Published in final edited form as:

Hepatology. 2017 April ; 65(4): 1181–1195. doi:10.1002/hep.28970.

Malondialdehyde epitopes are sterile mediators of hepatic inflammation in hypercholesterolemic mice

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Abstract

Background and Rationale—Diet-related health issues such as non-alcoholic 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.

Main Results—We identified biological processes related to innate immunity and oxidative stress as prime response pathways in livers of *Ldlr*^{-/-} mice on 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 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 scavenger receptors CD36 and MSR1. Moreover, *in vivo* neutralization of endogenously generated MDA epitopes by intravenous

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Author contributions: CJLB and TH designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. DW performed experiments and critically revised the manuscript. SJ, SMAW, AFR, AH, LG, SS, GH, NPM, JW, FP and VM conducted experiments or analyses. SK and CB provided intellectual input and critically revised the manuscript. CR, KB and RSS provided technical and material support and critically revised the manuscript. CJB conceived, designed, and supervised the study, wrote and critically revised the manuscript.

Disclosures: The authors have declared that no conflict of interest exists.

injection of a specific MDA antibody results in decreased hepatic inflammation in *Ldlr*^{-/-} mice on Western-type diet.

Conclusions—Accumulation of MDA epitopes plays a major role during diet-induced hepatic inflammation and can be ameliorated by administration of an anti-MDA antibody.

Keywords

oxidative stress; dyslipidemia; natural IgM antibodies; lipid peroxidation

Introduction

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, non-alcoholic fatty liver and cardiovascular disease has increased concordantly. However, the exact pathways that link diet-induced changes e.g. hyperlipidemia, and the ensuing inflammation ha 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 Western-type diet.

Experimental procedures

Animal experiments

C57BL/6J and *Ldlr*^{-/-} mice were purchased from Charles River Laboratories and The Jackson Laboratory, respectively. Mice were kept under specific-pathogen-free (SPF) conditions at the Medical University of Vienna or Maastricht University with ad libitum access to water and 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. SPF (CONV-R) or GF mice were fed a ND or a Western-

type diet (WD) containing 21% fat and 0.2% cholesterol for 1-4 weeks. For the antibody intervention study, baseline levels of lipids, MAA-binding, P2-binding and total 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 atherogenic diet (4), tested for endotoxin and microbial contaminations, kind gift of J.L. Witztum) or 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 RT-qPCR. Lipid and liver enzyme levels in plasma and liver homogenates were determined as described previously (8). Hepatic oxidative stress was assessed by histochemistry and flow cytometry. Sterile peritonitis was induced in 11-16-week-old, female C57BL/6J mice using 25 mg/kg MAA-BSA or unmodified (naive) BSA suspended in 200 µl PBS. After 2 hours, peritoneal fluid and cells were harvested and checked for leukocyte recruitment by flow cytometry and cytokine secretion by enzyme-linked immunosorbent assays.

Statistics

All data are displayed as mean ± SEM. For comparison of two groups, Student's paired (when applicable) or unpaired t-test was used. For comparison of > 2 groups, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used. All analyses were performed with GraphPad Prism v6.04. A p value of < 0.05 was considered significant.

Additional information can be found in the Supplementary Materials and Methods section.

Results

WD-induced steatohepatitis activates innate immune responses in *Ldlr*^{-/-} mice

We have previously shown that feeding a short-term 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 a 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 a WD, accompanied by hepatic leukocyte infiltrates (Figure S1AB, Suppl. Table 1), 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 of 0.96 to 0.98 (Figure 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 (Figure S2B, Figure S2C). When comparing the intersection of differentially regulated genes between the groups, we found that most genes differentially regulated in 2 week WD vs. ND comparisons were also different in 4 week WD vs. ND comparisons (> 400, Figure 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 (GO) analysis 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 (Figure 1A), including chemokine signaling pathway, leukocyte transendothelial migration, cytokine-cytokine receptor interaction (Figure 1B) and metabolic activity (Figure 1C), consistent with other high-fat-diet models (10, 11). Already 2 weeks of WD resulted in the significant enrichment of genes associated strongly with mechanisms of innate immunity such as Fc γ -mediated phagocytosis, complement system and TLR signaling pathway (Figure 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 (Figure 1D). Indeed, also pattern-recognition receptors (PRRs) such as *Tlr2*, *Tlr9*, *Clec12a* and *Nlrp3* were upregulated as well as regulators and transcription factors of the NF κ B pathway, a prominent signal transduction mechanism of innate immunity (Figure 1D). In addition, GO analysis of the significantly downregulated transcripts in livers after 2 weeks of WD revealed a strong enrichment of genes involved in the biological process „oxidation reduction“ (Figure 1C), suggesting the activation of oxidative stress response pathways. Indeed, a set of genes implicated in oxidative stress response was found to be deregulated upon WD in our global analysis (Figure 1E). Together, we show that 2 weeks of WD are sufficient to induce global changes in hepatic gene expression, which are still present and largely unchanged after 4 weeks of WD feeding, with a strong enrichment for transcripts involved in innate immunity and oxidative stress response.

Microbiota are not required for 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 conventionally-raised (CONV-R) and germ-free (GF) *Ldlr*^{-/-} mice a WD for 2 weeks and analyzed their livers for the presence of inflammation. Even in absence of microbiota WD induced hyperlipidemia, hepatic steatosis and inflammation in *Ldlr*^{-/-} mice (Figure 2A, B) characterized by infiltration of neutrophils and macrophages (Figure 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 upregulated due to WD in our global analysis was also upregulated in GF *Ldlr*^{-/-} mice upon WD (Figure 2D). Importantly, we confirmed that key deregulated genes involved in oxidative stress response such as *Cybb*, *Nrros*, *Irg1*, *Mmp12*, *Ncf1* and *Ncf2* were also significantly upregulated in livers of GF mice after 2 weeks of WD (Figure 2F). This observation suggests that WD evokes the development of increased oxidative stress and innate defense mechanisms independent of the presence of microbiota. Indeed, the induction of *Cxcl1* was comparable between GF and CONV-R mice (Figure 2E). Thus, sterile, non-bacterial 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 DHE staining indicating higher levels of ROS production compared to livers from ND-fed mice (Figure 3A), consistent with the oxidative stress response profile on a transcriptome level (Figure 1E). Additionally, flow cytometric analysis of both whole livers and isolated Kupffer cells using the oxidative stress indicator CM₂-DCFDA demonstrated a robustly elevated ROS production in the livers of WD-fed mice (Figure 3B). Importantly, we also detected elevated oxidative stress in livers of GF mice on WD compare to ND (Figure S3A). As a major consequence of oxidative stress is lipid peroxidation, we next investigated whether feeding *Ldlr*^{-/-} mice a 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 (Figure 3C and 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 (Figure 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 (Figure 3E and S3C). The density of MDA epitopes as calculated by mean fluorescence intensity (MFI) was also significantly increased in liver cells of WD mice (Figure S3D). Similar results were obtained using a polyclonal IgG antibody against MDA (Figure 3E, S3D). This data is consistent with previous studies showing the occurrence of MDA epitopes in chronic liver diseases including non-alcoholic steatohepatitis (NASH) (13–15). To determine the identity of the cells recognized by MDA antibodies, we co-stained 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-AAD staining (Figure 3F). Moreover, LR04⁺ cells were to a large extent F4/80⁺ Kupffer cells and LR04⁺F4/80⁺ cells were enriched in liver cell suspensions from mice on a WD (Figure 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 NADPH oxidase subunits *Cybb* and *Ncf2*, as well as upregulation of immunoresponsive gene 1 (*Irg1*), which mediates the production of mitochondrial ROS by oxidation of intracellular fatty acids (Figure 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 downregulated in Kupffer cells upon WD (Figure S3E). Thus, feeding a 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 pro-inflammatory effects, we performed immunopurification of homogenized liver samples from mice fed a WD for 4 weeks using LR04 and an isotype control antibody, and analyzed the obtained protein fraction by mass spectrometry. 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 (Figure 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 pro-inflammatory chemokine responses *in vitro* and *in vivo*

Data from several laboratories suggest that MDA epitopes are not merely markers of oxidative stress but also pro-inflammatory 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 malondialdehyde-acetaldehyde 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 an increased expression of *Cxcl1* after 2 hours of stimulation with MAA-BSA but not sham-BSA by real-time qPCR analysis (Figure 4A). Furthermore, CXCL1 secretion was also increased after stimulation with MAA-BSA (Figure 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 (Figure 4A, Figure 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- 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 (Figure 4B, Figure S4B). In addition, a time-course experiment showed that expression of *Cxcl1* as well as *Cxcl2* peaked around 1–2 hours after stimulation (Figure 4C). To evaluate whether inflammatory genes induced by WD in liver could also be upregulated 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 (Figure 4D).

To exclude the possibility that the modification of BSA per se - irrespective of the attached epitope - renders it pro-inflammatory, 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, FHP-BSA) and the aforementioned cyclic MDA structures termed MAA-BSA (4-methyl-1,4-dihydropyridine-3,5-dicarbaldehyde, MDHDC or malondialdehyde-acetaldehyde) 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 (Figure 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 are 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 previously shown to bind to MDA epitopes (24), are required for mediating MDA-induced signaling. We found that MSR1- but also CD36-deficient macrophages displayed markedly reduced CXCL1 secretion compared to wild-type macrophages (Figure 4F). Inhibition of endocytosis using Dynasore abolished CXCL1 secretion upon MDA stimulation (Figure 4F), further indicating the importance of uptake for MDA-induced signalling. Interestingly and in contrast to OxLDL, which is recognized by a heterotrimeric complex composed of TLR4/6/CD36 (22), TLR4 is not required for MDA signalling (Figure S5A). Moreover, MDA did not induce IL1 β secretion either alone or in macrophages primed with the LPS subunit Kdo2-Lipid A (KLA) - in contrast to OxLDL (25) (Figure S5B and data not shown). Finally, chemical inhibition of the NF κ B pathway using a selective inhibitor of I kappa B kinase diminished MDA-induced chemokine secretion in macrophages (Fig. S5C). Therefore, MDA epitopes are capable of activating signaling pathways that are mediated via scavenger receptors or dependent on scavenger receptor-mediated uptake and NF κ B 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 (Figure 5A) and recruitment of neutrophils and Ly-6C^{hi} inflammatory monocytes into the peritoneal cavity of MAA-injected mice, indicating that MDA epitopes have pro-inflammatory properties *in vivo* (Figure 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 μ g of the anti-MDA IgM antibody LR04, or of an isotype control antibody or PBS on day 1 and 4 (Figure 6A). On day 8, mice that received the LR04 antibody had significantly increased plasma levels of IgM antibodies reactive with the MDA mimotope P2 that was previously developed by us as a unique ligand for LR04 (4). Importantly, total IgM plasma levels were not affected (Figure 6B). After 1 week of WD all mice developed increased triglyceride and cholesterol levels in plasma and liver, which were not different among the 3 groups (Figure 6C, 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 (Figure 6D, 6E, Table 1). Remarkably, the anti-MDA treatment resulted in an approximately 30–40% reduction of leukocyte infiltrates (Figure 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 (Figure 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 OSEs 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-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 PRRs for bacterial ligands such as TLR2 and TLR9 in both CONV-R and GF livers. TLR9 has been previously suggested to mediate MCDD-induced NASH (28) by acting as sensor of bacterial products that accumulate in the bloodstream due to dysbiosis in the gut. However, a recent study proposed a non-bacterial TLR9 ligand, mitochondrial DNA, to be involved in driving NASH (36). We show that oxidation-derived MDA epitopes represent alternative, sterile PRR ligands that activate similar signaling pathways as microbial stimuli including NFκB signaling. In addition, inflammatory responses caused by other DAMPs or microbial danger signals resulting in oxidative stress and cell injury might also 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 pro-inflammatory 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 a 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 previously shown that MDA IgM are 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 non-alcoholic fatty liver disease (44). In line, we have shown that boosting natural IgM titers by active vaccination against OxLDL or by 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 pro-inflammatory, is sufficient to ameliorate hepatic inflammation by 30-40% despite equal levels of steatosis. This effect may be mediated either by neutralization of the pro-inflammatory 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 sub-acute, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We would like to 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.

Grant Support: This work was supported by the SFB Lipotox F30 of the Austrian Science Fund (to C. J. Binder); by a Boehringer Ingelheim PhD Fellowship (to C. J. Busch); by Cardiovascular Research the Netherlands (CVON 2012-03, IN-CONTROL), the Netherlands Organisation for Scientific Research (NWO) (Vidi 016.126.327) (to R. Shiri-Sverdllov); by an EMBO Short-term Fellowship, an Andrew K. Burroughs Short Term Fellowship, a Kootstra Fellowship (to T. Hendrikx); a Shared Expertise project grant from the German Center for Cardiovascular Research

(DZHK, AII MAP; to C. Reinhardt and S. Jäckel), a grant from the German Federal Ministry on Education and Research (BMBF 01EO1503) and DFG individual grants (RE 3450/3-1 and RE 3450/5-1; to C. Reinhardt). S. Jäckel and C. Reinhardt are members of Young DZHK. J. Weißer is recipient of a DOC fellowship of the Austrian Academy of Sciences.

Abbreviations

WD	Western-type diet
MDA	malondialdehyde
ND	normal diet
CONV-R	conventional-raised
GF	germ-free
DAMPs	danger-associated molecular patterns

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Figure 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 $-\log_{10}$ of modified Fisher Exact P value. (A) Gene ontology (GO) analysis of significantly upregulated genes using DAVID and showing biological processes. (B) KEGG pathway analysis of significantly

upregulated genes showing pathways. (C) GO analysis of significantly downregulated genes using DAVID. (D) Heatmap representation of selected genes significantly deregulated in livers after 2 and 4 weeks of WD expressed as $\log_2$ fold change over ND. Genes are classified as soluble mediators (brown), receptors and sensors (red); transcription and signalling factors (blue), others (grey). (E) Heatmap representation of selected oxidative stress response genes differentially regulated in livers after 2 and 4 weeks of WD expressed as $\log_2$ fold change over ND. Data from $n = 2$ mice per group.

Figure 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 cryo-sections 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 qPCR analysis of *Cxcl1* expression in livers of GF and CONV-R *Ldlr*^{-/-} mice on ND or WD. (F) Real-Time qPCR 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 ANOVA with Tukey's multiple comparisons test; (D, F): Student's unpaired t test; * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$; ns, not significant; scale bar: 50 μm .

Figure 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 CM₂-DCFDA in whole liver (left) or Kupffer cells (right) harvested from *Ldlr*^{-/-} mice fed a ND or a WD for 2 weeks. (C) Thiobarbituric-acid-reactive substances (TBARS) assay in Kupffer cells isolated from livers of mice fed ND, 2 or 4 weeks of WD. (D) Representative images of liver sections from *Ldlr*^{-/-} mice fed a 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 WD stained with LR04 or a monoclonal MDA-specific IgG antibody. (F) Flow cytometry analysis of liver cell suspensions harvested from mice on ND or 2-week-WD and stained with dead cell markers Annexin-V/7-AAD 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 $\pm$ SEM of n = 3-5 mice/group and were analyzed by Student's unpaired t test; *P < .05, **P < .01, ***P < .001; scale bar: 50 μ m.

Figure 4. MDA epitopes are pro-inflammatory 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 bone-marrow-derived macrophages (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 pro-inflammatory markers after 2 hours of incubation with sham-

BSA or MAA-BSA (100 μ g/ml). (E) CXCL1 secretion, LR04-binding and degree of lysine modification of different BSA modifications. (F) CXCL1 secretion by BMDMs from indicated KO mice after stimulation with MAA-BSA for 16 h (left and middle). CXCL1 secretion of BMDMs stimulated with indicated compounds after 30 minutes pre-treatment with Dynasore. Zymosan, positive control (right). Data in all panels are presented as mean $\pm$ SEM of triplicate stimulations and are representative of at least 2 (A, F) or 3 (B-E) independent experiments; *P < .05, **P < .01, ***P < .001, ****P < .0001.

Figure 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 scatterplot showing flow cytometric analysis of peritoneal cells stained with CD11b and Ly-6G for polymorphonuclear leukocytes (PMN) 2 hours after BSA (left) or MAA-BSA (right) injection. Newly recruited cells are marked with a black circle. (C) Quantification of PMN and inflammatory monocytes. Data shown in (A-C) are pooled from $n = 14$ mice/group from 2 independent experiments and are represented as mean $\pm$ SEM, each symbol indicates an individual mouse. All data were analyzed by Student's *t* test. *** $P < .001$, **** $P < .0001$.

Figure 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-6G⁺ in liver sections from intervention study. (E) Quantification of CD11b⁺ and Ly-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 the smallest and the largest values; * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$.

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

	ND		WD	
	PBS	PBS	Isotype Control	LR04
	(n = 5)	(n = 6)	(n = 11)	(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.