Model IgG Monoclonal Autoantibody–Anti-Idiotypic Pair for Dissecting the Humoral Immune Response to Oxidized Low Density Lipoprotein

Shang-Hung Chang,^{1,5} Michael Johns,¹ Joseph J. Boyle,^{1,6} Ellen McConnell,¹ Paul A. Kirkham,² Colin Bicknell,³ M. Zahoor-ul-Hassan Dogar,^{1,6} Robert J. Edwards,⁴ Oliver Gale-Grant,¹ Ramzi Khamis,¹ Kurrun V.V. Ramkhalawon,¹ and Dorian O. Haskard¹

Increasing evidence implicates IgG autoantibodies against oxidized forms of low density lipoprotein (oxLDL) in the pathophysiology of atherosclerotic arterial disease. However, insufficient knowledge of their structure and function is a key gap. Using an elderly LDL receptor-deficient atherosclerotic mouse, we isolated a novel IgG3k against oxLDL (designated MAb LO1). LO1 reacts with copper-oxidized LDL, but minimally with native LDL. Further analysis showed that MAb LO1 also reacts *in vitro* with malondialdehyde-conjugated LDL (MDA-LDL), a known key epitope in copper-oxidized LDL preparations. By screening a phage library expressing single chain variable region antibodies (scFv), we selected an anti-idiotypic scFv (designated H3) that neutralizes MAb LO1 binding to MDA-LDL. Amino acid substitutions between H3 and an irrelevant control scFv C12 showed that residues in the H3 CDRH2, CDRH3, and CDRL2 are all critical for MAb LO1 binding, consistent with a conformational epitope on H3 involving both heavy and light chains. Comparison of amino acids in H3 CDRH2 and CDRL2 with apoB, the major LDL protein, showed homologous sequences, suggesting H3 has structural similarities to the MAb LO1 binding site on MDA-LDL. Immunocytochemical staining showed that MAb LO1 binds epitopes in mouse and human atherosclerotic lesions. The MAb LO1-H3 combination therefore provides a very promising model for analyzing the structure and function of an individual IgG autoantibody in relation to atherosclerosis.

Introduction

ATHEROSCLEROSIS IS NOW WIDELY SEEN as a chronic inflammatory disease, driven in large part by the deposition and oxidative modification of low density lipoprotein (LDL) in the walls of arteries.^(1–3) Following oxidative modification, LDL becomes recognized by macrophage scavenger receptors, resulting in phagocytosis, foam cell formation, cell death, and eventually the generation of the lipid-rich core that characterizes atherosclerotic lesions.^(4,5) However, the humoral immune system provides an additional pathway for the disposal of modified LDL, mediated largely through the binding of IgM antibodies and complement.^(6,7) Besides contributing to oxidized LDL clearance, IgM “natural antibodies” can inhibit its uptake into macrophages and prevent foam cell formation in the arterial wall.⁽⁸⁾ The homeostatic role of IgM is well demonstrated by the marked acceleration of atherosclerosis caused by serum IgM deficiency in LDL receptor-deficient (*Ldlr*^{−/−}) mice.⁽⁹⁾

Although IgG antibodies that react with oxidized LDL (oxLDL) can readily be detected in serum, relatively little is known about their precise epitope specificities and functions.^(10,11) IgG anti-oxLDL in mice tend to be IgG1, IgG3, and IgG2a/c isotypes, while in humans they are predominantly IgG1 and IgG3.⁽¹²⁾ Theoretically, distinct IgG anti-oxLDL isotypes may have quite different consequences for atherogenesis, depending upon their relative capacities to activate complement and ligate proinflammatory Fcγ receptors (FcγR).^(13–18) The proinflammatory potential of IgG antibodies in atherosclerosis was supported by the marked protection from lesion development in LDL receptor-deficient mice lacking FcγRIII (CD16).⁽¹⁹⁾ On the other hand, passive immunization with human IgG1 or mouse IgG2b monoclonal antibodies (MAb) has been shown to be protective.^(20,21)

Antibodies that react with oxLDL probably recognize a range of epitopes, expressed at different levels of oxidative modification in plasma and in atherosclerotic lesions. Much of what we know about these antibodies has been

¹Vascular Sciences Section, ²Airway Disease Section, National Heart and Lung Institute; ³Vascular Surgery; ⁴Centre for Pharmacology & Therapeutics, Department of Medicine, Imperial College, Hammersmith Hospital, London United Kingdom.

⁵Present address: Department of Biochemistry, Sargodha Medical School, University of Sargodha, Pakistan.

discovered through the generation of MAb in mice, either following immunization⁽²²⁻²⁷⁾ or occurring spontaneously in hyperlipidemic mice.^(28,29) So far the spontaneously occurring autoantibodies that have been studied have been mostly, if not entirely, of the IgM isotype.^(28,29) With the aim to better understand the fine specificity and function of naturally occurring IgG anti-oxLDL autoantibodies, we generated hybridomas from the spleen of an elderly LDL receptor-deficient mouse that had not been immunized. This report describes the characterization of one of these new antibodies, together with an anti-idiotypic single chain variable region antibody (scFv) with which it specifically reacts.

Methods

LDL modification

Human LDL (Calbiochem, Beeston, United Kingdom) was dialyzed against PBS overnight at 4°C to remove EDTA. Following dialysis, copper-oxidized LDL (Cu-oxLDL) was prepared by incubating LDL with freshly prepared copper sulphate (10 µM) at 37°C for 24 h. The oxidation reaction was stopped by adding an excess amount of chelating resin Chelex 100 (Sigma-Aldrich, Poole, UK). The extent of LDL modification was estimated by the relative electrophoretic mobility of the LDL particles using pre-cast 0.6% agarose, 1.0% barbital buffer gels (Beckman Coulter, Fullerton, CA).

Malondialdehyde-conjugated LDL (MDA-LDL) was prepared as described by Palinski and colleagues.⁽³⁰⁾ Briefly, MDA was synthesized by acid hydrolysis of malondialdehyde bisdimethylacetal. Dialyzed human LDL was incubated for 3 h at 37°C with 0.5 M MDA at a ratio of 100 µL MDA/mg LDL. The extent of modification was estimated with a carbonyl assay.⁽³¹⁾ Briefly, 0.1 mg of modified LDL was added to 500 µL of 4 mg/mL bovine serum albumin (BSA). 700 µL of 0.1% (wt/vol) 2,4-dinitrophenylhydrazine (DNP) in 2 M HCl was then added and incubated for 1 h at RT. Following the addition of 500 µL 30% trichloroacetic acid, the mixture was centrifuged for 5 min at 18,000 g at 4°C, and then left on ice for 30 min. The precipitate was then washed three times with ethanol/ethyl acetate (1:1, vol/vol). The pellet was finally dissolved in 1 mL of 8 M guanidine hydrochloride, 13 mM EDTA, and 133 mM Tris (pH 7.4) and the OD read at 365 nm. The results were expressed as moles dinitrophenol (DNP)/mg of protein (mol/mg) using an extinction coefficient of 21 mM⁻¹/cm⁻¹. The synthesis and analysis of MDA conjugated to human serum albumin (HSA) were prepared using similar protocols.

Trypsinization of LDL was performed by adding 10 µL washed and diluted bovine pancreatic trypsin-conjugated agarose beads (60 mL beads in 100 µL of PBS, Sigma-Aldrich) to 0.5 mL of 342 µg/µL LDL. 45 µL aliquots were removed at intervals between 1 min and overnight, added to 3 µL of 1 mg/mL bovine pancreas-derived trypsin inhibitor (Sigma Aldrich) and centrifuged at 9100 g for 5 min. Control LDL was processed in the same way but without the addition of trypsin. The extent of LDL modification was confirmed by electrophoresis as above.

Hypochlorite modification of LDL (Calbiochem) was achieved by incubating LDL (1 mg protein/mL in PBS) with reagent-grade sodium hypochlorite (1 mM, Sigma-Aldrich) to a final concentration of 1 mg LD/mL of 1 mM hypochlorite solution for 24 h.

Hybridomas and selection of monoclonal antibody

Hybridomas were generated by fusing Sp2/0 myeloma cells with splenocytes from a one-year-old female LDL receptor-deficient mouse that had been fed a high fat diet from the age of 6 weeks old to give a serum cholesterol 25–30 mmol/L. Hybridoma culture supernatants were screened by ELISA for the presence of antibodies that bound with native LDL or oxLDL (each coated onto plates at 10 µg/mL). Hybridomas with differential reactivity to native LDL and oxLDL were then subcloned twice prior to further characterization. The isotypes of MAb were determined using a mouse MAb isotyping kit (IsoStrip, Roche Applied Science, Burgess Hill, UK). The hybridoma secreting an IgG3k isotype control MAb HK-PEG-1 (anti-influenza virus) was purchased from the European Collection of Cell Cultures (Porton Down, Salisbury, UK). MAB were purified from culture supernatant using a protein-G affinity chromatography column.

Enzyme-linked immunosorbent assay

Maxisorb 96-well plates (Nunc, ThermoFisher Scientific, Waltham, MA) were coated with 50 µL antigen/well at 4°C overnight. Non-adherent material was washed out, and then the plates were blocked with 2% BSA/PBS for 1 h at room temperature (RT). Appropriately diluted culture supernatant or purified MAB was added and incubated for 1 h at RT. Plates were then washed, and wells incubated with goat anti-mouse Ig (SouthernBiotech, Birmingham, AL) at 1:5000 dilution. After further washing, antibody binding was detected with 3,3',5,5'-tetramethylbenzidine (Sigma), and the reaction was stopped with 0.5 M H₂SO₄. The optical density was then measured with a Synergy HT microplate reader (BioTek, Winooski, VT) at wavelength 450 nm.

Variation in salt concentration was achieved by diluting MAB LO1 in different concentrations of NaCl ranging from 2.15 to 18 mM. To test the effect of altering pH, MAB LO1 was dialyzed into 0.1 M sodium acetate buffer using Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific, Rockford, IL) and then mixed with 0.1 M sodium acetate solutions prepared using combinations of 0.1 M sodium carbonate and 0.05 M sodium hydroxide solutions to produce working buffers of pH ranging from 3 to 11.

Equal loading of ELISA plates with native or modified LDL was confirmed in parallel wells using goat polyclonal anti-apoB (Abcam, Cambridge, UK). In the case of assessing MAB LO1 binding to immobilized scFv constructs, similar coating of wells with different constructs was checked by measuring binding of anti-myc MAB 9E10 (Sigma-Aldrich) in parallel wells.

Sequencing of MAB LO1 V_H and V_L variable regions

The MAB LO1 V_H variable region was amplified from hybridoma mRNA by RT-PCR using a series of degenerate oligonucleotide primers, which had been prepared by Eurofin MWG Operon (Ebersberg, Germany). The sense primer was based upon the leader sequence,⁽³²⁾ and used in combination with a universal antisense constant region oligonucleotide^(33,34) to amplify the leader, VDJ, and start of the constant region. The V_L variable region sequence was obtained in a similar manner. PCR products were cloned into pCRII (Invitrogen, Paisley, UK) and transformed into TOP10

E. coli. Individual colonies were selected and DNA isolated by miniprep (Qiagen, Hamburg, Germany) prior to sequence analysis using an ABI 3730 automated DNA analyzer. The sequences were analyzed by comparison with the International MunoGeneTics information system (IMG; www.imgt.org).⁽³⁵⁾ The veracity of the V_H and V_L sequences was confirmed by mass-spectrometry of tryptic peptides of purified MAb LO1 IgG.

Isolation of anti-idiotypic scFv antibody

The Tomlinson I library (Medical Research Council, Cambridge, UK) is a non-immunized human scFv phagemid library that was constructed in pIT2 (HIS6 myc tag) from V_H (V3-23/DP-47 and JH4b) and V_L (O12/O2/DPK9 and Jk1). The diversified residues were based mainly on positions that are diverse in the primary repertoire and which are known from crystallographic studies to make contact with antigen. These are V_H amino acids H50, H52, H52a, H53, H55, H56, H58, H95, H96, and H97; and V_L amino acids L50, L53, L91, L92, L93, L94, L95, and L96. The total diversity of the library is about 1.4×10^8 .^(36,37) The functional scFvs bind both proteins A and L, and either one of these or the myc or 6 histidine tags can be used for detection, purification, or immobilization.

The phagemid-containing TG1 (T-phage resistant *E. coli*) cells were amplified in a 2xYT microbial medium (Sigma) containing ampicillin (100 µg/mL) and glucose (1% w/v) and grown with shaking at 37°C until the OD₆₀₀ was about 0.4. The exponentially grown library-containing TG1 was then infected with KM13 helper phage and selected by ampicillin (100 µg/mL) and kanamycin (50 µg/mL; Sigma). The expanded phagemid library was then precipitated with PEG/NaCl (20% w/v polyethylene glycol 6000, 2.5 M NaCl) and titered on TYE plates (15 g Bacto-Agar, 8 g NaCl, 10 g Tryptone, 5 g yeast extract in 1 L dH₂O) containing ampicillin (100 µg/mL) and glucose (1%).

A magnetic bead-based method was employed to select LO1 binding phage-scFv. This was carried out in three steps. Initially an aliquot of the library phage (5×10^{12} PFU) was pre-cleared of irrelevant binding phage-scFv by rotating with blank human anti-mouse magnetic beads (Invitrogen) for 1 h at room temperature. The unbound fraction was then further pre-cleared against human anti-mouse Ig beads preincubated with control IgG3k (1.5 µg/200 µL beads). For positive selection of LO1 binding phage-scFv, the unbound fraction was then transferred to magnetic beads coated with LO1 (1.5 µg/200 µL beads) and incubated rotating for 1 h at room temperature. Selected phages were then eluted with trypsin (1 mg/mL in PBS) for 15 min at RT, and used to infect logarithmic grown TG1 (OD₆₀₀ about 0.4) by incubating at 37°C in a water bath without shaking. One tenth of the infected TG1 were then titrated in serial 10-fold dilution for phage titrating. The remaining infected TG1 were spread on a 15 cm × 15 cm TYE plates containing ampicillin (100 µg/mL) and grown at 37°C overnight. Phagemid-containing bacteria were scraped from the agar surface into 2xYT media containing ampicillin (100 mg/mL) and glucose (1%) and grown at 37°C with shaking until OD₆₀₀ reached 0.4. The propagated phagemid was then rescued by MK13 helper phage and precipitated with PEG/NaCl (20% (w/v) polyethylene glycol 6000, 2.5 M NaCl). The phage produced was then titrated for the next

round of selection. In total, three rounds of selection were carried out with a decreased concentration of LO-1 (1 µg/200 µL beads) used in rounds 2 and 3. Following incubation of human anti-mouse beads with LO-1, potential non-specific binding sites on the beads were blocked with 3% BSA/PBS. Beads were washed between stages with 0.05% Tween-20/PBS.

After each round of selection, 95 colonies on titrating plates were randomly chosen and grown in 100 µL 2xYT containing 100 µg/mL ampicillin and 1% glucose in microtiter plates. The ELISA plates were coated with LO1 or control IgG3 KH-PEG-1 (10 mg/µL in PBS) overnight at 4°C and were then blocked with 2% BSA/PBS for 2 h at RT. 10 µL PEG precipitated phage in 100 µL of 2% BSA/PBS was added into each well for 1 h of incubation. After three washes with PBS-Tween-20, the bound phage was probed with a horseradish peroxidase-conjugated monoclonal antibody that reacts specifically with the M13 phage major coat protein product of gene VIII, followed by TMB substrate. Phages giving an OD₄₅₀ in MAb LO1 coated wells three times higher than those in the control IgG3 coated wells were considered to be specifically reactive with MAb LO1.

All clones positive in the ELISA screen were sequenced. Phagemid double-stranded DNA was purified by miniprep kits (Qiagen, cat# 27104) and subjected to sequencing on an automated ABI3730 sequencing system operated by Imperial Central Sequencing Service. The primers used for sequencing were: V_H : CGA CCC GCC ACC GCC GCT G and V_L : CTA TGC GGC CCC ATT CA (Eurofins MWG Operon, Ebersberg Germany). The data were analyzed with open source software eBioX (www.ebioinformatics.org/ebiox/). Sequence comparisons of the CDR regions of the H3 anti-idiotypic with human and mouse apolipoprotein B-100 (Swiss-Prot: P04114.1) was performed with Clustal W algorithm to create multiple alignments.

As the TAG stop codon is suppressed in TG1 *E. coli*, both scFv and pIII are expressed. To produce scFv alone, selected phages were transferred to HB2151 (a non-suppressor strain), which was induced with 1 mM isopropyl β-D-thiogalactoside (Sigma). Antibodies were then isolated on Ni-NTA agarose (Qiagen) and eluted with imidazole (250 mM). Purity was confirmed by SDS-PAGE stained with Coomassie blue and a His-tag In-gel Staining kit (Invitrogen).

Generation of domain swaps between scFv

To construct the H3 V_H /C12 V_L and C12 V_H / H3 V_L , the light chains of each construct were swapped. To release the light chain from the vector, purified DNA from both H3 and C12 scFv was digested with Xho1 (Promega, Madison, WI) and Not1 (Promega) in the following reaction: 5 µg DNA, 2 µL 10x buffer, 2 µL BSA (1 mg/mL), 2.5 U Xho1, 2.5 U Not1 adjusted to 20 µL with dH₂O. Samples were incubated at 37°C for 1 h. Vector samples were subsequently treated with CIAP in the following reaction: 4 µL 10x buffer, restriction digested 20 µL DNA, 0.01 U CIAP, volume adjusted to 40 µL with dH₂O. Samples were incubated for 1 h at 37°C. Fragments were separated on 1% agarose gels, and the appropriate bands gel purified before ligation using the following reaction: 4 µL 5x ligation reaction buffer, 45 fmol ends of insert, 15 fmol ends of insert DNA, and 0.1 U T4 ligase (Invitrogen), with the final volume adjusted to 20 µL with dH₂O. After overnight incubation at 16°C, DNA was purified by phenol/chloroform/IAA extraction and ethanol precipitation. Purified,

ligated DNA was transformed into TOP10 *E. coli* and selected on ampicillin (100 µg/mL) plates.

Generation of mutant H3

Mutant constructs were created using the Site-directed MutagenesisTM kit (Agilent Technologies, Santa Clara, CA) on the C12V_L/H3V_H domain swap background. Mutagenic primers were designed by hand as specified in the kit's manual for single and multiple mutations. Forward and reverse mutagenic primers contained the desired mutation and annealed to the same sequence on opposite strands of the plasmid carrying the C12V_L/H3V_H domain swap insert. The primers were designed to be 25–45 bases in length with melting temperature (T_m) below 78°C to prevent secondary structure formation. The T_m for each primer was estimated using the formula: $T_m = 81.5 + 0.41 (\%GC) - 675/N - \%mismatch$, where N = the primer length, and %GC and %mismatch are whole numbers. Optimally primers contained a minimum 40% GC content and ended in two G or C bases. The desired mutation was located in the middle with 10–15 bases of correct sequence on both sides. All primers were manufactured by Eurofins MWG Operon (Ebersberg, Germany). To ensure that the mutagenic primers functioned adequately through annealing and amplification at the correct temperature, 25 cycles of PCR were performed for each primer pair on vector dsDNA containing the C12V_L/H3V_H domain swap scFv sequence. The results were analyzed through gel electrophoresis (0.7% agarose) for the presence of a band approximately 5600 bp in size that represented the amplified construct with the desired mutation. Finally, the fidelity of the mutation was validated by sequencing. Mutant scFv were amplified and isolated as above.

Peptide synthesis

Peptides representing sequences of H3 were synthesized by Cambridge Peptides (Cambridge, UK). All peptides were purified by HPLC and quality controlled by mass spectroscopy.

Immunocytochemistry

Sections of aortic roots from wild-type (WT) and *Ldlr*^{-/-} mice fed a low-fat diet to the age of 22 weeks were prepared as described previously.⁽⁹⁾ Human carotid endarterectomy specimens were collected with consent and approval from Institutional and National Ethics committees. Specimens were transported and dissected on ice, with a transport time of under 30 min. The tissue was inspected and areas of classical morphology with fibrous cap, lipid core, and shoulder were identified and snap-frozen in liquid nitrogen and stored at -80°C. Then the sections were cryosectioned at -20°C, embedded in OCT, and fixed in isopropanol. Cryosections were equilibrated in double-distilled water, then PBS, blocked in 10% normal goat serum for 30 min, and incubated in 10 µg/mL biotinylated LO1 or biotinylated isotype control at 4°C overnight in a humidified chamber. Antibody was then decanted, followed by a rinsing in PBS. Bound antibody was detected with goat anti-mouse AlexaFluor 568 (Molecular Probes, Invitrogen, Paisley, UK) at 1:200 for 120 min at 21°C. TOPRO-3 (Molecular Probes, Invitrogen) was used as a nuclear counterstain for general inspection, and macrophages

were detected by 1:100 anti-mouse CD68-AlexaFluor 488 (MCA1957, Serotec, Oxford, UK) or 1:50 anti-human CD68-FITC (green) (clone KP-1, F7135, Dako, Ely, UK). Sections were mounted in 80% glycerol / PBS and imaged by confocal microscopy (Zeiss LSM510 Meta, Jana, Germany) under standard settings as before.⁽³⁸⁾

Results

Generation and characterization of MAb LO1

Hybridomas were generated using splenocytes from a one-year-old female LDL receptor-deficient mouse that had not been immunized. Of 950 clones screened, we identified only 10 IgG antibodies with differential reactivity with native versus Cu-oxLDL. From these, we selected an IgG3k MAb, designated LO1, for further characterization. As shown in Figure 1A and B, MAb LO1 reacted with Cu-oxLDL but only minimally with native LDL, and reactivity with Cu-oxLDL was inhibited by including 50 µg/mL Cu-oxLDL in the buffer. We then tested MAb LO1 reactivity with malondialdehyde (MDA)-conjugated LDL, which is known to be a prominent epitope in copper-oxidized LDL preparations.⁽³⁰⁾ As shown in Figure 1C, MAb LO1 showed increasing binding over time of LDL exposure to MDA, in parallel with carbonyl adduction (Fig. 1D). The failure of MAb LO1 to react with MDA conjugated to human serum albumin (not shown) signifies that it does not simply react with MDA as a hapten.

We checked whether LDL stored over 2 months in the absence of EDTA would become "automodified" in the presence of atmospheric oxygen to reveal the LO1 epitope and found that this was not the case (data not shown). MAb LO1 also failed to react with LDL incubated with hypochlorite for 24 h (not shown). Furthermore, trypsin treatment for up to 2 h did not reveal the LO1 epitope, despite clear evidence for proteolysis upon electrophoresis (not shown). Trypsin treatment of MDA-LDL for up to 2 h failed to influence MAb LO1 binding (not shown).

The immunoglobulin variable regions of the heavy and light chains of LO1 were sequenced and then examined by searching the IMGT database. The V_H belongs to the VH1 family (V1-54) and is germline (Fig. 2). The V_L belongs to the V6 family (V6-20) and is germline apart from minor differences.

Generation of scFv anti-idiotypic H3

Failure to Western blot MDA-LDL with MAb LO1 suggested that it reacts with a conformational epitope. In view of the challenge of defining a conformational epitope on a molecule as complex as LDL, we decided to explore LO1 binding further by developing an anti-idiotypic antibody against the antigen binding site.^(36,39,40) Affinity-purified MAb LO1 was used to select human scFv antibodies from an unbiased phage library. Three rounds of selection resulted in the isolation of a single scFv antibody, designated H3, which reacted with MAb LO1 but not with IgG3k control MAb HK-PEG-1 (Fig. 3A). Also shown in Figure 3A is the failure of MAb LO1 to bind a control scFv, designated C12, isolated from the same scFv library. As shown in Figure 3B, H3 but not C12 was able to neutralize the binding of MAb LO1 to MDA-LDL with an IC₅₀ of 0.8–4.0 mg/mL, indicating that H3 binds MAb LO1 at or close to its recognition site for MDA-LDL. Changing the

FIG. 1. Initial characterization of MAb LO1. (A) Reactivity by ELISA of MAb LO1 culture supernatant and pooled sera from *Ldlr*^{-/-} mice (diluted 1:40) against Cu-oxLDL (white bars) or native LDL (black bars). (B) Competition ELISA showing binding of different concentrations of MAb LO1 in the presence and absence of fluid phase Cu-oxLDL. Values are mean $\pm$ SD of triplicates. (C) Binding by ELISA of MAb LO1 to LDL modified by MDA for durations up to 5 h. Also shown is the lack of binding of control IgG3 HK-PEG-1 at 1 and 10 μ g/mL (curves superimposed). Values are mean $\pm$ SD of triplicates. (D) The extent of carbonyl adduction to LDL following incubation with MDA was estimated using a carbonyl assay, with results expressed as the number of nmoles of DNP/mg of protein (nmol/mg).

salt concentration had a very similar effect on binding of MAb LO1 to H3 as to MDA-LDL (Fig. 3C). There was also a similar profile of pH sensitivities with optimal binding at pH 6.0. Interestingly MAb LO1 binding to H3 had a slightly broader pH range compared to MAb LO1 binding to MDA-LDL (Fig. 3D).

The CDR sequences of H3 were compared with the linear sequences of human and mouse apoB-100 using the Clustal W algorithm. As shown in detail in Figure 4, there are significant homologies between H3 CDRH2 and CDRL2 and peptide sequences of mouse and human apoB. Surprisingly, the relevant amino acids in H3 are situated not just among those that are diverse within the scFv library but also among constant determinants, particularly in the CDRL2 region.

As a prelude to site-directed mutagenesis of H3, we first swapped the V_H and V_L domains between H3 and C12. This established that the binding of MAb LO1 to H3V_H chain paired with C12V_L was similar to native H3, although the signal was slightly reduced at all concentrations of MAb LO1 (Fig. 5A). In contrast, no binding of LO1 occurred to a construct containing the H3V_L chain paired with the C12V_H (Fig. 5A). Specificity of the LO1-H3 interaction was demonstrated by the virtual absence of binding of control IgG3 MAb HK-PEG-1 to any of the four constructs (Fig. 5B).

The H3 and C12 scFv differ in 15 out of the 18 amino acids, which are diverse in the Tomlinson I library (Table 1A). Since the V_H of H3 was dominant for MAb LO1 binding, a series of individual point mutations were carried out on the H3V_H/

	CDR1	CDR2	CDR3
LO-1 V _H	QVQLQQSGAELVRPGTSVKVSKKASGYAFTN ^Y LEWVKQRPGQGLEWIGVINPGSGGTNYNEKFKGKATLTADKSSSTAYMQLSSLTSEDSAVVFCARSFKWKFDYWGQGTTLTVSS		
V1-54/JH2	QVQLQQSGAELVRPGTSVKVSKKASGYAFTN ^Y LEWVKQRPGQGLEWIGVINPGSGGTNYNEKFKGKATLTADKSSSTAYMQLSSLTSEDSAVVFCARSFKWYFDYWGQGTTLTVSS		
	CDR1	CDR2	CDR3
LO-1 V _L	NIVMTQSPKSMMSVSGKRVLTLSCKASENVGT ^Y VS ^W YQKPEQSPKLLIYGAT ^K RYIGV ^P DRFTGSGSATDFTLTISVQAEDLADYHCGQSY ^T YPYTFGGG ^T KL ^D IK		
V6-20/J2	NIVMTQSPKSMMSVSGRVLTLSCKASENVGT ^Y VS ^W YQKPEQSPKLLIYGAS ^N RYIGV ^P DRFTGSGSATDFTLTISVQAEDLADYHCGQSY ^S YPYTFGGG ^T KL ^E IK		

FIG. 2. Amino acid sequence of the MAb LO1 V_H and V_L. The panel shows the MAb LO1 V_H and V_L sequences and their similarity to the germline V1-54/JH2 and V6-20/J2 sequences. LO1 amino acids that differ from germline are shown in red.

FIG. 3. Reactivity of anti-idiotypic scFv H3. (A) Specific binding of MAb LO1 to scFv H3. Microtiter plates were coated with H3 or C12 and then tested for binding of MAb LO1 or control IgG3k HK-PEG-1 (each at 2 μg/mL). Values are expressed as mean and upper value of duplicates. (B) Neutralization of MAb LO1 binding to MDA-LDL by scFv H3. MAb LO1 (2 μg/mL) were incubated with plates coated with MDA-LDL (10 μg/mL) in the presence of varying concentrations of scFv H3 or control scFv C12. (C,D) Effects of changing salt concentration (C) and pH (D) on binding of MAb LO1 to H3 and MDA-LDL. (C,D) Data are expressed as percentage of the binding seen with PBS and percentage of maximal binding respectively.

C12V_L background, changing amino acids in the H3V_H to those of C12, and amino acids in the C12V_L to those of H3. Thus, this strategy looked for mutations in the H3 V_H that that might reduce MAb LO1 binding and mutations in the C12V_L as that might increase it. As summarized in Table 1B, MAb LO1 binding was severely reduced or completely abrogated by any change in the five H3 CDRH2 or four H3 CDRH3 amino acids to those found in C12. Although substituting the whole H3V_L with that of C12 had only a minor effect (Fig. 5A), individual amino acid swaps were surprisingly influential, with significant and paradoxical decreases in MAb LO1 binding caused by changing either of two amino acids in C12 CDRL2 to those on H3, but significant increases in binding of MAb LO1 to constructs with individual H3 substitutions at three of the four C12 CDRL3 sites (Table 1C). Taken together, the data strongly suggest that MAb LO1 recognizes H3 as a conformational structure rather than binding a particular linear peptide motif. This view is further supported by failed attempts by ELISA to bind MAb LO1 to linear peptides, alone or in combination, covering CDRH2 (VSDISGSNTITYA), CDRH2-FR2 interface (YADVKGFRFTIS), CDRH3 (CAKDDDAFDYWG), or CDRL2 (IYYASALQSGVP) (data not shown). Furthermore, these same peptides, either alone or in combination, failed to inhibit MAb LO1 binding to H3 (data not shown).

Immunocytochemistry of atherosclerosis

Preliminary ELISA established that MAb LO1 binding was unaffected by treatment of MDA-LDL with isopropanol, which was therefore used as fixative for immunostaining arterial tissue. As shown in Figure 6, MAb LO1, but not IgG3 control, showed obvious staining of both the macrophage-rich developing atherosclerotic lesion and the media of the aortic root of hyperlipidemic *Ldlr*^{-/-} mice but not normocholesterolemic WT mice, consistent with tissue accumulation of oxidized LDL in *Ldlr*^{-/-} mice. Importantly, staining of *Ldlr*^{-/-} mouse aortic root was inhibited by MDA-LDL or scFv H3 but not by scFv C12 (Fig. 6D–F). Taken together with the inhibition of MAb LO1 binding to MDA/Cu-oxLDL by Cu-oxLDL (Fig. 1B) or H3 (Fig. 3B), these data support the specific recognition of oxidized LDL by MAb LO1 through cognate antibody-antigen recognition rather than through non-specific binding.

When applied to human carotid endarterectomy tissue, MAb LO1 showed more restricted staining compared with staining of the *Ldlr*^{-/-} mouse tissue. Thus, MAb LO1 but not IgG3 control stained intracellular deposits in macrophages, and occasional extracellular deposits adjacent to the edge of the lipid necrotic core, but medial tissue was not stained (Fig.

FIG. 4. Similarities of sequences of LO-1 binding scFv and apoB-100; the figure shows the sequence of the V_H and V_L regions of scFv H3. Amino acids at each of the 18 points at which the Tomlinson I scFv library is diverse are in red. ApoB peptide sequences with similarities to H3 sequences are shown, with amino acids identical to H3 (green). Amino acid numbering is taken from NCBI protein sequences AAA51752.1 and NP_033823.2 for human and mouse apoB respectively.

FIG. 5. Effects of V_H and V_L domain swaps between scFv H3 and C12: H3, C12, H3V_H/C12V_L, and H3V_L/C12V_H were coated at 10 μ g/mL. Binding of MAb LO1 was tested by ELISA across a concentration range of 0.1–2000 ng/mL.

7). This pattern is consistent with that expected in the distribution of heavily oxidized LDL in a human atherosclerotic plaque.

Discussion

In this report, we describe the characterization of MAb LO1, a novel IgG autoantibody that was selected as reacting with oxidized LDL. The approach of generating MAb from the spleens of atherosclerotic mice provides a means to dissect the naturally occurring autoantibody repertoire against modified forms of LDL. Earlier reports have highlighted the high frequency of hybridomas secreting IgM germline encoded antibodies reacting with phosphorylcholine and other oxidation-specific determinants.^(28,29) Out of an interest in the IgG response, we deliberately fused splenocytes from a relatively old (i.e., one year) mouse and screened for clones secreting IgG rather than IgM antibodies. The low frequency of hybridomas secreting IgG autoantibodies against oxidized LDL is similar to the experience of Witztum and associates who failed to isolate such clones.⁽²⁸⁾ Why hybridomas making IgG anti-oxidized LDL antibodies are hard to generate is not clear, as these antibodies are readily detected in serum. Clearly, however, the antibodies found following splenocyte fusion are dependent upon the antigens used for screening, and ongoing work in our laboratory is also focused on other novel autoantibodies derived from *Ldlr*^{-/-} mice with specificities distinct from MAb LO1.

The copper-oxidized LDL preparation that was used to screen our hybridomas is a complex preparation likely to contain MDA-LDL as well as other oxidative modifications.⁽³⁰⁾ While MAb LO1 was subsequently found to react well, if not better, with LDL conjugated selectively with MDA compared to crude Cu-oxLDL, there was no reactivity with other forms of modified LDL tested, such as by trypsin digestion or incubation with hypochlorite. However we have not exhaustively tested binding of MAb LO1 to LDL with

TABLE 1. DIFFERENCES BETWEEN scFv H3 AND scFv C12 AND EFFECTS OF AMINO ACID SUBSTITUTIONS

VH CDR2				VH CDR3		VL CDR2	VH CDR3
A. VH and VL CDR sequences of scFv H3 and C12 with divergent residues (bold)							
scFv H3	SDISGSGN TT TYADSVKGR			DDDAFDYW		YASALQSGV	QQGDAAPT T
scFv C12	SDIS AGGY TSYADSVKGR			TST YFDYW		TASYLQSGV	QQSGSSPT T
	Position	H3 residue		C12 residue	Physiochemical properties		Effect on LO-1 binding
B. Effects of exchanging individual H3V _H amino acids in H3V _H /C12V _L construct with amino acids that differ in C12. Binding by MAb LO1 was tested by ELISA.							
CDR2	H52a	Glycine	→	Alanine	Both small hydrophobic		Complete loss
	H53	Serine	→	Glycine	Polar to hydrophobic		Complete loss
	H55	Asparagine	→	Tyrosine	Polar to aromatic hydrophobic		Complete loss
	H56	Threonine	→	Serine	Both polar		Significant decrease
	H58	Threonine	→	Serine	Both polar		Complete loss
CDR3	H95	Aspartate	→	Threonine	Acidic(-ve) to polar		Complete loss
	H96	Aspartate	→	Serine	Acidic(-ve) to polar		Complete loss
	H97	Aspartate	→	Threonine	Acidic(-ve) to polar		Complete loss
	H98	Alanine	→	Tyrosine	Aliphatic to aromatic hydrophobic		Significant decrease
	Position	C12 residue		H3 residue	Physiochemical properties		Effect on LO-1 binding
C. Effects of exchanging individual C12V _L amino acids in H3V _H /C12V _L construct with amino acids that differ in H3. Binding by MAb LO1 was tested by ELISA.							
CDR2	L50	Threonine	→	Tyrosine	Polar to aromatic hydrophobic		Significant decrease
	L53	Tyrosine	→	Alanine	Aromatic to aliphatic hydrophobic		Complete loss
CDR3	L92	Serine	→	Glycine	Polar to small hydrophobic		Slight increase
	L93	Glycine	→	Aspartate	Small to acidic(-ve)		Significant increase
	L94	Serine	→	Alanine	Polar to aliphatic hydrophobic		Significant increase
	L95	Serine	→	Alanine	Polar to aliphatic hydrophobic		No change

other possible adducts found in Cu-oxLDL, such as with 4-hydroxynonenal,⁽²⁸⁾ and we do not therefore yet know how necessary MDA-conjugation to LDL is for MAb LO1 reactivity. We did find, however, that MAb LO1 did not bind MDA-conjugated human serum albumin, indicating that the LDL carrier is as important as the MDA hapten. Furthermore, we cannot rule out the possibility that MDA is irrelevant to binding, and that what is critical is the unmasking of a cryptic epitope on ApoB with the structural changes to the LDL particle that occur with oxidation.

Staining of atherosclerotic plaques has been used by several groups to validate the binding of antibodies against modified LDL with naturally occurring epitopes in tissues.^(22–24,26,28,29) In a similar way, we have found that MAb LO1 specifically binds antigen accumulated in the aortic root of *Ldlr*^{-/-} mice and in foam cells and extracellular aggregates adjacent to the necrotic core within clinical pathological carotid tissue (AHA Type IV or so-called vulnerable plaque with an advanced lipid necrotic core and thin fibrous cap). In this area, it is thought that macrophages further modify mildly modified LDL, and are killed by their load of heavily modified LDL. The selectivity of LO1 staining for this zone of the plaque is consistent with current ideas about plaque LDL handling. Most of the extracellular LO1 staining (i.e., not obviously around nuclei) was also cell shaped, reflecting either that the nucleus was out of section (in the z-plane) or reflecting release from dying cells.⁽⁴¹⁾ Importantly, the isotype control did not share this pattern. Corroborating this interpretation was the colocalization of LO1 specific staining (not found in the isotype control) in the Alexafluor 568 (red emission) channel with classical oxidized LDL-associated

autofluorescence in the green emission channel (not shown).⁽⁴²⁾ It was notable that the diffuse staining, including the media that was observed in the *Ldlr*^{-/-} mouse tissue, was not seen in the human carotid, perhaps reflecting a more widespread microscopic accumulation of oxidized LDL in the hypercholesterolemic mouse.

It is interesting that MAb LO1 is of the IgG3 isotype, as mouse IgG3 is a relatively minor isotype in mouse serum (0.1–0.2 mg/mL, ~2% of total IgG; compared with IgG2a/c ~0.4 mg/mL). Mouse IgG3 activates complement well (IgG3,2a,2b > IgG1), probably as cooperative binding of its Fc domains gives IgG3 a functional polyvalency for C1q similar to IgM.^(43–45) In contrast to other mouse IgG isotypes, IgG3 does not ligate proinflammatory Fcγ receptors (IgG1,2a/c,2b >> IgG3 in mice).^(13,46,47) Thus, IgG3 antibodies may play a similar homeostatic role to IgM in scavenging modified LDL and, furthermore, are able to perform feedback enhancement of antibody responses.⁽⁴⁸⁾ By virtue of smaller monomeric size (~150 kDa versus ~900 kDa for IgM), IgG3 is likely to have greater tissue penetration and distribution than IgM.⁽⁴⁹⁾ Secretion of IgG3 is thought to be by B1 cells and to occur early in the adaptive immune response by thymus-independent (TI) class-switching with minimal hypermutations.^(45,50)

Low density lipoprotein is a 18–25 nm diameter particle (Mr 550 kDa) consisting of a hydrophobic triglyceride and cholesterol ester core, surrounded by a phospholipid shell wrapped by a single apolipoprotein apoB molecule, which in humans has 4,536 amino acids.⁽⁵¹⁾ The failure of isopropanol to influence the antigenicity of MDA-LDL for MAb LO1 suggests that the epitope is made up from protein rather than lipid determinants. To gain more insight into the binding

FIG. 6. Immunocytochemical staining of *Ldlr*^{-/-} mouse aortic root with MAb LO1. (A) Aortic valve cusp from a *Ldlr*^{-/-} mouse stained with Giemsa, a general purpose tissue stain. (B) Region indicated by the arrow is stained with the macrophage-specific marker CD68-AlexaFluor 488 (green) and the DNA-binding dye TOPRO (blue), revealing the localized area of macrophage infiltration and the unstained media (Med). Diffuse staining of the whole valve cusp is seen with MAb LO1 (C) but not with control IgG3 (D). (E) No equivalent staining was seen with MAb LO1 applied to the aortic root of a wild-type mouse. Preincubation of MAb LO1 with MDA-LDL (F) or scFv H3 (G), but not with scFv C12 inhibited staining (H), demonstrating specificity of MAb LO1 binding. Blue, TOPRO counterstain; red, AlexaFluor 568 fluorescence showing specific binding of MAb LO1. *Ldlr*^{-/-} mice fed a low-fat diet to the age of 22 weeks were chosen because their lesions are composed almost exclusively of macrophages, simplifying interpretation. Med, tunica media (vascular smooth muscle cell layer); Le, lesion (composed of macrophages); open arrow points to lesion.

interaction between MAb LO1 and the large and complex LDL particle, we screened an scFv library for an anti-idiotypic antibody that might mimic some or all of the epitope on MDA-LDL by carrying an “internal image” of the antigen. It should be noted that mutating amino acids in the binding site of a MAb against apoB has previously been used to gain insight into the molecular basis of LDL interaction with the LDL receptor,⁽⁵²⁾ but an anti-idiotypic approach to dissecting an anti-oxidized LDL autoantibody does not appear to have been previously adopted. We succeeded in isolating H3, a scFv antibody that specifically neutralizes the binding of MAb LO1 to MDA-LDL and which has amino acid sequences in the H3 CDRH2 and CDRL2 with strong similarity to peptide stretches in mouse and human ApoB. It is notable that while MAb LO1 showed similar physico-chemical constraints in binding H3 as MDA-LDL, the pH tolerance of H3 binding was greater, suggesting that H3 may actually be a better fit for the MAb LO1 antigen binding site than MDA-LDL. This may be related to MAb LO1 V_H being germline and lacking hypermutated sequences.

Further dissection of MAb LO1-H3 interactions in comparison with a non-binding control scFv C12 showed that the MAb LO1 bound well to H3V_H paired with C12V_L, demonstrating the importance of V_H residues. Site-directed mutagenesis failed to reveal particular H3V_H amino acids critical for binding, and the major reduction in binding that occurred with any substitution suggests that CDRH2 and CDRH3 are both involved. It is notable that CDRH3 contains a stretch of six amino acids with four aspartates (DDDAFD), which may well supply a critical negative charge. It should be noted that the failure of MAb LO1 to bind to H3V_L paired with C12V_H does not exclude involvement of the V_L since amino acid substitutions in the two diverse amino acids at CDRL2 abolished binding. Furthermore, SALQSG, which are the amino acids in CDRL2 with homology to apoB, are conserved between H3 and C12. Thus residues on H3V_L, particularly in CDRL2, may contribute to the putative conformational epitope that MAb LO1 reacts with.

Taken together, our observations raise the possibility that H3 has focal sequence and charge similarities to the site on oxidized LDL that binds MAb LO1. Linear peptides spanning H3 CDRH2, CDRH3, and CDRL2 were not able to inhibit MAb LO1 binding to H3, consistent with the importance of conformation. Therefore definitive evidence for the residues on H3 that MAb LO1 reacts with will require further studies. While there are precedents for co-crystallization of Fab with anti-idiotypic,⁽⁵³⁾ this would be technically extremely difficult for MAb LO1 bound to oxidized LDL. However an alternative approach for directly comparing MAb LO1 binding to H3 and oxidized LDL is the use of hydrogen-deuterium exchange mass spectrometry.⁽⁵⁴⁾

While phage display techniques have previously been used to isolate human IgG single-chain autoantibodies reacting with oxidized LDL,^(20,55) as far as we are aware MAb LO1 is the first spontaneously arising IgG anti-oxidized LDL autoantibody to be reported from mice. The unique autoantibody-anti-idiotypic combination provided by MAb LO1 and H3 will now be very useful for further structural and functional studies and will also allow us to perform *in vivo* experiments testing the capacity of passive immunization with MAb LO1 to influence oxLDL clearance *in vivo* and to retard atherosclerosis, and also examining the possibility that active immunization with H3 can elicit an endogenous LO1-like IgG autoantibody response.

FIG. 7. Immunocytochemical staining of human carotid atherosclerotic plaque with MAb LO1. (A) For orientation, low power view of a human carotid atherosclerotic plaque stained with Giemsa, with the area within the box magnified (B). (C–E) Confocal imaging of consecutive serial sections in the vicinity of the boxed area (B). Single stained with MAb LO1 (red AlexaFluor 568) (C), dual-stained by MAb LO1 (red AlexaFluor 568), and CD68-AlexaFluor 488 (green) with brilliant yellow-white indicating colocalization (D), and single stained with control IgG3 (E) with protocol and settings identical to (C). Lu, lumen; LC, lipid core; Med, media; Blue, TOPRO; nuclei (DNA); fine arrowheads, inflammatory cells at edge of lipid core; scale bars; distances are indicated.

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Author Disclosure Statement

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Address correspondence to:

*Prof. Dorian O. Haskard
Vascular Sciences Section
National Heart and Lung Institute
Imperial College
Hammersmith Hospital
Du Cane Road
London W12 0NN
United Kingdom*

E-mail: d.haskard@imperial.ac.uk

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