, immunization with MDA-LDL also induced antibodies that bound to MAA-modified peptides. This observation led to the conclusion that MAA epitopes are the immunodominant epitopes inducing atheroprotective immune responses. Thus, MAA hapten-specific immunity mediates the protective effect of immunization with MDA-LDL, independent of the carrier protein.

Several studies tried to characterize the mechanisms responsible for the protective effect of immunization. For example, as discussed earlier, we could show that immunization of both wild type and atherosclerosis-prone mice with homologous MDA-LDL and MAA-MSA induces a robust Th2-biased immune response that is dominated by MDA-specific IgG1 antibodies (Binder et al., 2004). High titers of antibodies may mediate atheroprotection, as infusion of IEI-E3, a human IgG1 antibody that was selected against MDA-modified peptides of human ApoB100, into

Apoe$^{-/-}$ mice for 4 weeks resulted in decreased atherosclerotic lesion formation compared to infusion of an isotype control (Schiopu et al., 2007). Moreover, infusion of another human recombinant IgG1 (2D03) recognizing the MDA modifications on the same human ApoB100 peptide also reduced atherosclerosis and constrictive injury-induced remodeling in the carotid artery of Ldlr$^{-/-}$ mice (Strom et al., 2007). Using the human-derived anti-OxLDL IgG Fab IK17 specific for MDA epitopes it was demonstrated that IK17 inhibits the uptake of OxLDL by macrophages in vitro (Shaw et al., 2001). In addition, infusion of an adenoviral construct overexpressing recombinant IK17 scFv into cholesterol-fed Ldlr$^{-/-}$Rag-1$^{-/-}$ mice resulted in decreased peritoneal foam-cell formation and reduced atherosclerosis (Tsimikas et al., 2011). These antibodies have been suggested to block foam cell formation and protect from atherosclerosis. On the other hand, Th2 cytokines, such as IL-5 and IL-13, induced by MDA-immunization possess important atheroprotective properties. For example, IL-5 stimulates B-1 cells to secrete IgM NAb in a noncognate manner. A large part of IgM NAbs have specificity for OSEs (Chou et al., 2009), and atherosclerosis-prone mice unable to secrete IgM antibodies develop accelerated atherosclerosis (Lewis et al., 2009). Hematopoietic IL-5 deficiency in Ldlr$^{-/-}$ mice leads to increased atherosclerosis and reduced levels of specific atheroprotective IgM NAb, T15/E06. Furthermore, we also showed that IL-13 protects mice from atherosclerotic lesion formation by promoting alternative activation of lesional macrophages that are more efficient in removal of OxLDL (Cardilo-Reis et al., 2012). Moreover, it has been suggested that immunization with MDA-LDL also induces regulatory T cells, which could suppress the secretion of the proatherogenic cytokine IFNγ by specific Th1 cells. Nevertheless, Zhou et al. showed that immunization of CD4$^{-/-}$Apoe$^{-/-}$ mice that lack Th cells with homologous MDA-LDL still induced atheroprotection (Zhou et al., 2005). On the other hand, adoptive transfer of CD4+ Th cells from MDA-LDL immunized mice into Apoe$^{-/-}$ SCID mice resulted in accelerated atherosclerosis compared to transfer of T cells from control immunized mice. Thus, the true effects of Th-dependent immunity to MDA-LDL remain to be elucidated in detail. Clearly, a feature common to all immunization studies is the production of MDA-specific antibodies at much higher levels than the ones occurring naturally and in the course of diet feeding.

In the past 25 years, many epidemiological studies assessed associations of IgG and IgM antibodies to OxLDL and MDA-LDL epitopes with the development of coronary, carotid, and peripheral artery disease (Salonen et al.,

1992; Tsimikas et al., 2003; Tsimikas, Witztum, et al., 2004; Wu et al., 1997). These studies have often been very inconsistent due to lack of standardized antigens or assays, and because tested cohorts of patients were simply too small or not followed prospectively (Tsimikas et al., 2012). Some of these inconsistencies could be overcome with the use of recombinant peptide mimotopes of MAA that serve as standardized antigens for MAA-specific antibodies (Amir et al., 2012). Nevertheless, studies in humans have provided insights into the role of these immune responses in CVD. While the role of IgG antibodies to MDA-LDL is less clear, there is now substantial evidence that IgM antibodies to MDA-LDL are inversely associated with atherosclerosis or clinical manifestations thereof (Gounopoulos, Merki, Hansen, Choi, & Tsimikas, 2007; Tsiantoulas et al., 2014). These data suggest that MDA-LDL-specific IgM antibodies may mediate protective functions in atherosclerosis. Interestingly, MDA-specific IgM antibodies have been found to be higher in people younger than 65, females, and nondiabetics (Fraley et al., 2009; Tsimikas, Lau, et al., 2004). Moreover, many if not most IgM antibodies with specificity for MDA-LDL may be IgM NAbs.

10.2 MDA-Specific Natural Immunity Protects from Atherosclerosis

As discussed earlier, IgM NAbs are important mediators of MDA-specific immunity, and several potential mechanisms on how they can mediate atheroprotective effects have been suggested. First, IgM with specificity for MDA/MAA have been shown to act antiinflammatory. We could demonstrate that the MAA-specific IgM NAb LR04 significantly decreased the ability of platelet-derived MV to induce IL-8 secretion by THP-1 cells and primary human monocytes (Tsiantoulas et al., 2015). IL-8 is a key mediator of leukocyte recruitment to the vascular wall and chief proatherogenic chemokine (Ait-Oufella et al., 2011). Thus, inhibition of MDA/MAA-induced IL-8 secretion by endothelial cells and monocytes may have an important modulatory function in atherogenesis. Furthermore, MDA-specific IgM may also protect by promoting clearance of dying cells. Indeed, defective phagocytic clearance of dying cells in atherosclerotic lesions has also been implicated in the progression of atherosclerosis (Tabas, 2010; Van Vre, Ait-Oufella, Tedgui, & Mallat, 2012). For example, $Ldlr^{-/-}$ mice reconstituted with bone marrow of mice deficient in the milk fat globule-EGF factor 8 protein ($Mfge8^{-/-}$), which facilitates PS-mediated clearance of apoptotic cells, developed increased lesion size with more

accumulating apoptotic cells and increased necrotic areas (Ait-Oufella et al., 2007). A similar effect has been observed in Apoe$^{-/-}$ mice also deficient in MER proto-oncogene tyrosine kinase (Thorp, Cui, Schrijvers, Kuriakose, & Tabas, 2008). The ability of MDA-specific IgM to modulate efferocytosis has been addressed in the studies by Chen et al. (2009) and Chou et al. (2009). For example, incubation of apoptotic thymocytes with wild-type mouse serum in the presence of MDA-BSA resulted in diminished deposition of IgM and C1q on apoptotic cell surfaces and consequently led to decrease in complement-mediated phagocytosis by dendritic cells (Chen et al., 2009). To explain this, the authors suggested that MDA-BSA competed for the binding to MDA epitopes on apoptotic cells, on which they may act as hubs for activation of complement-mediated efferocytosis via IgM and C1q axis. We could directly demonstrate a role for MDA-specific IgM NAbs in apoptotic cell clearance in an in vivo clearance assay in Rag1$^{-/-}$ mice, deficient in B- and T-cell populations. Intraperitoneal injection of apoptotic thymocytes preincubated with the IgM NAb NA17 resulted in significantly enhanced phagocytosis by macrophages compared to apoptotic thymocytes preincubated with control IgM (Chou et al., 2009). The importance of this mechanism is further supported by the fact that soluble IgM deficiency (Lewis et al., 2009) as well as C1q deficiency aggravates atherosclerosis in atherosclerosis-prone mice (Bhatia et al., 2007). Thus, MDA epitopes may act as marker of metabolic waste and dying cells in atherosclerotic lesions, allowing the immune system to mediate housekeeping functions (Chang et al., 1999; Chen et al., 2009; Chou et al., 2009; Weismann et al., 2011).

Not only IgM antibodies have the capacity to protect from MDA-induced inflammation. Additionally, CFH that colocalizes in human coronary lesions with MDA epitopes has been shown to neutralize MDA-induced expression of proinflammatory genes, including IL-8. Interestingly, a few studies have shown an association of the 402H allele, which reduces MDA binding of CFH (Weismann et al., 2011), with increased cardiovascular risk. However, a metaanalysis of eight different study populations failed to find a significant association of this gene variant with CVDs (Sofat et al., 2010). Nevertheless, additional SNPs of CFH that can affect its capacity to bind MDA have been identified and only functional data on MDA binding of CFH in plasma may provide insights into a potential association of this CFH function with CVD. In support of this, we could demonstrate that CFH bound on MDA-decorated surfaces still can mediate cofactor activity for Factor I to inactivate C3b into iC3b. In turn, freshly deposited inactive iC3b promotes antiinflammatory clearance of opsonized

particles (Weismann et al., 2011). Thus, CFH may protect from atherosclerosis by binding to MDA epitopes, thereby reducing inflammation and additionally increasing opsonization with iC3b, which facilitates efferocytosis. Studies in animal models of atherosclerosis may provide critical insights into these functions.

11. MDA EPITOPES AS THERAPEUTIC TARGETS IN CARDIOVASCULAR DISEASE

Current therapies in CVD mainly aim at lowering LDL plasma levels by increasing the availability of the LDL receptor at the cell surface using, for example, statins or PCSK9 antibodies. Nevertheless, an important contribution of the innate and adaptive immunity to the development of atherosclerosis has become evident. With two ongoing clinical studies, the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) and the Cardiovascular Inflammation Reduction Trial (CIRT), we are currently witnessing a premiere in regard to clinical trials addressing the role of inflammation in CVD. CANTOS and CIRT are the first studies which aim at preventing recurrent cardiovascular events in patients by reducing inflammation without affecting plasma cholesterol levels using low-dose methotrexate or a neutralizing anti-IL-1β antibody (canakinumab), respectively. Other potentially interesting though preclinical immunomodulatory approaches for therapeutic intervention in atherosclerosis have been reviewed elsewhere (Amir & Binder, 2010; Lichtman et al., 2013; Miller & Tsimikas, 2013; Witztum & Lichtman, 2014).

As OSEs have been implicated as potential antigens triggering innate and adaptive immune responses in atherosclerosis, targeting these structures by active or passive immunization in order to reduce the inflammatory burden could provide a novel therapeutic approach to treat atherosclerosis. Indeed, several lines of evidence point toward a protective effect of increasing antibody titers against OSEs. Interestingly, active immunization using a single OSE, MDA-LDL as an immunogen is sufficient to protect from atherosclerosis as demonstrated by a number of studies in animals (Binder et al., 2004; Freigang et al., 1998; George et al., 1998; Gonen et al., 2014; Palinski et al., 1995; Zhou et al., 2001). Even more intriguing is the fact that this protective effect seems to be independent of the carrier presenting MDA epitopes on its surface as several studies showed that immunization using various carrier proteins all modified with MDA epitopes results in less atherosclerosis, including MDA-modified ApoB100-peptides (Fredrikson et al., 2003), MDA-modified laminin (Duner et al., 2010) and fibronectin

(Duner et al., 2011), or killed *Porphyromonas gingivalis* (Turunen et al., 2012), a pathogen-carrying surface structures that mimic MDA epitopes. Therefore, the identification of MDA epitopes as critical structures conveying atheroprotection through immunization could allow for the development of more standardized immunogens benefiting from lower variability and thus higher reproducibility. Accordingly, using phage display libraries we have identified an MDA mimotope, the P2 peptide, and demonstrated that immunization with P2 induces MDA-specific antibodies, which bind to human atherosclerotic lesions (Amir et al., 2012). On the other hand, several studies reported that passive immunization by therapeutic infusion of anti-MDA antibodies also had beneficial effects by reducing plaque inflammation and regression of atherosclerotic lesions (Schiopu et al., 2007; Tsimikas et al., 2011). Even though animal studies have convincingly shown that vaccination using MDA epitopes is protective in atherosclerosis, translation to human settings has proven to be difficult. Not only do critical differences between rodents and humans exist in terms of lipoprotein metabolism during atherogenesis, which advises against a direct implementation of experimental setups to the clinics, but the ubiquitous presence of MDA epitopes in the body also complicates any human vaccination study because the generation of antibodies against continuously formed MDA epitopes could lead to unwanted side effects of the vaccination in the long term given that we still lack a deeper understanding of the mechanisms involved.

Meanwhile, other approaches offering protection from pathologically elevated levels of MDA epitopes might be worth of consideration. Several studies demonstrated that endogenous production of natural IgMs can be boosted by treatment with IL-18 (Kinoshita et al., 2006), IL-25 (Mantani et al., 2015), and IL-5 (Binder et al., 2004), by genetic deficiency of sialic acid-binding Ig-like lectin G (Siglec G), a negative regulator of the B cell receptor present also on B1a cells (Hoffmann et al., 2007), and by additional strategies yet to be elucidated. While many of these approaches are not specific or even potentially harmful, identification of pathways that specifically regulate IgM NAb production may provide novel therapeutic opportunities. In addition, the MDA-neutralizing properties of CFH could be harnessed for therapeutic purposes as we have shown that CFH protects from MDA-induced inflammation in a mouse model of wet AMD by binding to MDA epitopes (Weismann et al., 2011). Treatment of patients possessing a CFH variant with impaired binding using CFH or truncated CFH constructs which contain only a fraction of the protein including the critical MDA-binding region SCR19/20 could help protecting from

MDA-mediated inflammation. Indeed, such constructs, termed mini CFH, have demonstrated their therapeutic potential in mouse models of C3 glomerulopathy (Nichols et al., 2015) and we speculate that their neutralizing capabilities could be also beneficial in atherosclerosis. Regardless of the successes in animal models, the feasibility of these approaches in human patients has to be taken with caution as the treatment of a chronic disease that develops over decades might result in long-term complications. Nevertheless, in cases where only short-term treatment is required or for individuals at high risk, the benefits could outweigh the disadvantages.

12. CONCLUSIONS

Lipid peroxidation of cellular membranes results in the generation of various OSEs that act as downstream mediators of oxidative stress in tissues. In this chapter, we have discussed MDA epitopes as a prominent and important example. This includes evidence that they: (1) are proinflammatory danger signals for a wide range of cell types, (2) can be recognized by multiple arcs of innate immunity with critical roles in housekeeping functions, and (3) are potent immunogens, which elicit protective immune response in mouse models of atherosclerosis. We speculate that MDA epitopes present on dying cells and biomolecules damaged by oxidative stress serve as waste markers for the immune system, flagging their carriers for complement-mediated rapid and silent clearance by professional phagocytes. However, excessive production of MDA epitopes and/or impaired clearance capabilities results in their nonphysiological accumulation and subsequently elicits proinflammatory responses, which are sustained as long as the carriers of MDA epitopes cannot be neutralized or scavenged—a condition most likely occurring in many if not all chronic inflammatory diseases that are associated with impaired resolution, such as atherosclerosis.

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2.2 Review Article: Malondialdehyde Epitopes as Mediators of Sterile Inflammation

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Biochim Biophys Acta. 2017 Apr;1862(4):398-406. doi: 10.1016/j.bbalip.2016.06.016. Review. PubMed PMID: 27355566.

Biochimica et Biophysica Acta 1862 (2017) 398–406

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Review

Malondialdehyde epitopes as mediators of sterile inflammation☆

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ARTICLE INFO

Article history:
Received 6 April 2016
Received in revised form 21 June 2016
Accepted 24 June 2016
Available online 26 June 2016

Keywords:
Malondialdehyde
Innate immunity
Inflammation
Atherosclerosis
Lipid peroxidation
Oxidative stress

ABSTRACT

Enhanced lipid peroxidation occurs during oxidative stress and results in the generation of lipid peroxidation end products such as malondialdehyde (MDA), which can attach to autologous biomolecules, thereby generating neo-self epitopes capable of inducing potentially undesired biological responses. Therefore, the immune system has developed mechanisms to protect from MDA epitopes by binding and neutralizing them through both cellular and soluble effectors. Here, we briefly discuss innate immune responses targeting MDA epitopes and their pro-inflammatory properties, followed by a review of physiological carriers of MDA epitopes that are relevant in homeostasis and disease. Then we discuss in detail the evidence for cellular responses towards MDA epitopes mainly in lung, liver and the circulation as well as signal transduction mechanisms and receptors implicated in the response to MDA epitopes. Last, we hypothesize on the role of MDA epitopes as mediators of inflammation in diseases and speculate on their contribution to disease pathogenesis. This article is part of a Special Issue entitled: Lipid modification and lipid peroxidation products in innate immunity and inflammation edited by Christoph J. Binder.

1. Introduction

All cellular membranes consist to a large part of phospholipids that separate inside from outside, and cytoplasm from intracellular compartments by forming lipid bilayers, a consequence of their amphipathic nature. An important component in the hydrophobic part of some phospholipids are polyunsaturated fatty acids (PUFAs), which possess essential roles in membrane structure and cell physiology [1,2]. Furthermore, PUFA-containing phospholipids are also present in lipoprotein particles that transport triacylglycerols and cholesterol between tissues. However, PUFAs, owing to their bisallylic hydrogen atoms, are particularly susceptible to oxidative stress [3]. An increased generation of reactive oxygen species (ROS) during oxidative stress initiates a chain reaction of degradation steps termed lipid peroxidation, which particularly affects phospholipids that contain PUFAs. This results in the formation of lipid peroxides that can ultimately decompose into secondary products such as malondialdehyde (MDA) or 4-hydroxynonenal (4-HNE) [4,5]. MDA is commonly used as a measure of oxidative stress in biological materials and is a reactive aldehyde that can covalently attach to other biomolecules such as DNA, lipids or proteins, thereby forming neo-epitopes on autologous structures termed MDA epitopes [6]. Enhanced lipid peroxidation accompanies numerous pathologies with an inflammatory component, as evidenced by the detection of lipid peroxidation end products in cardiovascular, pulmonary, hepatic, retinal and neurodegenerative diseases [7–13]. Furthermore, certain end products of lipid peroxidation are recognized by different arms of immunity [6], adding support to the hypothesis that they may represent harmful danger signals requiring protection by both innate and adaptive immune mechanisms [14,15]. Here, we focus on MDA as an end product of lipid peroxidation that can serve as a mediator of both oxidative stress and inflammation, and address its role as a danger-associated molecular pattern (DAMP) by discussing cellular responses towards MDA epitopes presented by various carriers. Based on both common and cell-type-specific responses towards MDA epitopes, we finally highlight their role in the context of disease, focusing on inflammatory pathologies, and extrapolate implications for disorders where MDA epitopes occur but where their mechanistic involvement in driving the disease has yet to be studied.

2. Origin of MDA and formation of MDA epitopes

MDA together with 4-HNE and the less common acrolein comprise well-studied secondary, terminal degradation products as a result of PUFA oxidation, i.e. lipid peroxidation. In this process, oxygen-derived free radicals react readily with PUFAs such as arachidonic acid (20:4) and docosahexaenoic acid (22:6), which contain double bonds and allylic hydrogen atoms that are particularly susceptible to radical attack.

Abbreviations: DAMP, damage-associated molecular pattern; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MDA, malondialdehyde; MV, microvesicles; OSE, oxidation-specific epitope.

☆ This article is part of a Special Issue entitled: Lipid modification and lipid peroxidation products in innate immunity and inflammation edited by Christoph J. Binder.

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http://dx.doi.org/10.1016/j.bbalip.2016.06.016
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Although these lipid peroxidation end products are relatively long-lived compared to ROS, they possess reactive moieties that are capable of forming covalent adductions with target molecules such as proteins, with important physiological consequences [16]. MDA has been reported to be the most abundant aldehyde generated during lipid peroxidation as demonstrated by Esterbauer and Zollern in several in vitro oxidation studies, where the authors found that 4-HNE generation only amounts to 10% of that of MDA [17]. In the case of MDA (structure shown in Fig. 1A), subsequent protein modification occurs typically but not exclusively at the amino groups of a protein's side chain by Schiff base reaction and can result in the formation of various MDA epitopes, including linear and cyclic structures (Fig. 1). Importantly, several studies have shown that these epitopes display varying biological properties with regard to antibody reactivity [18–20] and immunogenicity [21,22] and there is accumulating evidence that a hybrid epitope composed of malondialdehyde and acetaldehyde in a 2:1 ratio termed MAA epitope (Fig. 1D) is immunodominant and biologically the most relevant epitope. Nevertheless, most studies do not explicitly investigate or compare the effects of the different epitopes and many MDA-modified carrier proteins used probably comprise a mix of epitopes, thus complicating the interpretation and side-by-side comparison of results.

3. Interactions of MDA epitopes with innate immunity

The innate immune system is the by far less sophisticated sibling of adaptive immunity, jointly occupied with the maintenance of homeostasis in an organism using cellular and humoral mechanisms. Contrary to adaptive immunity, which mounts a highly specific and tailor-made response against impending danger, the innate immune system is optimized for providing a wide recognition of endogenous and exogenous danger signals with the least effort possible. Innate immune cells and innate effector molecules are usually the first in place at sites of tissue damage and danger to initiate a principal though rudimentary immune response mostly against invading pathogens. Pattern-recognition receptors with broad specificities such as toll-like receptors (TLRs), scavenger receptors or nucleotide-binding-oligomerization-domain-(NOD)-like receptors expressed on macrophages serve as sensors of pathogen-associated molecular patterns of microbial origin but can at the same time recognize also DAMPs derived from endogenous sources. DAMPs are often retained intracellularly until tissue damage occurs upon which they are released into the extracellular milieu, thereby becoming visible to the immune system [23]. In other cases, DAMPs are generated from immunologically silent self-structures that upon tissue stress such as oxidative stress acquire neo-self epitopes with novel immunogenic properties [15,24]. One example for DAMPs are oxidation-specific epitopes (OSEs) including MDA epitopes generated upon lipid peroxidation. Consistent with the fact that DAMPs are by definition recognized by innate immunity, MDA epitopes are specifically bound by innate immune receptors such as the scavenger receptor SR-A1 [25,26] and humoral effectors, e.g. natural antibodies and complement factors. Natural antibodies are spontaneously secreted, germ-line-encoded antibodies with an evolutionary conserved repertoire of variable regions that are produced independently of antigenic stimulation by a B cell subset termed B1 cells [27]. They are specific for microbial but also self-antigens and possess antimicrobial as well as housekeeping functions due to their ability to promote clearance of apoptotic cells. We have previously demonstrated that OSEs are dominant targets of natural antibodies in mice and humans with around 15% of all natural antibodies recognizing MDA epitopes [28]. Several natural antibodies targeting MDA epitopes have been cloned and characterized in more detail, particularly E014, LR04 and NA17 [28–30]. Further support for the hypothesis that MDA epitopes are important targets in innate immunity came from the intriguing finding that also members of the complement system including complement factor H and C3a can bind to MDA epitopes [10,31]. Notably, the complement system is another safeguard mechanism of innate immunity to defend against invading pathogens but also to prevent pathological accumulation of dying cells and debris [32]. Thus, MDA epitopes are recognized by various arms of innate immunity that are involved in clearance mechanisms and tissue homeostasis, and we will speculate on their potential role as markers of damaged material in the concluding part of this review.

4. Physiological carriers of MDA epitopes in health and disease

As a result of lipid peroxidation, free MDA is generated, which can form adducts with biomolecules such as proteins, possibly with consequences for the functionality of the affected structure. On a molecular level, MDA generates condensation products with primary amines present in amino acids such as the terminal group of a lysine residue [5]. However, though less well described, also DNA or lipids could undergo similar MDA modifications upon lipid peroxidation. Here, we review a number of MDA carriers relevant in the context of physiology and disease.

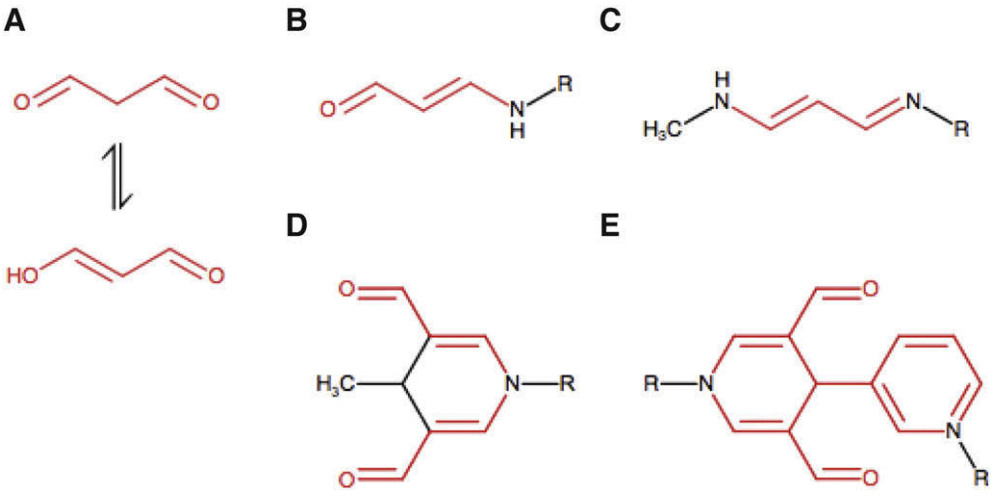

Fig. 1. Chemical structures of MDA before (A) and after (B-E) adduction (i.e., epitope formation) to amino groups (e.g. to lysine residues in proteins). MDA-derived parts are highlighted (red). R indicates rest of protein or other amino-group-bearing structure. (A) Structure of MDA ($C_3H_4O_2$) as *trans*- (top) and *cis*-isomer (bottom). (B) Linear epitope, *N*-ε-(2-propenal). (C) Linear epitope cross-linking to another amino group, 1-amino-3-iminopropene. (D) Cyclic epitope, 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde, also termed malondialdehyde-acetaldehyde (MAA) epitope. (E) Cyclic epitope cross-linking to another amino group, 3,5-diformyl-1,4-dihydropyridine-4-yl-pyridinium.

4.1. Membrane-enclosed carriers

4.1.1. Microvesicles

One example for physiological carriers of MDA epitopes are microvesicles (MV), membraneous particles of submicrometer size that are generated by activated or dying cells [33]. Easily detectable due to their subsequent release into the circulation, MV could serve as biomarkers for inflammatory conditions as MV levels were found to be increased in patients suffering from diabetes [34], liver diseases [35], acute coronary syndrome [36], atherosclerosis [37], venous thromboembolism [38] or ST-segment elevation myocardial infarction (STEMI) [39]. However, the contribution of MV content or their exposed membrane components to downstream effects is still not well understood. Several studies have suggested that MV-induced responses might be mediated via MDA epitopes found on the surface of MV. Huber et al. demonstrated that oxidation of human umbilical vein endothelial cells in vitro using *tert*-butyl hydroperoxide results in the generation of biologically active MV capable of inducing monocyte adhesion [40]. Importantly, these MV were recognized by IgM monoclonal antibodies specific for lipid peroxidation end products, including the natural antibody clone E014, which binds MDA epitopes. These results are similar to another study where MV were generated in vitro by treatment of human THP-1 monocytes with unesterified cholesterol (UCMV) [41]. UCMV induce adhesion of monocytes to the endothelium of murine aortic explants, an effect that is reduced when UCMV generation is preceded by incubation with an inhibitor of mitochondrial complex II, a member of the respiratory chain that is the major source of endogenous ROS in cells. Importantly, this inhibition also led to a decrease of MDA-positive UCMV. In the same manner, pre-treatment with high-density lipoprotein (HDL) or the HDL-associated antioxidant enzyme paraoxonase-1 reduces both monocyte adherence and the level of MDA-positive UCMV, which is suggested to be a result of chemical reduction of MDA epitopes. MDA epitopes can also be found in vivo on MV isolated from the circulation of healthy individuals. We showed previously that almost 50% of circulating MV isolated from healthy donors are recognized by the MDA-specific natural antibodies LR014 and NA17 [39], and about 50% of the isolated MDA-positive MV were covered with endogenous IgM found to be specific for MDA epitopes upon elution from lysed MV. In line with previous studies demonstrating pro-inflammatory properties of MDA epitopes, we could show that in vitro generated platelet-derived MV carrying MDA epitopes induce chemokine secretion in THP-1 cells and human peripheral blood mononuclear cells, an effect that was reduced by pre-treatment with the MDA-specific antibody LR04, indicating that MV may act as amplifiers of inflammatory responses in vitro that are at least partially mediated by MDA epitopes.

4.1.2. Dying cells

Apart from MV, many studies by us and others demonstrated that also apoptotic blebs and dying cells carry MDA epitopes. Initially, the presence of MDA epitopes was detected on serum-deprived, apoptotic endothelial cells stained with E014 [30]. Later on, it was found that other cells types including Jurkat T cells treated with UV, apoptotic THP-1 cells, and heat-induced necrotic retinal pigment epithelial cells displayed reactivity with E014 or LR04 antibodies when analyzed by flow cytometry or immunofluorescence microscopy [10,30,41,42], suggesting that MDA epitopes are formed on dying cells independent of the cell-type origin. However, only late apoptotic cells (positive for 7-AAD or propidium iodide, nucleic acid stains entering cells upon loss of membrane integrity) are typically bound by MDA-specific natural antibodies [28,42,43]. Cells undergoing apoptosis, an immunologically silent type of programmed cell death, generally do not induce inflammatory responses [44]. It has been speculated though that failure to remove apoptotic cells swiftly due to insufficient or impaired clearance mechanisms renders them pro-inflammatory and immunogenic, partly mediated by the exposure of OSEs [30]. Indeed, several reports demonstrate that apoptotic cells can induce pro-inflammatory responses characterized by leukocyte influx, monocyte adherence to endothelial cells or chemokine secretion [43,45–47]. Given that we showed that the MV-induced monocytic cytokine secretion is partially mediated by MDA epitopes as it was blocked with LR04 [39], we speculate that also the pro-inflammatory capacity of apoptotic cells could derive from the presence of MDA epitopes appearing at later stages of apoptosis. By binding to apoptotic cells, natural antibodies such as E014 and LR04 may facilitate a faster clearance of dying cells and cell debris, whose prolonged persistence in an organism is considered to be harmful and to result in chronic inflammatory and autoimmune responses [44,48,49]. Indeed, additional studies have shown the relevance of natural antibody-mediated clearance of apoptotic cells by complement-dependent mechanisms in vitro and in vivo [50–52], suggesting an important role for OSEs. Interestingly, we were able to show that NA17, another natural antibody specific for MDA epitopes, increases the uptake of apoptotic thymocytes by macrophages in vivo [28]. Thus, one physiological function of natural antibodies could be the facilitation of an enhanced clearance of dying cells, concurrently dampening their inflammatory potential to support the maintenance of tissue homeostasis. And, as it seems, one important target and ligand for natural antibodies on apoptotic cells are MDA epitopes.

4.2. Lipoprotein and protein carriers

In principle, any protein with amino groups can serve as a target for MDA modification if MDA is generated in its proximity in vivo or incubated with proteins in vitro. Modification of proteins by chemical derivatization in vitro has allowed studying MDA-mediated effects on a variety of carriers ranging from myelin proteins [53] to wheat peptides [54]. Reported observations of proteins modified in vivo with MDA were recently summarized by us elsewhere [16]. Here, we select several exemplary proteins shown to carry MDA epitopes in vivo for discussion with respect to their role in inflammatory diseases.

4.2.1. Low-density lipoprotein

Oxidative modification of low-density lipoprotein (LDL) is likely the best-studied modification involved in cholesterol accumulation by macrophages present in atherosclerotic lesions. Based on their discovery that LDL can be oxidized in vivo, Steinberg & Witztum laid the foundation for the so-called oxidation hypothesis, which states that different OSEs generated on LDL upon oxidation convert a normally harmless since autologous lipoprotein into an immunogenic form, thereby acquiring properties for initiating a perpetual immune and inflammatory response [55]. In the long term, the persistence of inflammation and lack of resolution result in the development of a chronic inflammation at the site where oxidized LDL (OxLDL) is accumulating, i.e. in the vascular wall, a condition known as atherosclerosis. It was soon recognized that MDA epitopes are abundant in atherosclerotic lesions of hyperlipidemic rabbits. Not only were the lesions strongly stained with MDA antibodies using immunohistochemical methods [13] but it was also found that LDL extracted from atherosclerotic lesions carries MDA epitopes [7,56]. Further characterization of LDL isolated from rabbit or human lesions revealed additional similarities with in vitro OxLDL including chemical and physical properties, an increased ability to stimulate cholesterol esterification, faster degradation in an MDA-dependent manner as well as a higher chemotactic potential compared to unoxidized LDL [56,57]. Circulating LDL from human plasma was also found to be oxidized to a certain extent, resulting in the formation of MDA epitopes [29,58,59].

Following these observations, a multitude of studies has since demonstrated that immunization with autologous proteins such as LDL modified in vitro with MDA protects from atherosclerosis by eliciting protective immune responses against MDA epitopes present in atherosclerotic lesions [21,60–63]. Evidently, this corroborates our hypothesis that MDA epitopes are prevalent OSEs in an inflammatory setting, with a fundamental role for LDL as a carrier in atherogenesis.

4.2.2. High-density lipoprotein

Another example showing that modification of a protein with MDA alters its functional properties is HDL. HDL participates in reverse cholesterol transport, a process thought to alleviate atherosclerosis by moving cholesterol from non-hepatic peripheral tissues to the liver for secretion into bile. Additionally, several other atheroprotective functions for HDL have been identified, including promotion of endothelial repair mechanisms, activation of endothelial nitric oxide production as well as anti-inflammatory actions on endothelial cells and leukocytes [64,65]. However, some of these functions are lost when HDL is oxidatively modified [66,67]. HDL isolated from plasma and atherosclerotic lesions of humans was bound by a monoclonal antibody against MDA epitopes, indicating that HDL can be modified in vivo by MDA [67]. Interestingly, HDL from atherosclerotic lesions contained 3.6-fold higher levels of MDA epitopes than circulating HDL as quantified by an immunoassay. Using in vitro modified HDL, the authors showed that the presence of MDA epitopes on HDL impairs its binding to ABCA1, preventing cholesterol efflux. Thus, cardioprotective functions of HDL can be blocked by modifications with MDA epitopes. In another study, Besler et al. found that HDL isolated from patients with stable coronary artery disease or acute coronary syndrome (HDL_{CAD}) lacked atheroprotective properties of HDL obtained from healthy donors ($HDL_{Healthy}$) such as induction of endothelial nitric oxide, endothelial repair and inhibition of endothelium-monocyte adhesion [66]. Importantly, HDL_{CAD} was enriched in both protein-associated MDA and MDA-lysine epitopes compared to $HDL_{Healthy}$, and in vitro modification of $HDL_{Healthy}$ with MDA epitopes resulted in loss of the beneficial effects of HDL, once more suggesting that modification of autologous proteins with MDA epitopes can abolish their functional properties.

4.2.3. Other proteins

Many more proteins have been identified as in vivo carriers of MDA epitopes (see [16] for an extensive list), including elongation factor-2 [68], cartilage collagen [69], and Surfactant Protein D (SPD, discussed below) [70]. However, we still lack a better understanding of the conditions under which these modifications occur in vivo and to what consequences they might lead.

5. Cellular responses to MDA epitopes

Both humoral and cellular mechanisms for recognition of MDA epitopes have been identified but here we will focus on cellular responses as humoral components with specificity for MDA epitopes have been discussed by us elsewhere in detail [16]. Cellular responses have been predominantly studied in three models or tissues, lung, liver and circulation, where MDA is generated due to oxidative stress caused by different external stimuli. An overview of cell types investigated so far with respect to their responses towards MDA epitopes can be found in Fig. 2.

5.1. In the lung

In the first model for examining effects of in vivo MDA generation, mice were exposed to tobacco smoke and alcohol, which induce oxidative stress in the lung and consequently high levels of two aldehydes in the bronchoalveolar lavage fluid, acetaldehyde and malondialdehyde [70]. These can react together to form hybrid adducts termed malondialdehyde-acetaldehyde (MAA) adducts with pulmonary proteins such as SPD. While unmodified SPD is an innate effector protein involved in host defense and phagocytosis, MAA-SPD induces inflammatory responses characterized by the secretion of the chemokines KC and MIP-2 in lung slices from wild-type mice in a protein-kinase-C-ε-dependent manner [71]. Furthermore, in vivo application of MAA-SPD by intranasal instillation results in elevated levels of KC and neutrophils in the bronchoalveolar lavage fluid, as well as leukocyte infiltrates in the lung in contrast to unmodified SPD. Importantly, these effects were also observed in the same model using bovine serum albumin (BSA) as a carrier protein, indicating that the inflammatory responses are mediated exclusively by MDA epitopes independent

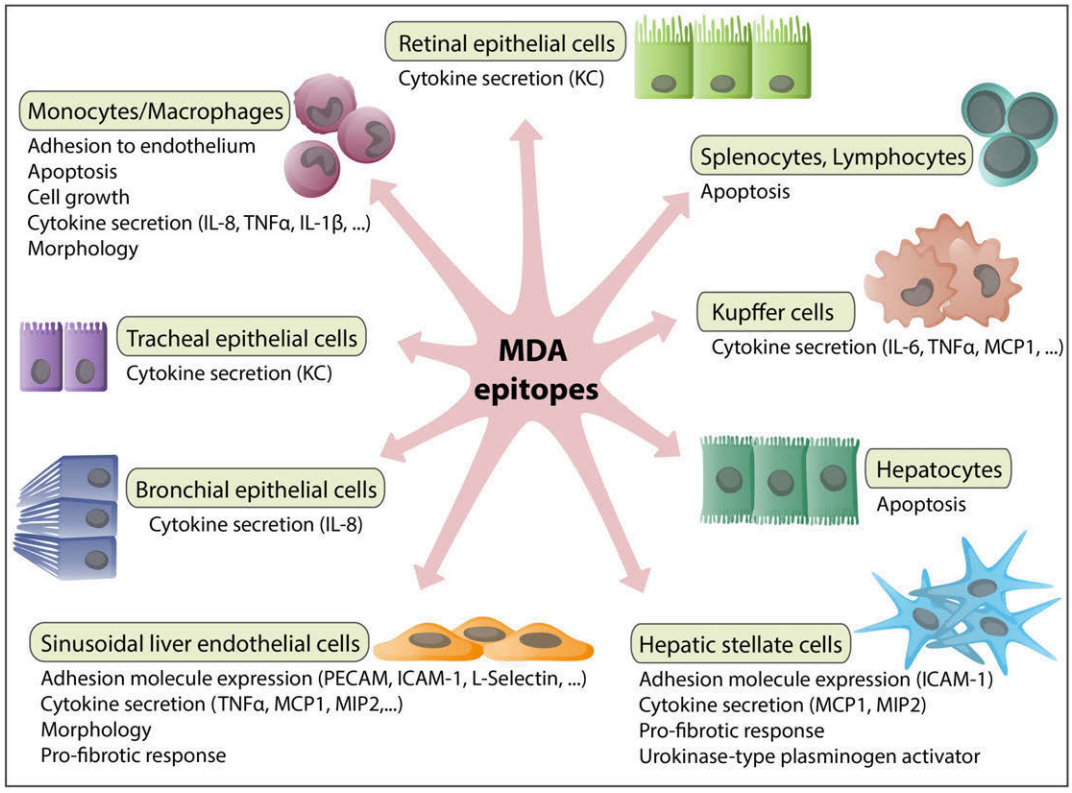

Fig. 2. Cellular responses towards MDA epitopes. MDA epitopes can have multiple effects on different cell types with cytokine secretion being the most common. References for cellular responses can be found in the text body.

of the carrier protein. Stimulation of bronchial epithelial cells or epithelial cells from the trachea with MAA-SPD or MAA-BSA resulted also in increased IL-8 and KC secretion compared to unmodified BSA, respectively [26,72]. This response was abrogated in epithelial cells from SR-A1-deficient mice, suggesting a role for SR-A1 in recognizing MDA epitopes in the lung.

5.2. In the liver

Another site where MDA is generated in vivo is the liver of patients or animals with a history of excessive alcohol consumption [73]. Here, both acetaldehyde and malondialdehyde are produced as metabolites of ethanol and accumulate during chronic alcohol intake, resulting in the formation of protein adducts in the liver [19,74,75]. Interestingly, in the same study also livers from patients with non-alcoholic steatohepatitis were found to be reactive with an MDA-specific antiserum [74]. A range of different cell types resident in the liver has been shown to respond to stimulation with MDA epitopes [76]. Sinusoidal endothelial cells (SECs) provide important homeostatic functions in the liver by acting as a barrier against pathogens and also as a selective filter for substances arriving via the bloodstream in the liver. Stimulation of SECs with MAA-BSA results in increased expression of adhesion molecules and the secretion of pro-inflammatory cytokines compared to unmodified BSA including tumor necrosis factor alpha (TNFα), monocyte chemoattractant protein 1 (MCP1) and macrophage inflammatory protein 2 (MIP2) [77]. In addition, MAA-stimulated SECs released fibronectin, which can activate hepatic stellate cells (HSCs) involved in liver fibrosis [78]. Furthermore, direct stimulation of HSCs results in secretion of MCP1, MIP-2, and urokinase-type plasminogen activator as well as upregulation of intercellular adhesion molecule 1 (ICAM-1) [79,80] but also collagen I, collagen III and fibronectin production [81]. This suggests that MDA epitopes can activate different cell types in the liver to initiate pro-inflammatory and pro-fibrotic responses that contribute to liver injury. Kupffer cells (KCs) are the hepatic representatives of macrophages located in liver sinusoids where they scan the surroundings for material to be phagocytosed. LPS-activated KCs also secrete pro-inflammatory cytokines when incubated with MAA-BSA but not with BSA alone [82,83]. In summary, the liver as an environment where MDA can be generated in large quantities contains various cell types that react to the increased presence of MDA epitopes by triggering pro-inflammatory and pro-fibrotic responses.

5.3. In the circulation

The detrimental effects of MDA epitopes in the (cardio)vascular system have been mostly investigated in regard to humoral responses from the adaptive immunity reviewed elsewhere [84–86]. Apart from the observation that MDA-LDL induces foam cell formation and cholesteryl ester accumulation in macrophages, which was recognized early after the discovery of oxidative modifications of LDL [87], not much is known about the responses of MDA epitopes in cells of the circulatory or vascular system. In addition to our finding that human THP-1 monocytic cells stimulated with MAA-BSA upregulated IL-8, TNFα and IL-1β expression [10], another study analyzed the secretome of THP-1 cells stimulated with MDA-lysine (i.e. MDA-modified Boc-L-lysine), compared to normal-glucose control using hybridization-based cytokine arrays [88]. Indeed, similar to other cell types, stimulation of THP-1 cells with MDA-lysine resulted in the secretion of 20 cytokines, including CC chemokine ligand (CCL) 11, CCL18, CCL28, tumor necrosis factor superfamily member 14 (TNFSF14), MCP1 and MCP2. Consistent with the fact that these chemokines are involved in biological processes such as immune response, cellular movement and cell-to-cell signaling, treatment with MDA-lysine also induced monocyte adhesion to smooth muscle and endothelial cells. Furthermore, the same group found that LDL modified with MDA induces secretion of TNFα, MCP1 and CCL5 in the murine macrophage cell line J774 [89]. Pro-inflammatory responses were also observed in heart endothelial cells stimulated with MAA-BSA, which resulted in increased TNFα secretion and adhesion molecule expression including ICAM-1, major histocompatibility complex (MHC) class II and vascular cell adhesion molecule (VCAM-1) compared to unmodified BSA [90]. Bone-marrow-derived macrophages stimulated with MDA-modified myelin oligodendrocyte glycoprotein also resulted in upregulation of pro-inflammatory cytokines including IL-12p35 and IL-23p19 [53].

5.4. In the eye

The retinal pigment epithelium provides a supportive function for retinal visual cells and is involved in the pathogenesis of age-related macular degeneration, where MDA epitopes are continuously generated and accumulate [10,91]. We could show that ARPE-19 cells, a retinal pigment epithelial cell line with high phagocytic activity, respond to MAA-BSA but not BSA by expressing IL-8 [10]. Additionally, intravitreal injection of MAA-BSA into wild-type mice resulted in a pro-inflammatory response in the choroid/retinal pigment epithelium fraction of the eye, characterized by a robust increase in KC expression, which is ameliorated when MAA-BSA was pre-incubated with complement factor H, while BSA injection did not induce a pro-inflammatory response.

From the available data reviewed here it is evident that most cellular responses towards MDA epitopes independent of the cell type or tissue are characterized by the expression of pro-inflammatory mediators to alarm the immune system about the presence of danger signals that require immediate attention. In the remaining part of this section we discuss other cellular responses than inflammation as well as signaling pathways demonstrated to be involved.

5.5. Morphological changes and cellular toxicity

Morphology can be an important indicator of the activation status of a cell but also of its viability. Several studies have noted morphological alterations in different cell types treated with MDA epitopes, though without analyzing the mechanism or the consequences in detail. These changes in cell shape and appearance were observed in J774 macrophages [92] and SECs [77] in the presence of MAA-BSA but not unmodified BSA, and are indicative of ongoing apoptosis. Indeed, MDA-modified hen egg lysozyme was found to induce cell death in antigen-presenting cells, lymphocytes and hepatocytes [93] as well as splenocytes [94]. Mechanistically, this seems to involve uptake of MDA epitopes, fusion with lysosomes, followed by disruption of lysosomal integrity and cellular leakage [92]. Furthermore, upon binding to MDA-BSA-coated plates SR-A-expressing human embryonic kidney cells display a spread morphology within minutes, accompanied by an increase in cell surface area and the formation of membrane extensions [95]. Whereas most studies indicate that MDA epitopes induce cell death, a more recent report, however, demonstrated that MDA-LDL induces cell growth in THP-1 cells [96]. While unmodified LDL was not used as a control in the latter study, the diverging observations might be also explained by the different cell types and concentrations used in these studies [93].

5.6. Signal transduction mechanisms and receptors for MDA epitopes

MDA epitopes have been shown to induce cellular responses including pro-inflammatory gene expression, which requires the presence of a receptor on the cell membrane to sense MDA epitopes as well as downstream signaling components capable of mediating the signal to the nucleus. Indeed, several studies demonstrated that the effects induced by various MDA epitopes are mediated by protein kinase C [71,72,80] but also PI3-kinase, Src-kinase [95], PL-C/IP3 [97], Erk1/2, Syk and NF-κB [83,88,89,98] have been implicated. On the extracellular side, SR-A1

has been suggested early on as a receptor required for binding of MDA epitopes to the cellular surface of human monocytes [25]. However, more recent studies reported that there might be at least two additional receptors next to SR-A1 for the recognition of MDA epitopes. CD16, also known as Fcγ receptor III, was found to inhibit binding of J774 macrophages to MDA-LDL-coated wells in a dose-dependent manner [89]. More strikingly, the pro-inflammatory response was abrogated in CD16-silenced J774 macrophages, suggesting a role for CD16 in MDA-epitope recognition. Last, Besler et al. found that the impairment of endothelial nitric oxide production by MDA-LDL could be rescued by blocking the lectin-type oxidized LDL receptor 1 (LOX-1) in endothelial cells [66]. Interestingly, LOX-1 is a scavenger receptor that was originally identified as the major receptor for OxLDL [99], while later studies demonstrated that it binds to many different ligands including apoptotic cells, platelets, bacteria as well as to other lipid peroxidation products such as 4-HNE [100,101]. Given that MDA epitopes are generated virtually in any tissue one can speculate that even more MDA receptors may exist, also in light of the fact that other OSEs have been found to bind to PRRs such as CD36, CD68, MARCO or TLRs. Different repertoires of receptors with cell-type-specific expression patterns or varying tissue tropism would allow the induction of certain responses to MDA that could be of advantage for identifying and reacting towards oxidative stress depending on the tissue context.

6. MDA epitopes as mediators of inflammation in disease

MDA is generated during oxidative stress and thus can be detected in plasma and tissue homogenates of patients suffering from a wide range of diseases [102]. We hypothesize that MDA produced during the course of these diseases would also result in the formation of neo-self epitopes on autologous proteins, which contribute to the pathogenesis (Fig. 3). Indeed, plasma antibodies against MDA epitopes have been found not only in patients with atherosclerosis [7] but also with many other diseases such as alcoholic and non-alcoholic liver disease, diabetes or systemic lupus erythematosus [103–107]. As atherosclerosis is in fact only one possible outcome of metabolically-induced inflammation affecting a single tissue [108], it will be interesting to see whether MDA epitopes also play a role in other diet-induced pathologies such as diabetes or non-alcoholic steatohepatitis. Given that so many cell types from different tissues are capable of recognizing MDA epitopes, we

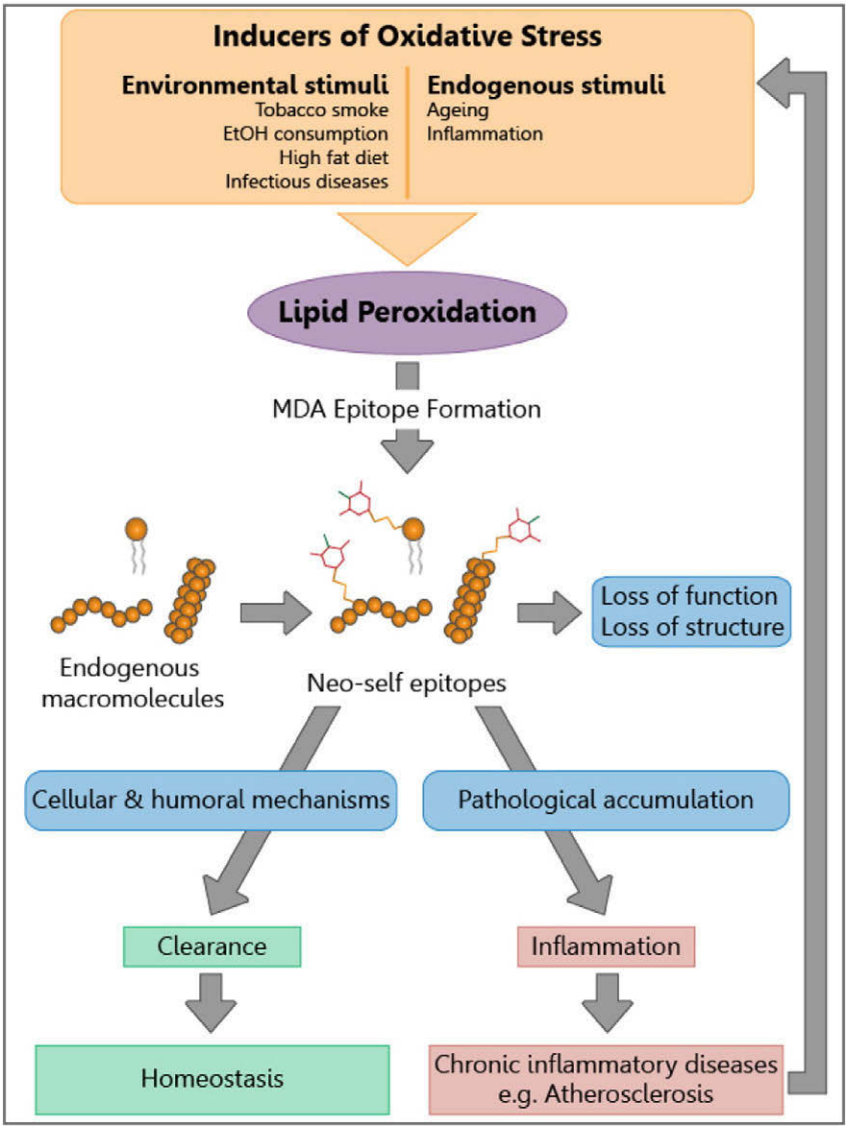

Fig. 3. Overview of the interplay between oxidative stress and inflammatory diseases. Various endogenous and environmental stimuli can induce oxidative stress, resulting in lipid peroxidation. MDA as one of its end products modifies readily endogenous proteins and lipids to give rise to neo-self epitopes with new biological functions. In a healthy state, cellular and humoral clearance mechanism allow the removal of MDA epitopes and aid in maintaining homeostasis. However, in their absence, pathological accumulation of MDA epitopes may result in an auto-amplifying loop leading to chronic inflammatory diseases.

like to speculate that there could be a tissue-independent, organism-wide role for MDA epitopes which accumulate during inflammatory settings when an imbalance between generation of apoptotic cells and their clearance occurs. In confirmation of this hypothesis, we found that not only MV numbers but also the frequency of MDA-positive MV were significantly increased at the coronary site of occlusion in STE-MI patients compared to peripheral blood [39]. To better understand the role of MDA epitopes in cardiovascular disease, we and others have employed active or passive immunization strategies against MDA epitopes to evaluate the benefit of targeting these structures [21, 61,62,109]. Indeed, a protective effect of either immunization with MDA-modified carrier proteins or infusion of *anti*-MDA antibodies has been observed. However, conclusive and well-controlled studies providing unambiguous evidence regarding a role for MDA epitopes in driving inflammatory processes are still to be awaited.

7. Conclusion

MDA epitopes can be detected as a biomarker for oxidative stress in many inflammatory pathologies due to an overwhelming production or deficient clearance of lipid-peroxidation products during conditions of oxidative stress. In addition, several important effects on various cell types including pro-inflammatory responses have been identified for MDA epitopes, indicating that they could be biologically active as mediators of both oxidative stress and inflammation. Consistently, MDA epitopes have been detected on MV, dying cells as well as on (lipo)proteins with critical consequences for their functionality. Both innate and adaptive immune responses exist to deal with situations where disturbed homeostasis results in a pathological accumulation of MDA epitopes. Therefore, several reasons suggest that MDA epitopes could be classified as DAMPs: 1) they are generated upon tissue damage and stress; 2) they have inflammatory properties and their presence is noticed by immune and non-immune cells; 3) they are targets of various arms of innate immunity. We hypothesize that MDA epitopes represent waste markers of damaged tissues or oxidized biomolecules and that their exposure on autologous structures alerts the immune system of impending danger as non-physiological accumulation can result in the induction of pro-inflammatory responses. Finally, we argue that lack of resolution of this response may result in the development of chronic inflammatory diseases.

Transparency document

The Transparency Document associated with this article can be found in the online version.

Acknowledgements

This work was supported by the SFB F30 “Lipotox” and the SFB F54 “InThro” of the Austrian Science Fund and a Boehringer Ingelheim PhD Fellowship. We are grateful to Vesna Krajina for preparing the illustrations.

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2.3 Sterile inflammation and its Sensors

While infection has been viewed for a long time as the only cause of inflammation, many other non-pathogen-derived sterile stimuli also exist, which include mineral particles, mechanical trauma or chemicals. In the latter case, the mere presence of many of these sterile stimuli does not necessarily cause harm. The damage rather comes from the fact that sterile stimuli initiate the same inflammatory responses such as recruitment of immune cells or secretion of cytokines, and it is the workings of these immune mechanisms that inflict damage on otherwise healthy tissue. Hence, a persistent sterile stimulus that cannot be resolved by host immune responses might lead to chronic inflammation with continued tissue damage (Rock et al., 2010). Indeed, many diseases have been associated with sterile ligands including atherosclerosis, Alzheimer's disease, gout but also type 2 diabetes (Chen and Nuñez, 2010). One apparent difference between sterile and infectious stimuli is the fact that sterile triggers are usually not proliferative and their ability to promote inflammation is outcompeted by inflammatory countermeasurements to dampen the inflammation and move to resolution. Conversely, infectious particles replicate intra- or extracellularly and compete with the host for energy resources, causing damage as long as the situation is unresolved.

To deal with infectious or sterile triggers, the immune system has evolved a wide panel of receptors to survey for the presence of undesired material, summarized under the term pattern-recognition receptors (PRRs) since they recognize generally a broad range of ligands (O'Neill et al., 2013). PRRs bind to both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and come in different flavours: soluble or membrane-bound, exposed to the extracellular space or intracellular (Iwasaki and Medzhitov, 2010). Soluble or secreted PRRs such as pentraxins, complement factors and antibodies both recognize ligands present on microbes and sterile ligands. For example, the pentraxin C-reactive protein (CRP), the complement factor H (CFH) and natural IgM antibodies bind also modified LDL (Papac-Milicevic et al., 2016). The PRR family further includes surface-bound or endosomal Toll-like receptors (TLRs), intracellular NOD-like receptors (NLRs), C-type lectin receptors (CLRs), scavenger receptors and others. These receptors are capable of recognizing exogenously derived crystals, silica or even asbestos (Chen and Nuñez, 2010). However, also endogenous proteins or nucleic acids that are under normal conditions kept within cells can be recognized by PRRs. Upon tissue damage, these molecules become released into the extracellular milieu and are thus visible to immune system components (Chen and Nuñez, 2010). For example, TLR3 recognizes double-stranded ribonucleic acid (RNA), TLR4 binds bacterial lipopolysaccharide (LPS) but also heparan sulphate or High-Mobility-Group-Protein B1 (HMGB1), TLR9 binds to unmethylated CpG of deoxyribonucleic acid (DNA), which is common in bacteria, but also to endogenous mitochondrial DNA, and scavenger receptors such as CD36, SR-A1

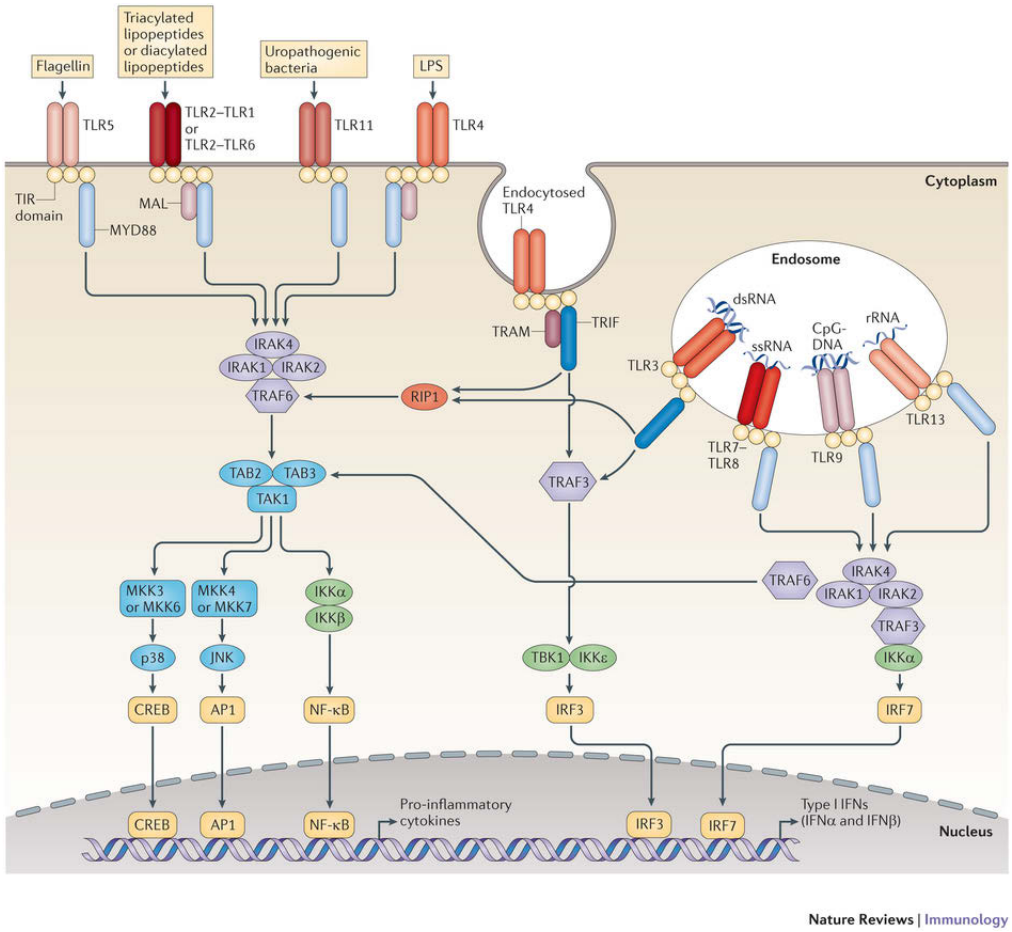

Figure 2.1: TLR signalling pathways as a prototypical example for signal transduction upon DAMP/PAMP recognition. TLRs initiate signalling upon receptor dimerization with the same (in case of TLR4) or a different partner (in case of TLR2). Recruitment of adaptors to TIR domains of TLRs results in the activation of a complex signalling cascade depending on the type of adaptor molecule but also TLR localization (cell surface or endosomal). TLR signalling induces different gene expression programs depending on the transcription factors activated. Image source O'Neill et al. (2013). Reprinted with permission.

and LOX-1 were identified as endocytic receptors of modified LDL. Hence, many receptors originally identified as broad-range microbial sensors are even more promiscuous than originally thought given they recognize also sterile ligands as long as pattern-matching is successful.

2.4 Signal Transduction in Sterile Inflammation

Independent of the nature of the ligand, similar downstream signalling pathways can be activated upon binding to receptors located on the cellular surface including nuclear factor-kB (NF-κB), type I interferon and mitogen-activated protein kinase (MAPK) signalling pathways.

Signal transduction by TLRs is initiated when two TLRs form homo- or hetero-dimers upon ligand binding (O'Neill et al., 2013). Specific TLR domains on the intracellular side named Toll-IL-1-resistance (TIR) domains allow recruitment of signalling adaptor proteins to the cell membrane. Depending on the TLR type, these adaptor molecules are either myeloid differentiation primary-response protein 88 (MyD88) and Myd88-adaptor-like protein (MAL), or TIR domain-containing adaptor protein inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). This induces the interaction of IL-1R-associated kinases (IRAKs) with TNF receptor-associated factors (TRAFs), which results in the activation of MAPKs, Jun N-terminal kinase (JNK), p38 or interferon-regulatory factors (IRFs). Ultimately, TLR signalling via Myd88/MAL leads to the activation of several transcription factors including NF-κB and activator protein 1 (AP1). Also type I interferon signalling pathways can be initiated via TRIF/TRAM activation when endosomal TLRs are engaged. Figure 2.1 illustrates TLR signalling pathways from both the cell surface and from endosomal compartments.

Less is known about the signalling pathways induced by scavenger receptors, even though many ligands have been discovered that are internalized by these PRRs including pathogens, modified lipoproteins, apoptotic cells, microvesicles and fatty acids (Canton et al., 2013). In many cases, scavenger receptors do not possess known signalling domains such as kinase-containing, G-protein-binding or scaffolding domains, which makes the dissection of downstream signal transduction difficult. For example, the scavenger receptor CD36 has two yet very short tails available for signal transduction because both the N- and the C-terminus of the protein are cytoplasmic, allowing the formation of a loop that reaches through the cell membrane into the extracellular space. The C-terminus has been identified to bind to Src family kinases such as Fyn but the exact mechanism of interaction is not known (Canton et al., 2013). Additionally, CD36 binding is associated with MAPK and JNK activation. Because of the short cytoplasmic tails and the lack of signalling domains identified so far it is not clear whether CD36 in general requires the formation of multi-meric complexes with other PRRs as co-receptors to induce signal transduction. As a matter of fact, complex formation has been demonstrated for CD36 with a variety of molecules such as TLRs, tetraspanins, Fc receptors and integrins. Also SR-A1 can form a complex with the tyrosine protein kinase Mer, thereby allowing uptake of apoptotic cells. Interestingly, for both SR-A1 and CD36, the nature of the co-receptor modulates the signalling pathways induced with opposing outcomes regarding the inflammatory status (Canton et al., 2013).

Overall, no difference between infectious or sterile stimuli has been observed regarding the type of signalling pathways induced but it is conceivable that an immune cell does possess mechanisms to differentiate between the binding of stimuli released due to tissue damage as opposed to stimuli that derive from an invading pathogen even when the signal is provided via the same receptor. Thus, although over 15 years have passed since the

discovery of TLRs, this question remains one of the many unanswered ones in the TLR field (Medzhitov et al., 2011).

2.5 Oxidation-specific Epitopes are Danger Signals Mediating Sterile Inflammation

Under certain circumstances, even self-molecules can be recognized as DAMPs, especially after being subjected to structural changes due to oxidation that render them non-self. As discussed in Section 2.1, oxidative modification is a common process in an aerobic world where reactive oxygen species (ROS) are produced as a by-product in oxidative reactions. ROS can damage bio-molecules that decompose into oxidative breakdown products collectively termed advanced lipoxidation end-products (ALE), which react with lipids, proteins or DNA to form so-called oxidation-specific epitopes (OSE). Though lipid peroxidation is generally a physiological process that occurs during inflammation but also apoptosis and aging, accelerated generation of ALE or impaired clearance can result in their pathological accumulation. One prominent example for the detrimental consequences of enhanced ALE production is the role of oxidized low-density lipoprotein (OxLDL) in atherosclerosis. Atherosclerosis is a degenerative vascular disease that results in stiffening and occlusion of arteries through the formation of atherosclerotic lesions in the arterial wall also called plaques (Witztum and Lichtman, 2014). Subsequently, either the narrowing of arteries due to plaque build-up or the downstream obstruction by instable, ruptured plaques prevents proper supply of all body parts with oxygenated blood and nutrients and may result, depending on the tissue type affected, in life-threatening complications such as myocardial or cerebral infarct. Due to the accumulation of macrophages in plaques at the arterial wall it has become clear that innate immune mechanisms play a major role in the early phase of atherogenesis, therefore many studies have focussed on understanding the nature of disease-specific PAMPs derived from microbes and suggested them to be the candidate antigens of immunity in atherosclerosis (Tufano et al., 2012). However, the involvement of innate immunity in atherogenesis could also be explained by the fact that the PRRs on macrophages recognize neo-self molecules carrying OSE on their surfaces, which trigger inflammatory responses (Miller et al., 2011). Considerable evidence has been gathered that points at a crucial role for OxLDL as a sterile stimulus for PRR-mediated inflammation in atherosclerosis (Hartvigsen et al., 2009; Miller et al., 2011; Witztum and Lichtman, 2014; Binder et al., 2016). This is supported by the observation that LDL oxidation occurs in vivo (see Section 2.1). To model OxLDL in vitro, LDL is extensively oxidized by exposure to $CuSO_4$, which generates a heterogeneous mixture of oxidized lipids and OSE. Alternative approaches include the generation of minimally-modified LDL (mmLDL) in presence of 15-lipoxygenase and chemical derivatization of LDL with ALE species such

as MDA or 4-hydroxynonenal (4-HNE). Compared to OxLDL, mmLDL carries both less and different OSE types and does not have the same functional properties also regarding receptor recognition (Witztum and Lichtman, 2014; Miller et al., 2003), whereas specific LDL modification with MDA or 4-HNE allows to dissect the contribution of individual OSE.

Rather than being a consequence of ageing and senescence, atherosclerosis appears to represent one potential outcome of unhealthy, fatty nutrition and sedentary lifestyle affecting the vascular system. Other organs such as the liver are also affected by a high-fat diet and the underlying mechanisms might be mediated by OxLDL in a similar manner. Indeed, a mechanistic contribution of OxLDL to the inflammatory component of non-alcoholic fatty liver disease (NAFLD) has been proposed and is supported by data from several studies (Walenbergh et al., 2013; Houben et al., 2017).

2.6 Non-alcoholic Fatty Liver Disease and Gut Microbiota

More than 35 years ago, Ludwig et al. (1980) described for the first time a liver phenotype of fat accumulation and inflammatory infiltrates in patients that was until then only associated with excessive alcohol consumption. The pathological changes in these patients, however, developed independently of alcohol consumption. The authors have termed this phenotype non-alcoholic steatohepatitis (NASH), which is today a prevalent liver disease in the developed world and belongs to the larger group of NAFLD. NAFLD encompasses a spectrum of diet-induced pathologies with hepatic tropism, ranging from reversible hepatosteatosis to non-alcoholic steatohepatitis, cirrhosis and hepatocellular cancer. The increasing incidence of NAFLD is primarily due to disadvantageous lifestyle choices, i.e. lack of exercise and a diet rich in fat, which results in the deposition of lipids in the liver (hepatosteatosis), followed by infiltration of immune cells and hepatic inflammation, elevated oxidative stress and tissue damage. While NASH is reversible, prolonged exposure to a high-fat diet results in liver fibrosis, which increases the risk for cirrhosis and hepatocellular carcinoma. As there is no treatment for NASH, patients have to resort to lifestyle changes including altering their diet preference. In fact, as with atherosclerosis, NASH is today considered to be merely the hepatic manifestation of a full panel of symptoms triggered by unhealthy, fat-rich nutrition that also affects other tissues such as the vascular system described before. These symptoms include hyperglycemia, hyperlipidemia and hypertension and are summarized under the term Metabolic Syndrome (MetS).

Different mechanisms have been proposed to be causative of NASH. In the “two-hit” model, two events are required to develop steatohepatitis. The first hit is simply the accumulation and deposition of lipids in the liver, the second hit consists of certain liver damage processes that result in hepatoxicity, for example due to increased oxidative stress and apoptosis. However, more recently, additional models have emerged where the two

hits are combined into one event (Hebbard and George, 2011), whereas other researchers favour a multi-factorial explanation of NASH pathogenesis (Walenbergh et al., 2013). Regardless of the number of hits required, the nature of the link between fat accumulation and hepatoxicity resulting in inflammation is not well understood and several hypotheses are prevalent. Firstly, the communication of the liver with adipose tissue has suggested the possibility that release of both cytokines and free fatty acids from the adipose tissue results in hepatic inflammation (Hebbard and George, 2011).

Secondly, the close connection between liver and the gastrointestinal tract (dubbed gut-liver axis) has recently received increasing attention as a contributing factor for NASH (Mehal, 2013). The liver receives its main blood supply via the portal vein, which transports bacterial products leaked from the intestine directly to the liver, where they could induce inflammation via initiation of TLR signalling in an organ that is already challenged by excessive fat accumulation. Changes in the intestinal permeability could also result in the translocation of bacteria-derived compounds into the circulation, where they cause a state of low-grade chronic inflammation characteristic of obesity (Lumeng and Saltiel, 2011). Characterization of gut microbiota species has revealed that the composition can be a determining factor for the development of obesity and NASH (Mehal, 2013).

Last, dietary cholesterol has been implicated in NASH development as mentioned in Section 2.5. Studies in hyperlipidemic animal models have shown that steatohepatitis and fibrosis can be induced after a few days of high-fat-high-cholesterol (atherogenic) diet (Shiri-Sverdlov et al., 2006; Wouters et al., 2008). Interestingly, only steatosis but no inflammation was observed when cholesterol was removed from the diet. Importantly, bone marrow transplants from mice deficient for scavenger receptors SR-A1 and CD36 into hyperlipidemic mice reduced hepatic inflammation after an atherogenic diet but again not steatosis, indicating that the ligand of these receptors has pro-inflammatory properties but that lipid deposition in the liver occurs independently of their presence (Bieghs et al., 2010).

2.7 Evidence for a Role for Malondialdehyde in Hyperlipidemia

In this thesis, I focus on the pro-inflammatory properties of epitopes derived from MDA as a prominent target of natural IgM antibodies and their potential use for developing therapeutic interventions in a mouse model of early-stage NASH. The importance of MDA as a subject of research in lipoprotein research became already apparent decades ago when researchers started to delineate the concepts underlying hyperlipidemia and atherogenesis.

Lipoprotein metabolism and atherogenesis were initially studied in the Watanabe heritable hyperlipidemic (WHHL) rabbit, which served as a model for familial hypercholesterolemia (FH) before the advent of genetic models and transgenic mice (Watanabe, 1980). WHHL rabbits contain a 12-nucleotide-long deletion in the LDL-binding region of the

LDLR gene, resulting in impaired LDLR surface expression and LDL accumulation in the circulation (Schneider et al., 1983; Yamamoto et al., 1986). These animals were important for advancing knowledge on lipoprotein metabolism in vivo and for developing treatments against hypercholesterolemia such as inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase (Shiomi and Ito, 2009). A number of studies over the next decades supported the hypothesis that the cause of atherosclerosis is not merely the accumulation of LDL particles in the bloodstream but rather the generation of an abnormal, oxidized form of LDL (Steinberg et al., 1989; Steinberg and Witztum, 2010; Glass and Witztum, 2015; Binder et al., 2016). In the early years of LDL research it was found that skin fibroblasts from FH patients that cannot take up LDL via the classical LDLR pathway were still capable of endocytosing cationized but not anionized LDL (Basu et al., 1976). Loading LDL with positive or negative charges was achieved by chemical treatment, in the case of anionization by modification of LDL with acetyl groups. It was then noticed that anionized LDL could be taken up by macrophages, which contain a high-affinity binding site for acetyl-LDL but not native LDL, in contrast to the earlier studied fibroblast. This acetyl- or scavenger receptor also recognized other modifications of LDL that confer negative charges to the modified molecule, including maleylation (Goldstein et al., 1979). Around the same time, investigations by other researchers revealed that LDL particles isolated from hyperlipidemic animals and analyzed by electrophoretic separation are larger than normal LDL molecules (St Clair and Leight, 1978; Rudel et al., 1977). Hence, Fogelman et al. (1980) hypothesized that the size of LDL particles could influence their uptake. In experiments using LDL particles treated with glutaraldehyde for cross-linking LDL molecules in order to form polymers they could show that modified LDL was taken up by human monocytes significantly more efficiently than native LDL. As the production of glutaraldehyde in the vicinity of atherosclerotic lesions had not been reported, the authors turned to modification by a related structure, malondialdehyde (MDA). MDA is generated via the arachidonic acid metabolism in platelets and also during lipid peroxidation, making its occurrence in atherosclerotic lesions more likely. MDA modification of LDL did not change particle size but increased net negative charges, with the same effect on LDL uptake by human monocytes. Based on these experiments the authors proposed that only modified LDL but not native LDL results in accumulation of cholesterol esters in macrophages and their conversion to foam cells. Similar to acetyl-LDL, MDA-LDL is taken up by a receptor different from the classic LDL receptor, namely the acetyl- or scavenger receptor (Shechter et al., 1981), known today as scavenger receptor A-1 (SR-A1) or macrophage scavenger receptor 1 (MSR-1). Comparable properties are acquired by LDL during exposure to monocytes, macrophages or endothelial cells, which are the major cell types in contact with trapped LDL due to their localization in the vascular wall (Steinberg et al., 1989).

Direct evidence for the occurrence of MDA in vivo was first demonstrated by Haber-

land et al. (1988). To perform studies on WHHL rabbit lesions, the authors generated a mouse antibody against MDA-LDL, MDAlys (IgG2a), which specifically recognized MDA-modified but not CuOx- or native LDL. This antibody was found to colocalize in WHHL rabbit lesions with antibodies for apolipoprotein B (ApoB), a component of LDL particles. Palinski et al. (1989) generated guinea pig antibodies against different models of OxLDL such as MDA-, 4-HNE and CuOX-LDL and found that similar areas in atherosclerotic lesions of WHHL rabbits are stained, while normal areas were not bound by the antibodies. Additionally, eluted LDL from aortic sections contained MDA epitopes demonstrated by MAL-2 or MDA2 antibody stainings specific for MDA-LDL (Ylä-Herttuala et al., 1989; Haberland et al., 1988). MDA epitopes could not be detected on plasma LDL, probably because only entrapped but not circulating LDL is subject to oxidation. Support for in vivo oxidation was further provided by the observation that every serum tested, be it from patients or rabbits, possesses autoantibodies against MDA-LDL independent of their plasma LDL concentration (Palinski et al., 1989). Today, the knowledge on the role of OxLDL in atherogenesis and the development of important tools such as natural IgM antibodies with specificity for certain OSE faciliates the investigation of the role of OxLDL and its distinct epitopes in other diet-related diseases such as NASH. To delineate the role of MDA epitopes in NASH, I use the monoclonal natural IgM antibody LR04 isolated from splenocytes of $Ldlr^{-/-}$ mice on atherogenic diet as a tool in my thesis project.

2.8 Natural IgM Antibodies in NASH

Natural IgM antibodies are naturally occurring, germ-line-encoded products of a specific B-cell subtype termed B1a cells, pre-existing in unchallenged individuals as a result of natural selection (Tsiantoulas et al., 2015b). They are considered to have important house-keeping functions by neutralizing and promoting the anti-inflammatory clearance of endogenous waste products. We and others have hypothesized that the protective functions of IgM derive mainly from their ability to recognize OSE that are also prominent on OxLDL (Miller et al., 2011; Tsiantoulas et al., 2015b). Previous studies have demonstrated an atheroprotective role for natural IgM antibodies (Tsiantoulas et al., 2015b). Splenectomized $Ldlr^{-/-}$ mice develop accelerated atherosclerosis as lack of proper B cell maturation results in lower peritoneal B-1a cell numbers and concomitantly reduced serum IgM levels (Kyaw et al., 2011). This phenotype could be rescued by adoptive transfer of wild-type B-1a cells but not B-1a cells deficient in IgM secretion ($sIgM^{-/-}$ mice). Consistently, $Ldlr^{-/-}sIgM^{-/-}$ mice were shown to suffer from more severe atherosclerosis than control mice (Lewis et al., 2009). Further supporting data for the atheroprotective role of IgM was obtained in immunization studies using $Ldlr^{-/-}$ mice injected with heat-inactivated pneumococci, which results in an increased production of a certain natural IgM clone (T15/E06) specific for epitopes on pneumococci but also OxLDL because of molecular

mimicry (Binder et al., 2003). The same strategy was selected to investigate whether E06 natural antibodies also exert protective effects in NASH. Indeed, pneumococci-immunized *Ldlr*$^{-/-}$ mice fed an atherogenic diet developed less severe NASH than not-immunized mice, characterized by reduced immune cell filtration into the liver and reduced pro-inflammatory cytokine expression (Bieghs et al., 2012). Additionally, the authors found that the liver-resident Kupffer cells (KC) contained less cholesterol crystals and were morphologically more similar to normal KC, while KC from NASH mice display a foam-cell like phenotype with large cytoplasmic inclusions and an overall increased cell size. In support of this hypothesis, additional studies by my thesis laboratory have shown that mice deficient in Siglec-G, a negative regulator of BCR signalling, have increased B1-cell-derived natural IgM antibody secretion with specificity for OxLDL and consequently are protected from atherosclerosis and NASH (Gruber et al., 2016). Furthermore, my thesis laboratory could show that the increased IgM antibody levels directly protect from OxLDL-induced inflammation in a model of sterile peritonitis. In conclusion, while many studies demonstrate the protective functions of natural IgM, the specific antigen(s) that is responsible for mediating the effects has not been identified so far.

3 Materials and Methods

The majority of experimental procedures used during the thesis project has been described and published as part of the manuscript and within its supplementary information, both of which are included in section 4.1 and 4.2, respectively. In the current section, a detailed description of methods with central importance to the project is provided. Furthermore, included below are methods used in the unpublished experiments described in section 4.3.

3.1 Proteins

3.1.1 Generation of MAA-BSA

MAA-BSA was generated as previously described (Xu et al., 1997; Gonen et al., 2014) with several modifications. BSA (2 mg/ml, BSA Fatty Acid Free, PAA Laboratories) was incubated with freshly prepared 0.1 M MDA (1,1,3,3-Tetramethoxypropane, Sigma) and 0.2 M acetaldehyde in phosphate-buffered saline (PBS) at pH 4.8 for 3 hours at 37°C. Under these reaction conditions, the formation of fluorescent 4-methyl-1,4-dihydropyridine-3,5-dicarbonyl (MDHDC)-type adducts is favoured. Low-molecular-weight reactants were removed by centrifugal filtration (Amicon Ultra-15 Centrifugal Filter Units, 10 kDa nominal molecular weight limit, Merck Millipore), followed by 3 washes with sterile PBS pH 7.4 and sterile-filtration using Acrodisc syringe filters (0.42/0.22 μm, Pall Corporation). Protein concentration was measured with the BCA Protein Assay Kit (Pierce). Briefly, for the microplate method, 25 μl of several protein dilutions (e.g. 1:5, 1:10, 1:20) or standards prepared according to the manufacturer's instructions (highest concentration 2 mg/ml) were mixed with 200 μl of working solution and incubated for 30 minutes at 37 °C. Absorbance was measured at 562 nm.

3.1.2 Quality control of MAA-BSA

Before use of MAA-BSA for in vitro stimulation assays or in vivo treatments, every new batch of sham-BSA and MAA-BSA was subjected to the following quality control tests to monitor and ensure consistent MAA-BSA preparations throughout the study.

Fluorescence. Preparation of MAA-BSA results in the generation of cyclic, highly fluorescent MAA structures (Xu et al., 1997). To assess fluorescence, 100 μl of 100 μg/ml

sham- or MAA-BSA were measured in duplicates at 394/462 nm (ex/em) using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments).

Degree of modification. The efficiency of MDA modification was assessed using the 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) assay, which measures the fraction of free amino groups in a given protein (Habeeb, 1966). TNBS reacts with primary amines to form a chromogenic molecule absorbing at 335 nm. Sham- or MAA-BSA was diluted in duplicates into 250 μl dH_2O (200 μg/ml) and combined with 500 μl bicarbonate buffer and 50 μl 0.1% TNBS. After incubation at 37 °C for 1 hour the reaction was stopped with 200 μl 1 N HCl and 100 μl 10% SDS. The tubes were vortexed and incubated for 15 minutes at room temperature. A 96-well plate with technical duplicates was read at 340 nm using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments).

Antibody binding. A chemiluminescent enzyme-linked immunosorbent assay (ELISA) was performed to assess the presence of MAA epitopes using the antibodies indicated in Table 3.1 and as previously described (Binder et al., 2003; Chou et al., 2009; Amir et al., 2012; Hendrikx et al., 2016). White 96-well round-bottom microtiter plates were coated with 1 μg/ml sham- or MAA-BSA in 35 μl tris-buffered saline (TBS) and incubated over night at 4°C. Plates were washed using TBS and an automatic plate washer (ELx405 Select Deep Well Microplate Washer, BioTek Instruments). Unspecific binding was blocked with 75 μl TBS containing 1% BSA for 1 hour at room temperature. Plates were incubated with antibodies against MDA epitopes or other OSE diluted as indicated in Table 3.1 in 35 μl TBS with 1% BSA for 2 hours at room temperature. Secondary antibody (alkaline-phosphatase-(AP)-coupled goat anti-mouse IgM or anti-mouse IgG, 1:20,000, Sigma-Aldrich) was incubated for 1 hour at room temperature to detect binding of primary antibodies to the plate. Generation of chemiluminescent signal by AP was detected after 30 minutes of incubation with 25 μl LumiPhos (Lumigen) at 37°C. Light emission was detected in a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments) and expressed as relative light units (RLU) per 100 ms.

Endotoxin. To exclude bacterial contamination, every batch was tested for the presence of endotoxin. For this, 100 μl of 100 μg/ml sham- or MAA-BSA in PBS were subjected to a chromogenic, limulus-amoebocyte-lysate-based assay with Endosafe-PTS cartridges (PTS20, Charles River Laboratories). All self-generated preparations used for stimulations with cells or in mice were tested for endotoxin and only used when containing < 1.5 ng endotoxin per mg protein (< 1 EU/ml).

Biological activity. To test the pro-inflammatory capacity of MAA-BSA, new batches

Table 3.1: OSE-specific antibodies used in this study.

Name	Antigen	Isotype	ELISA (μg/ml)	References
E06	POVPC	mouse IgM	0.5	Palinski et al. (1996)
E014	MDA-LDL	mouse IgM	0.5	Palinski et al. (1996)
LR04	MDA-LDL	mouse IgM	0.05	Amir et al. (2012)
MDA2	MDA-lysine	mouse IgG	1:100,000	Palinski et al. (1990)
NA17	MDA-LDL	mouse IgM	0.5	Chou et al. (2009)

were checked for induction of IL-8 secretion in THP-1 cells according to the descriptions in Section 3.3.2 and 3.4.1.

3.1.3 Mass spectrometric analyses

Mass spectrometric analyses of selected MAA-BSA preparations (5 min, 3 h, 24 h) were kindly provided by R. Birner-Grünberger, Institute of Pathology & Omics Center Graz, Medical University of Graz, Austria.

3.1.4 Alternative BSA modifications

Next to fluorescent MAA-BSA preparations, additional BSA modifications have been prepared and used as controls to exclude that the effects observed with MAA-BSA are due to modifications of the protein backbone itself, e.g. due to structural alterations as a result of the procedure. To generate AA-BSA, MDA-BSA, MAA-BSA and FHP-BSA preparations used in Section 4.1, BSA was modified with acetaldehyde, MDA, MAA and FHP using the protocol from Section 3.1.1 with changes to the reaction conditions as indicated in Table 3.2. To stabilize the respective modifications, sodium cyanoborohydride ($NaCNBH_3$) was added at the end of the reaction. $NaCNBH_3$ is a reducing agent, which can stabilize Schiff bases and prevent the degradation of less stable adducts such as acetaldehyde adducts.

While the degree of modification assessed by TNBS was comparable in all preparations, AA-BSA and MDA-BSA preparations had very low amounts of fluorescent adducts compared to MAA-BSA. Notably, MDA-BSA was slightly more fluorescent than sham- or AA-BSA because during the reaction MDA can decompose to acetaldehyde spontaneously and together with MDA produce fluorescent MAA adducts to a low extent (Gonen et al., 2014).

Table 3.2: Reaction conditions for alternative BSA modifications.

Modification	Reactants	pH	Duration (h)
sham-BSA	none	4.8	3.0
acetaldehyde-BSA	acetaldehyde	4.8	3.0
MDA-BSA	MDA	7.4	0.5
MAA-BSA	MDA, acetaldehyde	4.8	3.0
FHP-BSA	MDA	5.0	3.0

3.1.5 Preparation of MAA-BSA samples for time-course analysis

To perform a time-course analysis of MAA-BSA generation, BSA was modified with acetaldehyde and MDA as described in Section 3.1.1. To stop the reaction after 5, 15, 30, 60 minutes, 3 or 24 hours, acetaldehyde and MDA were removed at respective timepoints using centrifugal filtration. The resulting time-course samples were subjected to the same quality control measurements as before.

3.1.6 MDA modification of other carrier proteins

For modification of bMGL, ovalbumin or fibronectin, the modification was performed as described in Section 3.1.1 with the reaction volumes downscaled to 2 ml (instead of 20 ml) to adjust to the lower amounts of protein available for modification. Other than that, the same procedures were applied to modification of these proteins as described in Section 3.1.1, including all quality control steps.

3.2 RNA and cDNA

3.2.1 Total RNA extraction and cDNA synthesis

Total RNA was isolated using the Total RNA Kit peqGold (VWR Peqlab), followed by cDNA synthesis using 100-200 ng total RNA per reaction and the High-Capacity Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Typically, RNA was extracted from one confluent well of a 24- or 96-well-plate (seeded with 300,000 or 100,000 cells/well). Table 3.3 indicates the conditions used during the cDNA synthesis. Reactions were performed in PCR tubes in a total volume of 10 μl.

3.2.2 Quantitative PCR analysis

For quantitative PCR (qPCR) analysis, 4.5 μl of 1:10 diluted reverse transcribed cDNA were used per reaction, combined with 5 μl 2x Fast SYBR Green Master Mix and 0.5 μl

Table 3.3: Thermal cycling parameters for reverse transcription of mRNA.

Step	Temperature	Time
Annealing	25 °C	10 min.
Transcription	37 °C	30 min.
Inactivation	95 °C	5 min.
Storage	4 °C	forever

Table 3.4: Thermal cycling parameters for quantitative PCR.

Step		Temperature	Time
1	Activation	95 °C	10 min.
3	Denaturation	95 °C	15 sec.
4	Annealing	60 °C	15 sec.
5	Extension	72 °C	30 sec.
6	Storage	4 °C	forever

10 μM primer mix and incubated according to the protocol in Table 3.4 on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Steps 3-5 were repeated 39 times. Primer sequences are listed in Table 3.5. Induction of mRNA expression from genes of interest is displayed as relative expression compared to an untreated condition. The $\Delta\Delta$Ct mode was used to calculate fold inductions, expression of a house-keeping gene was used as a reference for normalization.

Table 3.5: qPCR primers used in this study.

ID	Name	Sequence
CJB011	mCycB-FW	CAGCAAGTTCCATCGTGTCATCAAGG
CJB012	mCycB-RV	GGAAGCGCTCACCATAGATGCTC
CJB013	mCxcl10-FW	GGTCTGAGTGGGACTCAAGG
CJB014	mCxcl10-RV	GTGGCAATGATCTCAACACG
CJB032	mCxcl1-FW	GCTGGGATTCACCTCAAGAA
CJB033	mCxcl1-RV	TCTCCGTTACTTGGGGACAC
CJB040	mTNFa-FW	CCCATATACCTGGGAGGAGTCTTC
CJB041	mTNFa-RV	CATTCCCTTCACAGAGCAATGAC
CJB042	mNlrp3-FW	CCACAGTGTAACTTGCAGAAGC

continued on next page

Table 3.5: qPCR primers used in this study.

ID	Name	Sequence
CJB043	mNlrp3-RV	GGTGTGTGAAGTTCTGGTTGG
CJB044	mNlrc4-FW	CTGGAAAAGGATGGGAATGA
CJB045	mNlrc4-RV	CCAAGGCAGCATCAATGTAG
CJB047	mCcl3-FW	GTGGAATCTTCCGGCTGTAG
CJB048	mCcl3-RV	ACCATGACACTCTGCAACCA
CJB049	mCcl2b-FW	GCTGGAGAGCTACAAGAGGATCA
CJB050	mCcl2b-RV	ACAGACCTCTCTCTTGAGCTTGGT
CJB051	mCxcl3b-FW	CAGCCACACTCCAGCCTA
CJB052	mCxcl3b-RV	CACAACAGCCCCTGTAGC
	mCcl24-FW	GCTGCACGTCCTTTATTTCC
	mCcl24-RV	TATGTGCCTCTGAACCCACA
	mIl10-FW	GCCAGAGCCACATGCTCCTA
	mIl10-RV	GTCCAGCTGGTCCTTTGTTTG
	mCcl1-FW	GGATGTTGACAGCAAGAGCA
	mCcl1-RV	TAGTTGAGGCGCAGCTTTCT
	mCcl9-FW	TACTGCCCTCTCCTTCCTCA
	mCcl9-RV	TTGAAAGCCCATGTGAAACA
	mIL6-FW	GTGGCTAAGGACCAAGACCATC
	mIL6-RV	ACGCACTAGGTTTGCCGAGTAG
	mCcl5-FW	GTGCCCACGTCAAGGAGTAT
	mCcl5-RV	TCCTTCGAGTGACAAACACG
	mF-IL1b	TGTGCAAGTGTCTGAAGCAGC
	mR-IL1b	TGGAAGCAGCCCTTCATCTT
	mCxcl2-FW	AGTGAACTGCGCTGTCAATG
	mCxcl2-RV	TTCAGGGTCAAGGCAAACTT
CJB055	mTLR9-FW	CAGTTTGTCAGAGGGAGCCT
CJB056	mTLR9-RV	CTGTACCAGGAGGGACAAGG
CJB057	mGpnmb_1-FW	GTCAGCATGGAAAGTCTCTGC
CJB058	mGpnmb_1-RV	GGATACTGTTCATGGCCCAG
CJB059	mGpnmb_2-FW	CGGAGTCAGCATGGAAAGTC
CJB060	mGpnmb_2-RV	GGATACTGTTCATGGCCCAG
CJB061	mMitf_1-FW	CATCATCAGCCTGGAATCAA
CJB062	mMitf_1-RV	TCAAGTTTCCAGAGACGGGT
CJB063	mMitf_2-FW	GAAATTTTGGGCTTGATGGA

continued on next page

Table 3.5: qPCR primers used in this study.

ID	Name	Sequence
CJB064	mMitf_2-RV	GGAGTTGCTGATGGTAAGGC
	mTLR2-FW	GTTTCTGATGGTGAAGGTTG
	mTLR2-RV	GCTGAAGAGGACTGTTATGG
	mTLR9_2-FW	GCACAGGAGCGGTGAAGGT
	mTLR9_2-RV	GCAGGGGTGCTCAGTGGAG
	mTLR9_3-FW	TGCAGGAGCTGAACATGAAC
	mTLR9_3-RV	TAGAAGCAGGGGTGCTCAGT
	mNrros-FW	ACTGCAGCTTCCCAAGGA
	mNrros-RV	TGGGTACCGAAGCAAGGT
	mEgr1-FW	GAGGAGATGATGCTGCTGAG
	mEgr1-RV	TGCTGCTGCTGCTATTACC

3.3 Cell Culture

Human monocytic THP-1 cells and human Jurkat T cells were cultured in RPMI-1640 (Thermo Fischer Scientific) supplemented with penicillin/streptomycin and 10% FCS (RPMI-full) at 37 °C, 5% CO_2. RAW264.7 macrophages, a macrophage-like cell line established from a Balb/c tumour induced by the Abelson murine leukemia virus, were cultured in DMEM-GlutaMax (Thermo Fisher Scientific) supplemented with penicillin/streptomycin and 10% FCS at 37 °C, 5% CO_2. Primary murine macrophages were cultured in RPMI-full supplemented with either 20 ng/ml murine recombinant M-CSF (Peprotech) or 10% conditioned medium at 37 °C. Conditioned medium was obtained from serum-free supernatants of M-CSF-producing L929 fibroblasts (gift from S. Knapp lab).

3.3.1 Isolation of primary cells from mouse

For isolation of bone marrow, femura and tibiae were removed from the euthanized mouse, separated from muscle, sterilized briefly in 70% ethanol and temporarily stored in RPMI with antibiotics on ice. For each bone, one 0.5-ml-microtube was prepared by piercing the bottom of the tube with a 20-G-needle and inserting it into an 1.5-ml eppendorf tube. The cleaned bones were opened (at the distal end of the tibia and by removing the greater trochanter of the femur) and placed into the microtubes with the opened end facing downwards. The bones were centrifuged at 5,000 g for 2 minutes to pellet the bone marrow into the 1.5-ml tube. Cell pellets were resuspended in 1 ml RPMI with antibiotics and passed through a 100 μm strainer to remove cell clumps and connective tissue. The strainer was rinsed with 20 ml RPMI plus antibiotics and the cells were pelleted at 300 g for

5 minutes at 4 °C. Erythrocyte lysis was performed with 2 ml hemolysis buffer (Morphisto) for 2 minutes at room temperature. The cell suspension was filled up to 20 ml with RPMI to stop the reaction and centrifuged at 300 g for 5 minutes. The resulting cell pellet was resuspended in 5-10 ml RPMI-full and a sample was removed for cell counting with the CASY counter (Roche). Cells were cultured as described in Section 3.3. Alternatively, bone marrow was isolated by the syringe method. For this, bones were opened at both ends and the bone marrow was flushed out of the bones by injecting several ml RPMI using a syringe with a 25-G needle inserted into one end of the bone. The subsequent steps were performed as described above. A third alternative involves crushing the bones using a pestle and a mortar. The resulting cell suspension is filtered using cell strainers to remove bone fragments.

Isolation of resident peritoneal macrophages was performed by peritoneal lavage. Euthanized mice were injected intraperitoneally with 10 ml cold RPMI using a 23-G needle. Adherent peritoneal macrophages were persuaded to detach from surfaces by applying repetitive taps to the abdomen. The 23-G needle was re-used to collect the peritoneal lavage fluid. Typically, 8-9 ml liquid could be recovered. The cell suspension was centrifuged at 300 g for 5 minutes, and, where applicable, erythrocytes were lysed with hemolysis buffer. Peritoneal cells were allowed to attach to culture plates and non-attaching cells (mainly B cells) were removed by sequential washes.

Isolation of thymocytes was performed on 4-6-week-old wild-type or gene-deficient mice. The thymus was removed from the euthanized mouse and passed through a 100 μm strainer. The strainer was washed with RPMI, followed by centrifugation. Cells were directly used for apoptosis induction in RPMI-full supplemented with apoptosis stimulants or as indicated.

Isolation of Kupffer cells from livers of wild-type and gene-deficient mice is described in detail in Section 4.2.

3.3.2 Stimulation assays

THP-1 cells were expanded in RPMI-full at 37 °C. For stimulation, cells were washed twice with PBS, seeded at a concentration of 500,000 cells/ml and stimulated in serum-free RPMI-1640 containing penicillin/streptomycin and 0.5% BSA for 16 hours with the respective stimuli. Cell supernatants were harvested by centrifugation and checked for secretion of chemokines (IL-8) by ELISA according to the manufacturer's instructions (BD Biosciences). Alternatively, THP-1 cells were stimulated for 2 hours, centrifuged and resuspended in 100 μl lysis buffer for total RNA extraction.

Bone-marrow-derived macrophages (BMDM) were generated by differentiation of bone-marrow cells isolated from the femura and tibiae of wild-type or gene-deficient mice using RPMI-full supplemented with 10% conditioned medium. Cells were re-fed with condi-

tioned medium on day 3. On day 7, cells were fed with RPMI with 10% FCS for 8 hours and stimulated in serum-free RPMI supplemented with 0.5% BSA for 16 hours with naive, sham-treated or MDA-modified BSA and controls as indicated. Cell supernatants were collected by centrifugation and screened for protein secretion (CXCL1 or CXCL2) by ELISA according to the manufacturer's instructions (R&D Systems). Alternatively, BMDM were stimulated for 2, 4, 6 or 8 hours and after removal of supernatant resuspended in 100 or 350 μl lysis buffer for total RNA extraction.

3.3.3 Generation of apoptotic cells

For irradiation, cell lines or primary cells were seeded on 10 cm TC-dishes, washed with PBS once and irradiated with 100 mJ/cm^2 UVC in 5 ml PBS. Then, cells were fed with fresh medium and incubated for 16 hours. Floating cells were collected and stained for flow cytometry. For chemical induction of apoptosis, drugs such as actinomycin D, cycloheximide, dexamethasone, etoposide and others (obtained from Millipore, APT800; or Sigma) were used at different concentrations to induce apoptosis. DMSO was used as a control treatment. Cells were incubated with apoptosis inducers for different timepoints and harvested by centrifugation for antibody staining. Inhibition of apoptosis was performed with the pan-caspase inhibitor Z-VAD-FMK (BD Biosciences, 550377) dissolved in DMSO. Blebbistatin was from Cayman Chemical.

3.3.4 Depletion of LR04-positive apoptotic cells

Jurkat T cells were irradiated with 100 mJ/cm^2 and incubated over night to induce apoptosis. Cells were harvested and incubated with 40 μg/ml LR04 in FACS buffer (PBS with 0.5% FCS) for 20 minutes on 4 °C. Cells were washed and resuspended in 80 μl FACS buffer and 20 μl anti-mouse IgM microbeads for MACS (Miltenyi, Biotec). After 15 minutes incubation at 4 °C, cells were washed, resuspended in FACS buffer and loaded on a column for magnetic-activated cell sorting (MACS). The column was washed 3 times with FACS buffer to elute unbound cells (LR04-negative). LR04-positive cells were harvested by removing the magnet and applying pressure to the column with a plunger. Cells were counted and stained for flow cytometry.

3.4 Immunoassays

3.4.1 Cytokine ELISA

Mouse CXCL1 and CXCL2 ELISA components were obtained from R&D Systems, human IL-8 ELISA components were obtained from BD Biosciences. Coating of Nunc 96-well flat-bottom Maxisorp Immunoplates was performed with 100 μl capture antibody (2 μg/mL

in PBS or 0.1 M $NaHCO_3$ buffer) over night at 4 °C. The coated plate was washed 3 times with PBS with 0.05% Tween and blocked with 1% BSA-PBS for 1 h at room temperature. After washing, samples (either directly from centrifuged cell supernatants or after dilution 1% BSA-PBS) and standards (from 1000 or 500 pg/mL to 15.6 or 7.8 pg/mL) were incubated for 2 h at room temperature or 4 °C over night. Biotinylated detection antibodies (200 ng/mL in PBS) were incubated for 2 h at room temperature, followed by incubation with streptavidin-coupled horseradish peroxidase (HRP) for 30 min at room temperature. Signal detection was performed after addition of 100 μl TMB solution containing a chromogenic peroxidase substrate. After 15 minutes, 50 μl of stop solution (1 N H_2SO_4) was added and the OD was read at 450 nm, with a wavelength correction (for subtracting background) set at 540 nm. Absolute concentrations were extrapolated using OD_{450} measurements of the standard curve.

3.4.2 CFH and C4b-binding protein ELISA

A chemiluminescent ELISA was performed to assess complement factor H (CFH) or C4b-binding protein (C4BP) binding. White 96-well round-bottom microtiter plates were coated with 1 μg/ml antigens such as sham- or MAA-BSA in 35 μl PBS and incubated over night at 4°C. Plates were washed using PBS (CFH) or PBS with 0.05% Tween (C4BP) and an automatic plate washer (ELx405 Select Deep Well Microplate Washer, BioTek Instruments) and unspecific binding was blocked with 75 μl TBS containing 1% BSA for 1 hour at room temperature. Wells were incubated with CFH at 0.75 μg/ml for 2 hours at room temperature, followed by 3 washes. Goat anti-CFH (1:10,000) or mouse anti-C4BP (1:687.5) antibodies was added to the wells in 35 μl TBS for 2 hours. To detect primary antibody binding, 35 μl of anti-goat ot anti-mouse IgG coupled to AP (1:20,000) were incubated for 2 hours. Chemiluminescence was detected after 30 minutes of incubation with 25 μl LumiPhos (Lumigen) at 37 °C. Light emission was detected in a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments) and expressed as relative light units (RLU) per 100 ms.

3.4.3 Flow cytometry

Single-cell suspensions were blocked with antibodies against murine CD16/32 (1:200, 15 minutes) or human IgG in FACS buffer (PBS with 1% BSA), followed by incubation with antibodies against OSE or isotype controls for mouse IgG and IgM (10 μg/ml, 20 minutes). Secondary antibody staining was performed with anti-mouse IgM or IgG antibodies coupled to APC or FITC (1:1000, 20 minutes). Then, viability stainings were performed in calcium-containing Annexin-V-buffer using an Annexin-V/7-AAD staining kit (eBioscience, 88-8102-74). Briefly, 5 μl Annexin-V-PE were incubated in 100 μl Annexin-V-buffer containing up to 1 mio cells for 15 minutes at 4 °C protected from light. After

washing with Annexin-V-buffer, cells were incubated for 5 minutes in 100 μl Annexin-V-buffer with 5 μl 7-AAD in the dark. Cells were recorded by flow cytometry (BD FACS Calibur) immediately after and analyzed with FlowJo software.

4 Results

4.1 Manuscript "Malondialdehyde epitopes are sterile mediators of hepatic inflammation in hypercholesterolemic mice"

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Published in: Hepatology. 2016 Dec 16. doi: 10.1002/hep.28970. [Epub ahead of print] PubMed PMID: 27981604.

HEPATOLOGY

HEPATOLOGY, VOL. 00, NO. 00, 2016

Malondialdehyde Epitopes Are Sterile Mediators of Hepatic Inflammation in Hypercholesterolemic Mice

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Diet-related health issues such as nonalcoholic fatty liver disease and cardiovascular disorders are known to have a major inflammatory component. However, the exact pathways linking diet-induced changes (e.g., hyperlipidemia) and the ensuing inflammation have remained elusive so far. We identified biological processes related to innate immunity and oxidative stress as prime response pathways in livers of low-density lipoprotein receptor-deficient mice on a Western-type diet using RNA sequencing and *in silico* functional analyses of transcriptome data. The observed changes were independent of the presence of microbiota and thus indicative of a role for sterile triggers. We further show that malondialdehyde (MDA) epitopes, products of lipid peroxidation and markers for enhanced oxidative stress, are detectable in hepatic inflammation predominantly on dying cells and stimulate cytokine secretion as well as leukocyte recruitment *in vitro* and *in vivo*. MDA-induced cytokine secretion *in vitro* was dependent on the presence of the scavenger receptors CD36 and MSR1. Moreover, *in vivo* neutralization of endogenously generated MDA epitopes by intravenous injection of a specific MDA antibody results in decreased hepatic inflammation in low-density lipoprotein receptor-deficient mice on a Western-type diet. *Conclusion*: Accumulation of MDA epitopes plays a major role during diet-induced hepatic inflammation and can be ameliorated by administration of an anti-MDA antibody. (HEPATOLOGY 2016; 00:000–000)

The combination of sedentary lifestyle and nutrient surplus has resulted in alarmingly high incidences of metabolic diseases that encompass an array of manifestations including obesity, hypertension, dyslipidemia, and insulin resistance.[1-4] As a consequence, the risk of developing type 2 diabetes, nonalcoholic fatty liver, and cardiovascular disease has increased. However, the exact

Abbreviations: BMDM, bone marrow–derived macrophage; BSA, bovine serum albumin; CD, cluster of differentiation; CONV-R, conventionally raised; CXCL1, chemokine (C-X-C motif) ligand 1; DAMP, danger-associated molecular pattern; GF, germ-free; Ig, immunoglobulin; Ldlr, low-density lipoprotein receptor; MAA, malondialdehyde-acetaldehyde; MAA-BSA, MAA-modified BSA; MDA, malondialdehyde; MSR1, macrophage scavenger receptor 1; NASH, nonalcoholic steatohepatitis; ND, normal diet; NFκB, nuclear factor κB; OSE, oxidation-specific epitope; OxLDL, oxidized low-density lipoprotein; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TBARS, thiobarbituric acid–reactive substances; TLR, Toll-like receptor; WD, Western-type diet.

Received June 24, 2016; accepted November 26, 2016.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28970/suppinfo.

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Supported by the SFB Lipotox F30 of the Austrian Science Fund (to C.J.B.); a Boehringer Ingelheim PhD fellowship (to C.J.-L.B.); Cardiovascular Research The Netherlands (CVON 2012-03, IN-CONTROL) and The Netherlands Organisation for Scientific Research (Vidi 016.126.327, to R.S.-S.); an EMBO Short-Term Fellowship, an Andrew K. Burroughs Short Term Fellowship, and a Kootstra Fellowship (to T.H.); a Shared Expertise project grant from the German Center for Cardiovascular Research (AIIMAP, to C.R. and S.J.); the German Federal Ministry on Education and Research (01EO1503) and Deutsche Forschungsgemeinschaft individual grants (RE 3450/3-1 and RE 3450/5-1, to C.R.). S.J. and C.R. are members of Young Deutsches Zentrum für Herz-Kreislauf-Forschung. J.W. is the recipient of a DOC fellowship of the Austrian Academy of Sciences.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.28970

Potential conflict of interest: Nothing to report.

pathways that link diet-induced changes (e.g., hyperlipidemia) and the ensuing inflammation have remained elusive so far.

Increased oxidative stress is associated with tissue injury and inflammation where an accumulation of free radicals results in the generation of lipid decomposition products such as malondialdehyde (MDA).[5] MDA can covalently modify self-molecules such as proteins or phospholipids and form hapten-like moieties termed "MDA epitopes." These and other oxidative modifications are collectively called "oxidation-specific epitopes" (OSEs) and have been found on the surface of dying cells *in vitro* as well as in many inflammatory pathologies including atherosclerosis.[6] We and others showed previously that OSEs are recognized by soluble and membrane-bound factors of the innate immune system.[6] For example, we demonstrated that up to 15% of all natural antibodies recognize MDA epitopes.[7] Consistent with the role of natural antibodies in promoting clearance of damaged cell structures, we hypothesize that MDA epitopes, being a major target of OSE-specific natural antibodies, constitute prominent endogenous danger-associated molecular patterns (DAMPs) that could propagate an existing inflammation and render it chronic.

In the current study, we provide several lines of evidence that MDA epitopes represent important DAMPs that initiate and perpetuate inflammatory processes in the liver triggered by a Western-type diet (WD).

Materials and Methods

ANIMAL EXPERIMENTS

C57BL/6J and low-density lipoprotein receptor–deficient ($Ldlr^{-/-}$) mice were purchased from Charles River Laboratories and The Jackson Laboratory, respectively. Mice were kept under specific pathogen-free conditions at the Medical University of Vienna or Maastricht University with *ad libitum* access to water and a regular chow diet (normal diet [ND]; ssniff Spezialdiäten GmbH). Age-matched germ-free (GF) $Ldlr^{-/-}$ mice were obtained by aseptic hysterectomy and maintained in sterile flexible film mouse isolators in the Translational Animal Research Center of the University Medical Center Mainz. Specific pathogen-free (conventionally raised [CONV-R]) or GF mice were fed an ND or a WD containing 21% fat and 0.2% cholesterol for 1-4 weeks. For the antibody intervention study, baseline levels of lipids, MDA-acetaldehyde (MAA) binding, P2-binding, and total immunoglobulin M (IgM) titers were evaluated before the diet. During the light cycle, $Ldlr^{-/-}$ mice received either 200 μg isotype control (LEAF Purified Mouse IgM, κ Isotype Ctrl Antibody, clone MM-3; 401604, BioLegend) or LR04 (monoclonal IgM antibody against MDA epitopes cloned from murine $Ldlr^{-/-}$ spleens on an atherogenic diet,[4] tested for endotoxin and microbial contaminations; kind gift of J.L. Witztum) or phosphate-buffered saline (PBS) intravenously on day 1 and day 4 after starting the diet. Hepatic inflammation was assessed by RNA sequencing and/or immunohistochemistry as well as gene expression analyses of selected genes using quantitative RT-PCR. Lipid and liver enzyme levels in plasma and liver homogenates were determined as described.[8] Hepatic oxidative stress was assessed by histochemistry and flow cytometry. Sterile peritonitis was induced in female C57BL/6J mice, 11-16 weeks old, using 25 mg/kg MAA-modified bovine serum albumin (MAA-BSA) or unmodified (naive) bovine serum albumin (BSA) suspended in 200 μL PBS. After 2 hours, peritoneal fluid and cells were harvested and

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checked for leukocyte recruitment by flow cytometry and cytokine secretion by enzyme-linked immunosorbent assays.

STATISTICS

All data are displayed as mean ± standard error of the mean. For comparison of two groups, the Student paired (when applicable) or unpaired *t* test was used. For comparison of more than two groups, one-way analysis of variance followed by Tukey's multiple comparison test was used. All analyses were performed with GraphPad Prism, version 6.04. $P < 0.05$ was considered significant.

Additional details can be found in the Supporting Information.

Results

WD-INDUCED STEATOHEPATITIS ACTIVATES INNATE IMMUNE RESPONSES IN *Ldlr*$^{-/-}$ MICE

We have previously shown that short-term feeding of a WD to atherosclerosis-prone *Ldlr*$^{-/-}$ mice that display a lipoprotein profile resembling the situation in dyslipidemic humans results in the development of hepatic steatosis and inflammation.[9] To investigate factors and mechanisms contributing to diet-induced hepatic inflammation in an unbiased manner, we fed *Ldlr*$^{-/-}$ mice a ND or WD for 2 or 4 weeks and performed transcriptomic profiling of liver tissue using RNA sequencing. Lipid levels in both plasma and liver were elevated in the mice fed WD, accompanied by hepatic leukocyte infiltrates (Supporting Fig. S1A,B and Table S1), in line with our previous study.[9] In general, all transcriptome data were similar across all samples and replicates, with Pearson correlation coefficients in the range 0.96-0.98 (Supporting Fig. S2A). Furthermore, RNA sequencing profiles for 2 and 4 weeks of WD-fed mice were more similar to each other than to those of ND-fed mice (Supporting Fig. S2B,C). When comparing the intersection of differentially regulated genes between the groups, we found that most genes differentially regulated in 2-week WD versus ND comparisons were also different in 4-week WD versus ND comparisons (>400; Supporting Fig. S2C, first vertical bar). Collectively, WD induces highly consistent and robust alterations of hepatic gene expression between biological replicates and time points. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology analyses of the transcripts differentially regulated upon 2 weeks of WD demonstrated enrichment of genes involved in pathways and biological processes mainly related to immune responses and responses to external stimulus or stress (Fig. 1A), including the chemokine signaling pathway, leukocyte transendothelial migration, cytokine–cytokine receptor interaction (Fig. 1B), and metabolic activity (Fig. 1C), consistent with other high-fat diet models.[10,11] Already 2-week WD resulted in significant enrichment of genes associated strongly with mechanisms of innate immunity such as Fcγ-mediated phagocytosis, complement system, and the Toll-like receptor (TLR) signaling pathway (Fig. 1B). Many inflammatory genes regulated by WD primarily belonged to the protein families of chemokines and complement components, indicating a predominant activation of innate immune responses in this model of diet-induced hepatic inflammation (Fig. 1D). Indeed, also pattern-recognition receptors such as *Tlr2, Tlr9, Clec12a*, and *Nlrp3* were up-regulated as well as regulators and transcription factors of the nuclear factor κB (NFκB) pathway, a prominent signal transduction mechanism of innate immunity (Fig. 1D). In addition, Gene Ontology analysis of the significantly down-regulated transcripts in livers after 2 weeks of WD revealed a strong enrichment of genes involved in the biological process "oxidation reduction" (Fig. 1C), suggesting activation of oxidative stress response pathways. Indeed, a set of genes implicated in the oxidative stress response was found to be deregulated upon WD in our global analysis (Fig. 1E). Together, we show that 2 weeks of WD are sufficient to induce global changes in the expression of hepatic genes, which are still present and largely unchanged after 4 weeks of WD feeding, with a strong enrichment for transcripts involved in innate immunity and the oxidative stress response.

MICROBIOTA ARE NOT REQUIRED FOR THE DEVELOPMENT OF HEPATIC STEATOSIS AND INFLAMMATION

To test whether the presence of microbiota is a requirement for diet-induced hepatic inflammation in hypercholesterolemic mice, we fed CONV-R and GF *Ldlr*$^{-/-}$ mice a WD for 2 weeks and analyzed their livers for the presence of inflammation. Even in the absence of microbiota, WD induced hyperlipidemia, hepatic steatosis, and inflammation in *Ldlr*$^{-/-}$ mice

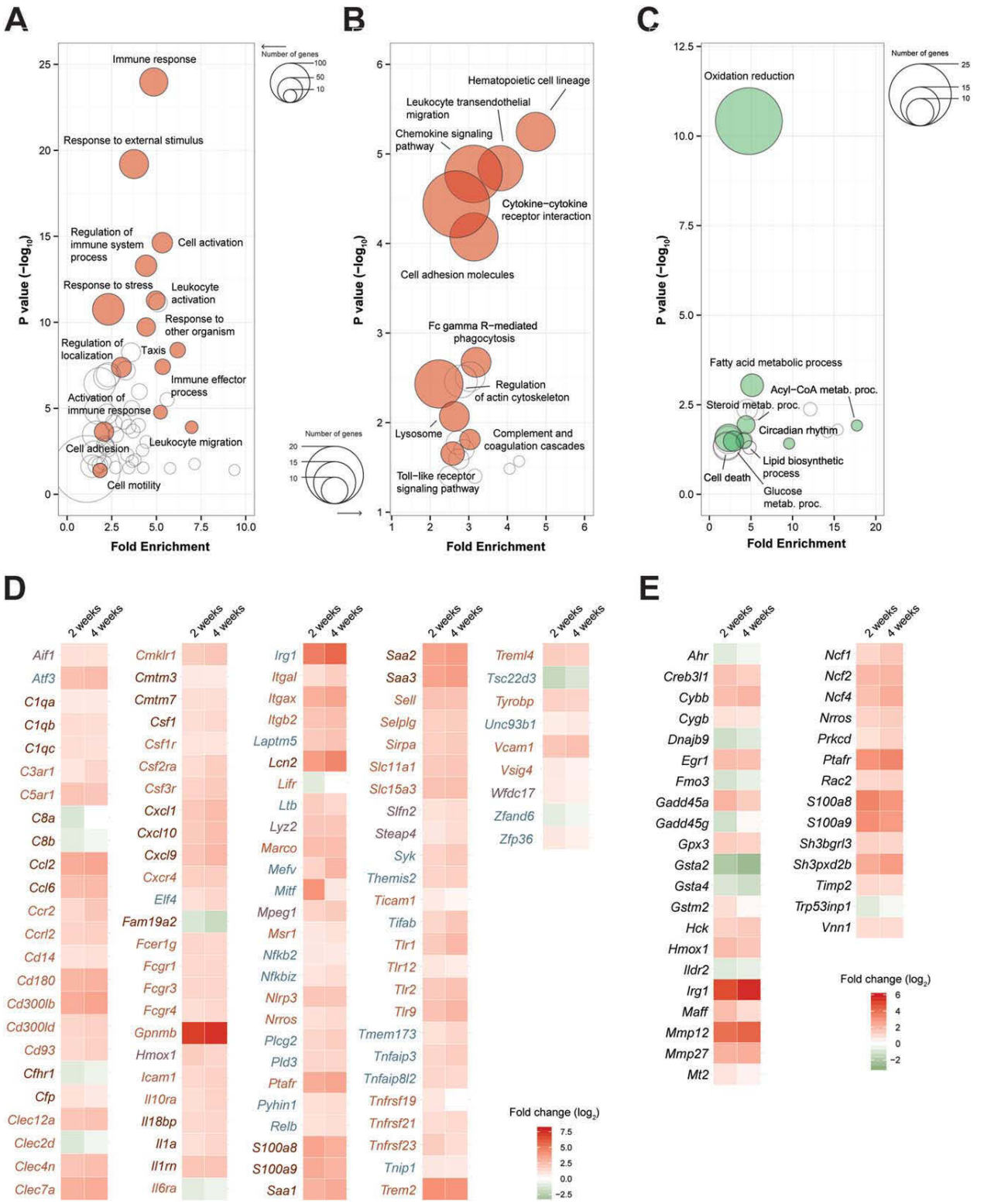
A
Immune response
Response to external stimulus
Regulation of immune system process
Cell activation
Response to stress
Leukocyte activation
Response to other organism
Regulation of localization
Taxis
Immune effector process
Activation of immune response
Leukocyte migration
Cell adhesion
Cell motility
Number of genes
100
50
10
P value (−log10)
Fold Enrichment
B
Hematopoietic cell lineage
Leukocyte transendothelial migration
Chemokine signaling pathway
Cytokine−cytokine receptor interaction
Cell adhesion molecules
Fc gamma R−mediated phagocytosis
Regulation of actin cytoskeleton
Lysosome
Complement and coagulation cascades
Toll−like receptor signaling pathway
Number of genes
20
15
10
P value (−log10)
Fold Enrichment
C
Oxidation reduction
Number of genes
25
15
10
Fatty acid metabolic process
Acyl−CoA metab. proc.
Steroid metab. proc.
Circadian rhythm
Lipid biosynthetic process
Cell death
Glucose metab. proc.
P value (−log10)
Fold Enrichment
D
2 weeks
4 weeks
Aif1
Atf3
C1qa
C1qb
C1qc
C3ar1
C5ar1
C8a
C8b
Ccl2
Ccl6
Ccr2
Ccrl2
Cd14
Cd180
Cd300lb
Cd300ld
Cd93
Cfhr1
Cfp
Clec12a
Clec2d
Clec4n
Clec7a
Cmklr1
Cmtm3
Cmtm7
Csf1
Csf1r
Csf2ra
Csf3r
Cxcl1
Cxcl10
Cxcl9
Cxcr4
Elf4
Fam19a2
Fcer1g
Fcgr1
Fcgr3
Fcgr4
Gpnmb
Hmox1
Icam1
Il10ra
Il18bp
Il1a
Il1rn
Il6ra
Irg1
Itgal
Itgax
Itgb2
Laptm5
Lcn2
Lifr
Ltb
Lyz2
Marco
Mefv
Mitf
Mpeg1
Msr1
Nfkb2
Nfkbiz
Nlrp3
Nrros
Plcg2
Pld3
Ptafr
Pyhin1
Relb
S100a8
S100a9
Saa1
Saa2
Saa3
Sell
Selplg
Sirpa
Slc11a1
Slc15a3
Slfn2
Steap4
Syk
Themis2
Ticam1
Tifab
Tlr1
Tlr12
Tlr2
Tlr9
Tmem173
Tnfaip3
Tnfaip8l2
Tnfrsf19
Tnfrsf21
Tnfrsf23
Tnip1
Trem2
Treml4
Tsc22d3
Tyrobp
Unc93b1
Vcam1
Vsig4
Wfdc17
Zfand6
Zfp36
Fold change (log2)
7.5
5.0
2.5
0.0
−2.5
E
2 weeks
4 weeks
Ahr
Creb3l1
Cybb
Cygb
Dnajb9
Egr1
Fmo3
Gadd45a
Gadd45g
Gpx3
Gsta2
Gsta4
Gstm2
Hck
Hmox1
Ildr2
Irg1
Maff
Mmp12
Mmp27
Mt2
Ncf1
Ncf2
Ncf4
Nrros
Prkcd
Ptafr
Rac2
S100a8
S100a9
Sh3bgrl3
Sh3pxd2b
Timp2
Trp53inp1
Vnn1
Fold change (log2)
6
4
2
0
−2

FIG. 1

(Fig. 2A,B) characterized by infiltration of neutrophils and macrophages (Fig. 2C). Of note, the numbers of WD-induced infiltrating cells were significantly lower in GF *Ldlr*$^{-/-}$ mice compared to their CONV-R counterparts, indicating that the presence of commensal microbiota has the capacity to augment phagocyte accumulation in the liver. We found that a range of transcripts involved in inflammatory responses that were highly up-regulated due to WD in our global analysis were also up-regulated in GF *Ldlr*$^{-/-}$ mice upon WD (Fig. 2D). Importantly, we confirmed that key deregulated genes involved in the oxidative stress response such as *Cybb, Nrros, Irg1, Mmp12, Ncf1*, and *Ncf2* were also significantly up-regulated in livers of GF mice after 2 weeks of WD (Fig. 2F). This observation suggests that WD evokes the development of increased oxidative stress and innate defense mechanisms independent of the presence of microbiota. Indeed, induction of the gene for chemokine (C-X-C motif) ligand 1 (*Cxcl1*) was comparable between GF and CONV-R mice (Fig. 2E). Thus, sterile, nonbacterial triggers have a major contribution to diet-induced hepatic inflammation.

WD CAUSES ENHANCED OXIDATIVE STRESS AND FORMATION OF MDA EPITOPES IN THE LIVER

Consistent with our findings, multiple lines of evidence document the occurrence of oxidative stress in hepatic inflammation.[10,12] Therefore, we investigated whether livers from WD-fed mice exhibited more oxidative stress by staining for hepatic reactive oxygen species (ROS) production. We found that these livers indeed displayed increased dihydroethidium staining, indicating higher levels of ROS production compared to livers from ND-fed mice (Fig. 3A), consistent with the oxidative stress response profile on the transcriptome level (Fig. 1E). Additionally, flow-cytometric analysis of both whole livers and isolated Kupffer cells using the oxidative stress indicator chloromethyl 2′,7′-dichlorofluorescein diacetate demonstrated a robustly elevated ROS production in the livers of WD-fed mice (Fig. 3B). Importantly, we also detected elevated oxidative stress in livers of GF mice on WD compared to ND (Supporting Fig. S3A). As a major consequence of oxidative stress is lipid peroxidation, we next investigated whether feeding *Ldlr*$^{-/-}$ mice WD also results in the production of thiobarbituric acid–reactive substances (TBARS). Increased TBARS were found in Kupffer cells isolated from mice after 2 or 4 weeks of WD, while no differences could be detected in whole-liver homogenates (Fig. 3C; Supporting Fig. S3B). In line with this, immunohistochemical analysis demonstrated the accumulation of lipid peroxidation–derived MDA epitopes in the livers of WD-fed, but not ND-fed, mice (Fig. 3D). Furthermore, using flow cytometry and a previously characterized monoclonal natural IgM antibody (LR04) with specificity for MDA,[4] we also found an increased frequency of cells carrying MDA epitopes in livers of WD mice, while an IgM isotype control antibody displayed only minimal binding (Fig. 3E; Supporting Fig. S3C). The density of MDA epitopes as calculated by mean fluorescence intensity was also significantly increased in liver cells of WD mice (Supporting Fig. S3D). Similar results were obtained using a polyclonal IgG antibody against MDA (Fig. 3E; Supporting Fig. S3D). These data are consistent with previous studies showing the occurrence of MDA epitopes in chronic liver diseases including nonalcoholic steatohepatitis (NASH).[13-15] To determine the identity of the cells recognized by MDA antibodies, we costained liver cell suspensions with markers for cell death and leukocytes. We found that LR04^{+} cells were detected within the population of dying cells, as characterized by Annexin V and 7-amino-actinomycin D staining (Fig. 3F). Moreover, LR04^{+} cells were to a

FIG. 1. WD induces hepatic inflammation and oxidative stress in *Ldlr*$^{-/-}$ mice. (A-C) Scatter plots of *in silico* functional analyses of differentially regulated genes in livers after 2 weeks of WD. Annotated processes are indicated in red (A,B) or green (C). Size of symbols represents numbers of genes enriched in a biological process. *x* axis: Fold enrichment describes the ratio of actual number of genes found in a pathway compared to expected number of genes. *y* axis: Significance represented as –log10 of modified Fisher exact *P* value. (A) Gene ontology analysis of significantly up-regulated genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) and showing biological processes. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of significantly up-regulated genes showing pathways. (C) Gene ontology analysis of significantly down-regulated genes using DAVID. (D) Heatmap representation of selected genes significantly deregulated in livers after 2 and 4 weeks of WD expressed as log2 fold change over ND. Genes are classified as soluble mediators (brown), receptors and sensors (red), transcription and signaling factors (blue), and others (gray). (E) Heatmap representation of selected oxidative stress response genes differentially regulated in livers after 2 and 4 weeks of WD expressed as log2 fold change over ND. Data from n = 2 mice/group. Abbreviations: CoA, coenzyme A; metab. proc., metabolic process.

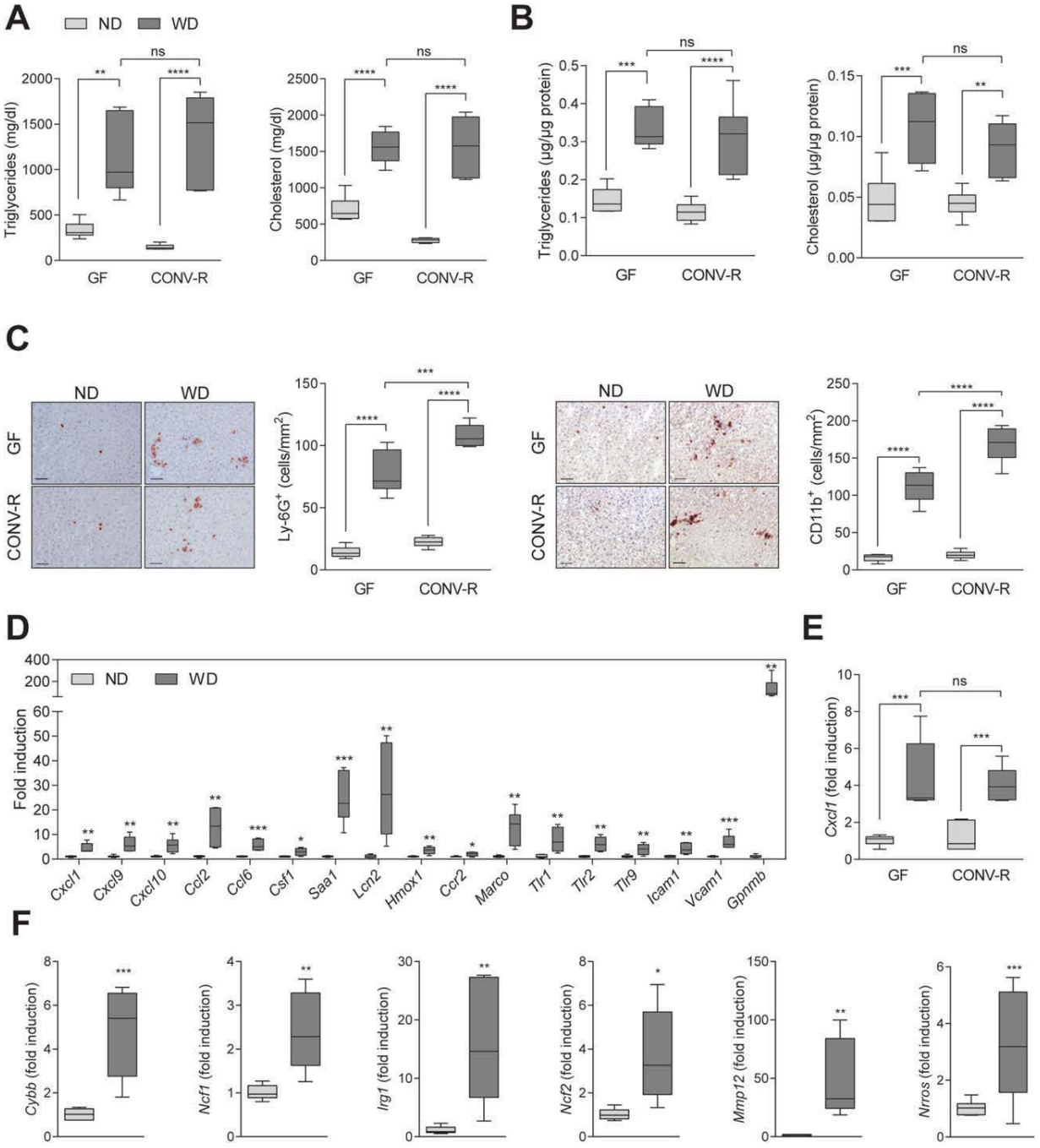

FIG. 2. WD drives hepatic steatosis and inflammation in the absence of microbiota. (A) Plasma and (B) liver triglyceride and cholesterol levels in GF and CONV-R *Ldlr*$^{-/-}$ mice on ND or WD for 2 weeks. (C) Representative images and quantification of hepatic neutrophils (anti-Ly-6G) and infiltrating macrophages (anti-CD11b) in liver cryosections of GF and CONV-R *Ldlr*$^{-/-}$ mice on ND or WD. (D) Inflammatory gene expression in liver tissue of GF *Ldlr*$^{-/-}$ mice on ND or WD, expressed as fold of ND. (E) Real-time quantitative PCR analysis of *Cxcl1* expression in livers of GF and CONV-R *Ldlr*$^{-/-}$ mice on ND or WD. (F) Real-time quantitative PCR analysis of oxidative stress response gene expression in livers of GF *Ldlr*$^{-/-}$ mice on ND or WD, expressed as fold of ND. In all panels, data of n = 6 (3 male, 3 female) mice/group are represented as boxplots, where the box extends from the 25th to 75th percentile; the line represents the median and the whiskers the smallest and the largest values. (A-C,E) One-way analysis of variance with Tukey's multiple comparisons test; (D,F) Student unpaired *t* test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Scale bar, 50 μm. Abbreviation: ns, not significant.

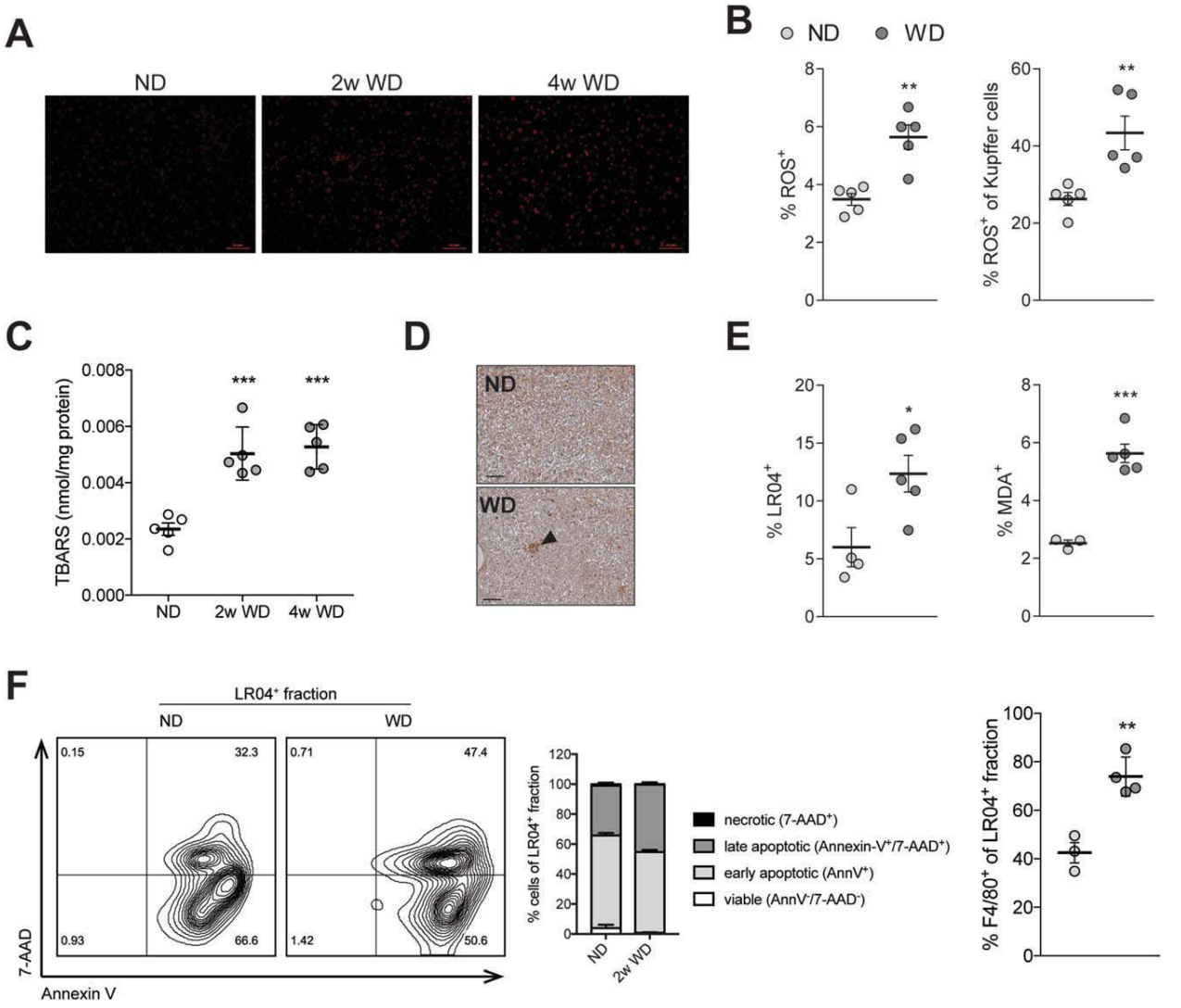

FIG. 3. WD results in increased oxidative stress and lipid peroxidation in the liver. (A) Representative images of sites of ROS production (red) detected by dihydroxyethidium staining in livers of *Ldlr*$^{-/-}$ mice fed ND or 2 or 4 weeks of WD. (B) Flow-cytometric analysis of ROS-producing cells stained with chloromethyl 2′,7′-dichlorofluorescein diacetate in whole liver (left) or Kupffer cells (right) harvested from *Ldlr*$^{-/-}$ mice fed ND or WD for 2 weeks. (C) TBARS assay in Kupffer cells isolated from livers of mice fed ND or 2 or 4 weeks of WD. (D) Representative images of liver sections from *Ldlr*$^{-/-}$ mice fed ND or WD for 2 weeks stained with anti-MDA antibody. Arrowhead denotes brown MDA staining. (E) Flow-cytometric analysis of cells harvested from whole livers of *Ldlr*$^{-/-}$ mice fed ND or 2 weeks of WD stained with LR04 or a monoclonal MDA-specific IgG antibody. (F) Flow-cytometric analysis of liver cell suspensions harvested from mice on ND or 2-week WD and stained with dead cell markers Annexin-V/7-amino-actinomycin D and LR04, gated on LR04-positive cells, showing representative graphs and quantification of dying cells (left). Quantification of F4/80^{+} cells within the LR04-positive fraction (right). Data are presented as mean ± standard error of the mean of n = 3-5 mice/group and were analyzed by Student unpaired *t* test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bar, 50 μm. Abbreviations: 7-AAD, 7-amino-actinomycin D; 2w/4w, 2 weeks/4 weeks.

large extent F4/80^{+} Kupffer cells, and LR04^{+}F4/80^{+} cells were enriched in liver cell suspensions from mice on WD (Fig. 3F). Additionally, mRNA expression of oxidative stress genes was examined in isolated Kupffer cells from livers of these mice. Consistent with increased TBARS in isolated Kupffer cells, we found increased expression of the nicotinamide adenine dinucleotide phosphate oxidase subunits Cybb and Ncf2 as well as up-regulation of immunoresponsive gene 1, which mediates the production of mitochondrial ROS

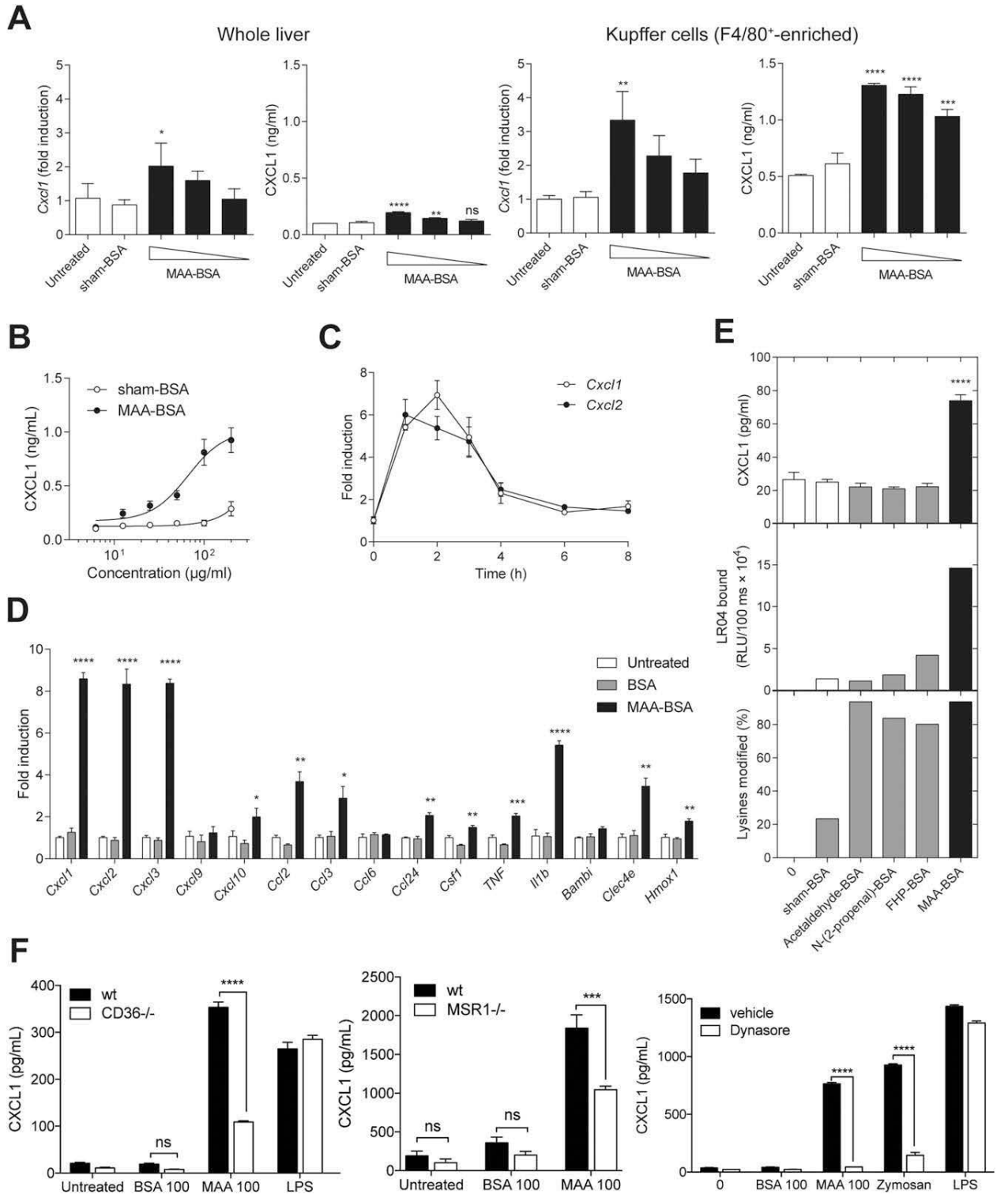
A
Whole liver
Kupffer cells (F4/80+-enriched)
Cxcl1 (fold induction)
CXCL1 (ng/ml)
Untreated
sham-BSA
MAA-BSA
B
sham-BSA
MAA-BSA
CXCL1 (ng/mL)
Concentration (µg/ml)
C
Cxcl1
Cxcl2
Fold induction
Time (h)
D
Untreated
BSA
MAA-BSA
Fold induction
Cxcl1
Cxcl2
Cxcl3
Cxcl9
Cxcl10
Ccl2
Ccl3
Ccl6
Ccl24
Csf1
TNF
Il1b
Bambi
Clec4e
Hmox1
E
CXCL1 (pg/ml)
LR04 bound (RLU/100 ms × 10^4)
Lysines modified (%)
0
sham-BSA
Acetaldehyde-BSA
N-(2-propenal)-BSA
FHP-BSA
MAA-BSA
F
wt
CD36-/-
CXCL1 (pg/mL)
Untreated
BSA 100
MAA 100
LPS
wt
MSR1-/-
vehicle
Dynasore
0
Zymosan

FIG. 4

by oxidation of intracellular fatty acids (Supporting Fig. S3E). On the other hand, members of the glutathione *S*-transferase family involved in detoxification responses such as Gsta1 and Gsta2 were found to be down-regulated in Kupffer cells upon WD (Supporting Fig. S3E). Thus, feeding WD to $Ldlr^{-/-}$ mice induces hepatic ROS production, and accumulation of MDA mainly presented on dying Kupffer cells.

To identify the specific proteins carrying MDA epitopes and thereby mediating proinflammatory effects, we performed immunopurification of homogenized liver samples from mice fed WD for 4 weeks using LR04 and an isotype control antibody and analyzed the obtained protein fraction by mass spectrometry. Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of 189 proteins found in the LR04 but not in the control pull-down demonstrated an enrichment for proteins located in the mitochondrial compartment and involved in metabolic and oxidative processes (Supporting Fig. S3F), in line with previous studies.[5] These findings are consistent with a documented role of oxidative stress in programmed cell death and our identification of dying cells as major carriers of MDA epitopes in the liver of WD-fed mice.

MDA EPITOPES INDUCE PROINFLAMMATORY CHEMOKINE RESPONSES *IN VITRO* AND *IN VIVO*

Data from several laboratories suggest that MDA epitopes are not merely markers of oxidative stress but also proinflammatory triggers in various cell types.[16-18] Thus, we asked whether MDA epitopes could also mediate or further propagate the inflammatory response in the liver. Therefore, we prepared MDA epitopes by *in vitro* chemical derivatization of BSA as a carrier protein with MDA using a method that leads to the preferential formation of MAA epitopes (MAA-BSA), the immunodominant subset of MDA epitopes.[7,19-21] Then we incubated cells harvested from whole-liver tissue of C57BL/6 mice with increasing concentrations of MAA-BSA and assessed the expression of *Cxcl1*, which is a prominent chemokine induced by lipid-derived danger signals such as oxidized phospholipids.[22,23] Indeed, we found increased expression of *Cxcl1* after 2 hours of stimulation with MAA-BSA but not sham-BSA by real-time quantitative PCR analysis (Fig. 4A). Furthermore, CXCL1 secretion was also increased after stimulation with MAA-BSA (Fig. 4A). Because of the critical role of Kupffer cells in the inflammatory response of the liver, we isolated $F4/80^{+}$ cells from wild-type livers and stimulated them with MAA-BSA. Compared to total liver cells, isolated Kupffer cells exhibited an even more pronounced and robust increase in *Cxcl1* expression and CXCL1 secretion in response to MDA epitopes (Fig. 4A; Supporting Fig. S4A). These data indicate that resident liver macrophages have the ability to sense and respond to MDA epitopes *in vitro*. To further characterize the inflammatory response mounted by macrophages after MAA-BSA stimulation, we incubated bone marrow–derived macrophages (BMDMs) with increasing concentrations of sham-BSA or MAA-BSA, which revealed a dose-dependent response in CXCL1 secretion after 8 hours of stimulation, while naive or sham-treated BSA did not have any effect (Fig. 4B; Supporting Fig. S4B). In addition, a time-course experiment showed that expression of *Cxcl1* as well as *Cxcl2* peaked around 1-2 hours after stimulation (Fig. 4C). To evaluate whether inflammatory genes induced by WD in liver could also be up-regulated by MAA-BSA in macrophages, we quantified the expression of selected genes in BMDMs after 2 hours of stimulation. Indeed, the expression of several chemokines and cytokines was found to be strongly induced following MAA-BSA stimulation (Fig. 4D).

FIG. 4. MDA epitopes are proinflammatory danger signals sensed by scavenger receptors on macrophages *in vitro*. (A) Gene and protein expression of *Cxcl1* in whole liver (left) and Kupffer cells (right) from C57BL/6 mice stimulated with 100 μg/mL sham-treated BSA or 50-200 μg/mL MAA-BSA for 2 and 7 hours, respectively. (B) Dose–response curve for CXCL1 secretion using BMDMs stimulated for 16 hours with sham-BSA (100 μg/mL) or indicated concentrations of MAA-BSA. (C) mRNA expression of *Cxcl1* and *Cxcl2* in BMDMs stimulated for 1-8 hours with 100 μg/mL sham-BSA or MAA-BSA. (D) Expression of selected proinflammatory markers after 2 hours of incubation with sham-BSA or MAA-BSA (100 μg/mL). (E) CXCL1 secretion, LR04-binding, and degree of lysine modification of different BSA modifications. (F) CXCL1 secretion by BMDMs from indicated knockout mice after stimulation with MAA-BSA for 16 hours (left and middle). CXCL1 secretion of BMDMs stimulated with indicated compounds after 30-minute pretreatment with Dynasore. Zymosan, positive control (right). Data in all panels are presented as mean ± standard error of the mean of triplicate stimulations and are representative of at least two (A,F) or three (B-E) independent experiments: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Abbreviations: FHP, 3,5-diformyl-1,4-dihydropyridine-4-yl-pyridinium; LPS, lipopolysaccharide; ns, nonsignificant; RLU, relative light unit; wt, wild type.

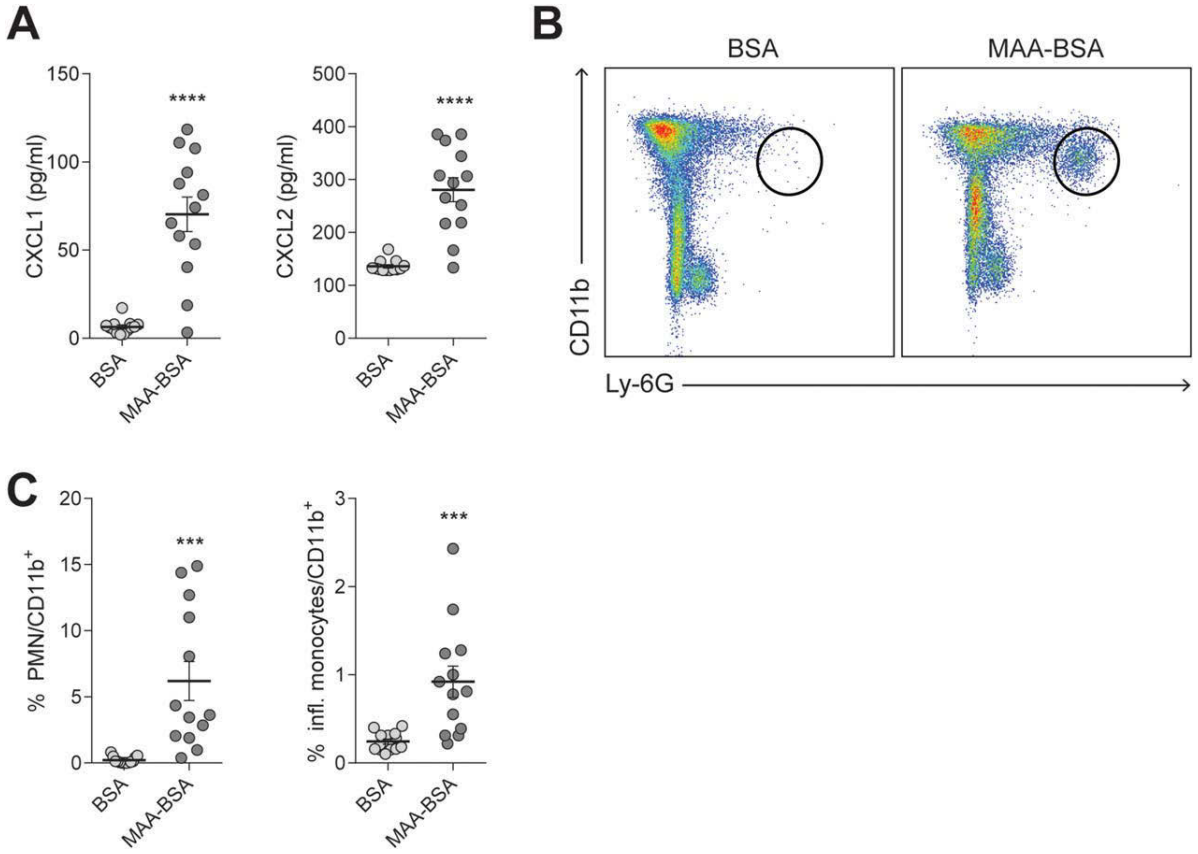

FIG. 5. MDA epitopes induce sterile peritonitis *in vivo*. (A) CXCL1 and CXCL2 levels in lavage fluid 2 hours after intraperitoneal injection of 25 mg/kg BSA or MAA-BSA. (B) Representative scatter plot showing flow-cytometric analysis of peritoneal cells stained with CD11b and Ly-6G for polymorphonuclear leukocytes 2 hours after BSA (left) or MAA-BSA (right) injection. Newly recruited cells are marked with a black circle. (C) Quantification of polymorphonuclear leukocytes and inflammatory monocytes. Data shown in (A-C) are pooled from n = 14 mice/group from two independent experiments and are represented as mean ± standard error of the mean; each symbol indicates an individual mouse. All data were analyzed by Student *t* test. ***$P < 0.001$, ****$P < 0.0001$. Abbreviations: infl., inflammatory; PMN, polymorphonuclear leukocyte.

To exclude the possibility that the modification of BSA *per se*—irrespective of the attached epitope—renders it proinflammatory, BSA was modified with different epitopes including acetaldehyde (acetaldehyde-BSA), linear MDA structures (*N*-[2-propenal]-BSA), crosslinking MDA structures (3,5-diformyl-1,4-dihydropyridine-4-yl-pyridinium), and the aforementioned cyclic MDA structures termed MAA-BSA (4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde or MAA) for stimulation of BMDMs with equal concentrations of each preparation. Only BSA modified with MAA but not with acetaldehyde, linear, or crosslinking MDA structures elicited a significantly increased CXCL1 response (Fig. 4E), despite a similar extent of modification as judged by the trinitrobenzenesulfonic acid assay. Among all modifications, MAA-BSA was also most strongly recognized by the natural IgM antibody LR04, consistent with previous observations by us and others that MAA epitopes are immunodominant structures generated by MDA.[7,19-21] If not indicated otherwise, all stimulation experiments were performed using the MAA type of MDA epitopes but referred to as MDA epitopes as this is the commonly used term for the entire class of these epitopes.

Following our observation that macrophages respond to MDA epitopes, we tested whether scavenger receptors, which have been shown to bind to MDA epitopes,[24] are required for mediating MDA-induced signaling. We found that MSR1-deficient, but also CD36–deficient, macrophages displayed markedly reduced CXCL1 secretion compared to wild-type macrophages (Fig. 4F). Inhibition of

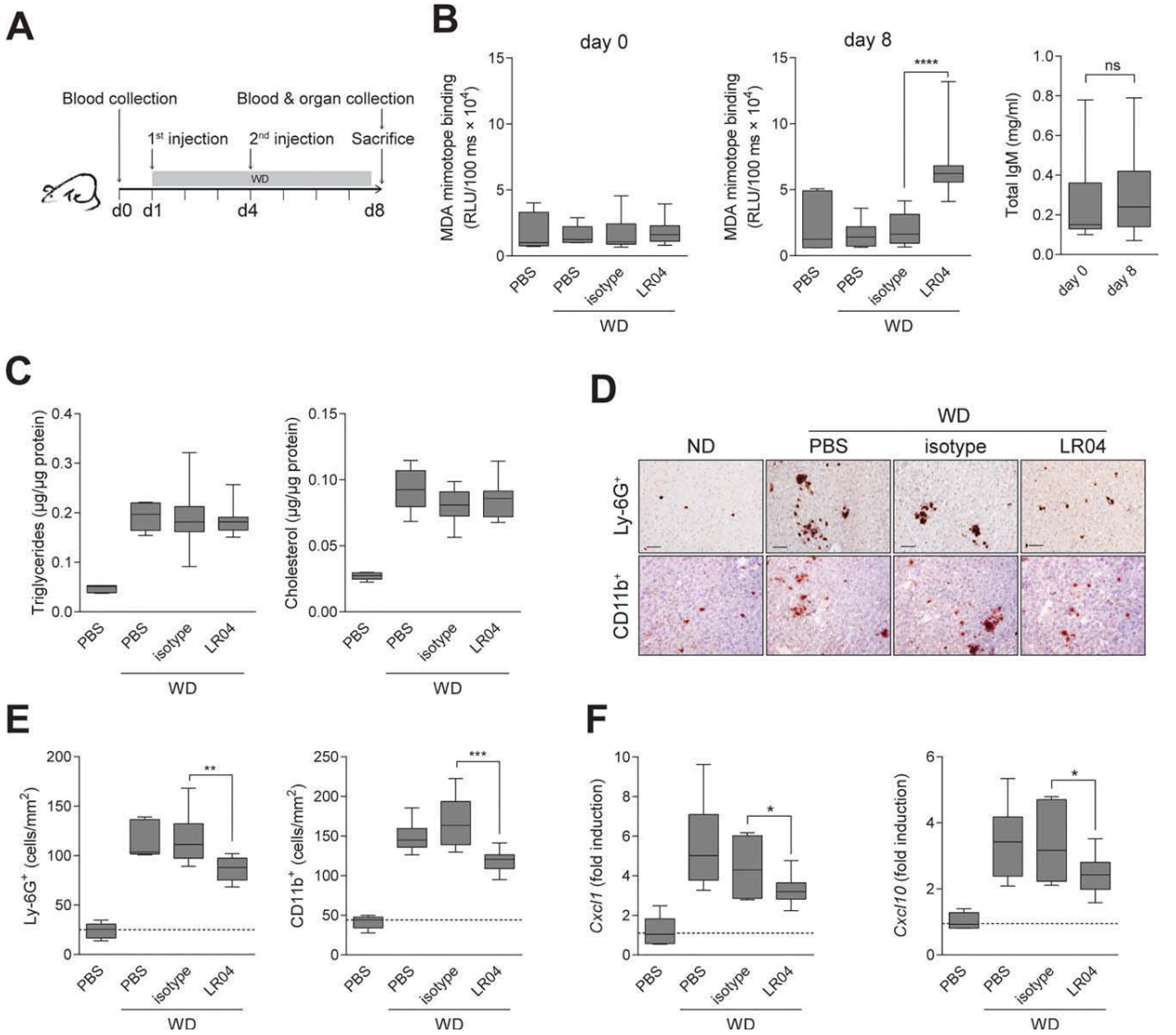

FIG. 6. Treatment with the anti-MDA IgM clone LR04 ameliorates diet-induced hepatic inflammation. (A) Experimental design and workflow of the intervention experiment. (B) IgM antibodies binding to P2 mimotope before and after injection of LR04 clone. Total IgM titers in the group treated with LR04 before and after the injection are shown on the right. (C) Liver lipid levels. (D) Representative histological stainings using $CD11b^+$ and $Ly\text{-}6G^+$ in liver sections from intervention study. (E) Quantification of $CD11b^+$ and $Ly\text{-}6G^+$ cells in liver sections. (F) Real-time PCR analysis of hepatic *Cxcl1* and *Cxcl10* expression. Data in all panels are of n = 5 (PBS/ND), n = 6 (PBS/WD), or n = 11 (isotype and LR04) mice per group and are represented as boxplots, where the box extends from the 25th to 75th percentile, the line represents the median, and the whiskers represent the smallest and the largest values: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Scale bar, 50 µm. Abbreviations: ns, nonsignificant; RLU, relative light unit.

endocytosis using Dynasore abolished CXCL1 secretion upon MDA stimulation (Fig. 4F), further indicating the importance of uptake for MDA-induced signaling. Interestingly and in contrast to oxidized low-density lipoprotein (OxLDL), which is recognized by a heterotrimeric complex composed of TLR4/6/CD36,[22] TLR4 is not required for MDA signaling (Supporting Fig. S5A). Moreover, MDA did not induce interleukin-1β secretion either alone or in macrophages primed with the lipopolysaccharide subunit Kdo2-Lipid A, in contrast to OxLDL[25] (Supporting Fig. S5B; data not shown). Finally, chemical inhibition of the NFkB pathway using a selective inhibitor of I kappa B kinase diminished MDA-induced chemokine

TABLE 1. Characteristics of *Ldlr*$^{-/-}$ Mice After 1 Week of WD (Intervention Study)

	ND	WD		
	PBS (n = 5)	PBS (n = 6)	Isotype Control (n = 11)	LR04 (n = 11)
Weight				
Total (g)	17.84 ± 0.4445	17.18 ± 0.3902	17.37 ± 0.2166	17.15 ± 0.3016
Liver (ratio)	0.0492 ± 0.0015	0.052 ± 0.0021	0.0519 ± 0.0020	0.0499 ± 0.0023
Spleen (ratio)	0.0044 ± 0.0005	0.0046 ± 0.0003	0.0045 ± 0.0002	0.0047 ± 0.0002
day 0				
Plasma measurements				
Triglycerides (mg/dl)	165.8 ± 15.16	134.5 ± 6.684	135.6 ± 7.252	122.6 ± 6.494
Cholesterol (mM)	7.391 ± 0.4258	7.679 ± 0.3	7.433 ± 0.2349	7.691 ± 0.1883
Total IgM (mg/mL)	0.27 ± 0.1098	0.2 ± 0.0121	0.18 ± 0.0201	0.2636 ± 0.0673
MAA-binding IgM (RLU/100 ms)	92,001 ± 40,947	93,266 ± 6,039	95,374 ± 8,950	106,149 ± 15,537
P2-binding IgM (RLU/100 ms)	18,295 ± 6,442	15,727 ± 3,031	16,809 ± 3,627	19,286 ± 3,386
day 8				
Plasma measurements				
Triglycerides (mg/dl)	131.6 ± 9.124	369.4 ± 33.51†	445.3 ± 37.05†	509.9 ± 46.37†
Cholesterol (mM)	8.402 ± 0.3451	32.23 ± 3.021†	32.76 ± 1.784†	36.84 ± 2.016†
Total IgM (mg/mL)	0.18 ± 0.07503	0.1617 ± 0.04126	0.2027 ± 0.05307	0.3 ± 0.06333
MAA-binding IgM (RLU/100 ms)	100,001 ± 22,186	94,691 ± 12,735	118,650 ± 9,952	158,994 ± 18,415
P2-binding IgM (RLU/100 ms)	24,642 ± 10,079	15,961 ± 4,369	20,959 ± 3,611	69,811 ± 7,803*†
Liver measurements				
Triglycerides (μg/μg protein)	0.047 ± 0.0034	0.1927 ± 0.0115†	0.1889 ± 0.0184†	0.1887 ± 0.0106†
Cholesterol (μg/μg protein)	0.0272 ± 0.0013	0.0926 ± 0.0066†	0.0807 ± 0.0037†	0.0866 ± 0.0045†
Other parameters				
Alanine aminotransferase	24.05 ± 3.875	23.18 ± 1.687	23.8 ± 1.707	25.98 ± 1.408

Data are shown as mean ± standard error of the mean. *Significantly different compared to isotype control; †significantly different compared to PBS/ND; data was analyzed by one-way analysis of variance followed by Tukey's multiple comparisons test.
Abbreviation: RLU, relative light unit.

secretion in macrophages (Supporting Fig. S5C). Therefore, MDA epitopes are capable of activating signaling pathways that are mediated through scavenger receptors or dependent on scavenger receptor–mediated uptake and NFkB but independent of inflammasome activation.

As CXCL1 is a major chemokine involved in the recruitment of neutrophils, we induced sterile peritonitis in C57BL/6 mice using MAA-BSA. Indeed, intraperitoneal injection with MAA-BSA, but not BSA, resulted in a robust secretion of CXCL1 and CXCL2 after 2 hours (Fig. 5A) and recruitment of neutrophils and Ly-6C^{hi} inflammatory monocytes into the peritoneal cavity of MAA-injected mice, indicating that MDA epitopes have proinflammatory properties *in vivo* (Fig. 5B,C).

PROTECTION FROM MDA EPITOPES RESULTS IN DECREASED HEPATIC INFLAMMATION

To investigate whether endogenously generated MDA epitopes can cause hepatic inflammation *in vivo*, we fed *Ldlr*$^{-/-}$ mice WD for 1 week, during which they received two intravenous injections of 200 μg of the anti-MDA IgM antibody LR04 or of an isotype control antibody or PBS on days 1 and 4 (Fig. 6A). On day 8, mice that received the LR04 antibody had significantly increased plasma levels of IgM antibodies reactive with the MDA mimotope P2, which was previously developed by us as a unique ligand for LR04.[4] Importantly, total IgM plasma levels were not affected (Fig. 6B). After 1 week of WD, all mice developed increased triglyceride and cholesterol levels in plasma and liver, which were not different among the three groups (Fig. 6C and Table 1). In contrast, LR04-treated mice developed significantly less hepatic inflammation as demonstrated by reduced infiltration of neutrophils and macrophages compared to mice receiving PBS or isotype control antibodies (Fig. 6D,E and Table 1). Remarkably, the anti-MDA treatment resulted in an approximately 30%-40% reduction of leukocyte infiltrates (Fig. 6E). Consistent with this, WD-induced *Cxcl1* and *Cxcl10* expression in the livers of LR04-treated mice was significantly reduced compared to livers of mice that received PBS or an isotype control (Fig. 6F). Thus, hepatic inflammation induced

by WD feeding is in part mediated by the accumulation of endogenously generated MDA epitopes triggering chemokine secretion and leukocyte recruitment.

Discussion

MDA epitopes are one specific type of OSE found on oxidized lipoproteins but also dying cells and microvesicles, which are membrane blebs that are shed from activated and dying cells.[26,27] Their role as an exemplary oxidation-derived modification present on oxidized lipoproteins is studied best in atherosclerosis, where multiple reports have shown that vaccination against MDA-modified LDL protects from atherosclerosis in mice.[5] Nevertheless, a thorough understanding of both the disease-promoting effects of MDA epitopes in atherosclerosis and the contribution of MDA epitopes to other chronic inflammatory diseases has been elusive. Here, we report that MDA epitopes are major drivers and propagators of diet-induced hepatic inflammation.

Although there is little doubt that microbiota and their metabolites contribute to diet-induced inflammation in various organs,[28-33] other sterile triggers such as cholesterol crystals,[34] OxLDL,[22] or advanced glycation end products[35] have also been suggested to promote sterile inflammatory responses. Our data demonstrate that WD-fed *Ldlr*$^{-/-}$ mice devoid of microbiota still develop hyperlipidemia, hepatic steatosis, and inflammation. Furthermore, WD-fed GF *Ldlr*$^{-/-}$ mice display enhanced oxidative stress and deregulation of oxidative stress response genes in the liver. Intriguingly, WD also induces the expression of classical pattern recognition receptors for bacterial ligands such as TLR2 and TLR9 in both CONV-R and GF livers. TLR9 has been suggested to mediate methionine/choline-deficient diet–induced NASH[28] by acting as a sensor of bacterial products that accumulate in the bloodstream due to dysbiosis in the gut. However, a recent study proposed a nonbacterial TLR9 ligand, mitochondrial DNA, to be involved in driving NASH.[36] We show that oxidation-derived MDA epitopes represent alternative, sterile pattern recognition receptor ligands that activate similar signaling pathways as microbial stimuli including NFkB signaling. In addition, inflammatory responses caused by other DAMPs or microbial danger signals resulting in oxidative stress and cell injury might lead to the formation of MDA epitopes that could act as amplifiers of an initial inflammation. Our findings describing that dying cells are major carriers of MDA epitopes in our model and that LR04-bound proteins in steatotic livers are prevalently of mitochondrial origin further suggest that MDA epitopes could also be associated with other DAMPs and thereby enhance their proinflammatory capacity.

Importantly, hepatic inflammation can be substantially improved by treatment with a specific MDA antibody, indicating that MDA epitopes contribute to the inflammatory phenotype in the liver of WD-fed *Ldlr*$^{-/-}$ mice. Of note, deficiency of CD36 and MSR1, which we found to be involved in MDA-induced inflammation, also results in reduced hepatic inflammation in this model,[37] and other receptors that have been implicated in the recognition of MDA epitopes may also contribute to it.[38-40] We show decreased expression of CXCL1 and CXCL10 in the livers of LR04-treated mice, which is consistent with the important roles of these chemokines and their receptors in other models of hepatic inflammation and their potential as biomarkers for fatty liver disease in humans.[41-43] The monoclonal MDA antibody LR04 used in the intervention study is part of the pool of natural IgM antibodies. Natural IgM antibodies perform important housekeeping functions by promoting the anti-inflammatory clearance of endogenous waste products.[6] Whereas one-third of natural IgM antibodies are targeted against OSEs, a considerable fraction of those (~50%) recognizes specifically MDA—but not other—epitopes,[7] which suggests selective pressure for the maintenance of high titers of MDA IgM by default. Consistent with the sterile origin of MDA epitopes, we have shown that MDA IgM is detected in both CONV-R and GF mice, and MDA IgM levels do not change upon reconstitution of GF mice with commensal bacteria.[7]

There is mounting evidence that low-MDA IgM titers are associated with an increased risk for cardiovascular disease[5] and with the presence of nonalcoholic fatty liver disease.[44] In line, we have shown that boosting natural IgM titers by active vaccination against OxLDL or using genetic mouse models results in reduced hypercholesterolemia and hepatic inflammation, suggesting that high-OSE IgM levels are protective.[8,45] Many different OSEs exist and are likely generated during such a diet treatment. Remarkably, passive transfer of monoclonal IgM antibodies targeting the MAA type of MDA epitopes, which are immunodominant and proinflammatory, is sufficient to ameliorate hepatic inflammation by 30%-40% despite equal levels of steatosis. This effect may be

mediated either by neutralization of the proinflammatory MDA moieties on cellular debris and microvesicles or by promoting IgM-mediated removal of dying cells tagged by MDA epitopes.[7,27] The latter would also prevent the continued release of other danger signals such as mitochondrial DAMPs by promoting the clearance of dying cells that would otherwise accumulate.

Although MDA antibody administration in our subacute, short-term model mimicking early NASH stages was clearly effective, a beneficial outcome of this treatment in both long-term and more severe NASH models cannot be directly extrapolated from our results and has to be evaluated in future studies. These may also include MDA-based vaccination strategies designed to preferentially boost protective immune responses.[5] In this regard, our study not only identifies MDA epitopes as a critical target but also suggests a strategy that corrects for deficiencies in protective IgM with a minimal degree of manipulation of host immunity. Particularly when designing treatments for chronically ill patients, selectively targeting disease-promoting structures is of great advantage. Thus, enhancing protective functions of natural host immunity by increasing MDA IgM titers could represent a novel approach for the development of therapeutic strategies.

Acknowledgment: We thank the team of the Biomedical Sequencing Facility at CeMM for their support with next generation sequencing. We are grateful to Klaus-Peter Derreth and Cecilia Perera for expert technical assistance.

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Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28970/suppinfo.

4.2 Supporting Tables, Figures and Methods to Manuscript

Supplementary information to: "Malondialdehyde epitopes are sterile mediators of hepatic inflammation in hypercholesterolemic mice"

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Published in: Hepatology. 2016 Dec 16. doi: 10.1002/hep.28970. [Epub ahead of print] PubMed PMID: 27981604.

Supplementary Table 1: Characteristics of $Ldlr^{-/-}$ mice after 2 or 4 weeks of Western-type diet (WD), and of germ-free (GF) vs. conventionally-raised (CONV-R) $Ldlr^{-/-}$ mice after 2 weeks of WD.

				GF		CONV-R	
	ND (n = 5)	2 weeks (n = 5)	4 weeks (n = 5)	ND (n = 6)	WD (n = 6)	ND (n = 6)	WD (n = 6)
Weight							
Total (g)	19.28±0.3484	21.08±0.481	20.98±0.3693	19.31±0.6918	21.12±1.07	21.73±0.9958	23.7±1.682
Liver (ratio)	0.0539±0.0023	0.0587±0.0014	0.0588±0.0026				
Plasma measurements							
Triglycerides (mg/dl)	126±12.79	1008±112.8*	858±119.1*	335.3±37.61	1130±174.8*	147.8±12.17	1366±203.2*
Cholesterol (mg/dl)	184.6±3.461	1301±75.86*	1176±51.31*	704±72.35#	1560±89.71*	277±12.05	1567±162.8*
Liver measurements							
Triglycerides (µg/µg protein)	0.1137±0.0142	0.3377±0.0382*	0.247±0.0293*	0.1458±0.0137	0.3339±0.0213*	0.1155±0.0105	0.3089±0.0383*
Cholesterol (µg/µg protein)	0.0236±0.0009	0.0746±0.0033*	0.0617±0.0039*	0.0481±0.0085	0.108±0.0121*	0.0448±0.0046	0.0904±0.0092*

Data are shown as mean ± SEM. * significantly different compared to ND of each group; # significantly different compared to CONV-R with same diet; one-way analysis of variance (ANOVA) followed by Tukey‘s multiple comparisons test.

Figure S1

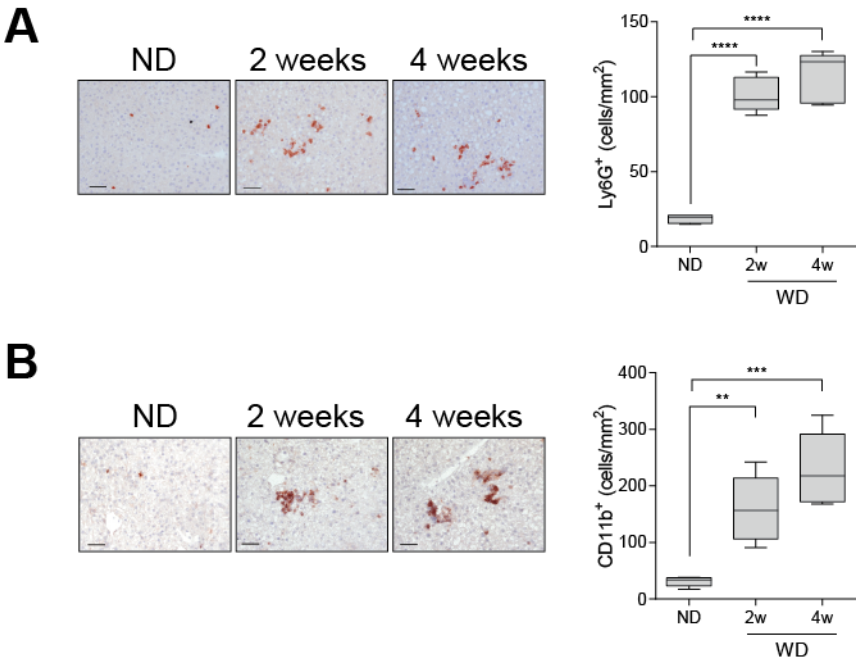

Figure S1: Diet-induced hepatic inflammation after 2 and 4 weeks of WD- feeding. *Ldlr*$^{-/-}$ mice were fed a normal diet (ND) or 2 or 4 weeks of Western-type diet (WD) and data of n = 5 mice/group are represented as box-and-whisker-plots, where the box extends from the 25th to 75th percentile, the line represents the median and the whiskers the smallest and the largest values. (A) Representative images of hepatic neutrophil infiltrates stained with anti-Ly-6G (shown in red) in *Ldlr*$^{-/-}$ mice on ND or WD for 2 or 4 weeks (left) and their quantification (right). (B) Representative images of hepatic macrophage infiltrates stained with anti-CD11b (shown in red) in *Ldlr*$^{-/-}$ mice on ND or WD for 2 or 4 weeks (left) and their quantification (right). Quantification was performed by averaging the count of positively stained cells in 6 microscope fields and is shown as average cell number per mm^2. (A,B) One-way ANOVA with Tukey's multiple comparisons test; **P < .01, ***P < .001, ****P < .0001; scale bar: 50 μm.

Figure S2

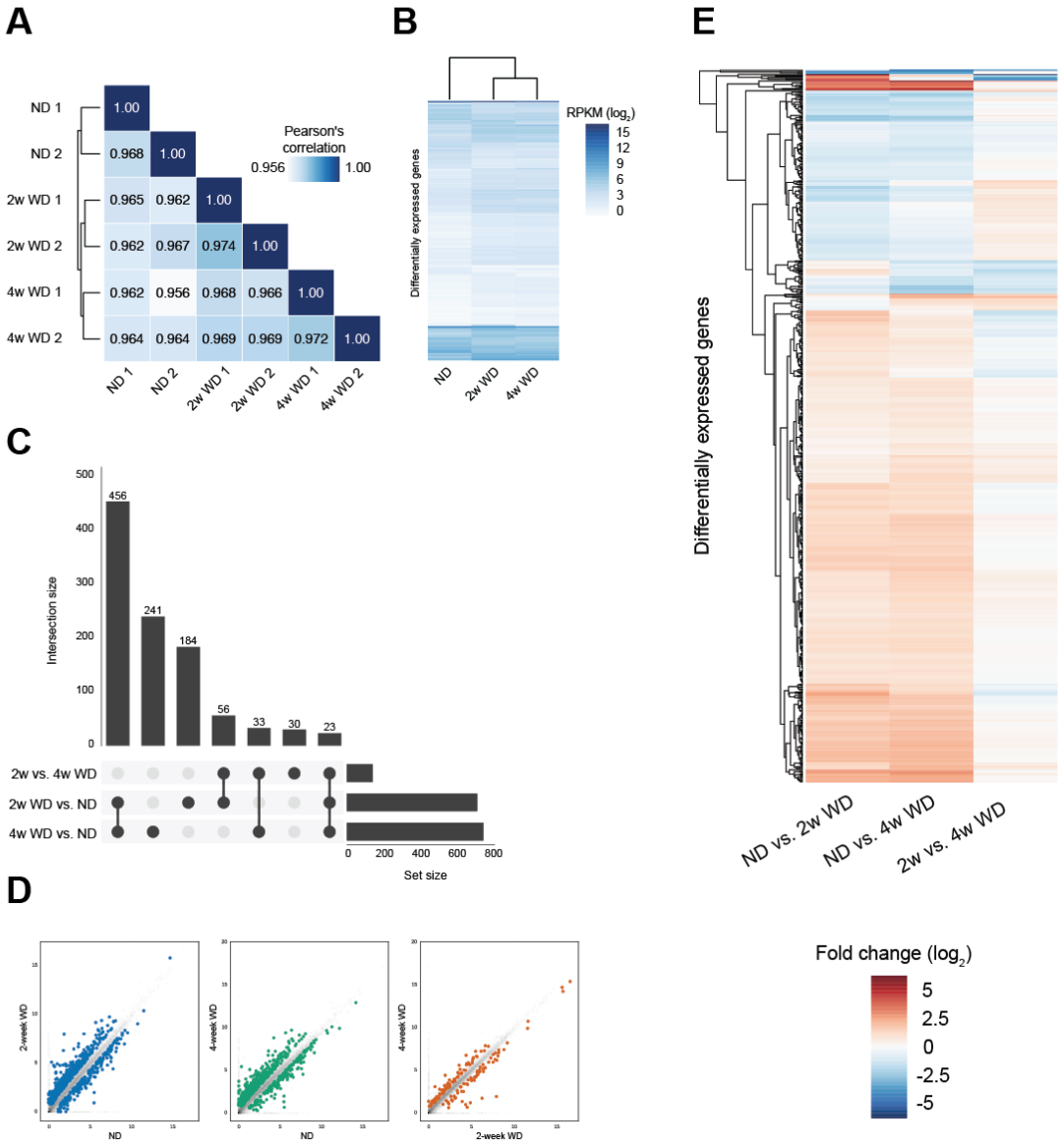

Figure S2. (A) Correlation matrix of sample pairwise Pearson's correlations showing the relationship between biological replicates of each liver sample and between liver samples from ND- or WD-fed animals. The correlation values (from white to blue) were clustered hierarchically based on the gene-level expression values of all genes. Values close to 1 indicate higher similarity. (B) Heatmap representation of gene-level expression (log2 of RPKM values, from white to blue) of the 1023 differentially expressed genes (in rows) between the three experimental conditions (columns). (C) Histogram with the amount of overlap in sets of differentially expressed genes between each experimental condition. The connected dots indicate which set(s) of differentially expressed genes is being compared. (D)

Scatterplots of gene level expression (log2 of RPKM values) between the investigated conditions. Differentially expressed genes are colored (blue, green or orange) and all others are plotted in black. (E) Heatmap representation of the 1023 differentially expressed genes (in rows) across the comparisons between three conditions (columns). Genes are colored according to the log2 of the fold change between each of the two conditions represented.

Figure S3

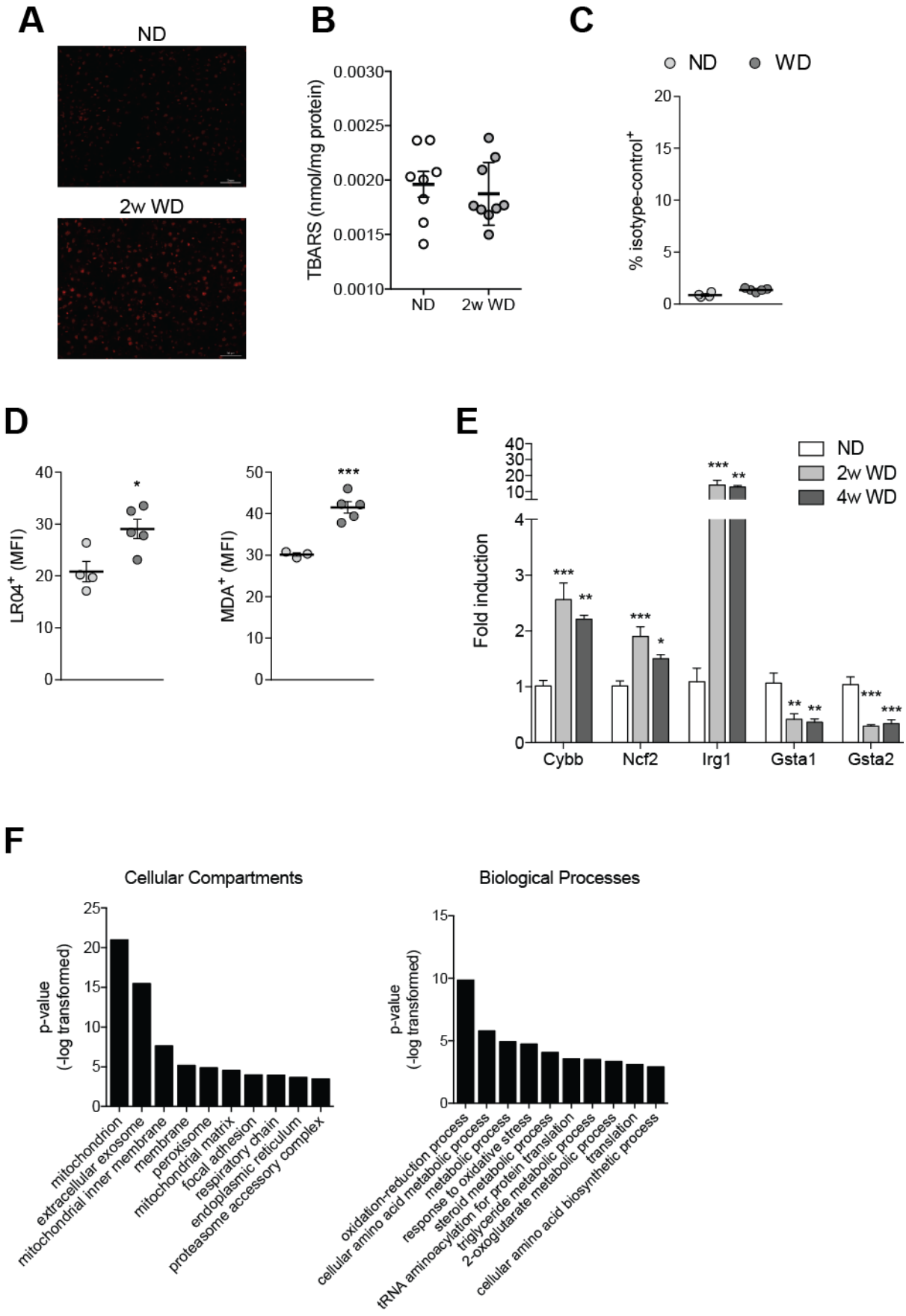
A
ND
2w WD
B
TBARS (nmol/mg protein)
0.0030
0.0025
0.0020
0.0015
0.0010
ND
2w WD
C
ND
WD
% isotype-control+
20
15
10
5
0
D
LR04+ (MFI)
MDA+ (MFI)
E
Fold induction
ND
2w WD
4w WD
Cybb
Ncf2
Irg1
Gsta1
Gsta2
F
Cellular Compartments
p-value
(-log transformed)
mitochondrion
extracellular exosome
mitochondrial inner membrane
membrane
peroxisome
mitochondrial matrix
focal adhesion
respiratory chain
endoplasmic reticulum
proteasome accessory complex
Biological Processes
oxidation-reduction process
cellular amino acid metabolic process
metabolic process
response to oxidative stress
steroid metabolic process
tRNA aminoacylation for protein translation
triglyceride metabolic process
2-oxoglutarate metabolic process
cellular amino acid biosynthetic process
translation

Figure S3: (A) Representative images of sites of ROS production (red) in livers of GF Ldlr-/- mice fed ND or 2 weeks of WD. (B) TBARS assay using whole liver homogenates from ND- or 2w-WD-fed $Ldlr^{-/-}$ mice. (C) Flow cytometric analysis of cells harvested from whole livers of $Ldlr^{-/-}$ mice fed ND or 2 weeks WD stained with an isotype control antibody. (D) Flow cytometric analysis as in (C) using LR04 (left) or a commercial antibody (right), expressed as mean fluorescence intensity (MFI). (E) mRNA expression of indicated genes involved in oxidative stress response in livers of mice fed ND, 2 or 4 weeks ND. (F) Functional annotation analysis of 189 proteins found in the LR04-pulldown using DAVID, showing enriched cellular components (left) and biological processes (right), sorted by p-value.

Figure S4

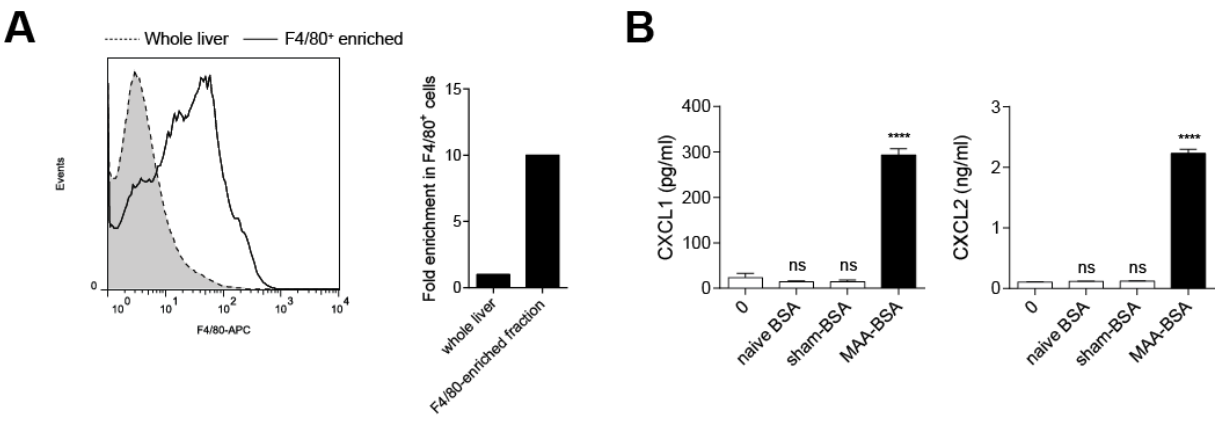

Figure S4. (A) Efficiency of enrichment for Kupffer cells using anti-F4/80 antibody. Histogram of cells from whole liver of F4/80-enriched fraction stained with F4/80-APC (x axis). Calculated fold enrichment compared to whole liver is shown on the right. (B) CXCL1 and CXCL2 secretion into the supernatant of bone-marrow-derived macrophage cultures after 16 hours of stimulation with naive, sham-treated or MAA-BSA,

Figure S5

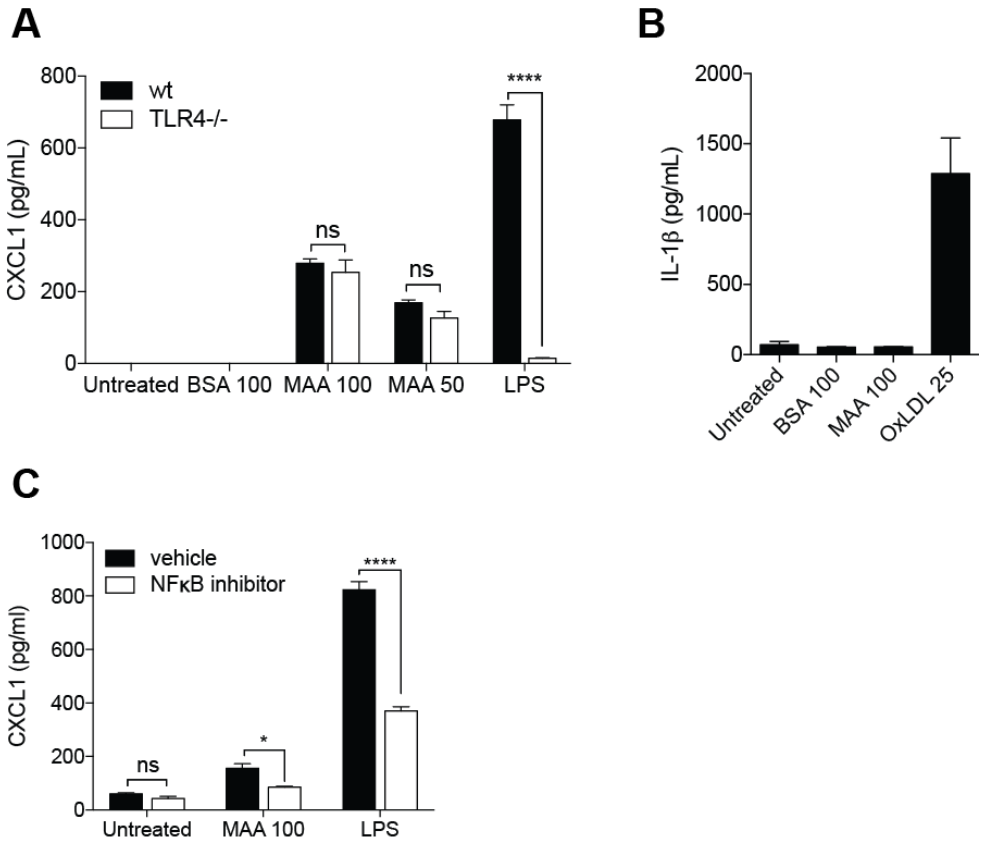

Figure S5: (A) Stimulation of TLR4-deficient BMDM with indicated ligands for 16 hours, followed by ELISA determination of CXCL1 in supernatants. (B) ELISA determination of IL1β in supernatants harvested from BMDMs stimulated for 16 h with indicated ligands. OxLDL treated BMDMs were primed with 1 µg/ml LPS for 30 min before stimulation with OxLDL. (C) ELISA determination of CXCL1 in supernatants harvested from BMDMs stimulated with indicated ligands for 16 h in presence of vehicle (DMSO) or the NFkB inhibitor BMS-345541.

Supplementary Materials and Methods

Animal experiments. To induce hepatic inflammation, 6-9-week-old, female *Ldlr*$^{-/-}$ mice were fed ND or a Western-type diet (WD) containing 21% fat and 0.2% cholesterol (EF R/M acc. TD88137 mod.; E15721-347, Ssniff Spezialdiäten GmbH) for 2 or 4 weeks. GF and SPF (CONV-R) mice received a standard autoclaved regular chow (ND; Mouse Chow, for autoclaving, 5021-3-RHI-S 17, PMI Nutrition International) or a WD (Adjusted Calories diet TD.88137; 21% fat, 0.2% cholesterol, vacuum packaged, irradiated and checked for microbial contamination; Envigo) for 2 weeks. The GF status was verified on the last day of experiment by 16S rDNA PCR and bacterial culture. All groups of mice were sex- and age-matched (9-16 weeks of age). For the antibody intervention study, 10-week-old, female *Ldlr*$^{-/-}$ mice were fed a WD (21% milk butter, 0.2% cholesterol; diet 1635, Scientific Animal Food and Engineering) for 1 week to induce hepatic inflammation. All mice were sacrificed using CO_2, followed by blood collection and tissue isolation as described previously (1, 2). For analysis of sterile peritonitis experiments, peritoneal cells were blocked with anti-mouse CD16/32 (1:100, clone 93; 14-0161-85, eBioscience), stained with Ly6C-FITC (1:200, clone HK1.4; 128006, BioLegend), Ly6G-PE (1:2000, clone 1A8; 127608, BioLegend), CD11b-APC (1:800, clone M1/70; 17-0112-82, eBioscience), B220-PerCP (1:600, clone RA3-6B2; 45-0452-82, eBioscience), CD5-PE (1:100, clone 53-7.3; 12-0051-82, eBioscience), and recorded on an LSR Fortessa (Becton, Dickinson and Company). Data were analyzed using FlowJo v10 (Treestar Inc.). Supernatants of peritoneal lavage fluids were directly analyzed for presence of CXCL1 and CXCL2 using enzyme-linked immunosorbent assays (R&D Systems, see below).

Study approval. All animal experiments were performed with the approval of the Intramural Committee for Animal Experimentation at a) Medical University of Vienna and the Federal Ministry of Science, Research and Economy of Austria, b) the Committee for Animal Welfare at Maastricht University, and c) local committee on

legislation on protection of animals (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany; 23177-07/G12-1-100).

RNA isolation and cDNA synthesis. RNA was extracted from ~25 mg frozen liver tissue using peqGOLD TriFast™ (30-2010, VWR International) after mechanical tissue disruption with TissueLyser II (Qiagen) and cleaned up with the peqGOLD Total RNA kit (12-6634-02, VWR International). Extraction of RNA from cell culture was performed with the peqGOLD Total RNA kit (12-6634-02, VWR International). Reverse transcription for cDNA synthesis of liver or cell culture RNA was done with the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific).

Real-time PCR. Reverse-transcribed cDNA from tissue or cell culture was amplified on a CFX96 system (Bio-Rad Laboratories) using KAPA SYBR® FAST qPCR Kit Master Mix (2X) (KK4606, Kapa Biosystems) and primers for indicated genes. Cyclin B was used as a reference gene for normalization.

RNA sequencing. Liver RNA from mice on ND or WD (n = 2/group) was extracted with peqGOLD TriFast™ (30-2010, VWR International) and RNA sequencing libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (RS-122-2101, Illumina). For RNA quantification and quality assessment, the Qubit 2.0 Fluorometric Quantitation system (Life Technologies) and the Experion Automated Electrophoresis System (Bio-Rad) were used. Pooled and diluted libraries were sequenced by the Biomedical Sequencing Facility at CeMM using the Illumina HiSeq 3000/4000 platform and the 25 base-pair single-read configuration. Base-calling was done using the Illumina Realtime Analysis software, followed by BAM conversion and demultiplexing using BamIndexDecoder (https://github.com/wtsi-npg/illumina2bam). Transcriptome analysis was performed with the Tuxedo Suite (3-5). Quality control was performed with the Bioconductor packages cummeRbund and biomaRt. For downstream analysis, differentially expressed genes were defined as having a q-

value (adjusted p-value) smaller than 0.01. Pairwise sample Pearson correlations were calculated based on all genes across all sample replicates. Heatmap clustering was performed on columns (samples or experimental conditions) and rows (genes) using Euclidean distances between either RPKM or $\log_2$ fold-change values and the complete linkage method. GO and KEGG pathway analysis were performed using DAVID (https://david.ncifcrf.gov/) (6).

Lipid analysis & aminotransferase measurements. Lipid levels in plasma and liver homogenates were determined using enzymatic colorimetric methods (CHOD-PAP, Cholesterol liquicolor, Human; GPO-PAP, Triglycerides liquicolor, Human) according to the manufacturer's instructions. ALT levels were determined using Refletron GPT (ALT) tests (Roche Diagnostics). Hepatic lipid levels are expressed as µg lipid/µg protein after determination of protein concentration using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

Liver histology. For determination of cell infiltrates, livers from ND- and WD-fed *Ldlr*$^{-/-}$ mice were embedded in O.C.T. compound and sectioned at 7-µm thickness. Liver sections were then stained with CD11b (1:500, clone M1/70; R&D Systems) or Ly-6G (1:200, clone 1A8; BD Biosciences) for infiltrating macrophages and neutrophils, respectively, as described previously (7, 8). Quantification was performed by averaging the number of positively-stained cells from 6 microscope fields per liver section (magnification of 200x).

Oxidative stress assays. ROS staining on liver sections was performed using dihydroxyethidium (DHE; D7008, Sigma-Aldrich). Briefly, 7-µm liver sections were fixed in 4% formalin and incubated with 20 mg/ml DHE (Ex/Em: 518⁄605) in DMSO for 30 minutes in darkness. Images were captured using a ZEISS Axio Imager 2 at a magnification of 200x. MDA staining was performed on paraffin-embedded liver tissue sectioned at 2 µm thickness. To reduce unspecific signals due to endogenous biotin, sections were treated with Biotin Blocking System (X0590, Dako) before incubation with a rabbit anti-MDA IgG antibody (1:1000; ab6463, abcam), followed by

secondary antibody incubation with a biotinylated goat anti-rabbit IgG antibody (1:300; BA1000, VectorLabs). VECTASTAIN Elite ABC Kit (PK-6100, Vector) and Vector Nova Red Substrate Kit for Peroxidase (SK-4800, Vector) were used for signal detection. Cell suspensions were stained with an oxidative stress indicator (CM-H2DCFDA; C6827, Thermo Fisher Scientific) for 30 min at 37°C or LR04 (10 µg/ml) and CD19-APC (1:200, clone 1D3; 557399, Becton, Dickinson, and Company) to exclude B cells for 30 min at 4°C, followed by anti-IgM-FITC (1:1000, clone II/41; 553437, Becton, Dickinson, and Company), and measured with FACSCalibur (Becton, Dickinson, and Company). Recorded data was analyzed with FlowJo v10 (Treestar Inc.).

For determining the degree of lipid peroxidation using the thiobarbituric-acid-reactive substances (TBARS) assay, proteins were precipitated from liver homogenates or cell lysates from Kupffer cells using sulfuric acid and phosphotungstic acid. The resulting protein pellet was resuspended in dH_2O and 2 mM butylated hydroxytoluene and mixed with thiobarbituric acid. After boiling the samples for 60 minutes at 95°C, TBARS were extracted using butanol and fluorescence was measured in a microplate reader (Ex/Em = 515/553).

Cytokine measurements in supernatants. Supernatant from cell culture or lavage fluid was analyzed for CXCL1 and CXCL2 secretion by an enzyme-linked immunosorbent assay using corresponding capture and detection antibodies and recombinant protein standards (CXCL1: MAB453, BAF453, 453-KC-010; CXCL2: MAB452, BAF452, 452-M2-010; R&D Systems), followed by Streptavidin-HRP (DY998, R&D Systems) and TMB Substrate incubation (555214, Becton, Dickinson and Company). Absorbance was measured using a Synergy 2 Multi-Mode Reader (Biotek).

Generation of MDA-modified proteins. To generate MAA-BSA, fatty-acid-free bovine serum albumin (2 mg/ml; K35-002, PAA Laboratories) was incubated with freshly prepared MDA (100 µmol/ml; 1,1,3,3-Tetramethoxypropane, 108383, Sigma) and

acetaldehyde (AA, 200 µmol/ml; 00070, Sigma-Aldrich) for 3 h at pH 4.8 and 37°C as described previously (9). Unreacted MDA and AA was removed by centrifugal filtration using Amicon Ultra-15 centrifugal filter units (cutoff 10 kDa; UFC901008, Millipore). Sham-BSA was generated under the same conditions with replacement of MDA and AA by PBS. Biotinylation of sham- or MAA-BSA was performed using EZ-Link Sulfo-NHS-Biotinylation Kit (21425, Thermo Fisher Scientific) according to the manufacturer's protocol. MAA-BSA was assayed for formation of fluorescent adducts by excitation at 394 nm and measuring emission at 462 nm. The extent of modified lysines was determined by the 2,4,6-trinitrobenzenesulfonic acid assay (10). All MAA-BSA preparations used for *in vitro* stimulations or *in vivo* experiments were tested for endotoxin by the Limulus amoebocyte assay (Endosafe–PTS, PTS20, Charles River Laboratories) and contained < 1.5 ng endotoxin per mg protein (< 1 EU/ml at standard concentration used). Formation of MDA epitopes on BSA was determined by an chemiluminescent enzyme-linked immunosorbent assay using MDA-specific natural antibodies E014 (11), LR04 (12), NA17 (13) and an IgG antibody, MDA2 (14), followed by secondary antibody incubation using alkaline-phosphatase-conjugated anti-mouse IgM (A9688, Sigma-Aldrich) and anti-mouse IgG (A3562, Sigma-Aldrich), respectively. Light emission was measured on a Synergy 2 Multi-Mode Reader (Biotek) after incubation with Lumi-Phos Plus (P-701, Lumigen) on 37°C.

Isolation of cells from liver tissue and enrichment for F4/80+-cells. Enrichment for F4/80-positive cells from C57BL/6J mice was performed as described previously (15). Briefly, livers from 3-4 mice (male or female, 8-12 weeks) were digested with Liberase (Liberase™ TM Research Grade; 5401119001, Sigma Aldrich) and DNase I (DNase I recombinant, RNase-free; 4716728001, Sigma Aldrich) at 37°C for 30 min, and passed through a 100 µm cell strainer to obtain single cell suspensions. Hepatocytes were removed by a low-spin centrifugation step at 20 g for 3 min and red blood cells were subsequently lysed using a hemolysis buffer (12146.001,

Morphisto). Cells were stained with anti-F4/80-APC (clone BM8; 123116, BioLegend) upon blocking with anti-mouse CD16/32 (14-0161-85, eBioscience), incubated with anti-APC MicroBeads (130-090-855, Miltenyi Biotec) and purified by magnetic-activated cell sorting using LS columns (130-042-401, Miltenyi Biotec) and a QuadroMACS Separator (130-091-051, Miltenyi Biotec). Bound cells were collected by flushing the columns and analyzed by flow cytometry for enrichment of F4/80-positive cells compared to total liver samples.

Generation of bone-marrow-derived macrophages. To generate of bone-marrow-derived macrophages, bone marrow was isolated from the femurs and tibiae of C57BL/6J mice and cultured in presence of RPMI-1640, 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and antimycotics, supplemented with 10% L929-conditioned medium for 7-8 days.

Stimulation experiments. Liver cell suspensions, isolated Kupffer cells or bone-marrow-derived macrophages were stimulated in culture medium without serum in presence of fatty-acid- and endotoxin-free 0.5% BSA with indicated concentrations of naïve BSA, sham-treated BSA or MDA-modified BSA preparations

Antibody measurements. MAA-BSA or P2 peptides (5 µg/ml) were coated on 96-well white round-bottom microtiter plates (7105, Thermo Fisher Scientific) and incubated over night at 4°C. Plates were blocked with TBS + 1% BSA for 1 h on RT and then incubated with diluted plasma (1:500 for MAA-BSA, 1:100 for P2) for overnight at 4°C. Binding of IgM antibodies was detected with alkaline-phosphatase-conjugated anti-mouse IgM (A9688, Sigma-Aldrich). Light emission was measured on a Synergy 2 Multi-Mode Reader (Biotek) after 30 min incubation with Lumi-Phos Plus (P-701, Lumigen) on 37°C. Results are represented as relative lights units per 100 ms (RLU/100 ms). For total IgM measurement, microtiter plates were coated with 2 µg/ml anti-mouse IgM antibody (M8644, Sigma-Aldrich) and blocked with TBS + 1% BSA, followed by incubation with plasma dilutions (1:30,000) at 4°C overnight and anti-mouse-IgM detection as before. An IgM standard curve using purified mouse

IgM (401602, BioLegend) was included to calculate the absolute amounts of antibodies present in plasma.

Immunopurification-MS. Flash frozen liver samples from mice fed a high fat diet for 4 weeks were homogenized in 50 mM Tris-HCL, 150 mM NaCl, 1 % NP-40, 5 mM EDTA, 5 mM EGTA supplemented with 1 mM PMSF and a protease inhibitor cocktail (Sigma, St.Louis, MO, USA). The protein concentration of the lysate was determined using the bicinchoninic acid protein assay kit (Pierce, Waltham, MA, USA) in accordance with the manufacturer's instructions. 4 µg of LRO4 antibody were added to 2 mg of protein lysate and incubated for 4 h at 4 °C with gentle rotation. An appropriate mouse isotype IgM antibody (clone MM-3) was used as a control in a separate immunopurification. 100 µl agarose bead slurry coupled to goat anti-mouse IgM antibodies (Sigma-Aldrich, St Louis, MO, USA) were added to the lysates and incubated for a minimum of 16 h at 4 °C with gentle rotation. The supernatant was removed and the beads washed with 3×500 µL 250 mM HEPES pH 8, 150 mM NaCl, 5 mM EDTA to remove unspecific interactors. Bound proteins were eluted from the beads by incubation with wash buffer supplemented with 2 % SDS under gentle rotation (16) for 20 min at room temperature. The immunopurified proteins were digested with trypsin according to the filter-aided sample preparation (FASP) protocol (17, 18). Proteins were denatured with 88 mM dithiothreitol for 5 min at 95°C, loaded on 30 kDa molecular-weight-cut-off (MWCO) filters (Microcon, Merck Millipore, Darmstadt, Germany) and rinsed with 8 M urea in 100 mM Tris-HCl pH 7.5. All centrifugation steps were performed at 14,000×g at RT. Cysteine residues were alkylated by incubation with 55 mM iodoacetamide for 30 min at RT in the dark. Alkylated proteins on the filters were washed 3× with 8 M urea in 100 mM Tris-HCl pH 7.5 and 3× with 50 mM TEAB pH 8.0. To digest the proteins into peptides, 1 µg sequencing grade porcine trypsin (Promega, Madison, WI, USA) in 40 µL 100 mM TEAB, pH 8.0 was added and the samples incubated for 16 h at 37 °C. Digested

peptides were collected by centrifugation through the filters. The filters were then washed 1× with 50 µL 0.5 M NaCl and 2× with 50 µL 50 mM TEAB and the filtrates were collected. Peptides were purified and concentrated via stop-and-go extraction (STAGE) tips (19). Organic solvent was evaporated by vacuum centrifugation and the peptides were dissolved in 5 % formic acid for LC-MSMS analysis.

LC-MSMS Analysis. Mass spectrometric analyses were performed on a Q Exactive mass spectrometer (ThermoFisher, Bremen, Germany) coupled to an Agilent 1200 series dual pump HPLC system (Agilent, Santa Clara, CA, USA). Samples were transferred from the thermostatted autosampler (4 °C) to a trap column (Zorbax 300SB-C18 5 µm, 5 × 0.3 mm, Agilent Biotechnologies, Santa Clara, CA, USA) by the binary pump at a flow rate of 45 µL/min. The analytes were then further transferred and separated on a 20 cm analytical column with a 75 µm inner diameter packed with Reprosil C18 (Dr. Maisch, Ammerbuch-Entringen, Germany) over a 60 minute gradient ranging from 3 % to 40 % organic phase at a constant flow rate of 250 nL/min. The mobile phases used for the HPLC were 0.4 % formic acid and 90 % acetonitrile plus 0.4 % formic acid. The mass spectrometer was operated in a data-dependent mode with each full scan followed by a maximum of 10 MSMS scans and a 10 s dynamic exclusion of previously fragmented ions. For MS and MSMS scans, 100 ms and 300 ms were allowed as the maximum ion injection time, respectively. The analyser resolution was set to 70,000 for MS scans and 17,500 for MSMS scans. Overfilling of the C-trap was prevented by setting the automatic gain control to 3 × 106 and 1 × 105 for MS and MSMS, respectively. The underfill ratio for MSMS was set to 12 %, which corresponds to an intensity threshold of 4 × 104 to accept a peptide for fragmentation. Fragmentation was performed by higher collision energy induced dissociation (HCD) at a normalized collision energy (NCE) of 28. The ubiquitous contaminating siloxane ion Si(CH3)2O)6 was used as a single lock mass

at m/z 445.120024 for internal mass calibration (20). All samples were analysed as technical replicates.

Data Analysis. Data was analysed using Proteome Discoverer version 1.4 (Thermo Scientific, Bremen, Germany). Spectra were searched with Mascot version 2.3.02 (Matrix Science, London, UK) against a SwissProt mouse protein database (downloaded on 20150601 and containing 24938 sequences). Common contaminants such as porcine trypsin were appended to the database. The precursor and fragment ion mass tolerances were ±10 ppm and ±0.1 Da, respectively. The data was searched with tryptic specificity, but up to 3 miscleavages were permitted. Cysteine carbamidomethylation was set as a static modification, oxidation of methionine residues was set as a dynamic modification. A 1% false discovery rate was enforced by the percolator node (21) and search results were subsequently filtered for high confidence peptides and a maximum search engine rank of 1. Protein identifications were compared between immunopurifications with LR04 and the corresponding isotype antibody. To be accepted as interactors of LR04 proteins were required to meet 2 conditions; i) to be only identified in the LRO4 immunopurification but not in the isotype and ii) to be present in at least 2 of 3 replicates. The resulting list of 189 protein identifications was then subjected to functional annotation analysis using DAVID bioinformatics resources (6, 22).

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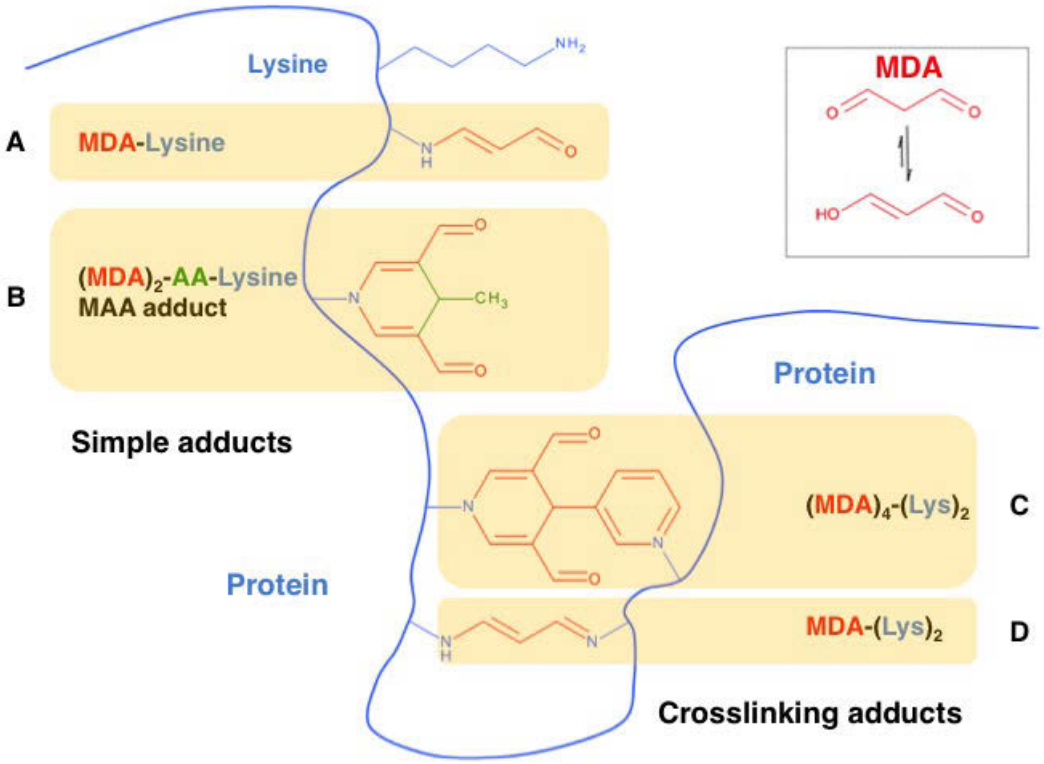

Figure 4.1: Adducts formed by BSA modification with MDA and acetaldehyde. (A) Linear, "simple" adduct. (B) Cyclic, "complex" and fluorescent adduct, also termed malondialdehyde-acetaldehyde (MAA) epitope. (C) Cyclic, cross-linking adduct. (D) Linear, cross-linking adduct. Figure adapted from Busch and Binder (2016).

4.3 Unpublished Results

4.3.1 Time-course analysis of MDA-epitope formation on BSA

MDA can react with amino groups of different biomolecules such as proteins to form covalent Schiff-base-type adducts. A common adduct is the linear and non-fluorescent N-ϵ-(2-propenal)lysine (Figure 4.1, Structure A). In presence of acetaldehyde and under acidic conditions, a more complex and fluorescent adduct can be formed on amino groups, namely a 4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde derivative (MDHDC or MAA adduct, Figure 4.1, Structure B). Both types of adducts can react further with a second amino group to crosslink two proteins or two domains of the same protein (Figure 4.1, Structures C and D). Studies have shown that the biologically relevant and immunodominant adduct is most likely the complex MAA-type (MDHDC) adduct (Tuma et al., 2001).

In a previous report by my thesis laboratory, CFH was identified as a major plasma protein binding to MAA-BSA in pull-down experiments (Weismann et al., 2011). Ad-

ditionally, it was shown that injection of MAA-BSA induces an inflammatory response in vivo that can be abolished by simultaneous treatment with CFH (Weismann et al., 2011). Interestingly, a CFH variant associated with an increased propensity for developing complement-related diseases such as age-related macular degeneration was demonstrated to possess lower binding affinities to MAA-BSA, indicating that the interaction between CFH and MAA-modified proteins may play an important role in chronic inflammatory diseases of the retina. Apart from CFH, also C4BP had been found to recognize MAA-BSA (unpublished observation). Hence, both binding of both CFH and C4BP to MDA-modified carrier proteins can serve as a proxy for the formation of biologically relevant MDA epitopes.

To characterize the formation of MDA-derived adducts on a carrier protein over time, BSA was modified with MDA and acetaldehyde for certain incubation times ranging from 5 minutes to 24 hours. For each timepoint, several measurements were performed: fluorescence as an indicator of cyclic MAA formation, binding of different antibody clones with specificity for MDA epitopes, binding of CFH, and biological activity in terms of IL-8 secretion by THP-1 cells incubated with the respective timepoint modifications.

To determine the degree of BSA modification, all samples were subjected to the TNBS assay (described in Section 3.1.1). As expected, short-term modifications were modified to a lower extent than samples with longer modification time (Figure 4.2, Panel A). Spectrophotometric analysis of all timepoints showed that fluorescence as a correlate of complex adduct formation increased with the time of modification, reaching a plateau level after 180 min (Figure 4.2, Panel B). Within minutes of incubation of BSA with MDA and acetaldehyde, adducts appeared that were strongly bound by MDA antibodies LR04 and MDA2 but not recognized by the antibodies E014 and NA17 (Figure 4.2, Panels C–F). Lack of E014 and NA17 binding was associated with lack of recognition by soluble PRRs CFH and C4BP (Figure 4.2, Panels G and H). Mass spectrometric analysis of the adducts formed on BSA demonstrated that short-term preparations contained non-fluorescent, linear adducts (Figure 4.2, Panel J). Long-term incubation resulted in the formation of more complex, cyclic and fluorescent adducts that were confirmed as MDHDC-type adducts by mass spectrometry. Complex adducts bound to all antibodies strongly (Figure 4.2, Panels C–F) and were also recognized by CFH and C4BP (Figure 4.2, Panels G and H). Stimulation of THP-1 cells with each respective timepoint modification demonstrated that only late, complex adducts on BSA had the capacity to induce a pro-inflammatory response characterized by IL-8 secretion (Figure 4.2, Panel I), consistent with the strong binding of CFH and C4BP to these late adducts. These studies indicate that both the natural IgM antibody LR04 and the IgG antibody MDA2 (generated by immunization with MDA-LDL) were more promiscuous in epitope recognition, while E014, NA17, CFH and C4BP only recognized those adducts that had progressed to a pro-inflammatory type. Interestingly, modification of BSA with MDA and acetaldehyde for 24 hours resulted in a preparation

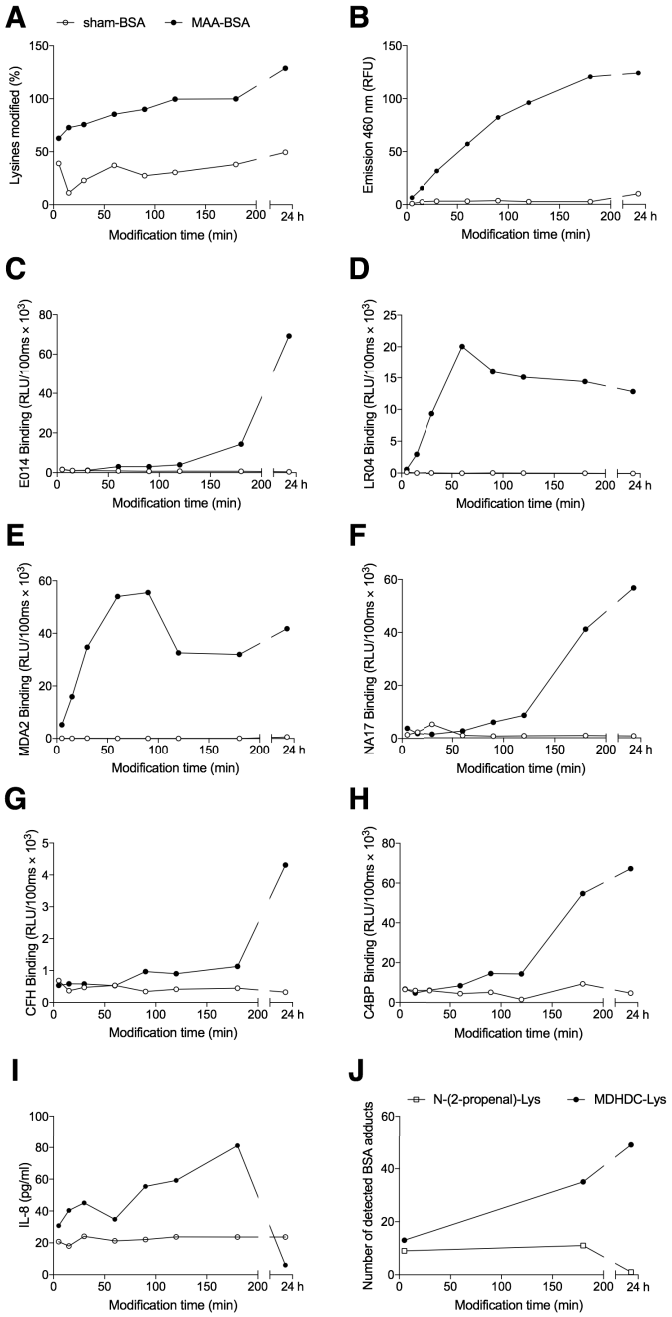

Figure 4.2: Time-course analysis of MAA-BSA. (A) Extent of BSA modification assessed by TNBS assay. (B) Relative fluorescence intensity at Ex/Em 394/462 nm. (C-F) Chemiluminescent ELISA with MDA-specific antibodies E014, LR04, MDA2 and NA17. (G, H) Binding of soluble PRRs CFH and C4BP. (I) Biological activity of MAA modification samples after stimulation of THP-1 cells with 100 μg/ml of each preparation and IL-8 secretion analysis by ELISA. (J) Mass-spectrometric analysis of MAA-BSA modified for different incubation times.

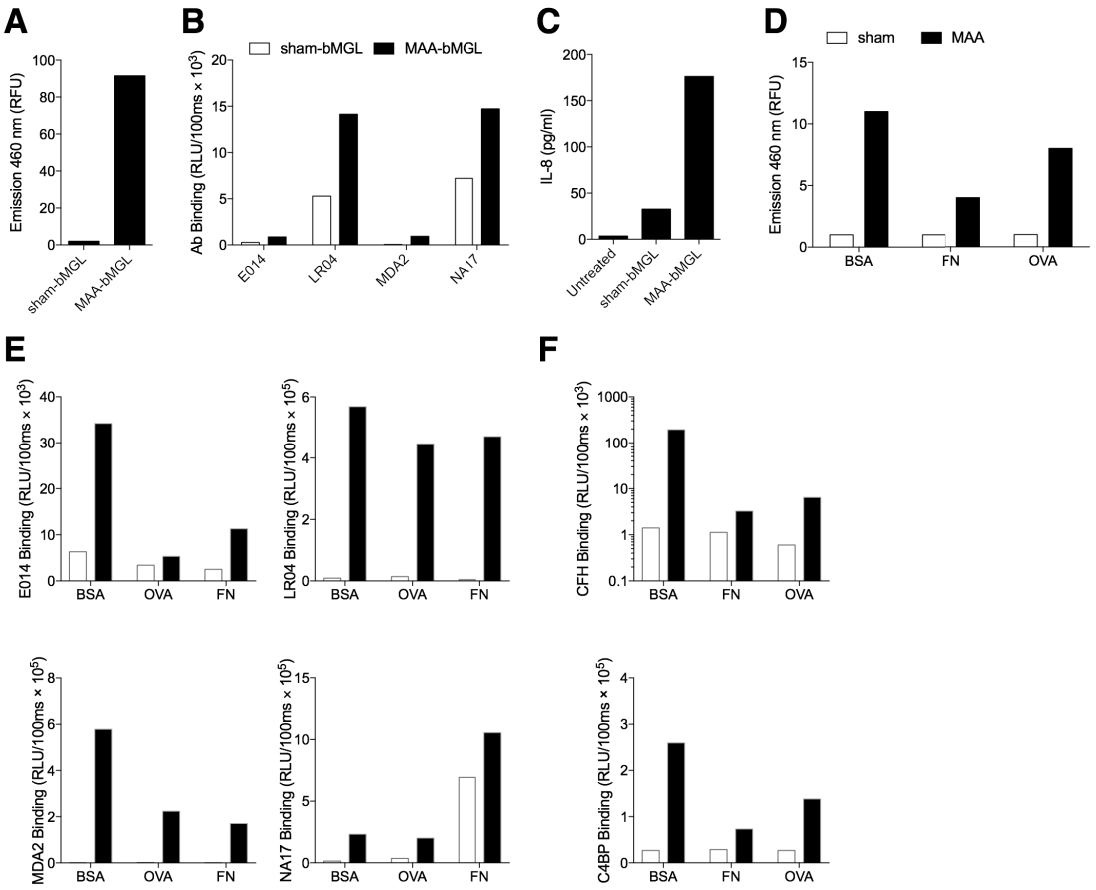

Figure 4.3: MDA modification of carrier proteins other than BSA. (A-C) Modification of bMGL. (D) Generation of fluorescent adducts on BSA, FN or OVA. (E) Binding of MDA-specific antibodies to MDA-modified BSA, OVA or FN. (F) Binding of soluble PRRs CFH and C4BP to BSA, OVA or FN. bMGL, bacterial monoacylglycerol lipase; OVA, ovalbumin; FN, fibronectin; CFH, complement factor H; C4BP, C4b-binding protein.

that was toxic to cells at the concentrations used for all other preparations (100 μg/ml). However, titration of the 24-hour-preparation showed that the toxic effect was abolished in high dilutions and became pro-inflammatory, indicating that the 24-hour preparation probably contained a higher density of MDHDC-type adducts. The mass spectrometric analysis is consistent with a recently published report characterizing MDA-modified peptides, which came to similar conclusions regarding the type of adducts formed in short- or long-term incubations (Weißer et al., 2017). In summary, I have shown that the MDHDC-type of MDA epitopes is most likely the one mediating pro-inflammatory effects in my in vitro and in vivo settings, as all stimulation and experiments with MAA-BSA were performed using the 3-hour preparation.

4.3.2 Formation of biologically relevant MDA epitopes does not depend on the carrier protein

Typically, LDL is used as a carrier protein for MDA modification due to its proposed contribution to atherogenesis (reviewed in chapter 2.7). In my thesis project, I have used BSA as a carrier to demonstrate that the pro-inflammatory effects observed are not restricted to LDL but independent of the lipid component in the protein carrier. To support the claim of carrier independency and to demonstrate that, in principle, MAA epitopes could be formed on any protein given sufficient amounts of accessible amino groups are present, I performed chemical derivatization of bacterial monoacylglycerol lipase (bMGL), ovalbumin (OVA) and human fibronectin (FN) with MDA and acetaldehyde. To test the preparations for the generation of biologically active, complex adducts I subjected each preparation to fluorescence measurements and chemiluminescent ELISAs to assay for binding by MDA antibodies. Figure 4.3 shows that all protein carriers tested could be modified with MDA. Furthermore, the reaction conditions allowed the formation of fluorescent MAA epitopes recognized by both the promiscuous LR04 and MDA2 antibodies as well as more stringent E014 and NA17 antibodies on all protein carriers tested (Figure 4.3, Panels A, B, D and E). MAA-FN and -OVA were also recognized by CFH and C4BP (Panel F), suggesting that they might have pro-inflammatory properties. Importantly, MAA-bMGL was capable of inducing a pro-inflammatory response in stimulated THP-1 cells (Figure 4.3, Panel C). Together, this shows that the pro-inflammatory effects of MAA epitopes presented in BSA are mediated by the epitopes themselves and independent of the protein backbone.

4.3.3 Characterization of MDA-epitope formation during apoptosis

Apoptosis, a type of programmed cell death, is a physiological process to remove unwanted cells during development but also in inflammation (Taylor et al., 2008). This step-by-step mechanism is strictly organized and occurs in a controlled, anti-inflammatory manner. Two pathways for apoptosis induction can be distinguished: the intrinsic and the extrinsic pathway. Central to both pathways is the involvement of cysteine aspartyl-specific proteases named caspases. Environmental cytotoxic stress triggers the intrinsic pathway, which leads to mirochondrial outer membrane permeabilization and the release of cytochrome C from the mitochondria, thereby inducing the formation of a complex called apoptosome. The extrinsic pathway is induced by binding of a ligand to membrane receptors such as tumour necrosis factor, which allows recruitment of adaptor proteins forming a different complex termed death-inducing signalling complex (DISC). Both intrinsic and extrinsic pathways ultimately lead to the activation of a cascade of caspases, which initiates a cellular program causing apoptosis of the cell, including cell shrinkage, nuclear fragmentation, chromatin condensation, DNA fragmentation and blebbing. The resultant

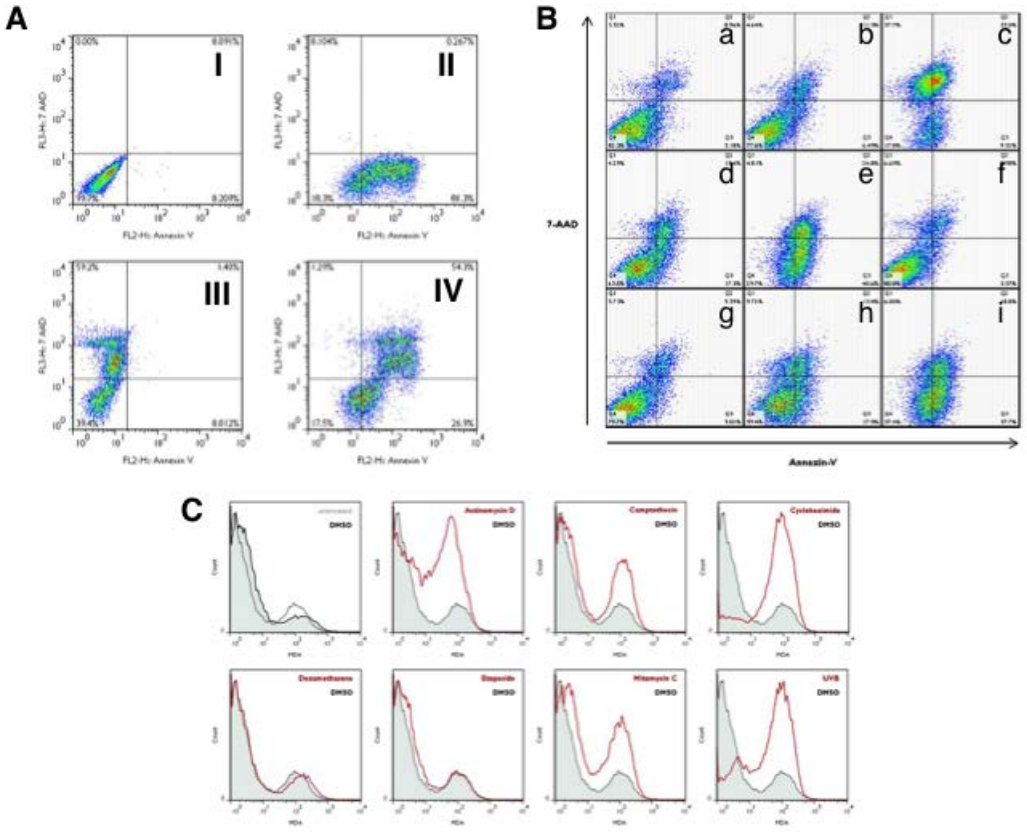

Figure 4.4: Apoptosis induction and MDA-epitope formation in RAW macrophages. (A) Cell populations (according to quadrant analysis) in dying macrophage cultures: I, Annexin-V$^-$7-AAD$^-$ viable cells; II, Annexin-V$^+$7-AAD$^-$ early apoptotic cells; III, Annexin-V$^-$7-AAD$^+$ necrotic cells; IV, Annexin-V$^+$7-AAD$^+$ late apoptotic cells. (B) Representative flow cytometry plots of Annexin-V/7-AAD-stained dying RAW macrophages treated with various apoptosis inducers for 16 hours: a, untreated; b, DMSO; c, actinomycin D; e, camptothecin; f, cycloheximide; f, dexamethasone; g, etoposide; h, mitomycin C; i, 100 mg/cm^2 UV irradiation. (C) Flow cytometric analysis of LR04-stained dying cells from (B) for detection of MDA epitopes. Dotted line, isotype control staining.

apoptotic bodies or microvesicles are removed from the organism by phagocytes.

Previous studies by us and others have shown that MDA-specific antibodies can recognize MDA epitopes on microvesicles (Tsiantoulas et al., 2015a) or dying cells (Chang et al., 1999; Amir et al., 2012). While these studies also addressed the specific dying sub-population bound by MDA-specific antibodies in the respective cell type using one method of apoptosis induction, there exists no systematic study of the generation of such epitopes on different cell types and using various apoptotic inducers. Here, I set out to analyze the dynamics of MDA generation during apoptosis on a variety of cell lines and primary cells using a range of apoptosis inducers.

First, RAW264.7 macrophages were treated with different apoptosis inducers or irradiated with 100 mJ/cm^2 UV. To confirm apoptosis induction, cells were stained with viability markers Annexin-V and 7-Aminoactinomycin D (7-AAD). Annexin-V binds to phosphatidylserine exposed on the surface due to loss of membrane asymmetry during apoptosis. 7-AAD is excluded by live, intact cells but intercalates into the DNA of dying or damaged cells due to increasing cell permeability during apoptosis. Thus, viable cells are Annexin-V$^-$7-AAD$^-$, early apoptotic cells are Annexin-V$^+$7-AAD$^-$, late apoptotic cells are Annexin-V$^+$7-AAD$^+$ and necrotic cells are Annexin-V$^-$7-AAD$^+$ (Figure 4.4, Panel A). The majority of treatments induced apoptosis to different extents (Figure 4.4, Panel B, a-i). Co-staining with LR04 revealed the generation of MDA epitopes on the surface of dying cells treated with actinomycin D, camptothecin, cycloheximide, mitomycin C or UV irradiation, while dexamethasone and etoposide did not induce detectable apoptosis or LR04 binding in RAW macrophages. Similar results were obtained by co-staining with other MDA antibodies, E014 and NA17 (not shown).

Next, I chose thymocytes, a classical cell-type for investigating apoptosis, to analyze the MDA-positive subpopulations in greater detail. Again, 16 hours of treatment with various apoptosis inducers resulted in different degrees of cell death within each population of thymocytes (Figure 4.5, Panel A). In contrast to the treatment of RAW macrophages, dexamethasone was the most potent apoptosis inducer in thymocytes using the same conditions. Only mitomycin C did not induce apoptosis at a detectable level. Interestingly, independent of the trigger used, E014- and LR04-positive cells were detected with all apoptotic drugs as soon as cell death occurred (Figure 4.5, Panels B and C). When analyzing the cell sub-population recognized by MDA antibodies, I found that both E014 and LR04 bound primarily late Annexin-V$^+$7-AAD$^+$ apoptotic cells (up to 80%), indicating that MDA epitopes are generated upon transition from early to late apoptotic stages (Figure 4.4, Panel C). To study the timing of MDA-epitope formation, I generated dying thymocytes using dexamethasone over a time period of 8 hours and analyzed the cells every two hours. The time-course analysis revealed that MDA epitopes are already present in untreated cells within the baseline-subset of late apoptotic cells (upper right quadrant in first timepoint of Figure 4.5, Panel D) and increase over time due to accumulation of dying

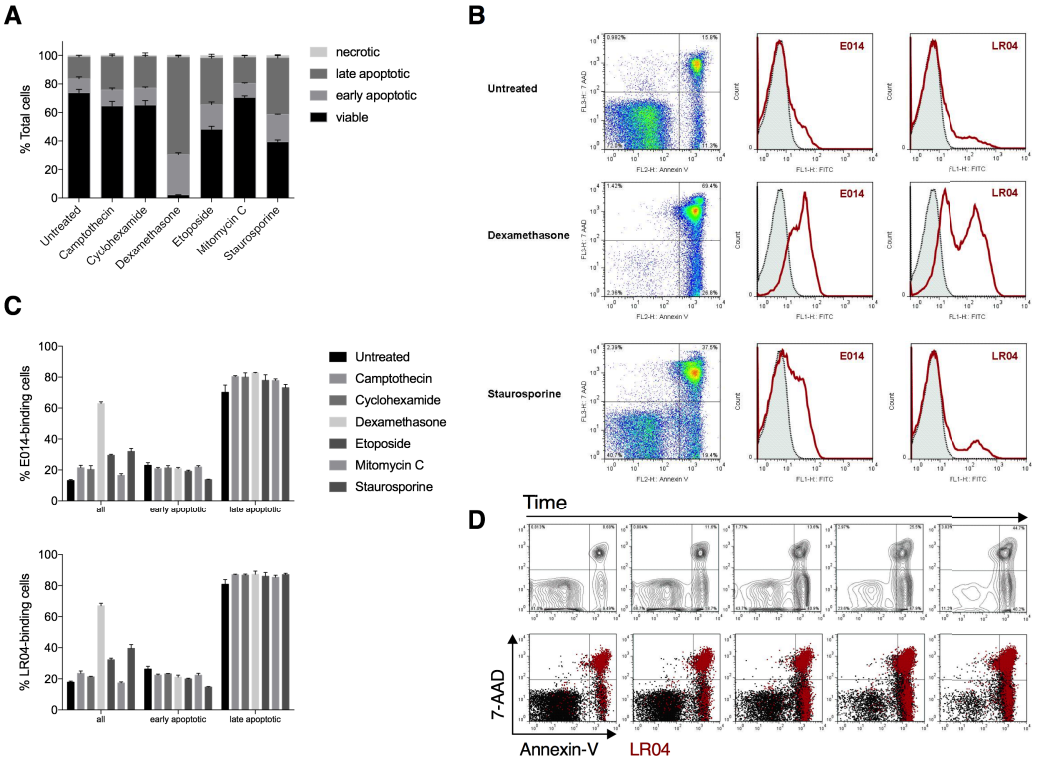

Figure 4.5: Apoptosis induction and MDA-epitope formation in thymocytes. (A) Flow cytometry analysis of dying thymocytes treated with indicated drugs for 16 hours and stained with Annexin-V and 7-AAD. (B) Representative flow cytometry plots of Annexin-V/7-AAD-stained dying thymocytes co-stained with MDA antibodies E014 and LR04. (C) Quantification of (B) gated on all, early or late apoptotic cells. (D) Time-course analysis of dying thymocytes treated with dexamethasone for 0, 2, 3, 6 or 8 hours, followed by flow cytometric analysis after Annexin-V/7-AAD/LR04 staining.

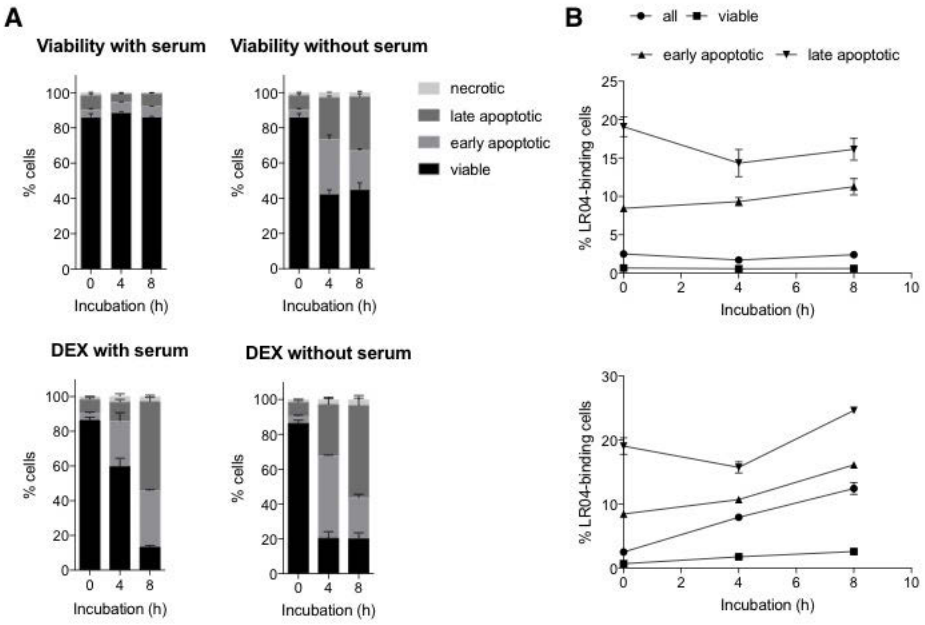

Figure 4.6: MDA-epitope formation on dying thymocytes occurs in presence or absence of serum. (A) Viability staining for thymocytes incubated for 0, 4 or 8 hours with or without dexamethasone in presence of absence of serum (left/right). (B) Flow cytometric analysis of LR04 positivity in presence (top) or absence (bottom) of serum.

cells in this quadrant, while the subset of viable cells (lower left quadrant) disappears over time. These observations were independent of the presence of serum during dexamethasone treatment (Figure 4.6). Of note, serum deprivation alone induced also apoptosis in thymocytes, which did not result in the overall increase of MDA epitopes observed with other treatments (Figure 4.6, Panel B, "all" gate of upper graph).

To systematically study the timing of MDA generation in thymocytes using different treatments, I performed a time-course analysis with the most potent apoptosis inducers, next to serum deprivation. Between 0 to 8 hours of treatment, the amount of E014- and LR04-positive cells within the late apoptotic population increased from 10 to 50 % (Figure 4.7, Panel B) in all treatment and to 30% in the serum-deprived late apoptotic cells. Notably, the overall MDA-positivity increased in all conditions up to 10% except for serum deprivation, where only a slight increase at the latest timepoint was observed, which did not reach 10%. Therefore, late apoptotic cells in absence of serum gain MDA epitopes, while no or very few cells undergoing apoptosis and becoming late apoptotic cells acquire MDA positivity at the timepoints investigated. This could be due to slower kinetics because of a weaker type of apoptosis induction or other unknown reasons. Importantly, induction of extrinsic apoptosis using Fas ligand (FasL) resulted also in the formation of

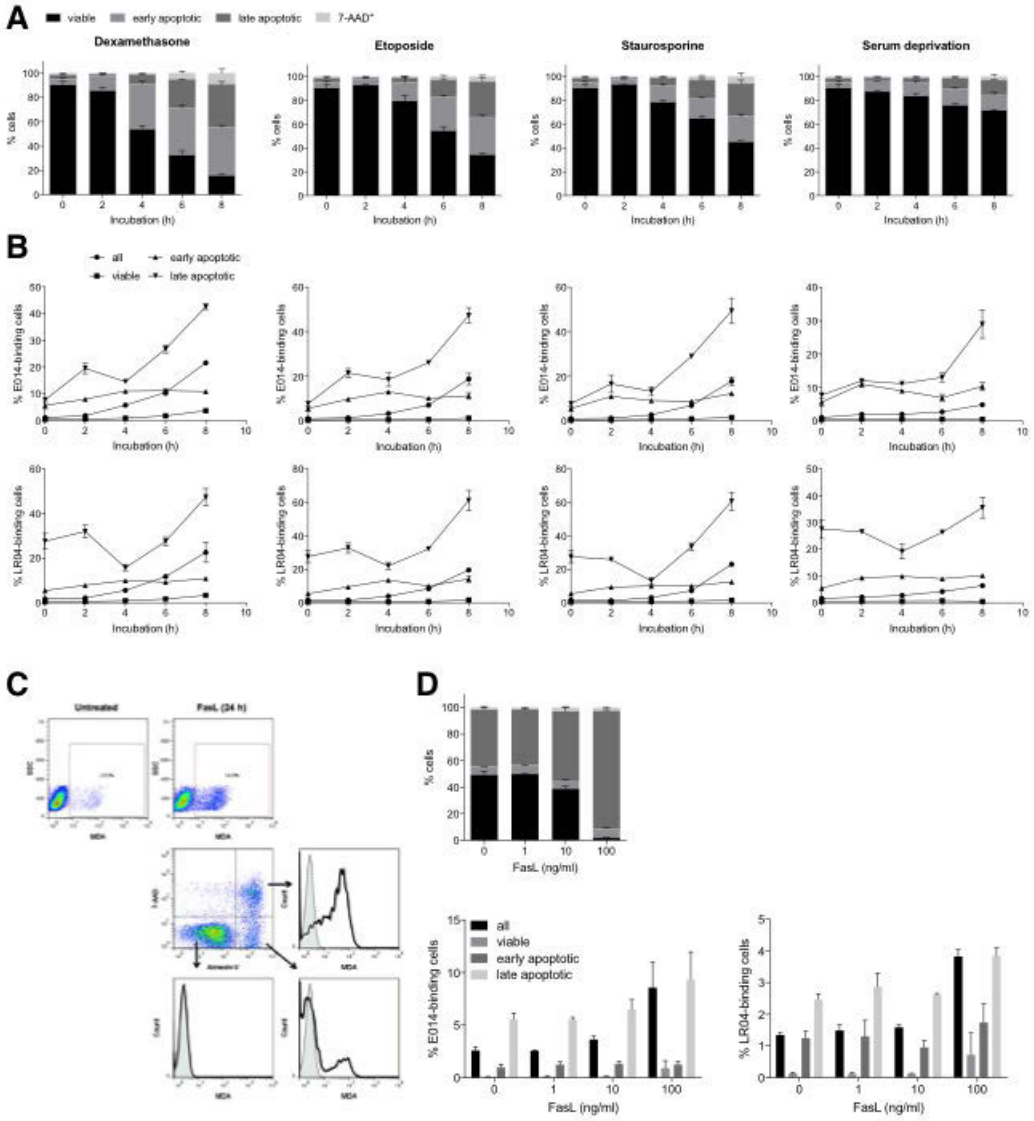

Figure 4.7: Time-course analysis of MDA-epitope formation in dying thymocytes. (A) Flow cytometry analysis of dying thymocytes treated with indicated drugs for 0 to 8 hours and stained with Annexin-V and 7-AAD. (B) Quantification of flow cytometric analysis of E014 (top) or LR04 (bottom) staining for each timepoint and treatment condition. (C) Representative flow cytometry dot plot for FasL-induced apoptosis in thymocytes treated for 24 hours. (D) Quantification of (C). Legend for viability plot as in (A).

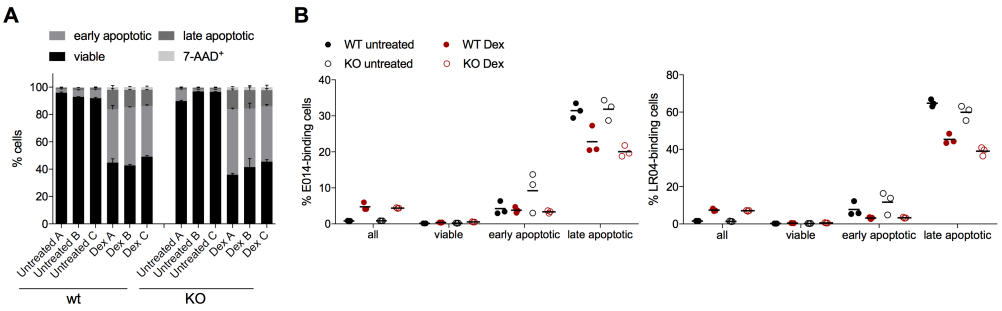

Figure 4.8: MDA epitope formation on dying thymocytes from Atgl-deficient mice. (A) Viability analysis by flow cytometry showing dying cell populations in three wild-type and Atgl-deficient mice (A, B, C) treated with or without dexamethasone (Dex). (B) Quantification of E014- and LR04 binding cells by flow cytometry in wild-type and knockout animals co-stained for viability markers. Each dot represents one mouse.

MDA epitopes primarily on late-apoptotic cells (Figure 4.7, Panel C and D) as indicated by enhanced E014 and LR04 staining in particularly this subset of dying cells.

Apart from RAW macrophages and thymocytes, treatment of Jurkat T cells, THP-1 cells and primary macrophages such as BMDM also resulted in the same outcome. Hence, independent of cell type or apoptosis inducer used, as soon as the Annexin-V$^+$7-AAD$^+$ stage was reached, the cells also acquired the ability to be bound by MDA antibodies. Interestingly, an antibody against a different OSE, 1-palmitoyl-2-(5-oxovaleroyl)-phosphatidylcholine (POVPC), namely E06, did not bind to this extent to dying cells, and neither did the isotype control antibody (not shown).

To better understand the generation of MDA epitopes on dying cells, I aimed at reducing oxidative stress by blocking ROS production or scavenging ROS by chemical and genetic means. I tested primary cells from four different genetically modified strains. Mice deficient in adipose triglyceride lipase (Atgl or Pnpla2 for patatin-like phospholipase domain-containing protein 2) have impaired hydrolytic activity on triglycerides, resulting in triglyceride accumulation in all organs and cell types analyzed (Zechner et al., 2009). Triglycerides are esters of glycerol with fatty acids, and the latter can be degraded to lipid peroxidation products in presence of oxygen radicals. Mice deficient for neutrophil cytosolic factor 1 (Ncf1, or p47phox) expression cannot assemble the NADPH oxidase due to lack of a critical subunit and thus have impaired superoxide production. Myeloperoxidase (Mpo) is an oxidative enzyme involved in the production of hypochlorous acid and also produces different radicals that may contribute to oxidative damage in tissue. Lipoxygenases such as Alox15 oxidize unsaturated fatty acids and Alox15 was also shown to oxidize LDL in vitro and in vivo (Rankin et al., 1991; Ylä-Herttuala et al., 1995).

To investigate the role of enzymes important for lipid peroxidation of ROS generation, I isolated primary cells including bone marrow cells and thymocytes from these knockout mice. Induction of apoptosis in Atgl-deficient mice did not reveal any differences

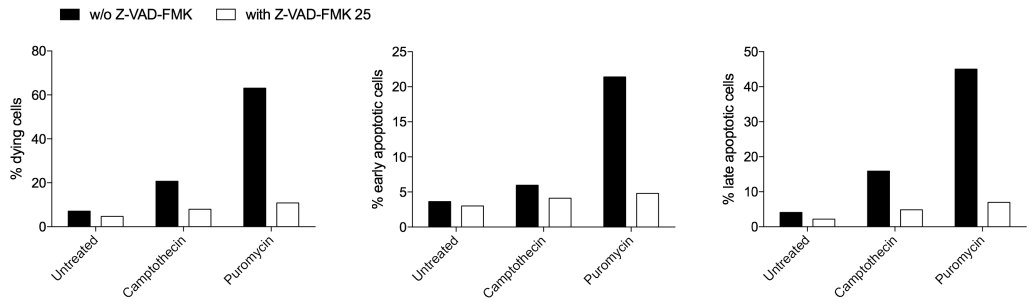

Figure 4.9: Inhibition of apoptosis in Jurkat T cells using a pan-caspase inhibitor. Flow cytometric analysis of Annexin-V/7-AAD-stained cells pre-treated with different concentrations of Z-VAD-FMK, followed by apoptosis induction with camptothecin or puromycin for 16 hours; gated on dying cells or on apoptotic cell subsets.

in MDA content compared to wild-type animals (Figure 4.8, Panel B). Similar results were observed with Ncf1-, Mpo- and Alox15-deficient mice (not shown). In summary, I could not observe a difference in MDA-epitope formation during apoptosis in any of these four strains, possibly due to either compensatory mechanisms acting in absence of each respective protein, or more likely due to the fact that a large amount of ROS is produced endogenously not only by NADPH oxidase complexes but also as a by-product during cellular respiration by the electron transport chain in mitochondria. Consistently, inhibition of NADPH oxidase with apocynin or pre-treatment of cells with glutathione and N-acetylcysteine, two antioxidants, also did not affect MDA formation during apoptosis at the concentrations tested (not shown).

Then, I tried to modulate cell death by chemical means using either an apoptotic inhibitor targeting the central proteases during apoptosis or an inhibitor of apoptotic-bleb formation, blebbistatin, which blocks myosin II ATPases. Treatment with blebbistatin was expected to increase the percentage of MDA-positive cells as the release of MDA-positive blebs or microvesicles during apoptosis would be inhibited by this drug. Inhibition of apoptosis by use of the pan-caspase inhibitor Z-VAD-FMK resulted in the generation of less apoptotic cells (Figures 4.9 and 4.10, Panel A) and slightly reduced MDA epitopes in total cells but not in apoptotic subsets (Figure 4.10, Panel B), whereas pre-incubation with different concentrations of blebbistatin increased the percentage of both E014- and LR04-positive cells both in total and when gated on dying cells but did not change viability (Figure 4.10).

Last, I depleted MDA-positive cells from apoptotic Jurkat T cells using LR04 antibodies and anti-mouse IgM microbeads and observed the remaining cell subset for MDA-positivity over time. Depletion of MDA-positive cells resulted in a reduction of apoptotic cells (Figure 4.11, compare 0 h timepoint of Panels A and B). However, cell death after 24 h was comparable between depleted and non-depleted samples. Interestingly, upon

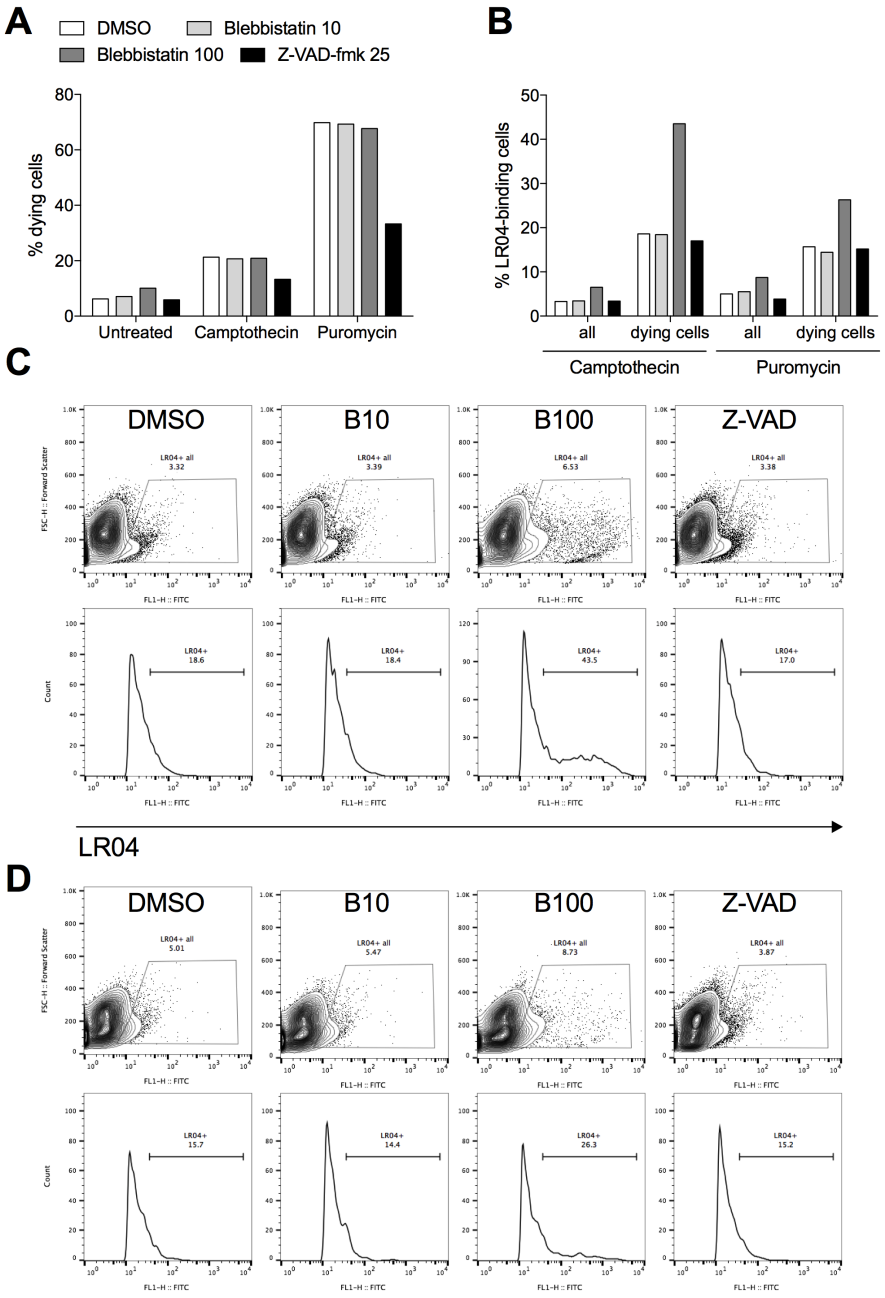

Figure 4.10: Inhibition of apoptosis or blebbing modulates MDA-epitope accumulation in dying Jurkat T cells. (A) Flow cytometric analysis of dying cells upon Z-VAD-FMK or blebbistatin treatment and apoptosis induction using camptothecin or puromycin, stained for Annexin V and with 7-AAD. (B) Flow cytometric analysis of LR04-stained dying cells after inhibition of blebbing or apoptosis and camptothecin/puromycin treatment. "Dying cells" includes Annexin-V$^+$7-AAD$^-$ and Annexin-V$^+$7-AAD$^+$ cells. (C) Representative FACS plots from (B) gated on all (top) or dying cells (bottom) using camptothecin. (D) Representative FACS plots from (B) gated on all (top) or dying cells (bottom) using puromycin. B10, Blebbistatin 10, B100, Blebbistatin 100.

MDA-depletion, the MDA-negative subset acquired MDA epitopes already 2 hours after depletion and continued to do so until 24 hours (Figure 4.11, Panel D). At 24 hours, the formerly MDA-depleted sample had acquired LR04 positivity similar to non-depleted samples after the same amount of time. This indicated, that MDA depletion can only temporarily result in a sample with reduced LR04 reactivity as the dying cells quickly generate novel MDA epitopes.

In summary, I could show that MDA epitopes are generated on a variety cell types independent of the type of apoptosis induction (both intrinsic and extrinsic). MDA epitopes appeared also on early but predominantly on late apoptotic cells, coinciding with the appearance of the 7-AAD stain, a marker of permeabilized cell membranes. It is possible that the permeabilized cellular state allows (part of the) MDA antibodies to access the cytosol and bind to internal MDA epitopes. My preliminary data demonstrates that permeabilization of cells using paraformaldehyde, methanol or detergent results in a shift towards higher E014- and LR04-positivity (but not E06, data not shown). Whether this observation is due to MDA epitopes present on cytosolic structures or due to exposure of MDA epitopes on the cellular surface as a cause of toxic treatment, remains to be shown by e.g. microscopy to clarify the localization of MDA antibody binding sites. Manipulation of the amount of MDA epitopes generated was possibly complicated by the fact that none of the approaches used could abrogate oxidative damage or ROS production to an extent sufficient for observing reduced lipid peroxidation and MDA generation. Lack of individual enzymes expected to be involved upstream of MDA generation clearly did not influence the amount of MDA epitopes on dying cells, and neither did treatment with antioxidants. However, I could show that inhibition of blebbing increases the fraction of MDA-positive cells, probably due to blocked shedding of MDA-positive microvesicles (Tsiantoulas et al., 2015a). Furthermore, inhibition of apoptosis by blocking caspases resulted in less MDA-positive cells. Many questions prevail: Can treatment with a more potent antioxidant cocktail influence MDA generation? Do other forms of programmed cell death (e.g. ferroptosis, necroptosis, pyroptosis) also induce MDA epitopes? Are MDA epitopes generated on dying cells pro-inflammatory, given a certain density of epitopes is reached? Can this pro-inflammatory capacity be enhanced when cells are treated with blebbistatin? Does the amount of MDA epitopes on dying cells influence the capacity of phagocytes to engulf them? To answer these questions, it will be very useful to design or obtain a tool to generate dying cells with reduced MDA epitopes, for example by enzymatic removal or masking of the epitopes by other means than MDA antibodies. Recently, a protocol was published describing how overexpression of Parkin enforces mitophagy, resulting in mitochondria-depleted cells (Correia-Melo et al., 2017). Generating a cell line without mitochondria that are one major place of ROS production could – though technically more challenging than testing different compounds – represent an interesting, novel tool to study MDA generation during apoptosis.

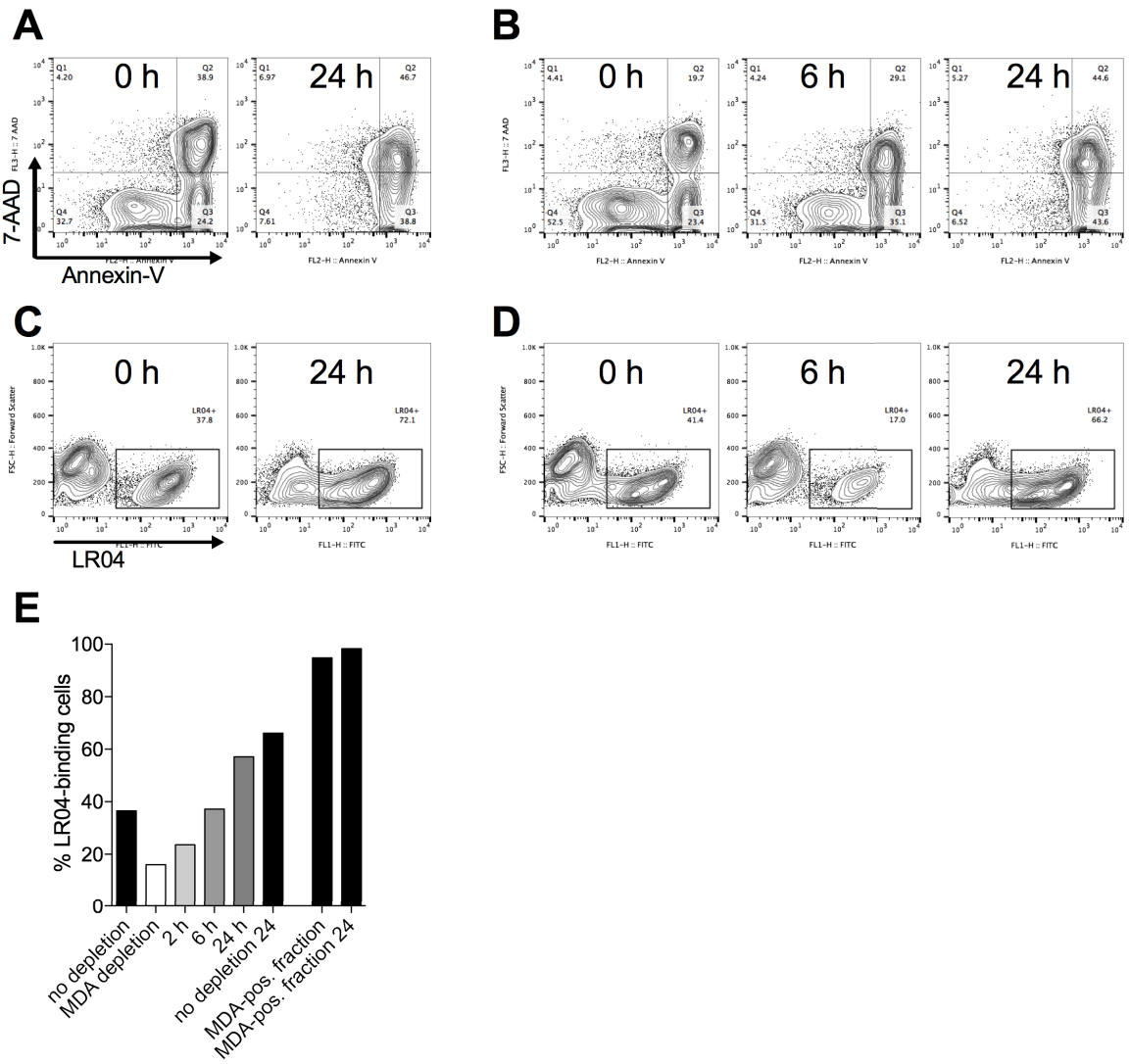

Figure 4.11: Antibody-mediated depletion of MDA-positive dying Jurkat T cells . (A) Flow cytometric analysis of viability-stained Jurkat T cells 0 or 24 h after apoptosis induction (100 mJ/cm^2 UV irradiation, 16 hours); without depletion. (B) Flow cytometric analysis of viability-stained Jurkat T cells 0, 6 or 24 h after apoptosis induction (100 mJ/cm^2 UV irradiation, 16 hours); after depletion of LR04-positive cells. (C,D) Flow cytometric analysis of LR04-positive cells among total cells after apoptosis induction (as above) without (C) or with (D) depletion (0, 6 or 24 hours after). (E) Quantification of LR04-positive cells from (C) and (D).

5 Discussion

In my thesis project, I have shown that hepatic inflammation can be induced and sustained under dyslipidemic conditions by non-bacterial, sterile triggers, namely MDA epitopes. MDA is generated during high oxidative stress by lipid peroxidation of poly-unsaturated fatty acids present in membrane phospholipids and can form covalent adducts with amino groups of proteins and lipids. In in vitro experiments, I demonstrated that MDA epitopes possess pro-inflammatory properties and induce the expression of a set of genes that was also found to be upregulated in livers of WD-fed animals. Using a model of sterile peritonitis, I showed that MDA epitopes elicit a robust influx of neutrophils and monocytes. Importantly, administration of an MDA-specific antibody resulted in a substantial improvement of hepatic inflammation in $Ldlr^{-/-}$ mice on WD, indicating that MDA epitopes significantly contribute to the inflammatory phenotype in the liver.

Pro-inflammatory effects in such models could also be exerted by microbial triggers, and there is little doubt that microbiota and their metabolites contribute to diet-induced inflammation in various organs (Turnbaugh et al., 2006; Henao-Mejia et al., 2012; Wang et al., 2011; Mehal, 2013). For example, germ-free (GF) C57BL/6 mice were found to have lower baseline hepatic triglyceride levels and to be resistant to development of diet-induced obesity compared to conventionalized GF mice (Bäckhed et al., 2004, 2007), which might be due to altered energy and lipid metabolism (Velagapudi et al., 2010). In the inflammation model used in my project, GF $Ldlr^{-/-}$ mice on WD do develop hyperlipidemia, steatosis and inflammation in the liver, indicating that the underlying mechanism is based on the pro-inflammatory properties of sterile triggers. The liver inflammation observed in this model is accompanied by enhanced oxidative stress, as indicated by elevated ROS production and an altered oxidative-stress response in the liver. Importantly, diet-induced hepatic inflammation was similar in GF and conventionally-raised (CONV-R) mice, indicating that the cause of inflammation in our model is independent of microbiota and suggesting the presence of non-bacterial inflammatory stimuli. The induction of inflammation in the liver is not only demonstrated by leukocyte infiltrates and enhanced inflammatory gene expression but also by the upregulation of classical PRRs known to recognize bacterial ligands, for example TLR2 and TLR9. As described earlier, while these PRRs were originally identified as receptor for exclusively bacterial products, today there is no doubt that many of them also bind to sterile triggers and mediate chronic inflammatory diseases (Chen and Nuñez, 2010). For instance, cholesterol crystals (Duewell et al., 2010), OxLDL

(Stewart et al., 2010), advanced-glycation-end-products (Harja et al., 2009) or mitochondrial DNA (mtDNA) (Garcia-Martinez et al., 2016) were identified as such ligands. In the latter case, mtDNA was found to bind to TLR9 and mediate MCD-diet-induced NASH. However, in support of the microbiota/dysbiosis-theory, an earlier study had shown that TLR9 ligands driving NASH are provided via the bloodstream due to dysbiosis in the gut caused by a high-fat diet (Henao-Mejia et al., 2012).

It is conceivable that the combination of both bacterial ligands derived from an altered microbiota and the subsequent cellular damage in the liver, leading to the release of DAMPs, contribute to liver inflammation. This is also a possible scenario in the liver inflammation model studied. While I could demonstrate that inflammation occurs also in absence of microbiota, indicating that sterile triggers are sufficient to induce an inflammatory response to a similar extent, an added effect of microbiota-derived ligands cannot be excluded and is – in my opinion – very likely, also given that the inflammation was slightly higher in CONV-R than in GF mice. Nevertheless, in this thesis, I described experiments that suggest that MDA epitopes as a model OSE for OxLDL could represent a DAMP generated upon cellular stress and tissue injury that can activate the same signalling pathways as microbial stimuli would, such as NFκB-signalling. The cause of cellular stress and tissue injury in CONV-R mice might be extensive ectopic lipid deposition. Because I found MDA epitopes primarily on the surface of dying cells in the liver and a pulldown using the MDA-specific antibody clone LR04 identified mainly mitochondrial proteins, I hypothesize that the generation and effect of MDA epitopes might be linked to the appearance of other DAMPs and synergistically enhance their inflammatory properties.

Interestingly, the role of other DAMPs in this diet-induced hepatic inflammation appears to be minor, given treatment with the natural IgM antibody clone LR04 specific for MDA epitopes can reduce inflammation to a large extent. This suggests that the inflammatory phenotype is mainly driven by the generation or presence of MDA epitopes, which could represent prominent marks of metabolic waste products, whose pathological accumulation during oxidative stress situations such as inflammation could result in disturbed homeostasis due to overwhelming production of oxidative danger signals that perpetuate inflammatory processes. Thus, the MDA-specific natural antibody LR04 is a promising tool for exploiting protective functions of natural antibodies towards sterile triggers.

In a collaboration, my thesis laboratory showed previously that active vaccination against OxLDL increased anti-OxLDL antibody titers and resulted in reduced hypercholesterolemia and reduced hepatic inflammation (Bieghs et al., 2012). Consistently, we found recently that expansion of the OSE-specific natural antibody pool beyond physiological antibody levels using Siglec-G-deficient mice on an $Ldlr^{-/-}$ background significantly reduces atherosclerotic burden and hepatic inflammation (Gruber et al., 2016). This thesis project provides evidence for a predominant role of a single OSE by passive transfer of monoclonal IgM antibodies targeted against MDA epitopes, and I could demonstrate that

targeting specifically MDA epitopes but no other OSE is sufficient to ameliorate hepatic inflammation by 30-40%. This is especially intriguing as it is known that many OSE exist and are generated during inflammation and lipid peroxidation (Binder et al., 2016).

For example, 4-hydroxynonenal (4-HNE) also possesses pro-inflammatory and pro-apoptotic properties via activation of NFκB- and p38- pathways (Yin et al., 2015; Kumagai et al., 2004). Additionally, it was shown that 4-HNE or 4-HNE-modified proteins can induce sterile peritonitis, similar to the results in the thesis project (Kumagai et al., 2004; Spite et al., 2009). A set of studies focussing on another oxidative breakdown product, 2-(ω-carboxyethyl)pyrrole (CEP) has produced interesting insights on its role in chronic inflammation as a sensor for oxidative stress (West et al., 2010; Cruz-Guilloty et al., 2014; Saeed et al., 2014; Kim et al., 2015). CEP is generated during inflammation and wound healing but also ageing and was found to mediate pro-angiogenic responses via MyD88-dependent TLR2 but not TLR4 signalling (West et al., 2010). Additionally, CEP plays a role in age-related macular degeneration by inducing M1 macrophage polarization and inflammation in the eye (Cruz-Guilloty et al., 2014), acting synergistically with TLR2 ligands (Saeed et al., 2014). A more recent study also implicated CD36 as a sensor for CEP-modified proteins (Kim et al., 2015). In summary, these studies show that additional OSE could play similar roles in other chronically inflamed tissues as MDA epitopes in this liver model.

5.1 Considerations on the Chosen Diet Model

The inflammation model used in this study employs a mouse strain deficient in the low-density lipoprotein receptor (Ldlr). Due to this defect, mice fed a high-fat-high-cholesterol diet, also known as atherogenic diet, develop hypercholesterolemia, hyperlipidemia and associated consequences depending on the duration of the diet. One to two weeks of Western-type diet (WD) result in early-stage hepatosteatosis and inflammation, while long-term feeding (e.g. 16 weeks) of WD induces arterial plaque formation and atherosclerosis (Wouters et al., 2008; Getz and Reardon, 2006). As a matter of fact, the $Ldlr^{-/-}$ mouse together with the $ApoE^{-/-}$ mouse represent the most widely used models in atherosclerosis and hyperlipidemia research. Ldlr is located on the surface membranes of liver cells and binds to several apolipoproteins including ApoE, which are important components of plasma lipoproteins that mediate the clearance of cholesterol and triglycerides from the circulation. Consequently, individuals lacking either functional LDLR or APOE suffer from hyperlipidemic disorders such as familial hypercholesterolemia. In contrast to wild-type mice, where the majority of cholesterol is transported in the HDL fraction, the hyperlipidemia profile of $Ldlr^{-/-}$ mice rather resembles human lipid profiles with much of the cholesterol being present in the LDL fraction (Ishibashi et al., 1993). Thus, the use of $Ldlr^{-/-}$ mice could be particularly advantageous in studies investigating early stages

of diet-induced tissue inflammation, where the ectopic lipid deposition in various organs including the liver causes disturbed tissue homeostasis and consequently inflammation such as NASH (Lumeng and Saltiel, 2011). In the case of NAFLD, there is still a lack of knowledge regarding underlying causes of the transition from a steatic liver to an inflamed liver. Several other diet models exist that reflect the pathology of human NASH to a certain extent, of which the methionine-choline-deficient (MCD) diet is widely used and well-known for inducing a severe liver phenotype within a relatively short timeframe. Whereas some features of NASH including serum aminotransferase elevation, histological changes in the liver and hepatic inflammation are observed in MCD-diet-fed mice, their metabolic profile is quite different from the typical situation in NASH patients, as these animals suffer from profound weight loss, display decreased plasma triglyceride levels and remain insulin-sensitive (Takahashi et al., 2012). Another important nutritional model known for inducing insulin resistance and early type 2 diabetes is based on feeding a high-fat diet (HFD), where 60% of the calories derive from fat, to C57BL/6 mice (Surwit et al., 1988). Using this diet, mice develop increased body and adipose tissue mass after 3 days, as well as glucose intolerance, insulin resistance and adipose tissue inflammation (Lee et al., 2011) but no or only very mild hypertriglyceridemia (Eisinger et al., 2014; Lai et al., 2015; Guo et al., 2009). While hepatic steatosis is induced after 3 days, hepatic inflammation only appears after 12-16 weeks of HFD feeding in this model (Lee et al., 2011; Lai et al., 2015). HFDs containing both cholesterol and cholate next to fat have also been used to study NASH-like phenotypes including hepatic steatosis and inflammation. Dietary cholate promotes the development of hypercholesterolemia, however, being a bile salt, it has various additional effects including hepatic toxicity, thus its usage is not recommended (Getz and Reardon, 2012). In a previous study, it was shown that dyslipidemic *Ldlr*$^{-/-}$ mice fed WD, a high-fat (21%) diet containing 0.2% cholesterol but no cholate, represent a physiological model for studying hepatic steatosis and inflammation reminiscent of early-stage human NASH (Bieghs et al., 2012). I demonstrate now that this model faithfully recapitulates also other diet-induced hepatic alterations apart from clinical features, such as hypercholesterolemia, dyslipidemia, steatosis and hepatitis. Indeed, by performing transcriptome analyses of whole livers, I have identified deregulation of certain sets of genes involved in biological processes including stress response, inflammation and metabolism that were also found to be altered in other diet models using unbiased approaches (Matsuzawa et al., 2007; Renaud et al., 2014; Shockley et al., 2009; Kim and Park, 2010). In contrast to many other studies, feeding WD to *Ldlr*$^{-/-}$ mice results in hepatic steatosis and inflammation already after a relatively short period of time of less than 1-2 weeks. Even though the phenotype of this model is driven by dyslipidemia, I found that mechanisms related to innate immunity are among the most deregulated pathways, revealing strong links between metabolic dysregulation and the subsequent mounting of immune responses, which underlines the concept of metabolically-induced inflammation.

Moreover, in this study I provide an example for using this model not only to study the relationship between hepatic lipid accumulation and inflammatory responses but also to examine the effect of therapeutic interventions aimed at reducing inflammation. In light of the fact that the lipoprotein profile of *Ldlr*$^{-/-}$ mice resembles the situation observed in human patients with hypercholesterolemia (Yin et al., 2012), this model is also particularly interesting with regard to metabolically-induced inflammation in humans, given metaflammation is described as a chronic, low-grade type of inflammation that is often not well reflected in many of the genetic or nutritional models resulting in more severe phenotypes.

In summary, the *Ldlr*$^{-/-}$ mouse serves as a useful tool for modelling the early stages of NASH development with the particular advantage of bearing a lipoprotein profile similar to the human situation. For studying late NASH events such as fibrosis this model is less well suited and the selection of the model should strongly depend on the research question to be addressed.

5.2 Conclusions and Future Directions

In my thesis I have shown that MDA epitopes generated during chronic inflammatory diseases such as NASH possess pro-inflammatory properties in vitro and in vivo, which are mediated by scavenger receptors, and can be neutralized by MDA-specific natural IgM antibodies. However, several questions remain unresolved.

As there is only limited knowledge on signalling pathways induced by scavenger receptors, it cannot be excluded that MDA-induced signalling is mediated via an assembly of several receptors similar to the OxLDL sensor (Stewart et al., 2010). Here, the authors found that OxLDL is recognized by a heterotrimeric complex composed of TLR4/6 and CD36 in macrophages, resulting in pro-inflammatory responses that trigger NFκB activation and prime the NLRP3 inflammasome for subsequent cholesterol-crystal-induced activation (Stewart et al., 2010; Sheedy et al., 2013). Additionally, the residual MDA-induced inflammation found in CD36- or MSR1-deficient macrophages suggests the existence of multiple MDA receptors, as the lack of a single receptor only partially abrogates the pro-inflammatory response. Indeed, other MDA receptors apart from MSR1 have been implicated in the literature, including CD16 (Zhu et al., 2014), LOX-1 (Besler et al., 2011) and more recently Stabilin-1 (Rantakari et al., 2016). Because MDA epitopes are ubiquitously found in inflammatory conditions I speculate that a functional redundancy of MDA receptors may be beneficial for allowing efficient clearance in multiple tissues. The findings that MDA epitopes mediate inflammation via CD36 and MSR1 are supported by a previous study in my thesis laboratory, which showed that deficiency of both scavenger receptors on myeloid cells reduces hepatic inflammation in WD-fed *Ldlr*$^{-/-}$ mice (Bieghs et al., 2010).

I have presented data in my manuscript demonstrating that WD-fed *Ldlr*$^{-/-}$ mice have a large population of LR04-positive cells in their livers and that LR04 recognizes predominantly late apoptotic (Annexin-V^{+}7-AAD^{+}) cells. In Section 4.3.3, I have attempted to characterize MDA-epitope generation during various apoptotic conditions and using different genetic strains. However, it is still unclear, which mechanism or enzyme(s) is involved in this process and whether MDA-epitope formation can be inhibited genetically or pharmacologically.

The MDA-mediated propagation of inflammation observed here might not be restricted to this model but could also explain part of the underlying mechanism of other chronic inflammatory diseases or even ageing. Recently, a study suggested that senescent cells carry MDA-modified vimentin, which might serve as a tag for these cells to enable swift clearance by humoral innate immunity, i.e., natural IgM antibodies or complement factors (Frescas et al., 2017). In light of the fact that elevated levels of natural IgM antibodies appear to be associated with beneficial outcomes both in mouse inflammation models (this study) and in human NASH patients (Hendrikx et al., 2016), future studies should continue to explore the potential of MDA epitopes as therapeutic targets.

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Presentations

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2014 Keystone Symposium on Innate Immunity, Metabolism and Vascular Injury (Poster)
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2013 9th Young Scientist Association PhD Symposium (Poster)
2013 Keystone Symposium on Metabolic Control of Inflammation and Immunity (Poster)
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Publications

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