Maternal Immunization Programs Postnatal Immune Responses and Reduces Atherosclerosis in Offspring

Tomoya Yamashita, Stefan Freigang, Claudia Eberle, Jennifer Pattison, Sachin Gupta, Claudio Napoli, Wulf Palinski

Abstract—Maternal hypercholesterolemia during pregnancy increases offspring susceptibility to atherosclerosis by an oxidation-dependent mechanism. The present studies investigated whether maternal immunization with oxidized LDL (OxLDL) before pregnancy protects the fetus from atherogenic in utero programming by maternal hypercholesterolemia. Maternal immunization of NZW rabbits and LDL receptor–deficient mice indeed reduced atherosclerosis in adult offspring by up to 56%, but the protective effect could not be attributed to a reduction of fetal exposure to hypercholesterolemia alone, and even nonspecific immune stimulation with adjuvant only provided some protection. Unexpectedly, offspring of immunized mothers developed increased IgM antibodies to selective OxLDL epitopes and increased IgM-LDL immune complexes, compared with offspring of nonimmunized controls. Even naïve offspring of OxLDL-immunized mothers never exposed to postnatal hypercholesterolemia responded to a one-time OxLDL and KLH challenge with greater OxLDL–specific IgM responses, increased OxLDL–specific IgM-secreting B cells, and more IgM-LDL immune complexes. In contrast, maternal immunization with KLH, a T cell–dependent nonmammalian antigen, did not influence postnatal immune responses. Effects of maternal OxLDL–immunization on offspring B cells and selective antibodies were independent of transplacental passage of maternal immunoglobulins. Results show that maternal immunization with antigens prevalent in atherosclerotic lesions reduces atherogenesis in their offspring by mechanisms that include, but are not limited to, reduced fetal exposure to maternal hypercholesterolemia and lipid peroxidation. More importantly, they demonstrate in principle that maternal adaptive immunity to selective antigens influences postnatal B cell and antibody responses in offspring, and that modulation of in utero immune programming may influence immune-modulated diseases later in life. (Circ Res. 2006;99:e51–e64.)

Key Words: adaptive immunity ■ arteriosclerosis ■ immunization ■ in utero programming ■ oxidized LDL ■ developmental programming ■ prevention

Hypercholesterolemia and the ensuing increased oxidative stress play an important role in the fetal programming of atherosclerosis. maternal hypercholesterolemia, including temporary hypercholesterolemia during pregnancy, is associated with markedly increased fatty streak formation in fetal arteries and accelerated progression of atherosclerosis during normocholesterolemic childhood. Although potential differences in the maternal susceptibility to atherosclerosis make it difficult to establish the impact of in utero programming by maternal hypercholesterolemia in humans, experiments in genetically more homogeneous animal models have demonstrated that diet-induced maternal hypercholesterolemia during pregnancy enhances fetal lesion formation in a dose–dependant manner, increases postnatal atherogenesis, and causes persistent changes in arterial gene expression. Detrimental effects of maternal hypercholesterolemia on endothelial function, a predictor of atherosclerosis, have also been established. An important corollary of in utero programming by maternal hypercholesterolemia is the possibility of achieving lifelong reduction of atherogenesis by brief interventions in mothers. In experimental models, maternal cholestyramine or vitamin E treatment reduced fetal and postnatal atherogenesis, thus establishing both the efficacy of maternal interventions and the pathogenic role of lipid peroxidation. However, in view of the potential risks of interventions with more powerful cholesterol-lowering or antioxidant drugs during pregnancy, other preventive approaches would be desirable. One such approach may be the induction of oxidation-specific antibodies in the mother before pregnancy.

Immune mechanisms play a complex role in atherogenesis. Proinflammatory cytokines and interleukins, such as IFNγ, CD40, and IL-8 clearly promote atherogenesis.
whereas some Th2 interleukins, eg, IL-10, inhibit its progression.13 OxLDL accumulating in atherosclerotic lesions is highly immunogenic, and low-titered autoantibodies to various oxidation-specific epitopes are present in humans and animals models.14,15 Although one would assume that such antibodies promote foam cell formation via Fc receptor–mediated uptake of immune complexes, immunizations of rabbits and mice with various models of OxLDL consistently reduced the progression of atherosclerosis.16–21 Furthermore, immunization with homologous MDA-LDL (ie, LDL conjugated in vitro with malondialdehyde, a reactive aldehyde generated during lipid peroxidation), or CuOx-LDL (ie, LDL oxidized by incubation with copper ions), induces high-titered antibodies which form immune complexes with circulating LDL particles bearing some oxidation-specific epitopes. This leads to the removal of such atherogenic particles from the circulation.19 Immunization may also promote a switch from Th1 cells secreting proatherogenic IFNγ to Th2 cells secreting antiatherogenic interleukins.9

The present experiments were designed to assess whether immunization of mothers before pregnancy also protects fetuses against atherogenic programming by maternal hypercholesterolemia, eg, by enhancing the removal from the circulation of minimally oxidized LDL or by reducing the amount of antigen present during the critical period of maturation and differentiation of the neonatal immune system. Maternal immunization indeed conveyed marked antiatherogenic protection to offspring, but the mechanism responsible was not limited to a reduction of fetal exposure to hypercholesterolemia and oxidative stress. Unexpectedly, results indicated that maternal immunization also programs specific B cell and IgM responses in offspring.

Materials and Methods
An overview of all rabbit and murine experiments is provided in Table 1.

Experimental Design: Rabbits
To investigate whether prior immunization with OxLDL reduces the atherogenic effect of maternal hypercholesterolemia during pregnancy, 7 groups of female NZW rabbits (Charles River, Wilmington, Mass) were used (“mothers”) (n=39, total). Three of these were nonimmunized controls: (1) Normocholesterolemic mothers fed regular rabbit chow; (2) Hypercholesterolemic mothers fed an atherogenic diet supplemented with individually adjusted cholesterol, to achieve plasma cholesterol levels of approximately 350 mg/dL before and during pregnancy; (3) Hypercholesterolemic + Vitamin E mothers fed the same individually adjusted cholesterol–enriched diet together with a high dose of vitamin E (10g a-tocopherol/kg diet, Sigma). The remaining groups were immunized with OxLDL or PBS before inducing hypercholesterolemia and pregnancy: (4) OxLDL-immunized Hypercholesterolemic mothers; and (5) OxLDL-immunized Marginally Hypercholesterolemic mothers. Originally, only one OxLDL-immunized hypercholesterolemic group was planned. However, early on it became apparent that hypercholesterolemia within the target range could not be achieved in some OxLDL-immunized mothers despite prolonged feeding of hypercholesterolemic diet, and these were therefore treated as a separate group: (6) OxLDL-immunized Normocholesterolemic mothers; and (7) PBS-immunized Hypercholesterolemic mothers immunized with PBS+Freund’s adjuvant (FA), to control for FA used in OxLDL immunizations.

Once a marked increase in antibody titer had been confirmed 2 weeks after the last boost, mothers in the hypercholesterolemic groups were fed an atherogenic diet containing ~9% fat, 17% protein, 57% carbohydrate, and 16% fibers (Harlan Teklad rabbit diet 7000 supplemented with 6.5% corn oil) with or without vitamin E. Initially, 0.15% cholesterol was added to the diet (dissolved in ether, sprayed onto food pellets, and thoroughly evaporated). Total plasma cholesterol (TC) was determined after 2 weeks and the cholesterol concentration added to the diet was individually adjusted (0.05 to 0.25%) until their TC was within the target range (300 to 400 mg/dL). Females were then mated and TC during pregnancy was determined after 2.5 weeks. After delivery, all mothers were reverted to the diet later fed to offspring (Harlan Teklad Rabbit Diet #7009 supplemented with 1.5% corn oil) containing 4% total fat, 17% protein, 57% carbohydrate, and 16% fibers.

Offspring were weaned at 4 weeks and fed the above 4% fat diet supplemented with individually adjusted cholesterol (range 0.05% to 0.4%, with most animals receiving 0.15 or 0.20%), to achieve plasma cholesterol levels of ~350 mg/dL. This approach yielded very similar time-averaged TC levels in all groups. Although the cholesterol amount administered to individual offspring varied, the cumulative dietary cholesterol exposure during the 5 months of dietary intervention was very similar in all groups (Normocholesterolemic, 0.175%; Hypercholesterolemic, 0.179%; Hypercholesterolemic+Vitamin E, 0.158%; OxLDL-immunized Hypercholesterolemic, 0.143%; OxLDL-immunized Marginally Hypercholesterolemic, 0.197%; PBS-immunized Hypercholesterolemic 0.159%). Blood samples for antibody and immune complex determinations were obtained at age 1, 3, 4.5, and 6 months. Experimental groups contained roughly equal numbers of males and females, and data were analyzed together because previous studies had not indicated significant gender differences.4,5,16

Experimental Design: Mice
To validate findings in the rabbit model in a different species, and in the absence of confounding effects stemming from dietary differences, analogous experiments were performed in LDL receptor–deficient (LDLR−/−) mice. Three groups of female LDLR−/− mice (from our colony established from Jackson Labs, Bar Harbor, Maine, LDLR−/− mice bred-back into C57BL/6 for 10 generations), age 6 to 8 weeks, were immunized with homologous OxLDL (an analogous mixture of MDA- and CuOx-LDL as for rabbits), or PBS+FA, and compared with nonimmunized controls. After the primary immunization and 2 to 3 biweekly boosts, immune response was ascertained by ELISA and mating occurred. All mothers were fed regular chow throughout immunization and pregnancy and had cholesterol levels of ~260 mg/dL before and 150 mg/dL during pregnancy. After weaning at age 4 weeks, offspring were fed a regular murine diet supplemented with 0.5% cholesterol for 16 weeks (females, n=35) or 30 weeks (males, n=35). One to three additional mice originally assigned to each group were excluded to match average cholesterol levels or because they developed health problems. The overall aim was to demonstrate that maternal immunization reduces atherogenesis, and that this is not limited to a particular gender, anatomical site, or stage of lesion, nor dependent on the parameter measured (cross-sectional lesion area versus surface area). In contrast to rabbits, gender- and site-specific differences in mice are well established, with females developing more extensive lesions in the aortic origin and males more extensive atherosclerosis in the aorta.22 Furthermore, extensive atherosclerosis in the entire aorta develops significantly later than in the aortic origin. Therefore, measurements in the aortic origin of 20-week-old females before the development of extensive aortic atherosclerosis and 34-week-old males displaying advanced aortic atherosclerosis provide a wide range of conditions with a limited number of offspring. Plasma cholesterol, antibodies, and immune complexes were measured in retro-orbital blood at age 4, 12, 20, and 34 weeks. All experiments were performed under approved UCSD animal protocols.

Maternal Immunization
To obtain a broad spectrum of oxidation-specific epitopes, including both malondialdehyde (MDA)-lysine epitopes and oxidized phos-
TABLE 1. Overview of Experimental Design and Groups

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<tr>
<th>Mothers</th>
<th>Offspring</th>
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<tr>
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<td>Immunization*</td>
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<td>NZW Rabbits</td>
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<td>Normocholesterolemic</td>
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<td>Hypercholesterolemic</td>
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<td>Hypercholesterolemic + Vitamin E</td>
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<td>OxLDL-immunized</td>
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<td>OxLDL-immunized Marginally Hypercholesterolemic</td>
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<td>LDLR−/− Mice</td>
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<td>PBS + FA-immunized</td>
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*Primary immunization at age 6 weeks. Two boosters at age 8 and 10 weeks. Determination of antibody titers at 12 weeks. All rabbit mothers were fed regular diet during immunization.†Cholesterol content of hypercholesterolemic WHHL rabbits or LDLR−/− mice fed a high-fat, high-cholesterol diet by sequential ultracentrifugation in the presence of antioxidants and antiproteolytic agents, and extensively modified with MDA or copper-ions, as previously described.23 LDL was tested for endotoxin levels by chromogenic Limulus amoebocyte assay (QCL-1000; BioWhittaker, Wakersville, MD) and contained less than 2 ng lipopolysaccharides/mg protein. The primary immunization consisted of inguinal subcutaneous injections of 200 μg (rabbits) or 60 μg (mice) of homologous OxLDL (protein) per kg body weight in PBS emulsified in an equal volume of complete FA (Sigma # F-5881). Two to three intramuscular booster immunizations with 200 (rabbits) or 30 μg antigen/kg (mice) in incomplete FA (Sigma # F-5506) were performed in biweekly intervals, and antibodies to MDA-LDL and CuOx-LDL verified 1 to 2 weeks thereafter. Control groups were immunized with PBS + FA.†Actual level reached: 109 mg/dl.‡Spontaneous level of chow-fed LDLR−/− mice. §Basic diet (4% fat, 17% protein, 57% carbohydrate,17% fibers) later supplemented with cholesterol and administered to offspring of the atherosclerosis experiment.

Phospholipid epitopes, an equal mixture of homologous MDA-modified LDL (MDA-LDL) and copper-oxidized LDL (CuOx-LDL) was used for immunization of rabbits and mice. LDL was isolated from spontaneously hypercholesterolemic WHHL rabbits or LDLR−/− mice fed a high-fat, high-cholesterol diet by sequential ultracentrifugation in the presence of antioxidants and antiproteolytic agents, and extensively modified with MDA or copper-ions, as previously described.23 LDL was tested for endotoxin levels by chromogenic Limulus amoebocyte assay (QCL-1000; BioWhittaker, Wakersville, MD) and contained less than 2 ng lipopolysaccharides/mg protein. The primary immunization consisted of inguinal subcutaneous injections of 200 μg (rabbits) or 60 μg (mice) of homologous OxLDL (protein) per kg body weight in PBS emulsified in an equal volume of complete FA (Sigma # F-5881). Two to three intramuscular booster immunizations with 200 (rabbits) or 30 μg antigen/kg (mice) in incomplete FA (Sigma # F-5506) were performed in biweekly intervals, and antibodies to MDA-LDL and CuOx-LDL verified 1 to 2 weeks thereafter. Control groups were immunized with PBS + FA.

Circulating Antibodies to Oxidation-Specific Epitopes, ApoB-Immune Complexes, and Total Immunoglobulins

Offspring plasma aliquots were stored at −80°C, and circulating antibodies, immune complexes, and total immunoglobulin concentrations at all time point were determined at the end of the intervention period by chemiluminescence ELISA.24,25 Circulating IgG and IgM antibodies in rabbit plasma (1:250 dilution) binding to human MDA-LDL, CuOx-LDL, and phosphorylcholine (PC) plated in 96-well microtiter plates (Thermo Labsystems) were detected using alkaline phosphatase (AP)-labeled goat anti-rabbit IgG (1:41,000 dilution; Sigma #A3812) or goat anti-rabbit IgM (1:4,000 dilution; Southern Biotechnology Associates #4020-4), and LumiPhos 530 (Lumigen). Although the use of homologous modified LDL it is essential for immunizations, to avoid generating antibodies to epitopes of heterologous native apoB, human modified LDL offers logistical advantages for ELISA measurements, and extensive characterization of natural and induced oxidation-specific rabbit and mouse antibodies has shown that they recognize the respective
epitopes on oxidized human LDL as well as on rabbit and murine LDL (or tissues).\textsuperscript{14,16,23,26} Circulating mouse antibodies binding to the same antigens were detected using AP-labeled goat anti-mouse IgG (γ-chain specific, Sigma #A-9688, dilution 1:36,000) or anti-mouse IgM (μ-chain specific) (Sigma #A-3438, dilution 1:56,000). Chemiluminescence (RLU/100 ms) was determined in a MLX Microtiter Plate Lumimeter (Dynex Technologies) and results reported as specific binding (eg, binding to CuOx-LDL divided by binding to native LDL). Circulating EO6/T15 antibodies in murine plasma (1:100 dilution) were determined by coating wells with 5 μg/mL of AB1–2 (anti-T15 idiotype, kindly provided by J. Kearney, University of Alabama) in TBS and detecting captured antibodies with biotinylated AB1–2 (1 μg/mL), followed by AP-labeled NeutrAvidin (Pierce Biotechnology) and Lumiphos (Lumigen).\textsuperscript{27} Purified EO6 was used for constructing a standard curve, and results were reported as μg/mL plasma.

Total rabbit plasma IgM was determined with a rabbit IgM ELISA Kit (Bethyl Laboratories #E120–110) and a 1:5000 plasma dilution. Total rabbit IgG was determined by a competitive binding ELISA, using rabbit IgG as the plated antigen (5 μg/mL) and incubating each well with 25 μL of a 1:5000 plasma dilution, or various dilutions of a standard rabbit IgG (Bethyl #P120–201) together with 25 μL of AP-labeled goat anti-rabbit IgG. Total mouse plasma IgG and IgM was assessed using mouse Ig ELISA Kit (Bethyl Laboratories #E90–131 for IgG; 1:50,000 plasma dilution; #E90–101 for IgM, 1:500 plasma dilution). Total IgG and IgM were reported as mg/mL. IgG and IgM immune complexes with LDL were measured by capturing rabbit apoB with monoclonal antibody MB47\textsuperscript{28} or mouse apoB with monoclonal antibody LFS.\textsuperscript{29} Capturing antibodies were plated overnight at 5 μg/mL of MB47 or LFS and incubated for 1 hour with rabbit or mouse plasma (1:50 and 1:100 dilution in BSA-TBS, respectively). IgG and IgM bound were detected using AP-labeled anti-rabbit or anti-mouse IgG or Ig Ig. Results were corrected for the amount of LDL captured from each plasma, determined in parallel wells, using a guinea pig antisera to rabbit apoB and AP-labeled anti-guinea pig IgG (Sigma, #A-5062) for rabbit plasma, and AP-labeled monoclonal antibody LFS (that binds to a single epitope per apoB particle) for murine plasma.\textsuperscript{29} Antibodies LFS and LFS were generous gifts from S.G. Young, University of California, Los Angeles.

**Offspring Immune Challenge**

Separate groups of offspring of OxLDL-immunized and nonimmunized NZW mothers aged 6 to 7 weeks, were fed the regular 4% fat diet not supplemented with cholesterol since weaning. In a first experiment, offspring received a single subcutaneous injection of two antigens: 300 μg of OxLDL in CFA and 100 μg keyhole limpet hemocyanin (KLH), a nonmammalian protein. The high dose of OxLDL was used because we were concerned that antibodies in offspring of OxLDL-immunized mothers might interfere with the immune response. In later experiments, rabbits were challenged with the same amount of KLH, but 2 mg of OxLDL. Blood was drawn after 4 days and 1, 2, 4, 6, and 8 weeks. Results with both doses of OxLDL were similar and were therefore pooled for statistical analysis. Immune challenge of offspring of KLH-immunized and nonimmunized LDLR−/− mice was performed with the same dose of KLH only, ie, without OxLDL or adjuvant, at the age of 6 weeks. Mice were fed regular chow, but are not termed “normocholesteremic” because of the spontaneous hypercholesterolemia in this strain.

**Enzyme-Linked Immunospot (ELISpot) Assay**

96-well MultiScreen-HA sterile nitrocellulose plates (Millipore) were coated overnight with/without antigen (rabbit or mouse OxLDL), or with capture antibodies for immunoglobulins, goat anti-rabbit IgM (Bethyl #A120–110A, 5 μg/mL in PBS, 50 μL/well), goat anti-rabbit IgG-Fc (Bethyl #A120–111A, 5 μg/mL), or rat anti-mouse IgM (BD-Pharmpingen #553406, 2 μg/mL in BSA-TBS), followed by HRP-Streptavidin (Zymed #43–4323, 1:1000 dilution). Plates were developed for 15 to 20 minutes using the Tetramethylbenzidine (TMB) Membrane Peroxidase Substrate System (Kirkegaard & Perry Laboratories), and spots were quantified under a dissecting microscope. Results were calculated as IgM- or IgG-secreting cells (ISC) per 100 cells and presented as antigen-specific ISC as percent of all ISC.

**FACS Analysis**

Murine peritoneal cells were harvested by lavage with ice-cold PBS supplemented with 1% BSA, centrifugation (300g for 5 minutes), and resuspension in PBS with BSA. Fc receptors were blocked by 10 minutes incubation at 4°C with a monoclonal antibody against CD16/CD64, and 106 cells were stained with FITC-labeled anti-IgM, PE-labeled anti-CD5 (Ly1), and APC-labeled anti-CD11b/Mac-1 in 100 μL volumes of staining buffer (FBS; Dulbecco’s PBS, MediTech Inc) for 30 minutes at 4°C in darkness, followed by two washes with staining buffer. Murine splenocytes were isolated by gently passing spleens through a 100 μm cell strainer (BD Falcon) into sterile RPMI medium containing 10% FCS, HEPES, and gentamicin. Cells were then resuspended 5 mL red blood cell lysis buffer for 5 minutes. After addition of 10 mL RPMI medium containing 2% FCS, cells were centrifuged at 300g for 5 minutes, resuspended in a fixed volume, and counted. Splenocytes were stained with the following monoclonal antibodies: FITC-labeled anti-IgM, PE-labeled anti-mouse IgCD43, APC-labeled anti-CD11b/Mac-1, PE-labeled anti-CD4, PerCP-labeled anti-CD8 (all from BD Biosciences-Pharmingen) and APC-labeled anti-B220, FITC-labeled anti-CD21 and PE-labeled anti-CD23 (all from eBioscience). Cell populations were analyzed on a Becton Dickinson FACScan using Cellquest software. More than 106 cells per sample were analyzed and dead cells excluded by forward and side scatter. Results were analyzed by FlowJo 6.3 software.

**Quantification of Atherosclerosis**

The extent of atherosclerosis in the entire aorta of rabbits and mice was determined by computer-assisted morphometry, as previously described.\textsuperscript{16,22,30} Atherosclerosis in the aortic origin of mice was determined in 11 equidistant cross-sections of the heart, using the aortic valve leaflets as anatomical reference. Sections were stained by a modified trichrome method,\textsuperscript{31} and images captured with a 11 megapixel Leitz DCX500 digital camera mounted on a Leitz DC5000 microscope. Results were expressed as percent of aortic surface covered by atherosclerotic lesions or mean cross-sectional lesion area.

**Statistics**

Data were analyzed with SSPS software version 13.0. Normal-distributed data were analyzed by ANOVA and groups compared by unpaired t test. Probability values less than 0.05 were considered significant.

**Results**

**Maternal Immunization Reduces Offspring Atherosclerosis By a Mechanism Independent of the Reduction of Fetal Cholesterol Exposure**

The effects of prior immunization were first assessed in offspring of normo- or hypercholesterolemic NZW mothers (Figure 1A). Postnatal cholesterol levels of all offspring groups were similar (Figure 1B). In agreement with previous findings,\textsuperscript{5} in offspring of nonimmunized mothers maternal...
hypercholesterolemia (339 mg/dL) increased aortic atherosclerosis by 48%, compared with offspring of normocholesterolemic mothers (43.7±4.5% of the surface area versus 29.6±4.4%, P<0.05) (Figure 1C). Vitamin E did not affect maternal cholesterol levels (Figure 1A), but abolished the atherogenic consequences of maternal hypercholesterolemia (25.6±5.3% atherosclerosis, ie, 41.5% less than in offspring of untreated hypercholesterolemic mothers; P<0.02).

Compared with offspring of similarly hypercholesterolemic, nonimmunized mothers, maternal immunization with OxLDL reduced atherosclerosis in offspring by 52% (20.9±3.8% of the aortic surface; P<0.001) (Figure 1C). Lesions in this group were even 29% smaller than those of offspring of nonimmunized normocholesterolemic mothers, although the difference did not reach statistical significance. The extent of atherosclerosis in offspring of marginally hypercholesterolemic mothers was almost identical (19.1±5.7% lesions, ie, 56% less than offspring of nonimmunized hypercholesterolemic mothers, P<0.005). Atherosclerosis in offspring of OxLDL-immunized normocholesterolemic mothers was 25% lower than in those of normocholesterolemic controls, though statistical significance was not reached (P=0.16). Immunization of hypercholesterolemic mothers with PBS+FA was far less effective than immunization with OxLDL, but also reduced atherogenesis (to 30±3.2%, P<0.05), indicating that the protective effect was not specific for OxLDL.

Given the strong atherogenic effect of maternal hypercholesterolemia previously established and confirmed here, the absence of differences in atherosclerosis between the three OxLDL-immunized groups was surprising, and indicated that the protective mechanism was not limited to the reduction of
fetal exposure to hypercholesterolemia. It was also not attributable to a reduction of fatty streak formation during fetal development, because in neonatal rabbits no difference in aortic lesions was detectable between offspring of OxLDL-immunized and nonimmunized hypercholesterolemic mothers (Figure 2).

Maternal Immunization of Rabbits Increases Circulating IgM-LDL Immune Complexes in Offspring
To assess the effects of immunization on postnatal immune responses, circulating antibodies and immune complexes in offspring were determined over time. IgG antibodies to oxidation-specific epitopes and IgG immune complexes with LDL (Figure 3A) showed no consistent differences between groups. In contrast, circulating IgM-LDL immune complexes were significantly higher in offspring of both hypercholesterolemic mothers, but did not appear to be more extensive in the controls (D, G) and PBS groups (E) than in the OxLDL-immunized group (F, H). The internal elastic lamina is outlined in the low-magnification images.

Figure 2. Reduction of atherosclerosis in adult offspring of OxLDL-immunized rabbit mothers cannot be attributed to reduced lesion formation during fetal development. To rule out that differences in postnatal atherogenesis stem from immune-modulation of fetal cholesterol during pregnancy, 15 offspring of hypercholesterolemic mothers were killed within 3 days of birth and atherosclerosis was compared in cross-sections through the aortic origin and aortic arch and in Sudan stained en face preparations of the entire aorta. At birth, all Sudan-stained aortas were free of macroscopic lesions, as shown for offspring of hypercholesterolemic control (A) and OxLDL-immunized (B) mothers. No lesions were seen in the aortic origin of either group (C). In contrast, microscopic lesions were prevalent in sections throughout the aortic arch of all newborn offspring of hypercholesterolemic mothers, but did not appear to be more extensive in the controls (D, G) and PBS groups (E) than in the OxLDL-immunized group (F, H). The internal elastic lamina is outlined in the low-magnification images.
terolemic controls (Figure 3C). Given that all offspring groups had similar plasma cholesterol levels, the consistent increase in IgM–LDL immune complexes can be attributed to increased antibodies, rather than increased amounts of antigen. However, plasma levels of IgM antibodies to MDA-LDL or CuOx-LDL were similar (not shown), and only IgM to phosphorylcholine (recognized by EO6/T15 idiotype IgM) showed a slight trend toward higher concentrations in offspring of OxLDL-immunized mothers (Figure 3D). This suggests that increased formation of LDL–IgM immune complexes must have compensated for any increase in specific IgM, or that the latter represents mainly IgM of different specificity than previously characterized oxidation-specific antibodies that were not detected by our ELISAs.

Maternal Immunization Increases Selected IgM and IgG Responses to an Immune Challenge With OxLDL and KLH in Naïve NZW Offspring

Static measurements of antibody titers and immune complexes represent the balance of antibody formation, amount of antigen present, and removal of immune complexes. Stronger evidence for an effect of maternal immunization on offspring immune responses was therefore sought from an immune challenge of naïve offspring of OxLDL-immunized mothers (hyper- and normocholesterolemic, n = 17) and control mothers (PBS-immunized hypercholesterolemic and nonimmunized hyper- and normocholesterolemic, n = 22). All of these had been fed the regular diet since weaning, to avoid confounding effects of postnatal hypercholesterolemia and atherogenesis, and received a simultaneous subcutaneous injection of OxLDL and KLH at age 6 to 7 weeks. Results are reported as the relative increase of antibodies over their preimmune level (Figure 4A through 4H). As expected, maternal immunization with OxLDL did not affect antibody responses to KLH. In both groups, IgG (Figure 4A) and IgM antibodies (Figure 4E) began to rise shortly after antigen injection and reached similar peaks after 2 to 3 weeks (IgM) or 6 weeks (IgG). Antibody responses to MDA-LDL, CuOx-LDL, and native LDL were slower and relatively weaker than those to KLH, but differences between groups began to emerge after 2 weeks. At 6 to 8 weeks, IgG and IgM responses to CuOx-LDL were markedly greater in offspring of OxLDL-immunized mothers than in controls (Figure 4C and 4G). The differences started earlier and were even more significant for IgG and IgM antibodies to “native” LDL (Figure 4D and 4H), showing that the epitopes recognized by antibodies of primed offspring occur on LDL that is not extensively oxidized. Native homologous LDL should not
Figure 4. Maternal immunization of rabbits influences responses to an immune challenge in offspring. Naïve, chow-fed offspring of OxLDL-immunized (n=17) and control mothers (n=22) received simultaneous subcutaneous injections of OxLDL and KLH at time zero. A through D, Circulating IgG and E through H, IgM antibodies to KLH, MDA–LDL, CuOx–LDL, and native LDL. Results are reported as relative increase over the prechallenge antibody level. I and J, Because antibody levels peaked at different times, results are also shown as the maximum increase in each animal, irrespective of when it was reached. K and L, Immune complexes of IgG and IgM antibodies with LDL, reported as relative increase over prechallenge levels. M, ELISpot analysis of splenic B cells secreting anti-OxLDL IgM, expressed as percentage of all IgM-secreting splenocytes. *P<0.005; **P<0.01, vs control.
elicit immune responses, and conversely, immunization with OxLDL should not yield antibodies binding to native apolipoprotein B. We therefore assume that the native LDL used in our assays was oxidized to some degree and that the antibodies primed by maternal immunization recognize oxidative neoepitopes. In fact, such epitopes are already present on circulating LDL and LDL isolated under stringent antioxidant precautions.23 In contrast, differences in antibodies to MDA-LDL did not reach significance (Figure 4B and 4F).

Similar results were obtained when data were compared on the basis of the maximum increase in antibody levels in each animal, irrespective of the time it was reached (Figure 4I and 4J). The fact that both IgM and IgG antibodies increased in parallel and to a much greater degree in offspring of OxLDL-immunized mothers than in controls is reminiscent of a secondary immune response, but the onset was slower than one would expect.

Circulating levels of IgG and IgM immune complexes with native LDL were also much greater in offspring of OxLDL-immunized mothers than in controls (Figure 4K and 4L). In analogy, differences in the maximum increase in immune complexes in each animal were highly significant (right-hand bars in Figure 4I and 4J). These results are consistent with the increase in IgM–LDL immune complexes seen in the atherosclerosis experiment (Figure 3B).

As expected from the IgM antibody and immune complex data, ELISpot analysis of splenic lymphocytes isolated from 30 animals at the end of the immune-challenge experiment indicated a significantly greater percentage of splenocytes secreting OxLDL-specific IgM in offspring of OxLDL-immunized mothers than in all controls (Figure 4M).

The immune challenge was performed in young animals, and data in Figure 4A through 4H are reported as relative increase of antibody titers over the prechallenge level in each animal. Nevertheless, results were not attributable to persistence of increased maternal IgG antibodies in offspring of OxLDL-immunized mothers, because the raw titers before the challenge were similar. Furthermore, a repetition of the entire experiment in 6-month-old naive NZW rabbits (n = 16), in which the persistence of maternal IgG is negligible, yielded very similar results (data not shown).

Maternal Immunization Influences IgM Antibody Responses, LDL Immune Complexes, and IgM-Secreting B Cells in Offspring of LDLR−/− Mice

Murine models are better suited to further elucidate fetal immune programming because of their genetic uniformity, well characterized immune system, and abundance of immune reagents. To verify the effects of maternal immunization, we compared 35 female and 35 male offspring of LDLR−/− mice immunized with homologous OxLDL, PBS+FA, and nonimmunized controls. All mothers were fed regular chow throughout pregnancy, to avoid confounding effects of different dietary cholesterol exposure. Offspring were fed regular chow supplemented with 0.5% cholesterol for 16 (females) or 30 weeks (males).

Circulating antibodies and immune complexes in female mice at age 4, 12, and 20 weeks indicated marked differences between offspring of OxLDL-immunized and control mothers (Figure 5). At all time points, IgG–LDL complexes were similar in all three offspring groups (Figure 5A), whereas IgM–LDL immune complexes were significantly greater in offspring of OxLDL-immunized than nonimmunized mothers (Figure 5B), in agreement with results in NZW rabbits (Figure 3B). As expected, IgG antibodies to MDA–LDL in offspring of OxLDL-immunized mothers exceeded those in both controls at 4 weeks, but not thereafter (Figure 5C). This reflects a much higher level of these antibodies in immunized mothers, their active transplacental transport, and their limited persistence in hypercholesterolemic offspring. In contrast, IgM antibodies to MDA–LDL were low and similar at 4 weeks, but began to differ at 12 weeks and were much greater in offspring of OxLDL-immunized than in nonimmunized mothers at 20 weeks (Figure 5D). Antibodies to phosphorylcholine (PC), an antigen recognized by innate IgM antibodies of the EO6/T15 idiotype, showed analogous differences (Figure 5F). However, direct measurements of circulating EO6/T15 antibodies revealed only a trend toward higher levels in offspring of OxLDL-immunized mothers at 20 weeks (not shown). Neither the increase in IgM-LDL immune complexes nor that of IgM antibodies to oxidation-specific epitopes was attributable to differences in total immunoglobulins (Figure 5G).

Consistent with the increase in IgM antibodies and immune complexes detected by ELISA, ELISpot analysis indicated a significantly greater percentage of OxLDL-specific IgM-secreting splenocytes in offspring of OxLDL-immunized mothers than in nonimmunized controls, whereas offspring of PBS-immunized mothers displayed an intermediate level (Figure 5H). Splenocytes of 20-week-old female offspring of OxLDL-immunized mothers also secreted significantly more IFNγ than those of nonimmunized controls (9,531 ± 3605 versus 2,426 ± 872 arbitrary units in BioRay mouse cytokine array I; P < 0.01), consistent with increased lymphocyte activation.

Overall, the effect of maternal OxLDL immunization on spontaneous postnatal IgM immune responses in markedly hypercholesterolemic murine offspring was more extensive than that in mildly hypercholesterolemic rabbit offspring, but similar to that observed in normcholesterolemic rabbits after exogenous immune challenge with OxLDL and KLH. Antibody and immune complex measurements in male LDLR−/− mice were similar to those in females (not shown).

The above results demonstrate increased B cell and antibody responses in offspring of OxLDL-immunized mothers, but FACS analysis indicated that this was not accompanied by relative increases of splenic B cells or CD4+ or CD8+ T cells, nor peritoneal B-1 cells (Table 2).

Maternal Immunization With OxLDL Reduces Offspring Atherosogenesis in LDLR−/− Mice

Time-averaged cholesterol levels in all 20-week-old female offspring was ∼800 mg/dL (Figure 6A). Maternal immunization reduced cross-sectional lesion areas by 26%, compared with nonimmunized controls (P < 0.05) (Figure 6B through 6D). In 34-week-old male offspring, atherosclerosis of non-
immunized controls covered 19.7% of the aortic surface, and maternal OxLDL immunization achieved an almost identical protective effect as in the aortic origin of 20-week-old females (Figure 6E through 6F). These results indicate that the antiatherogenic effect is not limited to a particular gender, anatomical site, stage of lesion, or parameter measured. Overall, the antiatherogenic effect of maternal immunization was less than that in rabbits, which may be because of the moderate maternal hypercholesterolemia and a lesser impact of immune mechanisms in offspring exposed to much greater postnatal hypercholesterolemia. The effects of maternal immunization on postnatal humoral immunity and atherosclerosis thus appear to be species-independent.

Maternal Immunization of LDLR−/− Mice With KLH Does Not Affect Offspring Immune Responses

The increased antibody binding to native LDL in atherosclerotic offspring of OxLDL-immunized mice and immune-challenged naïve NZW rabbits, and the partial antiatherogenic effect of maternal immunization with PBS + FA suggest that in utero programming is not specific for, or limited to, OxLDL. To assess whether the presence of the antigen in
IgM responses through maternal immunization with selected antigens, and may open a new preventive approach not only for atherosclerosis, but also for other immune-modulated diseases.

Given the marked atherogenic effect of maternal hypercholesterolemia,2–5,32 which was confirmed by the present data (Figure 1C), we expected maternal OxLDL immunization to protect against atherogenic programming by reducing fetal cholesterol exposure, eg, by the formation of immune complexes between induced antibodies and LDL bearing a limited number of oxidation-specific epitopes and their subsequent removal from the circulation.19 The observation of the greatest relative antiatherogenic effect in offspring of hypercholesterolemic rabbits suggests that this was indeed the primary mechanism. However, offspring of extensively and marginally hypercholesterolemic NZW mothers exposed to the same dietary cholesterol levels developed almost identical lesions. Thus, the antiatherogenic mechanism was not limited to a decrease in fetal cholesterol exposure (which enhances postnatal lesion formation in a dose-dependent manner4,5). In view of the well documented antiatherogenic effects of OxLDL immunization exerted in the immunized experimental subjects,16–21,33 it can be assumed that some of the immune responses primed in offspring contributed to the postnatal antiatherogenic effect by similar mechanisms, even though our experimental design does not permit to conclusively establish this. In fact, the observation that only selective humoral immune responses were enhanced in naïve offspring, whereas much broader cellular and humoral responses are triggered in immunized animals (mothers) should help identify the most effective antiatherogenic mechanisms/antibodies.

Nonspecific immune stimulation of hypercholesterolemic mothers with PBS+FA also reduced offspring atherogenesis in rabbits, albeit to a lesser degree than immunization with OxLDL (Figure 1C), and trends toward less atherosclerosis were also noted in mice (Figure 6). The underlying mechanism is not clear, but immunization with adjuvant could have enhanced immune responses to endogenously formed OxLDL, or promoted antigen formation by oxidizing LDL through local inflammation. Even modest increases in antibody titers to oxidation-specific epitopes have previously been associated with substantial decreases in atherogenesis.17,19

The most unexpected and fundamentally important finding was the programming of postnatal IgM responses. Evidence in support includes persistently increased IgM immune complexes in rabbit and mouse offspring subjected to postnatal hypercholesterolemia (Figures 3B and 5B), increased IgM antibodies to various oxidation-specific epitopes in mice (Figure 5D through 5F), and markedly greater humoral immune responses to OxLDL, but not KLH, in naïve normocholesterolemic rabbit offspring after an immune challenge with both antigens (Figure 4). The simultaneous increase of IgG and IgM to some epitopes of OxLDL after immune challenge of naïve rabbits (Figure 4C, 4D, 4G, and 4H) also supports the concept of immune programming. Although it is tempting to assume that the passing-on of maternal immune memory would convey an evolutionary advantage, to date the only well established mechanism consists of the temporary

**TABLE 2. FACS Analysis of Splenocytes and Peritoneal Cells From 6 to 7-Week-Old Offspring of OxLDL-Immunized and Nonimmunized LDLR<sup>−/−</sup> Mice**

<table>
<thead>
<tr>
<th></th>
<th>OxLDL-immunized</th>
<th>Non-immunized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peritoneal Cavity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM&lt;sup&gt;+&lt;/sup&gt; CD11b&lt;sup&gt;+&lt;/sup&gt; B cells, % of peritoneal cells</td>
<td>30.50±2.41</td>
<td>30.70±1.86</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM&lt;sup&gt;+&lt;/sup&gt; CD43&lt;sup&gt;+&lt;/sup&gt; B-1 cells, % of splenocytes</td>
<td>3.67±0.20</td>
<td>3.67±0.20</td>
</tr>
<tr>
<td>B220&lt;sup&gt;+&lt;/sup&gt; B cells, % of splenocytes</td>
<td>51.5±1.59</td>
<td>49.4±2.57</td>
</tr>
<tr>
<td>Follicular B cells, % of splenic B cells</td>
<td>70.1±1.72</td>
<td>73.3±0.49</td>
</tr>
<tr>
<td>Marginal Zone B cells, % of splenic B cells</td>
<td>4.0±0.23</td>
<td>4.3±0.35</td>
</tr>
<tr>
<td>CD8&lt;sup&gt;+&lt;/sup&gt; T cells, % of splenocytes</td>
<td>13.0±1.67</td>
<td>13.9±1.63</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; T cells, % of splenocytes</td>
<td>24.1±2.50</td>
<td>24.6±2.45</td>
</tr>
</tbody>
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arterial or other tissues of fetuses and offspring is a prerequisite of immune programming, analogous maternal immunization was performed with KLH, a T cell–dependent antigen that does not naturally occur in mammals. Six-week-old offspring of KLH-immunized and nonimmunized controls (n=11, each) were subjected to a single injection of KLH (without adjuvant), and antibodies to KLH and OxLDL followed for 6 weeks. In contrast to the analogous experiment in NZW rabbits, which yielded significantly greater responses to OxLDL, but not KLH, in offspring of OxLDL-immunized mothers, maternal immunization with KLH did not increase IgG or IgM responses to KLH (Figure 7A through 7E). In offspring of KLH-immunized mothers not subjected to the postnatal KLH injection, increased concentrations of IgG to KLH were evident during the first 2 weeks, compared with nonchallenged controls, reflecting an increased transfer of maternal IgG antibodies. KLH challenge lead to marked increases of both IgG and IgM, but no differences between maternal groups. As expected, in the absence of an OxLDL challenge, no significant increase and no differences between groups were seen for oxidation-specific IgG (Figure 7B and 7C) and IgM (Figure 7F and 7G). Thus, the programming of postnatal immune responses either depends on the presence of the antigen in fetuses or offspring or is limited to T cell independent antigens. Results of KLH immunization also provide additional evidence that the results in challenged offspring of OxLDL-immunized rabbits and mice were not attributable to persistence of maternal IgG.

**Discussion**

The present results demonstrate that maternal immunization with antigens prevalent in atherosclerotic lesions markedly reduces atherogenesis in adult offspring by mechanisms that include the protection of the fetus against atherogenic programming by maternal hypercholesterolemia. They also indicate that maternal immunization with OxLDL causes persistent changes in specific postnatal B cell and antibody responses that are independent of transplacental passage of immunoglobulins. This establishes, in principle, that it is
protection of neonates by maternal IgG antibodies carried across the placenta, or, in animals but not humans, antibody coating of enterocyte surfaces during lactation. All other active humoral immune defenses are thought to depend on mature B and T cells and to require “learning”, ie, clonal expansion of antigen-specific lymphocytes,34,35 with the possible exception of B-1 cell derived natural IgM antibodies that convey protection against a limited repertoire of bacterial antigens.36,37 Whereas offspring levels of IgG to MDA–LDL shortly after birth (Figure 5C) clearly reflected transplacental transport of high-titered maternal IgG antibodies induced by immunization (Figure 5A), this cannot account for later differences, eg, in immune-challenged rabbits, or differences in IgM antibodies, which do not cross the placental barrier (Figures 4 and 5D through 5F). The observation of increased splenic B cells secreting OxLDL-specific IgM in offspring of OxLDL-immunized mothers, both in normocholesterolemic immune-challenged rabbits (Figure 4M) and in hypercholesterolemic mice (Figure 5H), is also consistent with in utero immune programming, but not with placental or neonatal antibody transfer. However, it is possible that OxLDL spontaneously formed in vivo, in particular during hypercholesterolemia and atherogenesis, may have provided an essential natural boost enhancing the effect of in utero programming. Immunization with KLH indicated that fetal immune programming is dependent of the nature of the antigen, and that T cell–dependent antigens not present in fetuses and/or offspring do not affect postnatal immune responses. The present results suggest a prominent role of T cell–independent antigens. However, studies in immune-deficient models will have to establish the role of B cell subtypes and T cells as effectors or targets of in utero programming.

At present, we can only speculate about the nature of lymphocyte programming in utero. Fetal development of immune cells begins very early and hemopoietic cells in the mouse are already present at the end of the first trimester, but it remains unclear at what stage these progenitor cells differentiate into lymphocytes.36,39 Although adaptive immune defenses are generally thought to become fully functional only after birth, in humans mature CD4+ T cells already occur in the 17th week of gestation.40 OxLDL that could induce antigen-specific T cell responses is abundant in both plasma and arterial tissues of human fetuses by the end of the second trimester,7 and placental passage of maternal oxidized fatty acids may also increase fetal...
antibodies to OxLDL did not increase over time. Prohibitively increases, as in Figure 3). A, Circulating IgG antibodies to KLH challenge are included as additional controls. Therefore, results are reported as antibody concentrations (instead of relative increases, as in Figure 3). A, Circulating IgG antibodies to KLH, B and C, IgG to MDA-LDL and CuOx-LDL, respectively. D, IgM antibodies to KLH, E and F, IgM to MDA-LDL and CuOx-LDL, respectively. In the absence of an OxLDL challenge, antibodies to OxLDL did not increase over time.

antigens. It is therefore possible that maternal immunization modulates the amount of antigen in mothers and fetuses, and thus fetal lymphocyte differentiation. Postnatal immune memory could also be influenced by the amount of antigen persisting in lymphoid tissue.

The concept that postnatal immune responses are programmed in utero is contrary to the prevailing notion that modulation of adaptive immunity requires mature B and T cells. It is of course possible that no interactions of fetal lymphocytes with specific antigens or maternal antibodies are involved, and that the maternal immune response simply programs the reactivity of the fetal immune system by altering thresholds for activation or by influencing the development of subsets of immune cells. In this case, administration of cytokines, such as IFNγ, during pregnancy might have similar consequences. The weaker effects of maternal immunization with PBS + FA would be consistent with this assumption, but the failure of KLH immunization to influence postnatal immune responses does not support in utero effects of T cell cytokines. Our assumption that fetal immune cells are programmed in utero is also consistent with data suggesting that the in utero environment also plays an important role in the development of allergy later in life. In fact, allergen-specific T cells are already present in most newborns and seem to be of fetal origin. Evidence for an effect of prenatal immunization and for an involvement of maternal T and B cells in shaping allergic responses of adult offspring has also been reported. Finally, it is increasingly recognized that the differentiation and proliferation of B-1 cells secreting “natural” IgM antibodies is not limited to a brief postnatal period, but may already begin during fetal development, and that Th-independent antibody responses may be influenced by antigen exposure.

![Figure 7. Maternal immunization with KLH does not influence postnatal immune responses to KLH in mice. Offspring of LDLR−/−mothers immunized with KLH without adjuvant (n = 11) and nonimmunized controls (n = 12) of regular chow received a single subcutaneous injection of KLH at age 6 weeks. IgG and IgM antibodies to KLH and OxLDL were followed for 6 weeks. Three offspring of each maternal group that were not subjected to KLH challenge are included as additional controls. Therefore, results are reported as antibody concentrations (instead of relative increases, as in Figure 3). A, Circulating IgG antibodies to KLH, B and C, IgG to MDA-LDL and CuOx-LDL, respectively. D, IgM antibodies to KLH, E and F, IgM to MDA-LDL and CuOx-LDL, respectively. In the absence of an OxLDL challenge, antibodies to OxLDL did not increase over time.](image)

Disclosures

A patent based on the present work is pending. The authors have no other financial interests to disclose.

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