Atheroprotective immunization with malondialdehyde-modified LDL is hapten specific and dependent on advanced MDA adducts: implications for development of an atheroprotective vaccine

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Abstract

Immunization with homologous malondialdehyde (MDA)-modified LDL (MDA-LDL) leads to atheroprotection in experimental models supporting the concept that a vaccine to oxidation-specific epitopes (OSEs) of oxidized LDL could limit atherogenesis. However, modification of human LDL with OSE to use as an immunogen would be impractical for generalized use. Furthermore, when MDA is used to modify LDL, a wide variety of related MDA adducts are formed, both simple and more complex. To define the relevant epitopes that would reproduce the atheroprotective effects of immunization with MDA-LDL, we sought to determine the responsible immunodominant and atheroprotective adducts. We now demonstrate that fluorescent adducts of MDA involving the condensation of two or more MDA molecules with lysine to form malondialdehyde-acetaldehyde (MAA)-type adducts generate immunodominant epitopes that lead to atheroprotective responses. We further demonstrate that a T helper (Th) 2-biased hapten-specific humoral and cellular response is sufficient, and thus, MAA-modified homologous albumin is an equally effective immunogen. We further show that such Th2-biased humoral responses per se are not atheroprotective if they do not target relevant antigens. These data demonstrate the feasibility of development of a small-molecule immunogen that could stimulate MAA-specific immune responses, which could be used to develop a vaccine approach to retard or prevent atherogenesis.

It is now widely recognized that atherosclerosis is a chronic inflammatory disease and that immune modulation contains supplementary data in the form of eight figures and one table.

Abbreviations: AA, acetaldehyde; AP, alkaline phosphatase; Bt, bio-tin; CFH, complement factor H; FA, Freund’s adjuvant; GGDGDK, Gly-Asp-Gly-Asp-Gly-Lys(OH); glyLDL, glycated LDL; HC, high cholesterol; IL, interleukin; KLH, keyhole limpet hemocyanin; MAA, malondialdehyde-acetaldehyde; MAA-BSA, malondialdehyde-acetaldehyde-modified BSA; MAA-LDL, malondialdehyde-acetaldehyde-modified LDL; MAA-MSA, malondialdehyde-acetaldehyde-modified mouse serum albumin; mAb, monoclonal Ab; MDA, malondialdehyde; MDA-BSA, malondialdehyde-modified BSA; MDA-LDL, malondialdehyde-modified LDL; MDA-MSA, malondialdehyde-modified mouse serum albumin; MDHDC, 4-methyl-1,4-dihiydropyridine-5,5-dicarboxyl; MSA, mouse serum albumin; MWCO, molecular weight cutoff; NaB, natural Ab; OSE, oxidation-specific epitope; OxPL, oxidized phospholipid; OxLDL, oxidized LDL; PA, propanal; PA-BSA, propanal-modified BSA; PA-LDL, propanal-modified LDL; PA-MSA, propanal-modified mouse serum albumin; PC, phosphocholine; RLU, relative light unit; SFC, spot-forming cell; Th, T helper.

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is capable of altering the atherogenic process (1–4). While there are many potential antigens in the atherosclerotic lesion, much work has focused on oxidized LDL (OxLDL). During the oxidative modification of LDL, a wide variety of immunogenic, oxidation-specific neoepitopes are formed, leading to immune activation including auto-Ab formation (5). Indeed, in mouse models, titers to oxidation-specific epitopes (OSEs) of OxLDL parallel the development of lesion formation, as well as regression (6).

Our laboratory first demonstrated that immunization with malondialdehyde (MDA)-modified homologous LDL (MDA-LDL), an OxLDL model in which native LDL is heavily modified with MDA, led to atheroprotection in rabbits and mice (7–9). Similar data have been reported by others with MDA-LDL and with other oxidation-specific models leading to elevated Ab titers to epitopes of OxLDL (10, 11). These studies led us and others (5, 12–14) to propose the concept that a vaccine to OSEs of OxLDL could be developed to limit atherogenesis. Assuming the safety of such an approach, it would have the advantage of wide applicability and a relatively cost-efficient approach. However, modification of native human LDL to prepare an atheroprotective immunogen would be cumbersome and impractical for generalized use. Furthermore, when MDA is used to modify LDL, a wide variety of related MDA adducts are formed, both simple and more complex. In order to develop a small-molecule antigen that would reproduce the atheroprotective effects of immunization with MDA-LDL, we sought to determine the responsible immunodominant and atheroprotective adducts.

In this report, we demonstrate that MDA-LDL is not required to generate immunoprotective immunogens, and that complex adducts involving the condensation of two or more MDA molecules with lysine on homologous albumin are equally effective in generating an IgG1, T helper (Th) 2-biased humoral response that was atheroprotective. However, an IgG1, Th2-biased humoral response to an irrelevant antigen was insufficient in itself to limit atherogenesis. These data suggest that a hapten-specific humoral response to relevant disease-specific OSEs on OxLDL is sufficient to provide atheroprotection and that small-molecule immunogens bearing such OSEs could be used to provide atheroprotective immunity.

**METHODS**

**Animals and diets**

All mice were 10th-generation male Ldlr<sup>−/−</sup> mice on the C57BL/6J background. Mice were weaned at 21 days of age and fed regular chow consisting of 0.01% cholesterol and 4.4% fat (TD8604; Harlan-Teklad, Madison, WI) until initiation of the diet interventions. Mice were housed in cages equipped with rodent enrichments (igloo and gnawing bone; Bio-Serv, Frenchtown, NJ) in a facility with a 12 h light cycle and had ad libitum access to water and food. All animal experiments were performed according to National Institutes of Health guidelines and were approved by the University of California, San Diego Animal Subjects Committee.

**Protocol for induction of atherosclerosis and immunization interventions**

Six-month-old male Ldlr<sup>−/−</sup> mice (age 28.4 ± 2.6 weeks, n = 119) were bled for baseline evaluations and matched for age, litter, body weight, and plasma cholesterol and triglycerides and divided into seven immunization groups (n = 15–20/group) as described subsequently. Two weeks later, they were given a primary immunization, at which time a moderate high-cholesterol (HC) diet (regular chow supplemented with 0.5% cholesterol; TD97234; Harlan-Teklad) was initiated and continued for 28 weeks. Mice were immunized at baseline, which consisted of 75 μg immunogen (protein) in 100 μl sterile PBS emulsified with 50 μl of complete Freund’s adjuvant (FA; Sigma-Aldrich), and 50 μl was injected subcutaneously into both inguinal regions and intradermally at the tail base. Intraperitoneal booster immunizations were given at 2, 4, 8, 14, and 21 weeks following the primary and consisted of 50 μg immunogen in 100 μl sterile PBS emulsified with 50 μl of incomplete FA (Sigma-Aldrich). Mice were bled at baseline and at 5, 9, 15, and 22 weeks following initiation of diet and at 28 weeks, at the time of euthanization.

The seven intervention groups were injected with the following immunogens or vehicle: 1) MDA-modified mouse LDL (MDA-mLDL) and FA, 2) MDA-modified mouse serum albumin (MDA-MSA) and FA, 3) malondialdehyde-acetaldehyde (MAA)-modified MSA (MAA-MSA) and FA, 4) propanal (PA)-modified MSA (PA-MSA) and FA, 5) native MSA and FA, 6) PBS and FA, and 7) PBS without FA. (Definition and preparation of these immunogens is given subsequently.)

**Generation of fluorescent and nonfluorescent MDA-modified proteins**

MDA-modifications of keyhole limpet hemocyanin (KLH; Princeton BioMolecules, NJ), endotoxin-free BSA (BSA; Sigma-Aldrich), and fatty acid-free MSA (Alpha Diagnostics Intl. Inc., San Antonio, TX) were carried out to produce preparations that had either a minimal or maximal amount of fluorescent MDA-derived adducts (see Fig. 1). To produce preparations with minimal fluorescence, KLH, BSA, or MSA (2 mg/ml PBS) were reacted with freshly generated MDA (50 μmol/ml) for 30 min at pH 7.4 and 37°C. Protein content was isolated from free aldehydes using Amicon Ultra concentrators [molecular weight cutoff (MWCO) 10,000 Da; Millipore, Bedford, MA] and sterile filtered (0.22 μm; Millex GP, Millipore). These preparations are referred to as MDA-modified BSA (MDA-BSA) or MDA-MSA in this article and always have very low fluorescence as defined subsequently.

To produce high-fluorescence preparations, BSA or MSA (2 mg/ml PBS) were reacted with acetaldehyde (A; 200 μmol/ml) and freshly generated MDA (100 μmol/ml) for ~4 h at pH 4.80 and 37°C. This incubation of MDA and AA results in the formation of fluorescent adducts termed MAA adducts, of which the 4-methyl-1,4-dihydopyridine-3,5-dicarbonyl (MDHDC-type) adduct is a major product (see Fig. 1, MAA-MSA). The chemistry of such MAA adducts with proteins has been extensively described (see 15–17). The formation of fluorescent adducts was followed at λ<sub>max</sub> emission 462 nm (λ<sub>ex</sub> 394 nm), and modified proteins isolated as described above. These fluorescent preparations are referred to as MAA-modified BSA (MAA-BSA), MAA-MSA, or MAA-modified LDL (MDA-LDL) in this article and always have high fluorescence as shown in Fig. 1. Note that because small amounts of AA may spontaneously form as a decomposition product of MDA from its stable bis-diacetal form, there are correspondingly small numbers of MAA epitopes generated in MDA-MSA as well (see Fig. 1 and Results).

As a control for the various MDA-modified immunogens, we used PA-MSA. Like MDA and AA, PA also preferentially modifies...
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Americas Inc., Torrance, CA), were allowed to react with freshly generated MDA for 60 min at pH 7.4 and 37°C. The molar peptide-to-MDA ratio of the reactants was 1:0.1, 1:1, and 1:10 (mol/mol). All peptide preparations were dialyzed extensively in Spectra/Por dialysis membranes (MWCO 500 Da; Fisher Scientific) against PBS and sterile filtered (0.22 µm; Millex GP, Millipore).

We included a negative control for the peptide-to-MDA (1:10) modification, where the peptide was omitted as the only difference. MDA-modified poly-L-lysine (4–15 kDa; Sigma-Aldrich, St. Louis, MO) was prepared as described previously for MDA-LDL, except that Spectra/Por dialysis membranes with MWCO of 2,000 Da were used.

MAA-modified biotinylated peptide antigen

Other peptides were N-terminally biotinylated and had the following basal primary sequence: biotin-Gly-Asp-Gly-Asp-Gly-Lys(OH), hereafter referred to as Bt-GDGDGK (purchased from Biopeptide Co. Inc., San Diego, CA). It was modified with MAA by allowing the peptide (2 µmol primary amino groups/ml PBS) to react with AA (200 µmol/ml) and freshly generated MDA (100 µmol/ml) under conditions described previously, and formation of fluorescent adducts similarly monitored. PA-modified LDL (PA-LDL) was prepared as described previously for PA-MSA. Preparation of MDA-LDL with minimal fluorescence was made as described previously for MDA-MSA. All lipoprotein preparations were dialyzed extensively in Spectra/Por dialysis membranes (MWCO 12,000–14,000 Da; Fisher Scientific) against PBS and sterile filtered (0.45 µm; Millex HV, Millipore).

In MAA and MDA preparations, >90% of the lysines were modified as judged using the trinitrobenzenesulfonic acid assay (18), whereas in the PA-modified preparations the degree of modification was ~50%. All preparations used for immunization and in vitro T-cell assays were tested for endotoxin levels by the chromogenic Limulus amoebocyte assay (QCL-1000; BioWhittaker Inc., Walkersville, MD) and contained <1.5 ng lipopolysaccharides/mg protein.

MDA-modified peptide antigens

The peptides, Ac-SDKP(OH), Ac-DRLDS(OH), Ac-LKFSKKF(OH), Ac-MDKVLNRE(OH), and Ac-DRVYIHPFHL(OH) (Bachem Americas Inc., Torrance, CA), were allowed to react with freshly generated MDA for 60 min at pH 7.4 and 37°C. The molar peptide-to-MDA ratio of the reactants was 1:0.1, 1:1, and 1:10 (mol/mol). All peptide preparations were dialyzed extensively in Spectra/Por dialysis membranes (MWCO 500 Da; Fisher Scientific) against PBS and sterile filtered (0.22 µm; Millex GP, Millipore). We included a negative control for the peptide-to-MDA (1:10) modification, where the peptide was omitted as the only difference. MDA-modified poly-L-lysine (4–15 kDa; Sigma-Aldrich, St. Louis, MO) was prepared as described previously for MDA-LDL, except that Spectra/Por dialysis membranes with MWCO of 2,000 Da were used.

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high fluorescence were dried down and reconstituted in PBS. Mass spectral analysis confirmed that the GGDGDGK peptide contained the MDHDC adduct (data not shown).

**Synthesis of MDHDC-Lysine adduct containing peptides**

The pentapeptide Ac-Gd(OrBu)Gd(OrBu)Gd-GOH was prepared by standard Fmoc solid phase synthesis on a 2-chlorotrityl resin. Appropriately α-amino acid-protected lysine was converted to a Hantzsch dihydropyridine synthesis using AA and MDA to give Boc-K(MDHDC)-trimethylsilyl ester. The Boc was removed by trifluoroacetic acid (TFA), and the resulting aminolysine adduct was coupled to the pentapeptide using 3-(diethoxyphosphoryl)-1,2,3-benzotriazin-4(3H)-one. Global deprotection with TFA gave Ac-Gc-GDGDGK(MDHDC)-OH, which was purified by reverse-phase HPLC and characterized by NMR.

**Plasma lipid and cytokine analyses**

Mice were anesthetized with isoflurane, and retro-orbital blood was obtained via EDTA-coated microcapillary tubes. Plasma cholesterol and triglycerides levels were determined using automated enzymatic assays (Roche Diagnostics, Indianapolis, IN, and Equal Diagnostics, Exton, PA). Total cholesterol exposure was estimated as the area under the cholesterol curve over time divided by days of cholesterol feeding (Origin Pro 7; OriginLab Corporation).

**Antigen-specific cytokine release by splenic T cells**

Splenocytes from athymic lact/− mice after 28 weeks of intervention (without giving a recall boost) were suspended in culture media (RPMI 1640 media containing 10% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 2 mM l-glutamine, 0.1 mM nonessential amino acid solution, 50 µg/ml Gentamicin reagent solution, and 0.05 mM 2-mercaptoethanol; all Invitrogen, Carlsbad, CA) and seeded at 5 × 10⁵ cells per well in 96-well flat-bottom plates in a final culture volume of 200 µl. Triplicate cultures of splenocytes from individual mice were incubated for 72 h at 37°C/5% CO₂ in culture media with and without titrated amounts of antigens. In parallel wells, splenocytes were stimulated with suboptimal concentrations of Concanavalin A (EMD Chemicals, Gibbstown, NJ) to assess cytokine release capacity under identical and nonspecific conditions. ELISPOT kits for mouse IFN-γ, IL-5 (both BD Biosciences), and IL-17A (R and D Systems) were assayed according to manufacturers’ instructions, using the BD ELISPOT AEC Substrate Set for detection.

**Measurement of Ab levels and immune complexes by ELISA**

Specific Ab levels to given antigens in plasma were determined by chemiluminescent ELISA as previously described (9, 20). In brief, Microfluor® 2 White “U” Bottom Microtiter® plates (Thermo Labsystems, Franklin, MA) were coated with various antigens at 5 µg/ml PBS overnight at 4°C. The plates were blocked with 1% BSA in TBS, serially diluted plasma was added, and the plates incubated for 1.5 h at room temperature. Bound plasma Ig isotype levels were detected with various anti-mouse Ig isotype-specific AP conjugates using LumiPhos 530 solution and a Dynex Luminometer (Dynex Technologies, Chantilly, VA). The following goat AP-conjugated secondary Ig isotype-specific Abs were used: anti-mouse IgM (µ-chain specific) (Sigma-Aldrich) and anti-mouse IgG1, IgG2b, IgG2c, and IgG3 (all Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Data are expressed as relative light units counted per 100 milliseconds (RLUs/100 ms).

To investigate the fine specificity of the humoral immune responses, we developed a peptide-based ELISA using immobilized NeutrAvidin to capture biotinylated peptides, which were unmodified or MAA modified. In short, plates were coated with NeutrAvidin (10 µg/ml PBS) overnight at 4°C. The plates were blocked with 1% BSA in TBS and loaded with the unmodified or semipurified MAA-modified biotinylated peptides at 25 nmol/ml and incubated for 1 h at room temperature. After washing with PBS, serially diluted plasma was added and incubated for 1.5 h at room temperature. The remaining ELISA was performed as described previously.

Circulating apoB immune complexes (ICs) were measured by modifications of a sandwich ELISA previously described (9, 21), where apoB-100-containing lipoproteins were captured by the anti-mouse apoB-100-specific LF5 monoclonal Ab (mAb) (22). In short, plates were coated with LF5 mAb (a mouse IgG2a) at 5 µg/ml PBS overnight at 4°C. The plates were blocked with 1% BSA in TBS, and plasma at 1:100 dilution was added and incubated for 1.5 h at room temperature. Secondary Ig isotype-specific Abs and detection were as described previously. In parallel wells, captured apoB-100 was measured using biotinylated LF3 mAb [1 µg/ml (22)], which recognizes another epitope on mouse apoB-100. This was followed by AP-conjugated NeutrAvidin and LumiPhos as described previously. Data are expressed as the Ig/apoB ratio (both measured in RLU/100 ms). The LF3
and LF5 mAbs were kindly provided by Stephen Young of University of California, Los Angeles.

**Competition ELISA**

We have previously shown that MDA-mLDL immunization induces strong hapten-specific anti-MDA-epitope IgG1 humoral responses and confers atheroprotection in \( Ldlr^{-/-} \) mice (9). To test which MDA-modified amino acids were immunodominant in antisera from MDA-mLDL-immunized mice, we utilized the MDA-modified peptides described previously as competitors to inhibit IgG1 binding to plated MDA-LDL in competition ELISA. In brief, plates were coated with MDA-LDL at 1 \( \mu g/ml \) PBS overnight at 4°C. Plasma from C57BL/6J mice immunized with homologous MDA-mLDL (1:80,000 dilution) and competitors (250 \( \mu g/ml \)) were mixed and preincubated for 1 h at room temperature before addition to appropriate wells. After 1.5 h incubation, bound IgG1 was detected as described previously and expressed as a ratio of binding in the presence of competitor (B) divided by binding in the absence of competitor (B0).

In other competition ELISAs, competitors were serially diluted before overnight incubation at 4°C with fixed dilution of plasma. Plasma-competitor solutions were added to antigen-coated ELISA plates without pelleting of ICs in order to produce inhibition curves as B/B0 as described (9, 19).

**Atherosclerosis analysis and immunocytochemistry**

The extent of atherosclerosis was quantified by computer-assisted image morphometry in Sudan IV-stained en face preparations of the entire aorta and in cross-sections through the aortic origin of paraffin-embedded hearts, as previously described (23). It should be noted that among 119 total mice analyzed for atherosclerosis, 2 mice were statistical outliers (>3 SD) in the en face analysis, but not in any other analyses. Therefore, for this analysis only, one MDA-mLDL-immunized (36.7% lesion area) and one FA-immunized (43.5% lesion area) mouse were excluded. A modified trichrome stain, consisting of hematoxylin, fuschin red/picric acid, and aniline blue, was utilized for cross-sectional analysis of plaque burden and gross composition, as previously described (23). Techniques for the immunohistochemical analysis of immunoglobulin isotypes in lesions are presented in the supplementary figures.

**Antisera inhibition of modified LDL binding to macrophages**

Binding of biotinylated OxLDL ligands to J774 mouse macrophages plated in microtiter wells was assessed by a chemiluminescent binding assay as recently described by Montano et al. (24). In brief, isolated human LDL was biotinylated according to the manufacturer’s protocol (Cat# 21326; Pierce Biotechnology) prior to modifications to prepare biotinylated MAA-LDL, CuOxLDL, MDA-LDL, and PA-LDL ligands. The biotinylated MAA-LDL ligand at fixed concentrations (2.5 \( \mu g/ml \)) was incubated with serially diluted competitors (antisera) and controls in 1% BSA-PBS at concentrations/dilutions indicated in figures. The ligand-competitor solutions were incubated overnight at 4°C, and the following morning, the extent of binding of the biotinylated ligands was detected with Neutra/Avidin-conjugated AP (Pierce Biotecnology), LumiPhos 530 (Lumigen), and a Dynex Lumimeter (Dynex Technologies). Data were recorded as RLUs/100 ms and expressed as a ratio of binding in the presence of competitor (B) divided by binding in the absence of competitor (B0).

**Statistical analyses**

All experimental data are expressed as mean ± SEM unless otherwise noted. Differences in mean values were analyzed with the parametric one-way ANOVA with the Bonferroni’s or Newman-Keuls multiple comparison posttest unless otherwise noted (Prism v5; GraphPad Software). In some cases, data were log10-transformed to obtain equal variances among groups. Nonparametric data were expressed as box plots, where horizontal lines indicate median and 25–75 percentiles and whiskers indicate 10–90 percentiles, and analyzed using Kruskal-Wallis ANOVA with Dunn’s multiple comparisons posttest. \( P < 0.05 \) was considered significant.

**Immunogen preparation and characterization**

**Immune sera to MDA-modified proteins**

MDA-LDL contains MDA-derivated apoB as well as aminophospholipids. We previously demonstrated that the immune response to MDA-LDL was major histocompatibility complex (MHC) class II restricted (9), suggesting that MDA-modified protein, but not lipid per se, is the immunodominant epitope. To begin to determine the nature of the relevant epitope, we immunized C57BL/6J mice with MDA-mLDL as well as MDA-modified KLH (MDA-KLH), MDA-BSA, and MDA-MSA. With all immunogens, the IgG1 isotype dominated the responses (data not shown), as we previously reported (9), suggesting a Th2-biased response. Furthermore, >90% of the MDA-LDL-specific IgG1 titers in sera from mice immunized with MDA-mLDL could be competed by MDA-KLH (or other MDA-modified proteins) suggesting a hapten-specific response to the MDA adduct (data not shown). Because MDA is believed to preferentially modify lysine residues, we tested the specificity of antisera to MDA-LDL by competition ELISA with a series of MDA-modified arginine-, histidine-, and lysine-containing peptides (see Fig. 2). Only MDA-modified lysine-containing peptides competed. Collectively, these results indicated that MDA-LDL and proteins contained immunodominant MDA-derived lysine adducts, which induced a Th2-biased hapten (i.e., MDA-lysine)-specific humoral immune response.

Generation of nonfluorescent and fluorescent MDA-type adducts

MDA-LDL is a complex antigen. We therefore immunized seven groups of \( Ldlr^{-/-} \) mice with a variety of model MDA antigens to provide insight into the nature of its atheroprotective epitopes. Our first goal was to determine whether the molecular structure of the MDA-lysine adducts influenced their atheroprotective effects, and in particular, whether MDA-LDL itself was required to make an effective immunogen. We generated two different MDA-derived MSA preparations. MSA is a 67 kDa protein that contains 51 lysine residues and, when modified with MDA, most likely results in a mixture of MDA-derived adducts. Therefore, we focused on generating fluorescent and nonfluorescent MDA-derived MSA preparations (see Methods and Fig. 1). The nonfluorescent preparations are hereafter called MDA adducts (e.g., MDA-MSA) and represent simple MDA adducts with proteins prepared by a 30 min incubation with MDA at pH 7.4 (see Fig. 1). In contrast, the fluorescent preparations represent more...
Immunization protocol and impact on atherosclerosis

Experimental protocol. To determine which of the MDA-type adducts were atheroprotective, we immunized seven groups of \( \text{Ldlr}^{-/-} \) mice with various models. Five intervention groups (n = 15–20 mice/group) were immunized with modified proteins plus FA, one group with FA alone, and one group with PBS alone. Immunogens used in the five experimental groups were as follows: MDA-mLDL, MDA-MSA, MAA-MSA, PA-MSA, and MSA, respectively. We chose to study 28-week-old \( \text{Ldlr}^{-/-} \) mice because such mice have preexisting early to intermediate atherosclerotic lesions before modulation of their immunity and would therefore represent a model for the human target group of such atheroprotective immunizations. After baseline lipid evaluations, mice were given a primary immunization (with complete FA) and begun on a moderate HC diet (regular chow supplemented with 0.5% cholesterol), which was continued for 28 weeks. This diet generates an almost exclusive elevation of LDL-cholesterol, without associated hypertriglyceridemia, weight gain, and alterations of insulin/glucose associated with high-fat “Western”-style diets (23). Five booster immunizations (with incomplete FA) were given during the 28 weeks. Mice gained weight equally in all groups, and plasma cholesterol and triglycerides were similar throughout the study (Table 1). At the end of 28 weeks, terminal blood samples were obtained, and the extent of lesion formation was determined.

Atherosclerosis analyses. The extent of en face lesion formation in the entire aorta for all seven groups is graphed in Fig. 3 as percent of aortic lesion area, as well as percent of the complex malondialdehyde-acetaldehyde adducts, hereafter referred to as MAA adducts (e.g., MAA-MSA, Fig. 1), which exhibit a characteristic fluorescence profile (\( \lambda_{em,max} = 462 \text{ nm}, \lambda_{ex} = 394 \text{ nm} \)) as described in Methods. In both preparations, typically \( ~90\% \) of the lysines were modified. Fluorometric analysis indicated a >100-fold higher fluorescent intensity for MAA-MSA compared with the MDA-MSA or native MSA preparations (Fig. 1). It should be noted that although the MAA-MSA preparation was greatly enriched in MAA as judged by specific fluorescence, it is still likely that other MDA-derived adducts are also present. In turn, although MDA-MSA is termed a nonfluorescent preparation, Fig. 1 shows that very small amounts of fluorescent MDA-derived adducts are present.

Generation of PA-lysine adduct control. It has been suggested that a Th2-biased response per se might be atheroprotective independent of the immunogen (3). To study this, we generated a similar lipid peroxidation-derived adduct with PA, derivatized to MSA to yield PA-MSA (Fig. 1). PA contains only one aldehyde group, resulting in simpler chemistry than with MDA, and forms only adducts with primary amines via Schiff bases. We used PA-MSA as an “adduct-carrier” control in the intervention study, which we anticipated would serve as a nonrelevant Th2-biased immunogen control.

Thiele and colleagues (25) and we (26) have shown that MAA-modified proteins are proinflammatory. To confirm that our immunogens demonstrated this property, we incubated them with RAW cells, a mouse macrophage line. As shown in supplementary Fig. 1, MAA-LDL and the MDA-LDL preparation showing the most MAA-specific fluorescence resulted in the most robust macrophage inflammatory protein-2 (MIP-2) release from RAW cells. PA-LDL and MDA-LDL with minimal MAA fluorescence failed to increase MIP-2 above native LDL control. Thus, MAA epitopes, but not PA epitopes, are proinflammatory.

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| Immunization Groups | MDA-LDL | MDA-MSA | MAA-MSA | FA-MSA | MSA | FA | PBS | P     |
|---------------------|---------|---------|---------|--------|-----|-----|-----|-------|
| Reference (n = 20)  |         |         |         |        |     |     |     |       |
| Nonfluorescent (n = 18) |         |         |         |        |     |     |     |       |
| Fluorescent (n = 15) |         |         |         |        |     |     |     |       |
| Adduct-Carrier Control (n = 15) |         |         |         |        |     |     |     |       |
| Carrier Control (n = 16) |         |         |         |        |     |     |     |       |
| Adjuvant Control (n = 16) |         |         |         |        |     |     |     |       |
| Naïve (n = 19) |         |         |         |        |     |     |     |       |

Body weight (g)

| Weeks | Reference | Nonfluorescent | Fluorescent | Adduct-Carrier Control | Carrier Control | Adjuvant Control | Naïve |
|-------|-----------|----------------|-------------|------------------------|-----------------|-----------------|-------|
| 0     | 31.7 ± 0.6 | 30.9 ± 0.8     | 32.2 ± 0.4  | 32.3 ± 0.5             | 31.4 ± 0.6      | 32.0 ± 0.6      | 38.3 ± 0.9 | 0.29  |
| 28    | 33.2 ± 0.7 | 33.9 ± 0.7     | 34.8 ± 0.8  | 35.4 ± 0.9             | 34.0 ± 0.6      | 33.3 ± 0.6      | 35.1 ± 1.1 | 0.57  |

Triglycerides (mg/dl)

| Weeks | Reference | Nonfluorescent | Fluorescent | Adduct-Carrier Control | Carrier Control | Adjuvant Control | Naïve |
|-------|-----------|----------------|-------------|------------------------|-----------------|-----------------|-------|
| 0     | 136 ± 12  | 135 ± 8        | 151 ± 13    | 155 ± 15               | 188 ± 17        | 169 ± 14        | 161 ± 14 | 0.085 |
| 28    | 205 ± 16  | 229 ± 9        | 203 ± 21    | 197 ± 11               | 209 ± 11        | 218 ± 17        | 184 ± 12 | 0.37  |

Cholesterol (mg/dl)

| Weeks | Reference | Nonfluorescent | Fluorescent | Adduct-Carrier Control | Carrier Control | Adjuvant Control | Naïve |
|-------|-----------|----------------|-------------|------------------------|-----------------|-----------------|-------|
| 0     | 277 ± 12  | 285 ± 9        | 279 ± 11    | 279 ± 12               | 284 ± 17        | 292 ± 8         | 277 ± 10 | 0.97  |
| 5     | 680 ± 41  | 659 ± 39       | 650 ± 38    | 710 ± 37               | 705 ± 37        | 722 ± 25        | 761 ± 40 | 0.47  |
| 9     | 696 ± 34  | 721 ± 41       | 748 ± 37    | 730 ± 33               | 724 ± 46        | 712 ± 28        | 810 ± 37 | 0.66  |
| 15    | 746 ± 46  | 730 ± 38       | 750 ± 37    | 700 ± 42               | 745 ± 45        | 688 ± 17        | 727 ± 27 | 0.89  |
| 22    | 756 ± 40  | 813 ± 48       | 827 ± 33    | 703 ± 27               | 785 ± 36        | 773 ± 31        | 892 ± 65 | 0.094 |
| 28    | 841 ± 46  | 822 ± 27       | 849 ± 54    | 777 ± 32               | 795 ± 38        | 774 ± 47        | 779 ± 35 | 0.73  |

Total cholesterol exposure (mg/dl/day)

| Weeks | Reference | Nonfluorescent | Fluorescent | Adduct-Carrier Control | Carrier Control | Adjuvant Control | Naïve |
|-------|-----------|----------------|-------------|------------------------|-----------------|-----------------|-------|
| 28    | 0.349 ± 0.019 | 0.309 ± 0.020 | 0.299 ± 0.025 | 0.315 ± 0.016       | 0.351 ± 0.022   | 0.322 ± 0.020   | 0.393 ± 0.027 | 0.040 |

Aortic origin lesions (mm²/section)

| Weeks | Reference | Nonfluorescent | Fluorescent | Adduct-Carrier Control | Carrier Control | Adjuvant Control | Naïve |
|-------|-----------|----------------|-------------|------------------------|-----------------|-----------------|-------|
| 28    | 12.7 ± 1.1 | 11.9 ± 1.0    | 10.7 ± 0.8  | 14.6 ± 0.8             | 15.0 ± 1.2      | 14.5 ± 1.4      | 17.1 ± 1.4 | 0.0015|

Aortic surface lesions (% of total area)

Data shown as mean ± SEM. Statistical differences were analyzed using ANOVA with Newman-Keuls multiple comparisons posttest.

\*P < 0.05 versus PBS mice.

\*\*P < 0.05 versus PA-MSA, MSA, and FA mice.

\*\*\*P < 0.01 versus PBS mice.
PBS control (actual data presented in Table 1). These data indicate that the most effective atheroprotective immunogen was the MAA-MSA, leading to a 37% reduction of lesion formation compared with the PBS control (10.7 ± 0.8% vs. 17.1 ± 1.4% lesion area, \( P < 0.01 \)). As these mice were already 28 weeks old at the initiation of the protocol, and the intervention phase lasted another 28 weeks, this represents a substantial reduction in the rate of progression. Lesion formation in the MAA-MSA group was also significantly decreased from three other control groups, FA, MSA, and PA-MSA controls \( (P < 0.05) \). Although the MAA-MSA group had less atherosclerosis than the two MDA groups, this difference was not significant. The FA group showed a small (though nonsignificant) protective effect, consistent with prior observations by us (20) and others (27) that FA and other adjuvants have minor atheroprotective properties. The MDA groups had significantly less atherosclerosis than the PBS group, but not compared with the FA control in this experiment.

Quantification of lesion formation at the aortic root revealed that only the MAA-MSA group showed significantly reduced lesion formation at this site, a 24% reduction compared with PBS (0.299 ± 0.025 vs. 0.393 ± 0.027 mm\(^2\)/section, \( P < 0.05 \)) (Table 1).

We investigated lesion composition by a modified trichrome stain (as described in Methods) of the MAA-MSA, MDA-LDL, and PBS and MSA control mice but did not find any obvious differences in composition between lesions of comparable size. We also investigated the deposition of endogenous IgG isotypes in the aortas of the variously immunized mice, and significant differences were found, as described subsequently.

Analysis of plasma Ab titers and lipoprotein ICs

To ensure the effectiveness of the immunizations in each mouse, titers were measured after primary and two booster injections (week 5) for IgG1, IgG2c, and IgM titers to respective immunogens. All mice immunized with MDA-mLDL, MDA-MSA, MAA-MSA, and PA-MSA showed high-titered IgG1 reactivity to respective immunogens, whereas titers in MSA- and FA-injected mice were similar to PBS-injected mice (data not shown). Thus, after only 5 weeks of immunization, there were high-titered, hapten-specific IgG1 responses against respective adducts in each immunized group, indicating Th2-biased cellular immunity.

At the end of the study, a detailed analysis of final Ab titers and specificity was conducted. Formal Ab dilution curves for individual mice were conducted for each isotype and each antigen (supplementary Figs. II and III). IgG1 titers to respective OSEs were uniformly high, while IgG2c titers were low, indicative of Th2-biased responses, and pertinent results of the IgG1/IgG2c ratios to each antigen are summarized in Fig. 4. In general, MAA-MSA immunization generated and sustained a stronger and relatively more IgG1-dominant hapten-specific humoral immune response to both MAA-BSA and MDA-LDL than the other immunogens. For example, the IgG1/IgG2c ratio was 40 for the MAA-MSA-immunized mice versus ~8 for the MDA-mLDL-immunized group. By analogy, PA-MSA immunization induced high IgG1/IgG2c ratios for binding to propanal-modified BSA (PA-BSA), indicating strong Th2 bias for this immunization strategy also.

Mice immunized with MAA-MSA showed high IgG1 titers to the complex MDA/MAA epitopes as exemplified by binding to MAA-BSA and MDA-LDL, but much lower binding to the nonfluorescent MDA epitopes on MDA-BSA (supplementary Fig. III). Importantly, MDA-MSA-immunized mice showed higher binding to MAA-BSA than to MDA-BSA (note different scales), while the binding to MDA-BSA was not different than that with sera from MAA-MSA-immunized mice. Collectively, these data indicate that complex, fluorescent MDA/MAA epitopes are immunodominant.

Because of the specific modification protocol used, MDA-LDL contains a greater distribution of MDA-derived epitopes, both nonfluorescent and fluorescent epitopes, than MDA-MSA and MAA-MSA (Fig. 1). It was therefore expected that mice immunized with MDA-mLDL would show hapten-specific IgG titers to the entire spectrum of MDA-derived epitopes, that is, binding to MDA-BSA, MDA-LDL, and MAA-BSA (supplementary Fig. III). In contrast, mice immunized with PA-MSA showed high titers to PA-BSA, but only weak titers to MDA or MAA epitopes. At this high dilution, no titers to any OSEs were seen in MSA-, FA-, or PBS-immunized mice, although titers were measured at much lower dilutions (supplementary Fig. III).

We also analyzed pooled antisera for responses of other isotypes. IgG2b titers to MDA/MAA were relatively strongly induced by MDA-mLDL immunization, while in contrast, IgG3 titers were not induced (data not shown). IgM titers appeared to follow the same pattern with highest IgM binding to antigens in MDA-mLDL-immunized mice, less
Atheroprotective immunization with oxidation-specific epitopes

MAA-lysine, while mice immunized with MDA-mLDL and MDA-MSA showed lesser titers (Fig. 5B). None of the other groups showed any MAA-specific binding, including the PA-MSA-immunized mice. Similarly, low titers of IgG2b and IgG2c to MAA were seen in the MAA-immunized and, to lesser extent, MDA-immunized mice (Fig. 5C, D). In contrast, the highest titers of IgM Abs were seen in the MDA-mLDL-immunized group (Fig. 5F). Combined, the data in Fig. 5A–F show that MAA-MSA immunization induces a very strong Th2-biased IgG1-dominant MAA-epitope-specific humoral response with a formal titer >>1:50,000 in the MAA-MSA-immunized mice. In further studies, we used the synthetic MDHDC-lysine peptide to perform a competitive ELISA using pooled plasma from MAA-MSA-immunized mice. The MDHDC peptide was able to fully compete the IgG1 binding to the plated MDHDC peptide (Fig. 5G) and was nearly as effective in competing binding to plated MAA-BSA (Fig. 5H). Thus, MDHDC is an immunodominant epitope of antisera of MAA-MSA-immunized mice.

binding in MAA-MSA-immunized mice, and the least IgM binding in the remaining five groups, which had fairly equal IgM binding (supplementary Fig. III). We also measured titers of the atheroprotective IgM natural Ab (NAb) E06, which binds to the phosphocholine (PC) of oxidized phospholipids (OxPLs). Levels were highest in the MAA-MSA group among the active immunizations, and nearly two to three times as high as seen with other modified LDLs or MSA immunogens (supplementary Fig. IV). Interestingly, the E06 titers were as high as that seen after atheroprotective immunization with Streptococcus pneumoniae (20).

**MAA-specific Ab titers**

To characterize the fine specificity of the induced Abs, we coated microtiter plates with a small lysine-containing peptide bearing a chemically synthesized MAA (MDHDC) epitope (shown in Fig. 5) and measured isotype-specific binding to the MAA-lysine epitope (Fig. 5). Mice immunized with MAA-MSA showed very high-titered IgG1 binding to MAA-lysine, while mice immunized with MDA-mLDL and MDA-MSA showed lesser titers (Fig. 5B). None of the other groups showed any MAA-specific binding, including the PA-MSA-immunized mice. Similarly, low titers of IgG2b and IgG2c to MAA were seen in the MAA-immunized and, to lesser extent, MDA-immunized mice (Fig. 5C, D). In contrast, the highest titers of IgM Abs were seen in the MDA-mLDL-immunized group (Fig. 5F). Combined, the data in Fig. 5A–F show that MAA-MSA immunization induces a very strong Th2-biased IgG1-dominant MAA-epitope-specific humoral response with a formal titer >>1:50,000 in the MAA-MSA-immunized mice. In further studies, we used the synthetic MDHDC-lysine peptide to perform a competitive ELISA using pooled plasma from MAA-MSA-immunized mice. The MDHDC peptide was able to fully compete the IgG1 binding to the plated MDHDC peptide (Fig. 5G) and was nearly as effective in competing binding to plated MAA-BSA (Fig. 5H). Thus, MDHDC is an immunodominant epitope of antisera of MAA-MSA-immunized mice.
other mice were similar. No other important discriminatory findings were observed with the other IC analyses.

MAA-specific antisera inhibit binding of MAA-LDL to macrophages and immunostain atherosclerotic plaques

We previously showed that mAbs to OSEs of OxLDL inhibit the binding and uptake of OxLDL or MDA-LDL to macrophages. We measured isotype-specific ICs with apoB-containing lipoproteins (supplementary Fig. V). Levels of IgM/apoB ICs were significantly higher in mice immunized with MDA-mLDL, MDA-MSA, MAA-MSA, PA-MSA, and MSA than in mice injected with FA and PBS. Interestingly, mice immunized with MAA-MSA had the highest IgG1/apoB levels, while levels of these ICs in other mice were similar. No other important discriminatory findings were observed with the other IC analyses.

Presence of ICs with apoB lipoproteins in plasma

We measured isotype-specific ICs with apoB-containing lipoproteins (supplementary Fig. V). Levels of IgM/apoB ICs were significantly higher in mice immunized with MDA-mLDL, MDA-MSA, MAA-MSA, PA-MSA, and MSA than in mice injected with FA and PBS. Interestingly, mice immunized with MAA-MSA had the highest IgG1/apoB levels, while levels of these ICs in other mice were similar. No other important discriminatory findings were observed with the other IC analyses.

Fig. 5. MAA-MSA immunization induces and sustains an IgG1-dominant hapten-specific humoral immune response against MDHDC-lysine, demonstrating that MDHDC-lysine is an immunodominant epitope. A–F: Terminal antisera were pooled according to immunization groups, formally diluted, and tested for IgG1 (A, B), IgG2c (C), IgG2b (D), IgG3 (E), and IgM (F) binding to a semisynthetic MAA-modified biotinylated GDGDGK peptide [Bt-GDGDG-K(MAA)] (B–F) or the control peptide (A) by ELISA. The Bt-GDGDG-K(MAA) peptide was captured with plate-immobilized avidin to ensure reproducible epitope presentation and density. Data are curves of averaged triplicate determination in RLU. G: A fixed 1:15,000 dilution of pooled terminal MAA-MSA antisera was preincubated in the presence or absence of synthetic GDGDG-K(MDHDC) (green diamonds; chemical structure shown in I) or GDGDG-K control (circles) peptide prior to direct plating of mixture on immobilized Bt-GDGDG-K(MAA) or MAA-BSA and detection of IgG1 binding by competition ELISA. Synthetic GDGDG-K(MDHDC) completely competes all IgG1 binding to immobilized Bt-GDGDG-K(MAA) illustrating that binding is specific to MDHDC-lysine. IgG1 binding to the more complex MAA-BSA antigen is less efficiently competed by GDGDG-K(MDHDC), showing that the MAA-MSA antisera contains Abs specific to MAA epitopes other than the MDHDC-lysine. Data are B/B, where each data point is the average of triplicate determination.
Atheroprotective immunization with oxidation-specific epitopes (2147).

We observed with plasma from PA-MSA-immunized mice (Fig. 6C). We then assessed the ability of plasma from each of the seven immunized groups to compete, using antisera pools at 1:100 dilution (Fig. 6D). Even at this high dilution, plasma from MAA-MSA mice was a highly effective competitor, followed closely by plasma from MDA-mLDL-immunized mice, competing as effectively as a 40-fold excess of unlabeled MAA-LDL, and much more effectively than any of the other plasma pools. In part, the degree of competition of the other plasmas is due to presence of endogenous anti-MAA Abs, and in part to the presence of nonimmunoglobulin plasma proteins that bind MDA, such as complement factor H (CFH), which we recently reported is a major MDA/MAA binding protein.

To determine whether the antiserum from the immunized mice had this capacity, we added varying dilutions of plasma to macrophages in microtiter wells and tested their ability to inhibit the binding of MAA-LDL (Fig. 6). MDA-LDL is well known to bind to macrophage scavenger receptors (29), and in a preliminary study, we showed that biotinylated MAA-LDL also bound to J774 macrophages in a saturable and specific manner (Fig. 6A, B), whereas PA-LDL did not. Next, we showed that plasma from MAA-MSA mice inhibited MAA-LDL binding in a dose-dependent manner, consistent with their high MAA-specific Ab titer. At equivalent dilutions of plasma, this competition was much greater than that observed with plasma from PA-MSA-immunized mice (Fig. 6C).

Fig. 6. Pooled MAA-MSA antisera compete for modified LDL binding to macrophages. A: In vitro binding experiment showing that biotinylated MDA-LDL (circles), CuOxLDL (squares), and MAA-LDL (black/filled/solid diamonds) bound in a dose-dependent and saturable manner to J774 macrophages, whereas native LDL (reversed triangles) and PA-LDL (triangles) did not. Data are RLUs per 100 ms. Plot is a representative experiment of more than four. B: MAA-LDL, but not native LDL, competes for Bt-MAA-LDL (1.5 μg/ml) binding to J774 macrophages. Data are B/Bo for a representative experiment of more than four. C: Plasma dilution curves showing that pooled antisera from MAA-MSA-immunized mice, but not PA-MSA-immunized mice, compete for Bt-MAA-LDL (2.5 μg/ml) binding to J774 macrophages. Data are mean ± SD of B/Bo from two independent experiments for a total of four replicates. §, PA-MSA versus no antisera, P < 0.0001; #, MAA-MSA versus no antisera, P < 0.05; $, MAA-MSA versus PA-MSA, P < 0.001. D: The pooled antisera from MAA-MSA-immunized mice compete significantly more Bt-MAA-LDL (2.5 μg/ml) binding to J774 macrophages than the other six pooled antisera at 1:100. Data are mean ± SD of B/Bo from two independent experiments for a total of four replicates. Statistical differences were analyzed using ANOVA with Bonferroni’s multiple comparisons posttest. P values are given in plot.
protein (26). These data strongly suggest that the induced anti-MAA Abs have the ability to block modified LDL binding.

To demonstrate that the induced immune sera recognized endogenous epitopes in lesions, we immunostained aortic sections from mice of the various groups for deposition of endogenous isotype-specific Abs (supplementary Fig. VIA). Interestingly, the aortas of mice immunized with MAA-MAA showed the greatest content of IgG1 Abs, consistent with the high IgG1 titers to MAA. A semiquantitation of these data and other Ab isotypes is presented in supplementary Fig. VIB, which shows that IgG1 deposition in MDA-MSA- and MAA-MSA-immunized mice was the most prominent, consistent with the high IgG1 titers. Interestingly, the MAA-MSA showed the least IgG2c titers, possibly reflecting an actual decrease in this Th1-biased IgG isotype. It should be noted that the PA-MSA-immunized mice also showed considerable IgG1 deposition, presumably to a PA-like epitope.

T-cell responses to immunizations

The humoral Ab responses to the MAA-lysine and other modifications were dominated by IgG1 isotypes, suggesting a Th2-biased response. To directly examine the T-cell response, we measured IFN-γ and IL-5 cytokine responses in splenic T-cell cultures prepared from the immunized and atherosclerotic Ldlr−/− mice after 28 weeks of intervention. We did not give the mice a recall injection 3 days prior to euthanization, as is common practice, because we wanted to evaluate the actual immune status in the mice at time of euthanization. Initially, we examined the T-cell memory response to generalized activation with anti-CD3 (Fig. 7). Remarkably, the most prominent response was a generalized suppression of IFN-γ release in mice immunized with MDA-mLDL, MDA-MSA, MAA-MSA, and PA-MSA (Fig. 7A). There did not appear to be differences in IL-5 release (Fig. 7B), and a comparison of the two expressed as the IFN-γ/IL-5 ratio (Fig. 7C) shows the marked suppression of IFN-γ by MAA immunization.

We next examined antigen-specific cytokine release to each of the different antigens (Fig. 7D–G). Only splenocytes of mice immunized with MDA-mLDL (Fig. 7D) or MAA-MSA (Fig. 7F) showed antigen-specific release of IL-5 in this assay. IFN-γ responses were mixed and unremarkable, though again, there appeared to be a blunted response to MAA-MSA (Fig. 7G).

We also measured cytokines in plasma after 28 weeks of intervention (Fig. 8). In general, there were large variations in each of the cytokines measured even within a given immunization group. Increased IFN-γ levels were observed in mice immunized with MDA-MSA and MDA-mLDL and, to a lesser extent, PA-MSA, but remarkably, levels of IFN-γ were statistically suppressed in the MAA-MSA group (Fig. 8A). With respect to Th2 cytokines, IL-5 levels were highest in MDA-MSA- and MDA-mLDL-immunized mice (Fig. 8B), and IL-4 and IL-10 were also increased significantly only in the two groups of MDA-immunized mice (supplementary Fig. VII). A comparison of the IFN-γ/IL-5 ratio in individual mice again reveals that the MAA-MSA-immunized mice had a remarkably blunted IFN-γ response (Fig. 8C).

Such whole spleen cytokine release assays are subject to a number of experimental variables that may limit their sensitivity to detection of antigen-specific responses. Therefore, we immunized C57BL/6J mice with MAA-MSA and PA-MSA, MAA, and adjuvant and again examined the ability of splenic T cells from these mice to release IL-5 and IFN-γ into the supernatant in dose-dependent, antigen-specific recall assays under more optimal in vivo and in vitro conditions. Preliminary studies validated that the expected humoral Ab responses occurred as were observed with the immunized Ldlr−/− mice (data not shown). Splenocytes were harvested 7 days after the last booster injection and were costimulated with an anti-CD28 Ab. Splenocytes responded with dose-dependent, antigen-specific release of IL-5 (Fig. 9A), IFN-γ (Fig. 9B), and IL-17A (supplementary Fig. VIII A) in response to MAA-MSA, which generally was more stimulatory than PA-MSA. Because there is a differential capacity for release of different cytokines in response to stimulation, the data were normalized and plotted as the ratio of antigen-specific responses of IFN-γ and IL-5 as a percentage of the responses to stimulation with anti-CD3/anti-CD28, indicative of maximal capacity of cytokine release by T cells in the splenic culture (Fig. 9C). Clearly, there was a heightened release of IL-5 compared with IFN-γ in MAA-immunized mice, and in a similar comparative plot of IL-5 versus IL-17A, again there was a strong IL-5 bias (supplementary Fig. VIII B).

As an alternative approach to assess the Th bias of antigen-specific T cells, we performed ELISpot assays of splenic cells from the same groups of immunized mice and counted splenic cells that released IL-5 and IFN-γ after stimulation with their respective immunogens. To normalize the antigen-specific ELISpot data in a similar manner, we compared each value with the number of spot-forming cells (SFCs) after stimulation with the nonspecific T-cell stimulant, Concanavalin A, at a limiting concentration. There were considerably more IL-5 SFCs compared with IFN-γ SFCs (Fig. 9D) in the MAA-MSA-immunized mice. In contrast, IL-5 SFCs were only minimally increased after PA-MSA stimulation. A similar analysis for IL-17A SFCs also revealed strong bias toward IL-5 SFCs (supplementary Fig. VII C) in MAA-MSA-immunized mice.

These data strongly support a strong antigen-specific Th2-biased response for T cells of MAA-MSA-immunized mice, which also was associated with a suppression of IFN-γ release.

Impact of immunizations on B-cell and T-cell populations

We also examined the impact of MAA-MSA and PA-MSA immunizations on T- and B-cell populations compared with adjuvant-immunized controls (supplementary Table I). We did not observe differences in the distribution of T cells (Total, CD4+; CD8+) or in B cells (Total, B-2, MZ-B, or B-1) in the spleens or inguinal draining lymph nodes of the immunized mice. We also did not find differences in Treg cells (CD4+FoxP3+) in these two sites (supplementary Table 1), nor did we find differences in Treg cells that were CD25+ or CD25− (data not shown).
MMA epitopes and NAbs to MAA are present in humans

The work presented here supports the concept that MAA is an immunodominant epitope among the many MDA-derived adducts and its use as an immunogen can lead to atheroprotective immunity. Using a recently cloned human monoclonal Fab Ab specific for MAA, we demonstrate the presence of MAA epitopes in human atherosclerotic lesions (Fig. 10A, B), confirming similar observations recently reported by others (17, 30). We have postulated that because such OSEs are proinflammatory [e.g., see Methods and supplementary Fig. I and (25, 26)], multiple innate pattern recognition receptors, such as NAbs, have been conserved to mediate homeostasis (5). We have recently shown that in mice, up to 20% of innate IgM NAbs bind to
molecules with lysine to form MAA-type adducts generate immunodominant epitopes that lead to atheroprotective responses. We further demonstrate that a hapten-specific humoral response is sufficient, and thus, MDA-LDL is not a requisite immunogen, as MDA- and MAA-modified homologous albumin were equally effective immunogens. This report provides a detailed investigation into the nature of the Abs that appear to provide atheroprotection and the cellular immune responses associated with the immunizations. From these studies, a number of relevant observations on the desirable atheroprotective properties of a vaccine approach can be deduced.

First: a hapten-specific humoral response against a disease-relevant OSE is sufficient to provide atheroprotective immunity

Our laboratory first demonstrated that immunization with homologous MDA-LDL led to atheroprotection in rabbits and mice, data confirmed and extended by others [reviewed in (3, 5)]. Assuming the safety of such an approach, these studies led us to propose the concept that a vaccine to OSEs of OxLDL could be developed to limit atherogenesis. However, modification of native human LDL to use as an atheroprotective immunogen would be cumbersome and impractical for generalized use. Furthermore, when MDA is used to modify LDL, a wide variety of related MDA adducts are formed, both simple and more complex. In order to develop a small-molecule antigen that would reproduce the atheroprotective effects of immunization with MDA-LDL, we sought to determine the responsible immunodominant and atheroprotective adducts.

In this report, we demonstrate that fluorescent adducts of MDA involving the condensation of two or more MDA-type epitopes (19). To demonstrate that humans also possess MAA-specific NAbs, we examined IgM of newborn umbilical cord plasma. Because this IgM is solely derived from the newborn, it is thought to represent naïve NAbs in humans. As shown in Fig. 10C, newborn IgM bound specifically to MAA epitopes in a hapten-specific manner with relatively high avidity ($K_d$ of $2.6 \times 10^{-3}$ M) and were also competed for in part by MDA-LDL.

**DISCUSSION**

Fig. 8. Plasma cytokines indicate strong systemic Th2-biased immunity in MAA-MSA-immunized $Ldlr^{-/-}$ mice after 28 weeks of cholesterol feeding. Plasma IFN-γ (A) is markedly reduced in MAA-MSA compared with MDA-mLDL-, MDA-MSA-, and PA-MSA-immunized $Ldlr^{-/-}$ mice, whereas plasma IL-5 (B) is identical. C: MAA-MSA-immunized mice also have a markedly reduced IFN-γ/IL-5 ratio. Plasma cytokines were reported as box plots of 15–20 mice/group (as indicated in Table 1, except that only 11 of 19 PBS plasmas were analyzed). P values are given in plots as * $P<0.05$; ** $P<0.01$; and *** $P<0.001$. 

Molecules with lysine to form MAA-type adducts generate immunodominant epitopes that lead to atheroprotective responses. We further demonstrate that a hapten-specific humoral response is sufficient, and thus, MDA-LDL is not a requisite immunogen, as MDA- and MAA-modified homologous albumin were equally effective immunogens. This report provides a detailed investigation into the nature of the Abs that appear to provide atheroprotection and the cellular immune responses associated with the immunizations. From these studies, a number of relevant observations on the desirable atheroprotective properties of a vaccine approach can be deduced.
humoral responses to a variety of such OSEs may be of potential benefit.

**Second: MAA epitopes are immunodominant structures of the MDA-LDL immunogen and lead to a Th2-biased IgG1 response and atheroprotection**

We previously showed that immunization of mice with homologous MDA-LDL was MHC-II restricted and was strongly Th2 biased (9). In the current studies, we found that immunization with MDA-mLDL, MDA-MSA, MAA-MSA, and PA-MSA all lead to similar high-titered, almost exclusive IgG1 titers to their respective hapten immunogens, compared with IgG2c titers, again suggesting strong Th2-biased immune responses (Fig. 4 and supplementary Fig. III). The Th2 bias is all the more remarkable in that immunization with MDA-modified heterologous carrier proteins such as MDA-BSA and MDA-KLH lead to hapten-specific anti-MDA IgG1 titers, but mixed IgG1 and IgG2c anti-carrier titers (data not shown). Moreover the use of complete FA, particularly in C57BL/6 mice, is known to strongly stimulate Th1-biased responses (34).

Because preparations of MDA-LDL yield both simple (nonfluorescent) and complex (fluorescent) adducts, we sought in this study to define which of these were immunodominant among the MDA-LDL-specific Abs. We first showed that MDA-type modifications of lysine were the relevant immunogenic modifications (Fig. 2) and that, among these, the highly fluorescent MAA-type lysine

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**Fig. 9.** Th2-biased response in MAA-MSA-immunized C57BL/6j mice. Mice were immunized with homologous MAA-MSA and relevant controls in FA, and 1 week after the third injection, a single-cell suspension of whole spleen was cultured with various stimuli (n = 8 per group). A, B: Concentration-dependent antigen-specific release after 72 h of IL-5 (A) and IFN-γ (B) in eight of eight MAA-MSA-immunized mice, but not in PA-MSA-immunized (IL-5 and IFN-γ: zero and four of eight mice, respectively) and MSA-immunized (IL-5 and IFN-γ: zero and two of eight mice, respectively) mice, upon in vitro recall stimulation with indicated immunogens (mean ± SEM). C: To “normalize” antigen-specific cytokine release, we plotted the relative cytokine release in response to antigen stimulation with 50 μg/ml MAA-MSA + anti-CD28 divided by cytokine release in parallel cultures stimulated with anti-CD3 and anti-CD28. D: Increased frequency of MAA-MSA-specific IL-5 secreting cells in spleens of MAA-MSA-immunized mice as assessed by ELISpot assay. Splenocytes (2 × 10^6) of immunized and control mice were incubated overnight with 50 μg/ml immunogen (FA splenocytes were stimulated with MSA) or with a limiting concentration of the nonspecific stimuli Concanavalin A (1 μg/ml), and the frequencies of IL-5 and IFN-γ SFCs were assessed. The plot shows individual mice as the number of antigen-specific SFC divided by number of Concanavalin A SFCs, after subtraction of background spots.
human IgM NAbs. As shown in Fig. 10C, these human IgM bound to MAA in a hapten-specific manner and with relatively high avidity. We have also recently shown that the innate protein CFH bound strongly to MAA-LDL and MAA-BSA, neutralizing their proinflammatory effects (26). Further, we show that MAA-LDL is specifically bound by macrophage scavenger receptors (Fig. 6A, B) consistent with prior reports showing that other MAA-modified proteins are also bound by scavenger receptors (35, 36).

These data demonstrate that MAA is a danger-associated molecular pattern (DAMP), the target of multiple innate pattern recognition receptors: NAbs, scavenger receptors, and CFH. This implies that MAA-specific innate responses have been conserved via natural selection to maintain homeostasis, as previously suggested (5). Indeed, we [supplementary Fig. I and (26)] and others (25) have shown that MAA-modified proteins induce strong proinflammatory responses, and thus, NAbs and CFH likely serve to neutralize some of these proinflammatory responses, and scavenger receptors likely effect removal of these modified structures (albeit in the context of also initiating inflammatory responses). We also show the prominent presence of MAA epitopes in human atherosclerotic lesions (Fig. 10A), confirming recent reports (17, 30). Importantly, MAA-epitopes are also prominent OSEs on apoptotic cells, as demonstrated by the binding

epitopes were immunodominant. For instance, antisera from MAA-MSA immunization bound to both MAA-BSA and MDA-LDL (supplementary Fig. III), and conversely, MDA-MSA immunization also induced both MDA- and MAA-specific titers, even though the MDA-MSA preparation had only low amounts of fluorescent MAA-lysine adducts (~2 FLU; Fig. 1). Furthermore, using the pure, chemically synthesized MAA (MDHDC) epitope (shown in Fig. 5), we show that in the MAA-MSA antisera, the anti-MDHDC IgG1 titers were >100-fold higher compared with MDA-MSA antisera (Fig. 5B), and in turn, in the MDA-MSA antisera they were higher than any of the four control groups. The induced IgG1 in the MAA-MSA-immunized mice were remarkably specific as shown by competition immunoassays (Fig. 5G). These data support the conclusion that MAA-lysine adducts are immunodominant, leading to a Th2-biased IgG1 response.

Third: MAA is a DAMP and a major target of multiple innate immune pattern recognition receptors; therefore, adaptive responses to this OSE are likely to be beneficial

We pursued an approach to target adaptive responses to MAA as we previously found that up to 20% or more of all IgM in both mouse plasma and newborn human cord blood specifically bound to MAA (19). IgM in newborn cord blood is fully of fetal origin, and therefore, represent human IgM NAbs. As shown in Fig. 10C, these human IgM bound to MAA in a hapten-specific manner and with relatively high avidity. We have also recently shown that the innate protein CFH bound strongly to MAA-LDL and MAA-BSA, neutralizing their proinflammatory effects (26). Further, we show that MAA-LDL is specifically bound by macrophage scavenger receptors (Fig. 6A, B) consistent with prior reports showing that other MAA-modified proteins are also bound by scavenger receptors (35, 36).

These data demonstrate that MAA is a danger-associated molecular pattern (DAMP), the target of multiple innate pattern recognition receptors (PRRs): NAbs, scavenger receptors, and CFH. This implies that MAA-specific innate responses have been conserved via natural selection to maintain homeostasis, as previously suggested (5). Indeed, we [supplementary Fig. I and (26)] and others (25) have shown that MAA-modified proteins induce strong proinflammatory responses, and thus, NAbs and CFH likely serve to neutralize some of these proinflammatory responses, and scavenger receptors likely effect removal of these modified structures (albeit in the context of also initiating inflammatory responses). We also show the prominent presence of MAA epitopes in human atherosclerotic lesions (Fig. 10A), confirming recent reports (17, 30). Importantly, MAA-epitopes are also prominent OSEs on apoptotic cells, as demonstrated by the binding
of LRO4, an MAA-specific mouse NAb (37), and such apoptotic cells are proinflammatory if not promptly cleared (38). Indeed, as shown in Fig. 10C, MAA is a prominent target of human IgM NAbs in newborn cord blood. We have cloned a panel of MAA-specific NAbS from a phage display library prepared from lymphocytes isolated from the cord blood, and remarkably, each of these MAA-specific NAbS bind apoptotic cells (data not shown), observations similar to those recently reported by Wang and colleagues (30). All these data suggest that MAA-epitopes are prominent OSEs that mediate proinflammatory events and play a major role in atherogenesis, as well as other inflammatory diseases (26, 39). Such natural selection of PPRs infers that immune responses to MAA, a DAMP, confers overall benefit and thus is desirable. Our current data are thus consistent with the concept that MAAs are indeed immunodominant epitopes of MDA-type modifications, and that targeting them by induction of adaptive responses, as occurs with this immunization strategy, should not only provide a degree of atheroprotection, but will likely also provide overall beneficial effects.

**Fourth: immunization with MDA/MAA confers atheroprotection via both humoral and cellular mechanisms**

Immunizations with MDA-LDL and MDA-MSA, as well as MAA-MSA, reduced atherosclerosis equally, compared with PBS (Fig. 3 and Table 1). However, among these, MAA-MSA appeared to provide better atheroprotection, as this was also statistically different from the FA- as well as PA-MSA-immunized mice, and among all the immunogens, it was the only one that provided atheroprotection when examined at the aortic root (Table 1). It should be realized that the intervention in these mice only began when the mice were already 28 weeks old and already had atherosclerotic lesions, and thus, the bar to show effective reduction of progression was fairly high. We chose this strategy as this might be the situation in adult humans who likely already have existing lesions. Even under these circumstances, there was substantial reduction in progression over a prolonged 28-week course of HC feeding.

Although these studies do not provide a definitive answer as to the mechanisms by which this immunization strategy provided atheroprotection, we think it is likely a combination of induced changes in both humoral and cellular immunity.

We have previously shown that many Abs to OSEs have the capacity to block the binding and uptake of OxLDL to macrophages. This is true for the atheroprotective IgM NAb E06 (20), as well as the human Fab Ab IK17, which binds to OxLDL as well as both MDA-LDL and MAA-LDL (21). Plasma from these mice strongly inhibited the binding of OxLDL to macrophages in parallel with the atheroprotection. In the latter study, IK17 was injected intravenously and thus directly demonstrates the potential of such an Ab to inhibit atherosogenesis. Further, because the IK17 Fab lacks the Fc effector portion of Abs, this strongly suggests that IK17’s binding and blocking ability alone is sufficient to provide atheroprotection. In the current work, antisera from MAA-immunized mice were the most effective in inhibiting macrophage binding, followed closely by that of MDA-mLDL-immunized mice. Indeed, the induced antisera were as effective as a 40-fold excess of unlabeled MAA-LDL (Fig. 6).

On the other hand, we were surprised that the antisera from the MAA-MSA immunizations were not as effective in blocking MAA-LDL binding, and yet this immunization reduced atherogenesis, obviously implying that other properties of the Abs, and/or changes in cellular immunity, were also important to the atheroprotective effects of immunizations. We noted above that MAA-modified proteins were proinflammatory, and that CFH had the capacity to block some of these effects. In a similar manner, it is also possible that an important if not major effect of the MAA-specific Abs is related to their ability to bind and block such proinflammatory effects.

To characterize the cellular immune responses, we measured cytokine release (IFN-γ, IL-4, IL-5, IL-10, and IL-17) following dose-dependent 72 h stimulation of primary splenic cultures with each of the modified antigens and MAA. In response to nonspecific stimulation with anti-CD3 (Fig. 7), we found impaired splenic capacity to release the Th1-cytokine IFN-γ, a well-known proatherogenic cytokine, in T cells of mice obtained from all four adduct-carrying immunogens, but did not see any impairment of Th2-cytokine release. This suggests that immunizations with the adduct-carrying immunogens in general induced a Th2 response that blunted a Th1 response (e.g., via IL-4). On the other hand, in response to antigen-specific stimulation, we saw a pronounced Th2 response with antigen-specific cytokine release for IL-5, and this only occurred following MDA-mLDL and MAA-MSA stimulation (Figs. 7D, F, and 9). The released IL-5 corresponds to 60%–80% of the maximal amount released following anti-CD3 stimulation. These data with MAA-MSA recapitulate what we previously reported for MDA-mLDL immunization (9). Indeed, in that report we showed that the pronounced IL-5 release, a known B-1 cell stimulant, subsequently led to stimulation of the E06 NAb. In the current studies, MAA-MSA immunization led to the strongest stimulation of IL-5 and, similarly, led to a strong induction of E06 titers (supplementary Fig. IV); indeed, we speculate that these titers contributed significantly to the atheroprotection seen with the MAA immunizations. Finally, we note that we did not see an obvious impact of the immunizations on changes in T-cell populations (supplementary Table 1), and in particular, we did not see changes in various Treg populations, as has been reported by others, with other immunization strategies (40, 41).

Thus, we believe that the protective effects of immunization with MAA were due to the induction of IgG1 Abs that both bound to and blocked the uptake of OxLDL by macrophages and also blocked proinflammatory effects of MAA-modified proteins. One might speculate that the predominant IgG1 IgGs formed with the induced Abs (e.g., supplementary Fig. VIB), which are known to bind with higher affinity to inhibitory Fcγ receptor (FcγR)IIb receptors versus those of IgG2 isotypes, for example, might contribute also to the anti-inflammatory activity (42). In addition, the prominent Th2-biased cellular response appeared to
dampen IFN-γ release and led to increases in IL-5, which led to expansion of atheroprotective NAbs such as E06. (As discussed subsequently, the Th2 responses by themselves were not atheroprotective.) At the same time, we recognize that many questions remain to fully understand the mechanisms by which such immunizations are atheroprotective. For example, what is the fate of the OxLDL bearing OSE to which the induced IgG1 binds? Are they cleared by the reticuloendothelial system outside the artery? Our previous studies with immune-mediated clearance of nonenzymatically glycated LDL (glyLDL) may be of relevance. We previously showed that LDL in euglycemic humans, and to a greater extent in diabetics, is nonenzymatically glycated. We further showed that there is a prominent hapten-specific IgG response to the glucitolysine adduct formed, which leads to enhanced plasma clearance of glyLDL (43, 44). To determine the fate of the glyLDL bound by anti-glucitolysine Abs, we studied the tissue sites of degradation of apoB of glyLDL in rabbits immunized with homologous glyLDL. The high plasma IgG titers to glyLDL redirected degradation of the modified LDL primarily to the reticuloendothelial cells of the liver and other tissues, and consequently, the net deposition in the artery was reduced (45). In a similar manner, it is possible that LDLs bearing OSEs are similarly cleared and contribute to the atheroprotective effects noted, but future studies will be needed in this setting to test this speculation.

Fifth: nonspecific IgG1-Th2-biased immune responses per se are not atheroprotective

It has been suggested that a Th2-biased response per se might be atheroprotective independent of the immunogen (46). To study this, we generated a similar, Th2-biased response to a lipid peroxidation-derived adduct, but one that we did not expect to be abundant in vivo, nor relevant to atherogenesis. We derivatized MSA with PA because it is a lipid peroxidation product of n-3 polysaturated fatty acids and a small 3-carbon aldehyde like MDA but contains only one aldehyde group, resulting in simpler chemistry. PA modifies lysines via the Schiff base reaction and thus forms PA-lysine adducts (Fig. 1). In this study, immunization with PA-MSA conferred no atheroprotective benefit, even though it generated a pronounced IgG1, Th2-biased humoral response (Fig. 4), though the cellular Th2 response (Fig. 9D) was quite weak. The PA-MSA antisera did immunostain atherosclerotic tissue to some extent, but it was unable to inhibit binding of MAA-LDL to macrophages (Fig. 6). Notably, PA-LDL did not appear to be proinflammatory as indicated by the failure to stimulate MIP-2 release when incubated with macrophages (supplementary Fig. 1). These data suggest that an irrelevant IgG1, Th2-biased humoral immune response per se is not atheroprotective.

Sixth: implications regarding autoimmunity to apoB

Recently, a number of investigators have reported a variety of immunization strategies to essentially abrogate presumed immune responses to epitopes of native apoB (3, 14, 40, 41, 47, 48). We emphasize that our approach is quite different in that the principle of the immunization strategy here is to generate an active humoral and cellular response to OSEs (e.g., to haptens of proteins generated by adduct formation between products of lipid peroxidation and various proteins, including apoB of OxLDL). The principle of this strategy is to generate humoral and cellular responses that effectively provide protective immunity against OSEs, in much the same way that immunization to pathogenic epitopes helps prevent and/or ameliorate infectious diseases. However, it is possible that the two basic strategies are related in that the generation of OSEs on apoB, for example, might well lead to the formation of conformational epitopes on apoB at a distance from the oxidized lipid-apoB adduct, to which an adaptive response is initiated that has proinflammatory and atherogenic consequences. Thus, theoretically, enhanced adaptive responses to the OSEs might indirectly lead to a reduction in the formation of such conformational epitopes. Obviously, a great deal is left to learn about these complex immune responses and their relationship to both enhancement and amelioration of inflammation and atherogenesis.

Summary

Our report demonstrates the feasibility of development of a small-molecule immunogen that could stimulate the atheroprotective properties of MAA-specific Abs and their associated cellular immune responses, which could eventually lead to the generation of a vaccine that could be used to retard or even prevent the development of atherosclerosis.

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