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PRECLINICAL RESEARCH

Malondialdehyde Conjugated With Albumin Induces Pro-Inflammatory Activation of T Cells Isolated From Human Atherosclerotic Plaques Both Directly and Via Dendritic Cell–Mediated Mechanism

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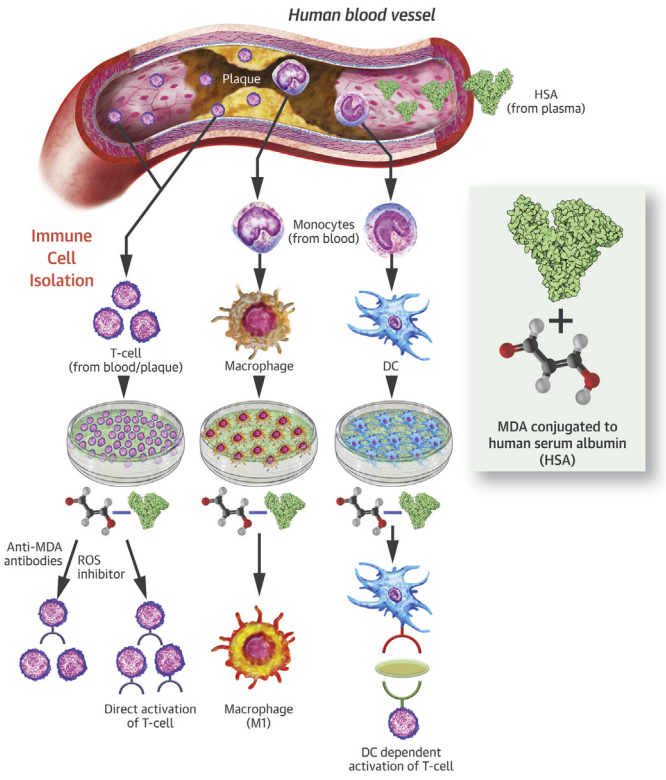
VISUAL ABSTRACT



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HIGHLIGHTS

- MDA conjugated with HSA activates T cells from human atherosclerotic plaques through a direct mechanism.
- MDA-HSA also induces maturation and activation of human dendritic cells, which in turn promote activation of autologous T cells from the same donor's plaques, although this effect appears somewhat weaker than the direct activation.
- M1 polarization of macrophages, potentially an atherogenic effect, were also induced by MDA-HSA.
- Heat shock protein 60 was induced in T cells by MDA-HSA, is atherogenic, and could promote dendritic cell/T cell activation.
- Two peptide modifications of serum albumin in atherosclerotic patients' HSA were similar to those generated by treatment of HSA with MDA in vitro.

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SUMMARY

Human dendritic cells were differentiated from blood monocytes and treated with malondialdehyde (MDA) conjugated with human serum albumin (HSA). Autologous T cells from human plaques or blood were co-cultured with the pre-treated dendritic cells or treated directly. MDA modifications were studied by mass spectrometry. MDA-HSA induced a pro-inflammatory DC-mediated T-cell activation and also a strong direct effect on T cells, inhibited by an inhibitor of oxidative stress and antibodies against MDA. Atherogenic heat shock protein-60 was strongly induced in T cells activated by MDA-HSA. Two peptide modifications in atherosclerotic patients' HSA were similar to those present in in vitro MDA-modified HSA.

ABBREVIATIONS AND ACRONYMS

ATP = adenosine triphosphate
CVD = cardiovascular disease
DC = dendritic cell
GM-CSF = granulocyte-macrophage colony-stimulating factor
HLA = human leukocyte antigen
HSA = human serum albumin
HSP = heat shock protein
IFN = interferon
IgM = immunoglobulin M
IL = interleukin
LDL = low-density lipoprotein
MDA = malondialdehyde
MS = mass spectrometry
OxLDL = oxidized low-density lipoprotein
PCR = polymerase chain reaction
TCR = T-cell receptor
TGF = transforming growth factor
TLR = Toll-like receptor
TNF = tumor necrosis factor

Typical of atherosclerosis, the major cause of cardiovascular disease (CVD) is the presence in atherosclerotic plaques of activated immune competent cells including T cells, dendritic cells (DCs) and monocytes/macrophages, dead cells (in a necrotic core), and oxidized low-density lipoprotein (OxLDL), commonly present in inert mainly macrophage-derived foam cells (1,2). In previous studies, findings have indicated that OxLDL can activate T cells (3-6), including those from human atherosclerotic plaques (3,6,7). Moreover, we have shown that the oxidized phospholipid moiety of OxLDL promotes immune activation, including production of interferon (IFN)-γ (8,9), but the underlying mechanisms and possible activation of T cells by other components of OxLDL remain unclear. In contrast to the situation in mice (10), we observed no low-density lipoprotein (LDL)-mediated activation of human T cells, including any that might be found isolated in plaques (6).

Both murine models of atherosclerosis and human ex vivo investigation indicate that T cells play major roles in atherosclerosis and ensuing CVD.

Transfer of $CD4^+$ T cells to atherosclerotic mice exacerbates atherosclerosis, possibly by abrogation of T-cell transforming growth factor-beta (TGF-β) signaling (11,12).

In humans, an association between the $CD8^+$ subset of T cells and CVD has been reported (13). The DCs and T cells co-localized in plaques may play a role in local immune reactions within lesions (14). DCs may be of major importance during different stages of the development of atherosclerosis in humans (15–17).

Oxidation of LDL generates a variety of compounds from both protein (apolipoprotein B [apoB]) and lipids. Two of the products from lipid malondialdehyde (MDA) and phosphorylcholine (PC) appear to be of particular interest and elicit a pronounced antibody response in humans that may have atheroprotective properties (2).

We reported recently that production of antibodies against both PC and MDA conjugated with an albumin is dependent on T cells in humans. Moreover, these antibodies together strongly and negatively correlated with atherosclerosis and vulnerable plaques in systemic lupus erythematosus (where the risk of CVD is exceedingly high) and also with the risk of CVD in a general population (18,19).

Heat shock protein (HSP) 60 may be involved in activation of T cells by OxLDL, as well as the atherosclerotic immunity in general (20), and more specifically in T-cell activation (6,21).

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All authors attest they are in compliance with human studies committees and animal welfare regulations of the author's institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* author instructions page.

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We report that MDA-human serum albumin (HSA) activates DCs and T cells, the latter even directly, and that this effect is inhibited by anti-MDA antibodies. The implications of these findings are discussed.

METHODS

DC DIFFERENTIATION. Monocytes were isolated using human enrichment cocktail (STEMCELL Technologies, Grenoble, France) and thereafter were cultured in Roswell Park Memorial Institute (RPMI) medium containing 50 ng/ml each of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 (ImmunoTools, Friesoythe, Germany). After 72 h of incubation, half of the medium was replaced with fresh medium. After 6 days of culture, cells were collected and 2×10^6/ml were cultured in the presence or absence of 10 μg/ml MDA-HSA prepared as described previously (18). Endotoxin was not detected in MDA and HSA by the Limulus Amebocyte Lysate test.

The cells collected after 24 h were ≥90% viable. DCs were stained with antibodies against CD11C-PE/BV421, CD86-Percp-Cy5.5, CD80-FITC, and CD40-FITC (BD Bioscience, San Jose, California).

CO-CULTURE OF DCs AND T CELLS. CD3 T cells were isolated from the buffy coat of healthy donors with the human T-cell enrichment cocktail (STEMCELL Technologies) in accordance with the manufacturer's instructions. Differentiated DCs were cultured as described earlier in the presence or absence of MDA-HSA for overnight or for 12 h, then washed and resuspended in complete RPMI medium; thereafter, they were co-cultured with 4×10^5 CD3 T cells for 48 h. To block human leukocyte antigen II (HLA-II), 8 μg/ml of low-endotoxin, azide-free purified anti-HLA-II antibodies (Biolegend, Dedham, Massachusetts) were added to DCs before co-culturing with T cells. Cells collected after 48 h of co-culture were stained with anti-CD3-Percp Cy5.5, anti-CD 25-PE/FITC, anti-CD69-APC/FITC, and anti-CD71-BV421/FITC (BD Bioscience) antibodies.

ISOLATION AND CULTURE OF PLAQUE CELLS. Atherosclerotic plaques were obtained from patients who were undergoing carotid endarterectomy at the Department of Surgery, Vascular Surgery, Södersjukhuset, Stockholm, Sweden. This study was pre-approved by the research ethics committee of Karolinska Institutet and in accordance with the Declaration of Helsinki. All subjects gave their written informed consent before entering the study.

Cells were isolated from atherosclerotic plaques as described earlier (22). In brief, the plaques were first dissected into small pieces, which were then incubated with 1.25 mg/ml collagenase IV (Life Technologies Europe BV, Stockholm, Sweden), 25 μg/ml Liberase DL (Roche Applied Science, Stockholm, Sweden), and 0.2 mg/ml DNase I (Roche Applied Science, Stockholm, Sweden) for 1 h at 37°C. The dissociated plaque cells were then passed through a 100-μm Celltrics filter (Millipore AB, Stockholm, Sweden) to remove unwanted fat and debris, and T cells were purified with the EasySep T-cell enrichment kit (STEMCELL Technologies). DCs obtained from the peripheral blood of the plaque donors were treated with or without MDA-HSA as described above and thereafter co-cultured with plaque T cells for 48 h. In addition, plaque T cells were cultured in the presence or absence of MDA-HSA for 24 h.

T CELL CULTURES. Isolated CD3 T cells were cultured in the presence or absence of MDA-HSA and/or of 2 μg/ml anti-MDA antibodies (Academy Biomedical, Houston, Texas) for 24 h. Furthermore, CD3 T cells were incubated with 20 μmol/l/ml MitoTEMPO (Sigma Aldrich, St. Louis, Missouri) for 30 min to inhibit mitochondrial reactive oxygen species (ROS) production and subsequently cultured with or without MDA-HSA for a maximum of 20 h.

MACROPHAGE CULTURE. Isolated monocytes were cultured with 50 ng/ml recombinant human GM-CSF in RPMI medium, which was replaced on day 3 with fresh medium. On day 5, these cells were treated with MDA-HSA for 48 h to investigate M1 (pro-inflammatory) macrophage differentiation. Further, macrophages were co-cultured with T cells (pre-treated with MDA-HSA for 6 h, and then for 48 h).

CELL PROLIFERATION. Cell proliferation was determined using the colorimetric BrdU kit in accordance with the manufacturer's protocol (Sigma Aldrich). In brief, DCs cultured with or without 10 μg/ml MDA-HSA were cultured for 12 h, washed, and resuspended in the complete RPMI medium, and then 0.5×10^5 DCs were co-cultured with 2×10^5 autologous T cells in a 96-well round-bottom plate (Becton Dickinson, Franklin Lakes, New Jersey). In addition, T cells were also cultured alone with or without MDA-HSA. After 72 h of incubation, the cells were labeled with BrdU, incubated for 20 more hours, centrifuged, and dried at 60 °C for 1 h. The dried cells were fixed with a FixDenat (Roche) solution before incubation with anti-BrdU peroxidase antibodies. After 2 h of incubation, the cells were washed and substrate solution added for development of color. To stop the reaction, 1 mol/l H_2SO_4 was added and absorption at 450-nm wavelength (with 690 nm as reference) determined.

STAINING OF INTRACELLULAR CYTOKINES. CD3 T cells with or without MDA-HSA were incubated for 24 h; lysed with 0.1% saponin; stained for IFN-γ, IL-4, and IL-17A; and analyzed by flow cytometry.

CYTOKINE QUANTIFICATION. The levels of INF-γ, IL-6, IL-4, tumor necrosis factor (TNF)-α, IL-10, and TGF-β collected after 18 h were assessed by enzyme-linked immunosorbent assay (R&D systems, United Kingdom). In the same manner, HSP60 was determined in supernatant from CD3 T cells or DCs after 12 h of stimulation with MDA-HSA. In the case of CD3 T cells, the same was found from patients after 24 h of such stimulation.

GENE SILENCING. The gene encoding Toll-like receptor 4 (TLR4) or T-cell receptor (TCR) was silenced in T cells with a specific short hairpin RNA (shRNA) plasmid (Santa Cruz Biotechnology, Heidelberg, Germany). After 72 h of shRNA transfection, gene silencing was investigated at the protein level using flow cytometry. Nonspecific shRNA was used as a control.

ADENOSINE TRIPHOSPHATE ASSAY. T cells were stimulated with MDA-HSA for 2 h, then with cellular adenosine triphosphate (ATP), after which they were quantified with the ATP measurement kit (Promega, Madison, Wisconsin) in accordance with the manufacturer's instructions.

APOPTOSIS. After culturing DCs or CD3 T cells with 10 μg/ml MDA-HSA for 2, 4, 6, 18, and 24 h, apoptosis was assessed by flow cytometry using Annexin AV-FITC.

MDA-HSA BINDING. CD3 T cells were cultured in the presence or absence of MDA-HSA and/or anti-MDA (antibodies without fluorochrome conjugation) antibodies for 1 h and then centrifuged, washed, and resuspended in 100 μl phosphate-buffered saline. Fluorescein isothiocyanate-conjugated anti-MDA antibody (5 μg/ml) was added to both control and MDA-HSA–treated cells; after 30 min of incubation, the cells were washed and analyzed by flow cytometry.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION. Total RNA was extracted with Qiagen mini (Qiagen, Hilden, Germany), and cDNA was synthesized from total RNA using the cDNA synthesis high capacity kit (Applied Biosystem, Foster City, California). One microliter of cDNA was used in each reaction of quantitative real-time polymerase chain reaction (PCR). Transcription factors TBET (Th1), GATA3 (Th2), RORC (Th17), and FOXP3 (T-reg) genes were analyzed. Housekeeping gene glyceraldehyde 3-phosphate dehydrogenase was used as a reference gene or to normalize the difference. Taq-man PCR master mix was used to run the reaction (Applied Biosystems) in 7500 Real-Time PCR system (Applied Biosystems). The ΔΔCT method was used to calculate the relative difference in gene expression.

MASS SPECTROMETRY SAMPLE PREPARATION AND ANALYSIS. In vitro, MDA-modified HSA was analyzed for peptide modification. HSA was first alkylated with dithiothreitol (Sigma) and iodoacetamide (Sigma), precipitated, and thereafter digested by sequencing grade trypsin in ammonium bicarbonate and dimethyl sulfoxide. The peptides thus obtained were desalted on a C18 column and analyzed by liquid chromatography–mass spectrometry (MS).

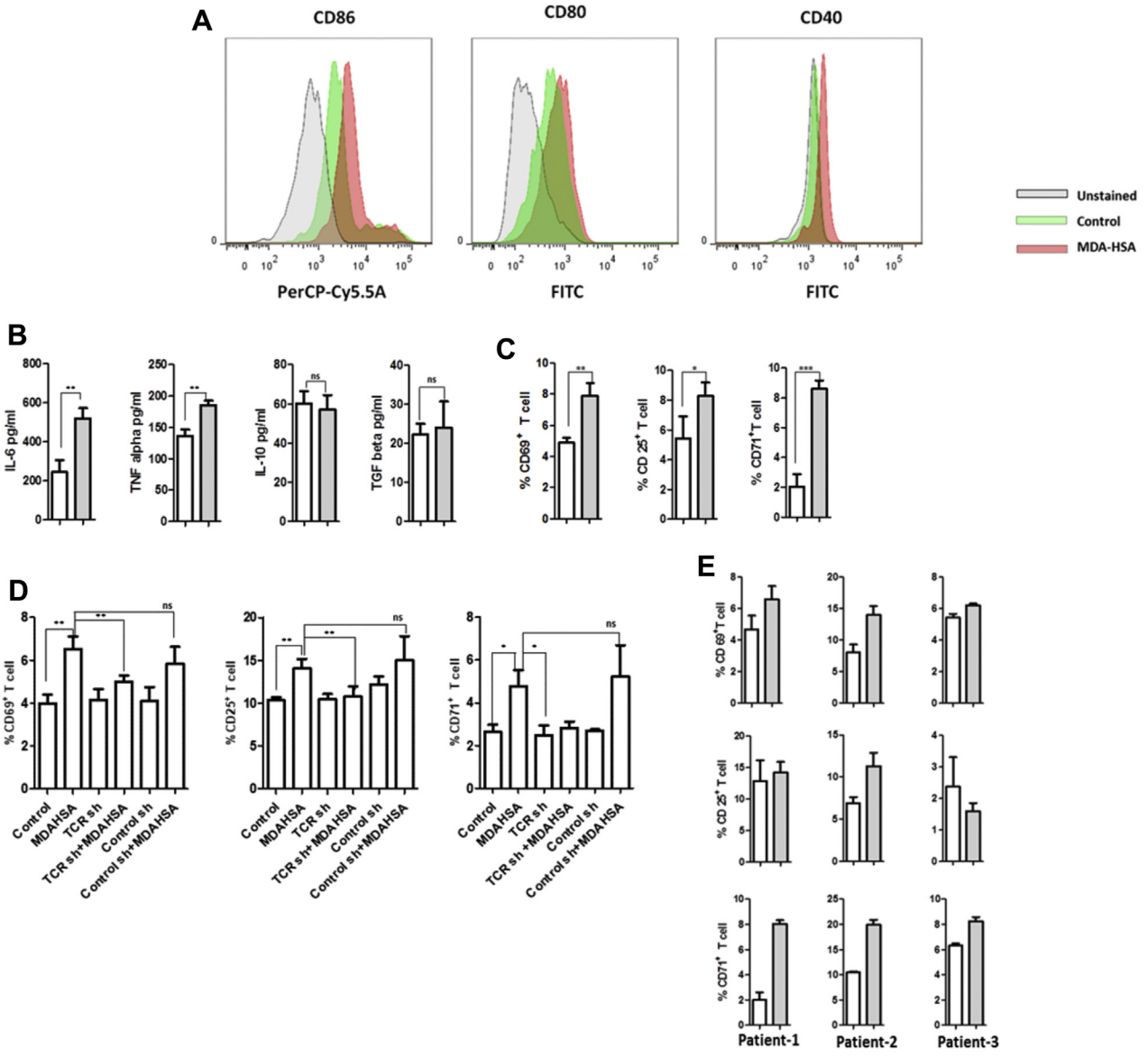
Plasma proteins (10 μg) from each of 10 patients' samples were dissolved in 50 mmol/l ammonium bicarbonate; reduced with 20 mmol/l DTT (Sigma) for 30 min at 56°C. Iodoacetamide (66 mmol/l) in 50 mmol/l ammonium bicarbonate was added for alkylation at room temperature for 30 min. Sequencing-grade trypsin (1:3, trypsin: protein; Promega) was incubated with each sample (1:33 trypsin: protein) at 37°C, and formic (final concentration of 5%) added to stop this digestion. After 20 min, the samples were placed on a C18 Hypersep plate (Thermo Scientific), dried using a Speedvac, and re-suspended in 25 μl 2% Acetonitrile/0.1% formic acid.

LIQUID CHROMATOGRAPHY–MS/MS ANALYSIS. Peptides were separated on a 50-cm Easy C18 chromatography column connected to an nLC1000 system (Thermo Fisher Scientific). The peptides were loaded onto this column at a flow rate of 1000 nl/min, and then eluted at 300 nl/min flow rate for 120 min with linear gradient from 4% to 26% AcN in 0.1% formic acid. After ionization by electrospray, the peptides eluted were analyzed in an orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). The MS spectrum was acquired at a resolution of 60,000 in the range of m/z 200 to 2,000. MS/MS data were obtained with a higher-energy collisional dissociation for ions with charge $z > 1$ at a resolution of 15,000.

MS DATA ANALYSIS. The raw data obtained were converted into the Mascot Generic Format (mgf) using a written Raw2mgf program written in-house. Proteins were identified by searching the SwissProt database (Porcine) with the Mascot v 2.4 (Matrix Science) database search engine.

STATISTICAL ANALYSIS. Statistical analysis was performed by Student's t-test; $p \leq 0.05$ was considered as statistically significant. $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ were expressed as *, **, and ***, respectively. The bar diagrams are expressed as mean ± SD.

FIGURE 1 MDA-HSA Mediated Activation of DCs From CVD Patients or Healthy Blood Donors of DC Activation and Ensuing Activation of T Cells Exposed to the DCs

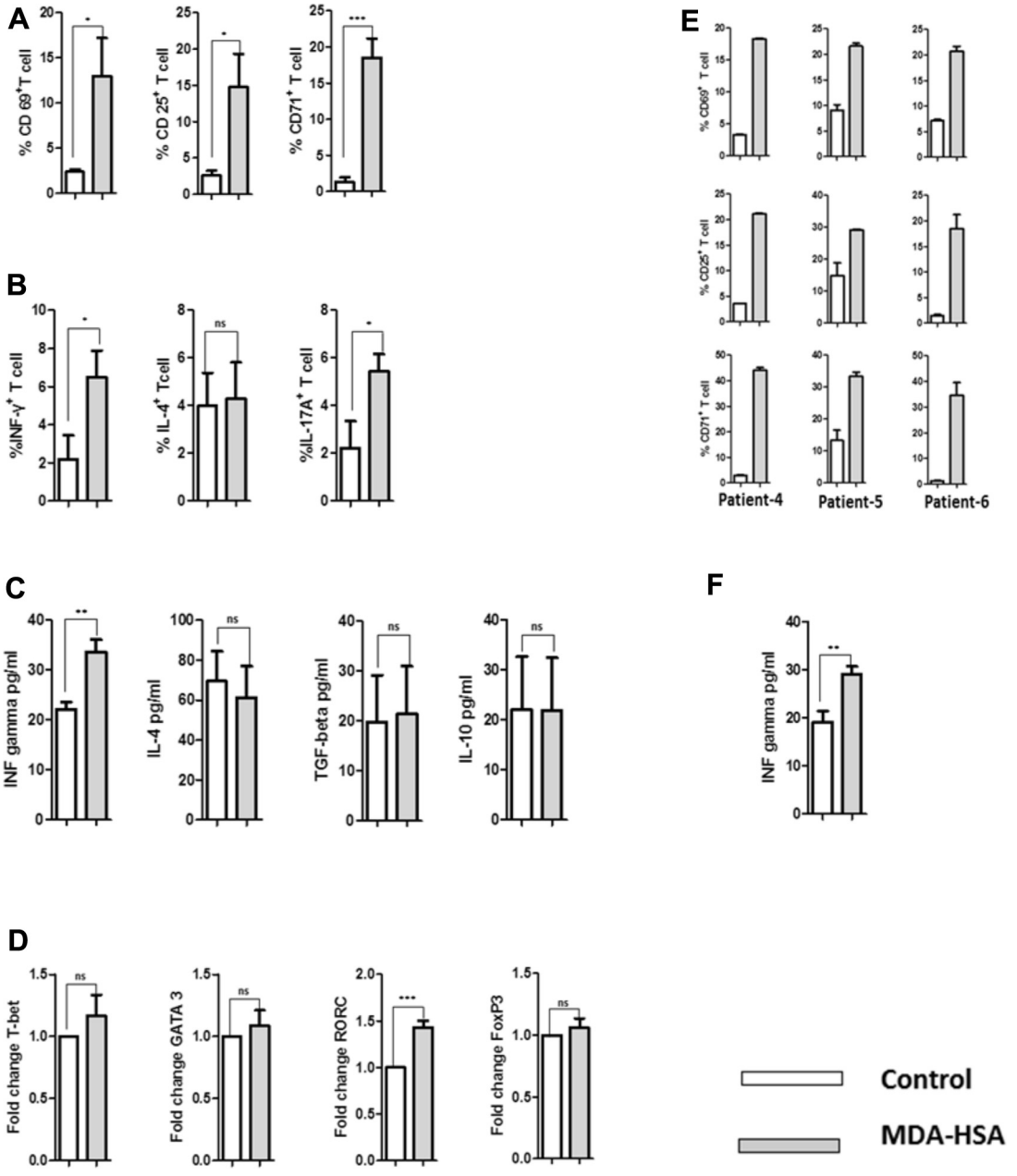


(A) DCs were stimulated with 10 μg/ml MDA-HSA for 24 h. Expression of the surface markers CD86, CD80, and CD40 was induced, as shown by 1 of 3 independent experiments. **(B)** MDA-HSA–stimulated DCs promoted production of pro-inflammatory but not anti-inflammatory cytokines, with no change in the level of TGF-β (mean value of 3 independent experiments). **(C)** MDA-HSA–induced DCs promoted T-cell activation (mean value of 3 independent experiments). **(D)** MDA-HSA–induced DC–mediated T-cell activation was inhibited when TCR (α and β) had been silenced (mean of 3 independent experiments). **(E)** MDA-HSA–treated peripheral blood DCs from atherosclerotic patients activated plaque T cells from same patients. DC = dendritic cell; FITC = fluorescein isothiocyanate; HSA = human serum albumin; IL = interleukin; MDA = malondialdehyde; sh = short hairpin; TCR = T cell receptor; TGF = transforming growth factor; TNF = tumor necrosis factor.

RESULTS

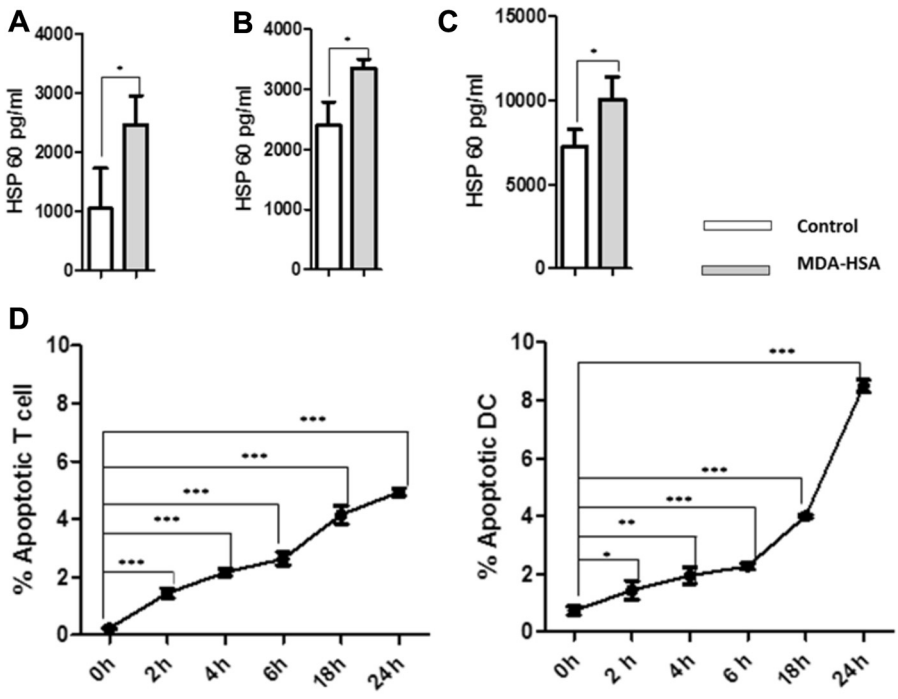
INDUCTION OF DC-MEDIATED T-CELL ACTIVATION BY MDA. After testing 1, 5, and 10 μg/ml MDA-HSA, we chose the highest dose for use. MDA-HSA enhanced the expression of markers of DC activation and several co-stimulatory proteins (Figure 1A) (median fluorescence intensity from 3 individual experiments are presented in Supplemental Figure 1A), as well as the levels of IL-6 and TNF-α. In contrast,

FIGURE 2 **MDA-HSA Induces Pro-Inflammatory Activation on T Cells From Both Plaques and Healthy Blood Donors**



(A) CD3 T cells were activated by incubation with 10 μg/ml MDA-HSA and the treatment-induced T-cell activation. **(B)** MDA-HSA induced differentiation of INF-gamma- and IL-17A-positive cells but no significant change in IL-4-positive T cells. **(C)** MDA-HSA induced pro-inflammatory but not anti-inflammatory cytokines in plaque T cells. **(D)** MDA-HSA induced the transcription factors RORC, but not T-bet, GATA3, or FoxP3. Mean of 3 independent experiments **(A-D).** **(E)** T cells from atherosclerotic plaques duplicates were activated by MDA-HSA. **(F)** The level of IFN-gamma in the supernatant of plaque T cells from patients was elevated by MDA-HSA. (Mean of 3 patients.) FoxP3 = forkhead box P3; IFN = interferon; other abbreviations as in Figure 1.

FIGURE 3 Effects of MDA-HSA on HSP60 Production and Activation in DCs and T Cells



CD3 T cells or DCs were stimulated with MDA-HSA for 12 h, leading to much higher production of HSP60 in the former **(A)** than latter **(B)**. Three individual experiments are shown. **(C)** MDA-HSA elevated HSP60 production by plaque T cells. **(D)** Exposure of CD3 T cells or DCs to MDA-HSA for different periods promoted apoptosis among both. In both DCs and T cells, the percentage of apoptotic cells was increased by the time (mean of 3 individual samples). HSP = heat shock protein; other abbreviations as in Figure 1.

TGF-β levels were not significantly altered, and IL-10 levels declined (Figure 1B). HSA itself was without effect (Supplemental Figures 1B to 1D).

Furthermore, MDA-HSA promoted activation of T cells by DCs (Figure 1C). To investigate HLA-II-mediated T-cell activation, MDA-HSA-induced DCs were cultured with T-cell presence or absence of HLA-II blocking antibodies. Blockage of HLA-II with specific antibodies did not inhibit induction of CD25, a marker of activation, by MDA-HSA (not shown); whereas silencing of TCR-α and -β inhibited MDA-HSA-induced DC-mediated activation of T cells (Figure 1D).

Treatment of DCs derived from peripheral monocytes of patients with MDA-HSA and subsequent co-culture with T cells obtained from plaques of the same patients gave similar results (Figure 1E).

THE EFFECT OF MDA-HSA ON DC-INDEPENENT ACATIVATION OF T CELLS. MDA-HSA (Figure 2A) caused potent activation of T cells, whereas once again HSA alone had no effect (Supplemental Figure 2A). Silencing or inhibition of TLR2, TLR4, or TCR (α/β) did not alter this response to MDA-HSA (Supplemental Figure 2B). MDA-HSA was found to bind to the cell/cell membrane directly and/or penetrate into the cell (Supplemental Figure 2C), and intracellular staining showed activation of pro-inflammatory Th1 and Th17 but not Th2 T cells (Figure 2B). In the supernatants from cells treated with MDA-HSA, the levels of IFN-γ were increased; IL-4 and TGF-β showed no significant change (Figure 2C). IL-17 was undetectable in our enzyme-linked immunosorbent assay.

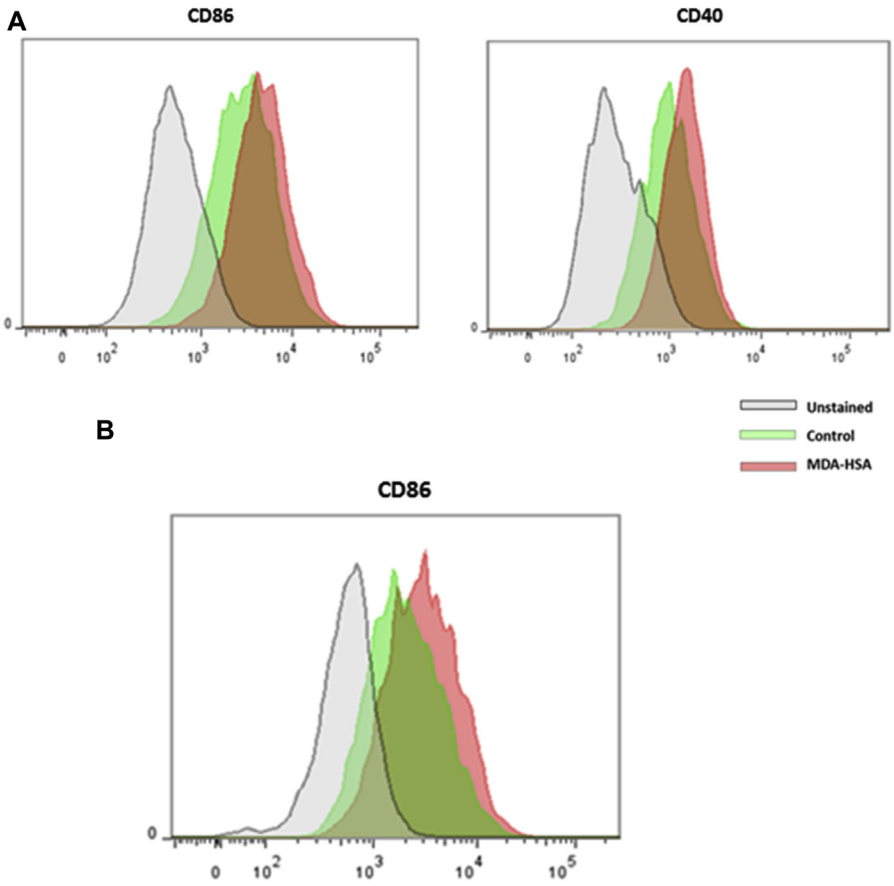
Transcription factors for Th17 cells (RORC) were induced by MDA-HSA with no alteration in the case of GATA3, Tbet-1, or Fox P3 (Figure 2D).

As with peripheral blood T cells, plaque T cells were also activated by MDA-HSA (Figure 2E), and the level of IFN-γ in the cells supernatant was elevated (Figure 2F).

CELL PROLIFERATION. MDA-HSA did not stimulate DCs and T cell proliferation (Supplemental Figure 2D).

INDUCTION OF HSP60. MDA-HSA induced HSP60 in both T-cells (Figure 3A) and DCs (Figure 3B) from healthy donors as well as plaque T cells (Figure 3C).

FIGURE 4 Macrophage Activation



(A) One of 3 individual experiments. When monocytes were first cultured with GM-CSF for 5 days and then stimulated with MDA-HSA, expression of surface proteins CD86 and CD40 by macrophage was enhanced. **(B)** CD86 expression increased when MDA-HSA–stimulated T cells were co-cultured with monocytes derived macrophages for 48 h of incubation. GM-CSF = granulocyte-macrophage colony-stimulating factor; other abbreviations as in Figure 1.

ACTIVATION INDUCES APOPTOSIS IN DCs AND T CELLS. The concentration of MDA-HSA used, which is not highly toxic to the cells, induced apoptosis in both DCs and T cells that increased with time (Figure 3D).

M1 MACROPHAGE DIFFERENTIATION BY MDA-HSA. When macrophages (identified by CD11b) were treated with MDA-HSA, the percentage expressing M1-specific marker CD86 was increased (Figure 4A).

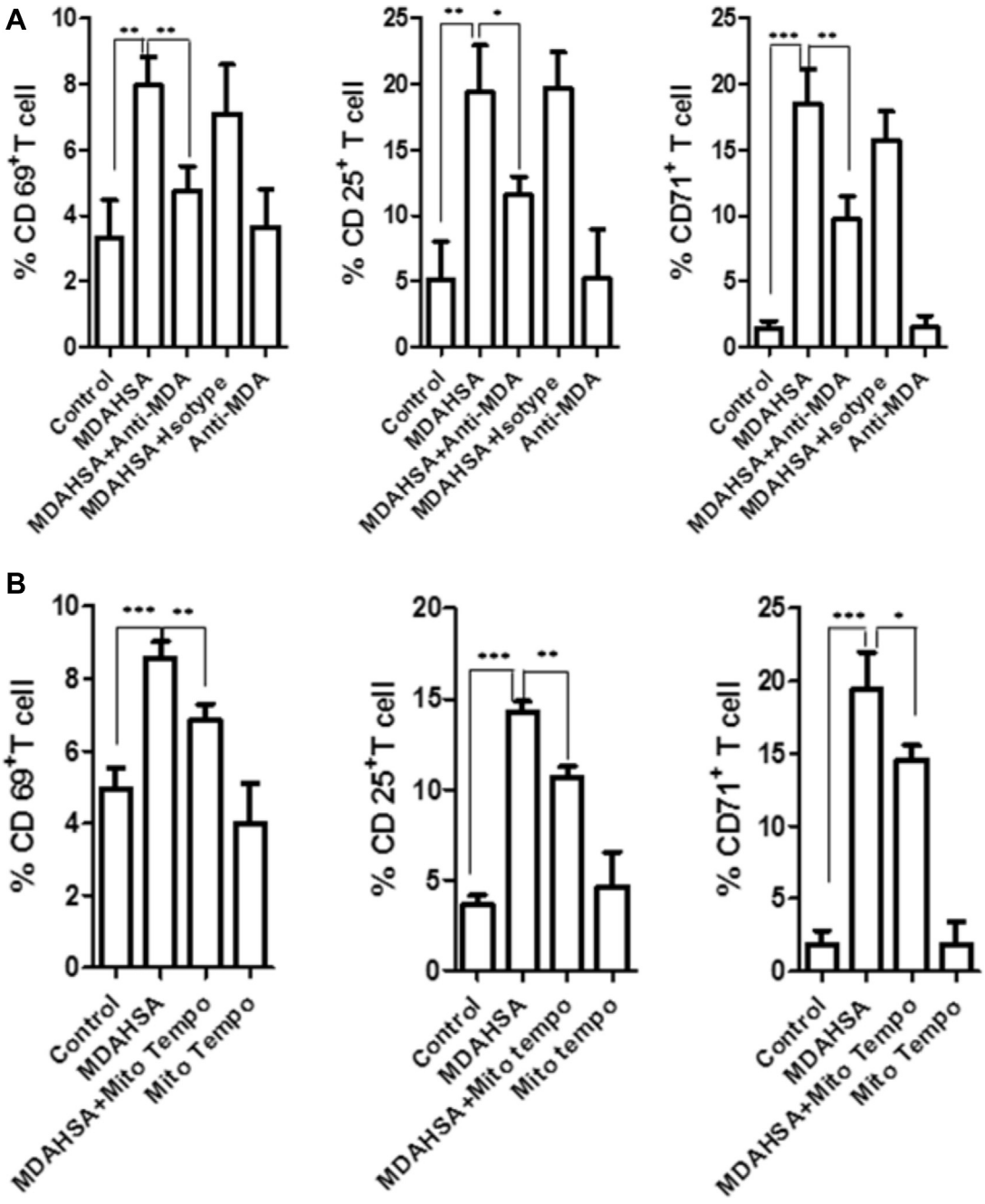
CD40 ligation plays an important role in the inflammation associated with CVDs; this protein was also induced by MDA-HSA (Figure 4A), and mean fluorescence intensity of 3 individual experiments is shown (Supplemental Figure 3A).

Our hypothesis that MDA-HSA-induced T cells influence M1 development in atherosclerotic lesions was supported by the finding that MDA-HSA-treated T cells induced expression of CD86 in macrophages (Figure 4B); MFI from 3 individual experiments are shown (Supplemental Figure 3B). Only mild apoptosis occurred in the macrophages treated with MDA-HSA (Supplemental Figure 3C).

INHIBITION OF T-CELL ACTIVATION. Direct activation of T cells by MDA-HSA was inhibited by anti-MDA antibodies (Figure 5A), as was the binding of MDA-HSA to T cells (Supplemental Figure 3D). Mito-TEMPO inhibited mitochondrial ROS as expected (Supplemental Figure 3E), and partially reduced the activation of T cells in response to MDA-HSA (Figure 5B).

MDA-HSA INDUCES P38 MITOGEN-ACTIVATED PROTEIN KINASE, TLR2, AND TLR4. MDA-HSA increased the activation of p38 mitogen-activated protein kinase (MAPKp38), as well as expression

FIGURE 5 Means of 3 Independent Experiments

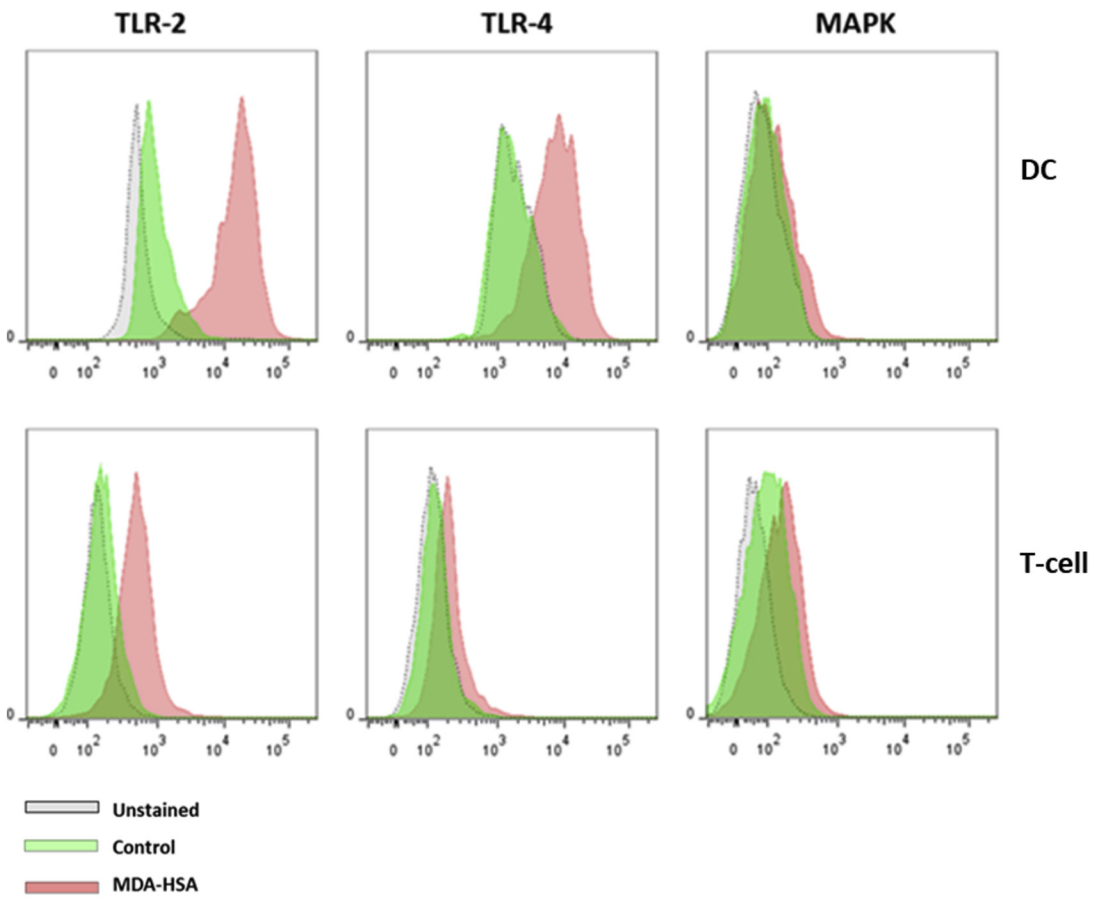


(A) The activation of T cells in response to MDA-HSA was inhibited by anti-MDA antibodies. **(B)** Pre-incubation of CD3 T cells Mito-TEMPO (Sigma Aldrich, St. Louis, Missouri), an inhibitor of mitochondrial ROS production, inhibited their subsequent activation by MDA-HSA slightly. ROS = reactive oxygen species; other abbreviations as in Figure 1.

levels of TLR2 and TLR4 in cells from each individual (Figure 6); MFI are shown from 3 individuals' experiments (Supplemental Figure 4), but not of the P65 subunit of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (not shown).

MS ANALYSIS. After treating HSA in vitro, 31 peptides could be identified (Table 1) (see Supplemental

FIGURE 6 MAPK p38 Was Elevated in Response to MDA-HSA in Both DCs and T cells



TLR2 expression was potently induced in both DCs and T cells, and TLR4 also increased. (One of 3 individuals' experiments is shown.)
MAPK = mitogen-activated protein kinase; TLR = Toll-like receptor; other abbreviations as in Figure 1.

Figure 5 for a representation of MS tracings). In the HSA of atherosclerotic patients, there were 9 MDA-modified peptides (Table 2), 2 of which were similar to those obtained in in vitro modification (set in boldface in Tables 1 and 2).

DISCUSSION

OxLDL has a complex structure, the different parts of which might play varying roles in immune activation. Apparently, mice and humans differ in this respect; LDL in a murine model activates T cells.

We have shown that OxLDL activates T cells in humans (4,5), which is a finding confirmed by others (7) that has recently been further developed (6,21).

Therefore, we chose here to study T cells isolated directly from human atherosclerotic plaques in relation to DCs from the same individuals, that is, an ex vivo human model similar to that described previously (6,21).

We report for the first time that MDA, conjugated with autologous human albumin, activates T cells, including those isolated from plaques, by both direct and, to some extent, indirect mechanisms via DCs. As expected, albumin itself has no effect.

MDA-HSA induced a pro-inflammatory activation of DCs, indicated by increased expression of CD86, CD80, and CD40 markers and production of cytokines. The direct effect on T cells from plaques (and peripheral blood) was strong, whereas DC-mediated MDA-HSA-induced T-cell activation was weaker. MDA-HSA induced secretion of pro-inflammatory cytokine secretion as IFN-γ, but not IL-17. At the same time, cell proliferation was unaltered, which is not the case with DC-mediated activation of T cells in response to OxLDL (6,21).

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TABLE 1 Mass Spectrometric Analysis of MDA-Modified-HSA In Vitro

MDA Modified Peptide		
Sequence (HSA)	Peptide Position in Protein/MDA Site in Peptide	Mascot Scores
LVnEVTEFAK	66–75/N3	29.88
SLhTLFGDK	89–97/H3	23.33
LVnEVTEFAKTcVADESAENcDK	66–88/N3	71.82
SLhTLFGDKLcTVATLR	89–105/Q1	56.61
qEPERNEcFLQHK	118–130/Q1	28.78
qEPERNEcFLQHKDDNPNLPR	118–138/Q1	15.34
nEcFLQHK	123–130/N1	39.93
nEcFLQHKDDNPNLPR	123–138/N1	43.00
DDnPNLPR	131–138/N3	11.11
RhPYFYAPELLFFAKR	169–184/H2	35.63
AEFAEVSKLVTDLTKVhTEccHGDLLEcADDR	250–281/H17	31.06
LVTDLTKVHTEcchGDLLEcADDR	**258–281/H14**	**1.25**
VhTEccHGDLLEcADDR	265–281/H2	120.50
VhTEccHGDLLEcADDRADLAK	265–286/H2	68.46
LKEccEKPLLEKSHcIAEVEnDEMPADLPSLAADFVESK	299–337/N21	14.62
EccEKPLLEKShcIAEVENDEMPADLPSLAADFVESKDVcK	301–341/H12	4.20
nYAEAK	342–347/N1	23.17
RhPDYSVVLLLR	361–37/H2	77.98
hPDYSVVLLLR	362–372/H1	37.38
ccAAADPhEcYAKVFDEFKPLVEEPQNLIK	384–413/H8	7.40
QncELFEQLGEYK	414–426/N2	69.33
FqNALLVR	427–434/Q2	38.27
YTKKVPqVSTPTLVEVSR	435–452/Q7	39.37
KVPqVSTPTLVEVSR	438–452/Q4	38.21
VPqVSTPTLVEVSR	439–452/Q3	20.88
nLGKVGSK	453–460/N1	34.56
nLGKVGSKccK	453–463/N1	17.75
EFnAETFTFHADIcTLSEK	525–543/N3	114.90
EFnAETFTFHADIcTLSEKER	525–545/N3	41.65
qIKKQTALVELVK	546–558/Q1	69.53
QIKKqTALVELVK	546–558/Q5	38.42
KqTALVELVK	449–458/Q2	39.75
KqTALVELVKHKPK	449–462/Q2	25.23
qTALVELVK	550–558/Q1	51.03
LVAASqAALGL	599–609/Q6	24.37
TcVADESAEncDKSLHTLFGDK	**76–97/N10**	**3.92**

Two peptides from patients' plasma had similar modification as in vitro modification **(bold)**.
MDA = malondialdehyde; HSA = human serum albumin.

Because MDA is exposed extensively in atherosclerotic lesions (not only on proteins and OxLDL but also on the dead cells present) (23), this finding could have important implications for plaque rupture and thus CVD. Activation of pro-inflammatory T cells and DCs is a typical phenomenon in vulnerable plaques (1,14–17), and MDA has a danger-associated molecule pattern to promote inflammation (23). The presence of MDA-modified proteins in atherosclerotic plaques of animal models (Watanabe rabbits) (24), apoE knockout mice (25), and in the human atherosclerosis (23,26) has long been known. Furthermore, circulating levels of MDA are elevated in many conditions with increased oxidative stress and chronic inflammation, not only atherosclerosis and CVD but also diabetes, Alzheimer's disease, and systemic lupus erythematosus (23,27–29).

Previously, immunization with MDA-modified LDL (which is also produced during oxidation of LDL by copper ions among different methods) was found to inhibit the development of atherosclerosis in animal models (30,31). The MDA in OxLDL may play an important role in connection with pro-inflammatory effects and sterile inflammation (23,32). Little is known about the direct effects of MDA conjugates on T-cell activation (33), although in an earlier investigation, Jurkat cells developed a more pronounced pro-inflammatory phenotype in response to MDA (34).

DCs activated by MDA-HSA could promote T-cell activation induced by other antigens, presented to T cells by DCs. In addition, to some extent, T cells could be activated indirectly by MDA-HSA, although the underlying mechanism remains to be elucidated. However, 2 h of MDA-HSA stimulation caused a mild reduction in the level of ATP in T cells (Supplemental Figure 6), indicating an energy-dependent mechanism, possibly involving active diffusion. It is thus possible that MDA-HSA enters cells through diffusion and activates cells by mitochondrial ROS. This change in the ATP level was not due to cell death. Direct activation of T cells in plaques by pro-inflammatory MDA-modified proteins could thus, in principle, promote plaque rupture and ensuing CVD, stroke, and myocardial infarction because both activated DCs and T cells are localized in vulnerable plaques, that is, plaque especially close to areas prone to rupture or where rupture has already occurred.

Peptides of serum albumin from atherosclerotic patients' plasma are modified by MDA.

Circulating levels of antibodies against OxLDL (anti-OxLDL) have been reported to be both negatively and positively associated with atherosclerosis and CVD (2,18).

Different components of OxLDL could be involved here. For example, OxLDL cross-reacts with cardiolipin, and antibodies against cardiolipin are well known to be associated with an increased risk of CVD, most likely through oxidized phospholipids (35). Antibodies against apoB or peptides thereof have also produced varying results, sometimes as a protection, and in other cases they have provided to be risk markers (2,19).

We focused on the phospholipid portion of OxLDL, where compounds such as PC and MDA are typically

generated and exposed, the latter binding especially readily to proteins.

Both anti-PC and anti-MDA immunoglobulin M (IgM) antibodies are markers of protection, even when exposed on compounds other than those related to oxLDL such as albumin (2,18). Surprisingly, IgM anti-MDAs are T-cell dependent (18); therefore, we have focused on the effects of MDA conjugated with albumin on activation of T cells in human blood and plaques.

We hypothesized that OxLDLs promote immune activation and inflammation indirectly by inducing HSP60 in monocytes/macrophages (36) and recently confirmed and extended this notion by showing that OxLDL-induced activation of T cells (also from plaques) depends on HSP60 because silencing of HSP60 inhibited OxLDL-induced T-cell activation through DCs. Moreover, we recently reported that HSP60 is a classic T-cell antigen, presented by DCs to plaque T cells through major histocompatibility complex class II (22).

Our findings do not support the proposal that OxLDL induction of HSP in DCs is caused largely by MDA epitopes because this compound itself did not induce HSP60 strongly in DCs.

However, the direct induction of HSP60 in both plaque and blood T cells by MDA-HSA was pronounced.

This could contribute to activation of plaque T cells through DC-mediated activation as well as other mechanisms because HSP60 also elicits a more nonspecific pro-inflammatory state, such as activation of monocytes/macrophages.

We reported that HSP60 is essential for oxLDL-induced DC-mediated activation of T cells (6); however, it is also implicated in statin-induced inhibition of this effect (21) and could in principle be a target for atherogenic anti-HSP60 antibodies (37,38).

Moreover, we have recently shown that a classic T-cell antigen HSP60 elicits a pro-inflammatory response from atherosclerotic plaque cells (22). Induction of HSP60 in T cells by MDA-HSA could thus contribute to atherogenesis, as well as immune activation and inflammation in plaques.

We have recently shown that MDA-HSA induces oxidative stress in peripheral blood mononuclear cells/monocytes, and, furthermore, that anti-MDA IgM antibodies inhibit this effect (18). Such anti-MDA IgM (directed against MDA conjugated with HSA) are markers of protection in patients with SLE (especially in relation to atherosclerosis) and also for risk of CVD among 60-year-old patients (18,19). The underlying mechanisms proposed include enhanced clearance of dead or dying cells and inhibition of the uptake of OxLDL by macrophages/foam cells (2,18).

TABLE 2 Identification of MDA-Modified-HSA Peptides in Atherosclerotic Patients

MDA Modified Peptide		
Sequence (HSA)	Peptide Position in Protein/MDA Site in Peptide	Mascot Scores
AAFTECCQAADk	187–198/K12	4.29
VTkCCTESLVNR	497–508/K3	7.36
EQLkAVMDDFAAFVEK	566–581/K4	20.79
TCVADESAEnCDkSLhTLFGDK	**76–97/N10 K13 H16**	**17.42**
LVRPEVDVMCTAFhDNEETFLkk	139–16/1H14 K22 K23	17.01
LVTDLTkVhTECChGDLLECADDR	**258–281/K7 H9 H14**	**5.01**
QNCELFEQLGEYkFQnALLVrYTK	414–437/K13 N16 R21	1.17
TCVADESAEnCDkSLhTLFGDKLCTVATLR	76–105/N10 K13 H16	14.46
VHTECCHGDLLECADDrADLAkYICEnQDSISSK	265–298/R17 K22 N27	17.40

Two peptides from patients' plasma had similar modification as in vitro modification **(bold)**.
Abbreviations as in Table 1.

We show here that specific anti-MDA IgGs inhibited direct activation of T cells by MDA-HSA, indicating a potential protective property of these antibodies and providing support for elevation of anti-MDA levels through active or passive immunization as a therapeutic intervention.

In addition, direct activation of T cells by MDA-HSA was attenuated by an inhibitor of oxidative stress, MitoTEMPO. In general, ROS are chemically very active, small, and short-lived oxygen-containing molecules in which a common denominator is that their unpaired electrons (i.e., free radicals) may play various roles in both health and disease, for example, by damaging proteins, DNA, and lipid bilayers. ROS play a direct role in innate immune responses to pathogens and may also be common signaling molecules in cells, tissues, and organs.

Mitochondria are a source of ROS. Although the relationship between ROS and immunity in general has been studied extensively, the role of ROS in the activation of T cells is less well described. In general, ROS may participate and modulate various processes, including hyporesponsiveness and apoptosis in a complex and situation-dependent manner (39,40).

Our finding that MDA-HSA-induced apoptosis in both DCs and T cells after directly, indicates that MDA-HSA might contribute to cell death in plaques, in which accumulating dead cells is a typical characteristic.

TLR2 and TLR4 were induced in both T cells and DCs by MDA-HSA. Neither is involved T-cell activation, but they play a role in the inflammatory response to MDA-HSA.

Moreover, MDA-HSA promoted polarization of macrophages toward the M1 phenotype. Much attention has been paid to the macrophage subtypes M1 and M2, where M1 is involved in pro-inflammatory kill responses and M2 in repair.

M1 is associated with Th1 and M2 more with Th2 responses, whereas M1 may both be induced by and promote the Th1 phenotype (41).

Macrophages play major roles in all stages of atherogenesis, from the initiation with fatty streak formation and foam cells derived from macrophages, to later complex lesions with a necrotic core and inflammation with macrophages and other immune-competent cells producing primarily pro-inflammatory cytokines (1). However, apoptosis among macrophages' response to MDA-HSA was much less pronounced than that among T cells and DCs. Thus, macrophages may reprogram in response to MDA-HSA to adapt.

MDA-HSA induced activation of MAPKp38 but not NF-Kb in both DCs and T cells. MAPKp38 are signaling molecules of major importance, regulating cellular response to stressors. Because they regulate pro-inflammatory cytokines, these may be underlying factors in chronic inflammatory conditions, including rheumatic and autoimmune diseases, as well as atherosclerosis. Accordingly, inhibition of MAPKp38 is being tested in various clinical settings. Induction of MAPKp38 could thus represent yet another pro-inflammatory property of MDA-HSA (42).

TLRs, especially TLR2 and TLR4, are implicated in innate immune responses, and inhibition of these to ameliorate atherosclerosis, CVD, and other chronic inflammatory conditions is being discussed as a therapeutic possibility.

Although not directly involved here in MDA-HSA-mediated T-cell activation, TLR2 and TLR4 were induced by MDA-HSA and may thereby promote atherosclerosis and CVD through innate immune responses and inflammation in plaque (43). Furthermore, TLR2 may be involved in in plaque erosion, a potentially important underlying factor in CVD (44).

In addition, macrophages of the M1 and M2 phenotypes have been divided into further subtypes, and several stimuli have been described that could promote such processes. In general, the M1 subtype is believed to be atherogenic, and in light of its pro-inflammatory features, it may also promote CVD. Whereas the M1 phenotype is known to be induced by OxLDL and cholesterol crystals, little is known about the effect of MDA in this context. MDA coupled with carriers such as HSA might promote atherosclerosis and other complications by promoting the M1 phenotype.

The present investigation reveals that MDA modified HSA peptides in vitro and, moreover, that the serum of albumin of atherosclerotic patients contains MDA-modified HSA peptides.

Importantly, 2 of the modifications in atherosclerotic patients were similar to those in vitro. This is an indication that the in vitro MDA-modified HSA is of biological relevance and might cause T-cell activation in human atherosclerotic plaques.

Taken together, the novel properties of MDA-HSA observed here may play a role in inflammation and immune activation in atherosclerotic plaques. Both DCs and, directly, T cells were activated, in the latter case with the apparent involvement of ROS.

However, DC-mediated T-cell activation was modest. HSP60 was induced potently in T cells by MDA-HSA, which is interesting because HSP60 is a much-discussed possible contributing cause to atherosclerosis at different stages of disease development. IgG anti-MDA antibodies inhibited the effects of MDA-HSA on T cells, a finding with therapeutic implications. Amelioration of MDA-mediated immune activation in connection with atherosclerosis by immunization or by other means could be a valuable therapeutic option.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: In this paper, we delineate a possible new pathway and mechanism by which T cells could be activated in human atherosclerotic plaques. It is possible that immune modulation could be implemented in the future in this field, but there are also consequences of our study for current clinical practice.

TRANSITIONAL OUTLOOK: From a translational point of view, our findings provide information for further development aimed at ameliorating and modulating immune responses related to MDA in atherosclerotic plaques, where immunization is one interesting possibility we think deserves more attention.

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KEY WORDS atherosclerosis, dendritic cells, T cells, malondialdehyde, oxidized low-density lipoprotein

APPENDIX For supplemental figures, see the online version of this paper.