Atherosclerosis Susceptibility in Mice Is Independent of the V1 Immunoglobulin Heavy Chain Gene


Objective—The V1 (VH107.1.42) immunoglobulin heavy chain gene is thought to be critical in producing IgM natural antibodies of the T15-idiotype that protect against both atherosclerosis and infection from Streptococcus pneumoniae. Our aim was to determine whether genetic loss of the V1 gene increased atherosclerotic plaque burden in vivo because of a reduction in the T15-idiotype or other atheroprotective antibodies.

Approach and Results—We crossed Vh107.1.42-deficient mice with the atherosclerosis-prone Apoe−/− and Ldlr−/− strains. Although these double knockout strains manifested no defects in B-cell development, we did observe a substantial reduction in early immune responses against phosphocholine after immunization. However, the titers of plasma antibodies reacting against defined atherosclerotic antigens such as oxidized low-density lipoprotein, as well as the T15-idiotype, were unaffected by loss of the Vh107.1.42 gene in hypercholesterolemic mice. Furthermore, we observed no increase in atherosclerotic lesion formation, either within the aortic arch or aortic root. Robust deposition of IgM within atherosclerotic plaques could also be readily observed in both control and experimental mice.

Conclusions—Our data indicate that IgM-dependent protection against atherosclerosis is unlikely to be dependent on antibodies that use the Vh107.1.42 gene, in contrast to the acute immune response conferred by this heavy chain in the response to phosphocholine and in providing resistance against lethal S pneumoniae infection. (Arterioscler Thromb Vase Biol. 2016;36:25-36. DOI: 10.1161/ATVBAHA.115.305990.)

Key Words: aorta, thoracic ■ atherosclerosis ■ B-lymphocytes ■ mice ■ phosphorylcholine

There is a general consensus that the pathology of atherosclerotic plaque formation includes a strong inflammatory component and that selective amelioration of this inflammation could be of therapeutic benefit. One of the immune cell subsets implicated in atherosclerosis disease progression are B lymphocytes, which as antibody-secreting cells form the humoral arm of immunity. B cells have been shown to have ambivalent functions in atherosclerotic plaque formation. Splenectomy or genetic ablation of B cells resulted in an overall increase in atherosclerosis in susceptible animal models, whereas acute B-cell depletion via anti-CD20 therapy indicated possible proatherogenic functions. However, a major humoral function of B cells is the secretion of the so-called natural antibodies that mediate protection from atherosclerosis because T15-id IgM antibodies of the T15-idiotype that protect against both atherosclerosis and infection from S pneumoniae that is observed after splenectomy, whereas transfer of wild-type B-1 cells fully restored plasma IgM levels and prevented accelerated lesion formation in these mice.

Many antigens have been implicated in the process of atherosclerotic lesion formation. Prominent among these is the modification of low-density lipoprotein (LDL) into oxidized low-density lipoprotein (OxLDL). This modification unmasks the phosphocholine epitope of oxidized phospholipids that is subsequently recognized by the natural antibody identified by the T15-idiotype (T15-id). The recognition of OxLDL by T15-id antibodies is thought to mediate protection from atherosclerosis because T15-id IgM antibodies have been shown to limit proinflammatory cytokine secretion by oxidized phospholipids in macrophages, block OxLDL-induced foam cell formation, and promote apoptotic cell clearance.

In addition, transfer of B-1 cells that cannot secrete IgM fails to protect against the increase in atherosclerosis in Apoe−/− mice that is observed after splenectomy, whereas transfer of wild-type B-1 cells fully restored plasma IgM levels and prevented accelerated lesion formation in these mice. Natural antibodies are generally germline encoded and of the IgM isotype and are thought to be predominately produced by the B-1 type of mature B cells. Mice unable to secrete IgM are more prone to diet-induced atherosclerosis. In conclusion, atherogenic functions. However, a major humoral function of B cells is the secretion of the so-called natural antibodies that mediate protection from atherosclerosis because T15-id IgM antibodies of the T15-idiotype that protect against both atherosclerosis and infection from S pneumoniae that is observed after splenectomy, whereas transfer of wild-type B-1 cells fully restored plasma IgM levels and prevented accelerated lesion formation in these mice. Many antigens have been implicated in the process of atherosclerotic lesion formation. Prominent among these is the modification of low-density lipoprotein (LDL) into oxidized low-density lipoprotein (OxLDL). This modification unmasks the phosphocholine epitope of oxidized phospholipids that is subsequently recognized by the natural antibody identified by the T15-idiotype (T15-id). The recognition of OxLDL by T15-id antibodies is thought to mediate protection from atherosclerosis because T15-id IgM antibodies have been shown to limit proinflammatory cytokine secretion by oxidized phospholipids in macrophages, block OxLDL-induced foam cell formation, and promote apoptotic cell clearance.
The T15-id was originally discovered as a phosphocholine binding antibody of the IgA subtype from hybridomas obtained after pristane injection. The production of the T15-id is thought to be absolutely dependent on a successful V(D)J recombination event between the V$\mu$S107.1.42 (also known as V17), D$\mu$FL16.1 and the J$\mu$1 gene segments. The immune reaction to phosphocholine in a hapten form is characterized by an early response dominated by T15-id B cell clones. The secondary immune response against phosphocholine differs as the humoral response consists of antibodies that have incorporated V$\mu$S107.1.42 immunoglobulin heavy chains in their rearrangement but are T15-id negative or alternatively uses other V$\mu$ genes in a productive V(D)J rearrangement but nevertheless bind phosphocholine.

A well-characterized protective physiological role of T15-id antibodies is the recognition of phosphocholine present in the capsular polysaccharide of Streptococcus pneumoniae. The T15-id is produced in both germfree and conventional mice from $\approx$ 1 week after birth, thereby providing protection against S pneumoniae infection as a part of the natural immunoglobulin repertoire. Nonimmunized mice deficient for the V$\mu$S107.1.42 gene are highly susceptible to death after S pneumonia infection. This is thought to be because of the lack of T15-id antibodies induced in the early immune response that would normally prevent binding of S pneumoniae to the platelet-activating factor receptor and subsequent transport across host cell membranes. Immunization of Ldlr$^{−/−}$ mice with heat killed S pneumoniae increases the titers of T15-id IgM antibodies that bind OxLDL and decreases the extent of atherosclerosis. Moreover, infusion of T15-id IgM antibodies reduces plaque formation within grafted veins of Apoe$^{−/−}$ mice, whereas this was not sufficient to affect the extent of native atherosclerosis in the aortic origin. In addition, infusion of T15-Id IgM had no effect on accelerated atherosclerosis in a carotid cuff model in Apoe$^{−/−}$ mice fed an atherogenic diet. However, the duration of the T15-id IgM intervention or the levels achieved by the infusion strategy may not have been sufficient to mediate a protective effect in these studies.

In addition to the T15-id encoding V$\mu$S107.1.42 gene segment, many other V$\mu$ genes can be incorporated into antiphosphocholine-binding specificities. Noticeably, mice that are deficient for D$\mu$FL16.1 lose T15-id IgM production and have increased susceptibility to S pneumoniae infection, yet have normal levels of serum anti-OxLDL antibodies. Therefore, we wished to determine whether the loss of the V$\mu$S107.1.42 gene is similarly critical against the development of atherosclerosis as it is for the protection against lethal S pneumoniae infection. We present evidence that the atheroprotective effects of IgM do not depend on serum immunoglobulins that require the V$\mu$S107.1.42 gene.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Normal B-Cell Development in the Absence of the V$\mu$S107.1.42 Gene

The production of T15-id antibodies is thought to be critically dependent on the V$\mu$S107.1.42 gene. However, the in vivo function of V$\mu$S107.1.42 with regard to B-cell development and atherosclerosis has to date not been determined. We first quantified the expression levels of the V$\mu$S107.1.42 gene compared with those of the neighboring V$\mu$ genes in splenic B-1 cells using previously published mRNA-seq analysis of sorted cell populations from the spleen (Figure 1A). When compared with the most highly expressed B-1 V$\mu$ gene, namely V$\mu$1-J2.53, V$\mu$S107.1.42 is expressed 77-fold lower (Figure 1B) and is only the 61st most highly expressed functional V$\mu$ gene of the 113 measured in splenic B-1 lymphocytes. Next, we crossed V$\mu$S107.1.42$^{−/−}$ mice onto the Apoe$^{−/−}$ background, so that effects of V$\mu$S107.1.42 in the context of hyperlipidemia and spontaneous atherosclerotic plaque formation could be ascertained. We confirmed these mice were deficient for the V$\mu$S107.1.42 gene by designing PCR primers specific for this gene segment. Whereas we could readily detect a positive PCR band in control Apoe$^{−/−}$ V$\mu$S107.1.42$^{+/−}$ mice for both the V$\mu$S107.1.42 and the related V$\mu$S107.3.62 genes, we could only detect the presence of V$\mu$S107.3.62 in Apoe$^{−/−}$ S107.1.42$^{−/−}$ experimental mice (Figure 1C), consistent with the fact that the V$\mu$S107.1.42 has been deleted in experimental mice. Noticeably, we could also confirm the presence of the most 5’ and 3’ proximal genes to V$\mu$S107.1.42, namely V$\mu$Q52.13.40 and the V$\mu$S107.2pg.43 pseudogene, respectively (Figure 1C). Therefore, the Apoe$^{−/−}$ V$\mu$S107.1.42$^{−/−}$ experimental mice are deleted for the V$\mu$S107.1.42 gene in a minimally intrusive manner that has not lead to spurious deletions within the neighboring immunoglobulin heavy chain environment.

In the mouse, bone marrow lymphocytes commit to the B-cell lineage at the pro-B stage of development (defined CD19$^{+}$c-Kit$^{+}$) with V$\mu$-D$\mu$J$\mu$ recombination of the immunoglobulin heavy chain also occurring at this developmental stage. Successful recombination of one of the IgH alleles results in the formation of the pre–B-cell receptor at the immature stage. Finally, B lymphocytes exit from the immature stage into the pre-B-cell stage (CD19$^{+}$c-Kit$^{−}$) with V$\mu$J$\mu$ recombination and progression to the pre-B stage of development (CD19$^{+}$CD25$^{+}$IgM$^{−}$) followed by immunoglobulin light chain recombination and progression to the immature stage. Finally, B lymphocytes exit from the bone marrow to the spleen for the final maturation stages but can return as recirculating B cells. It has previously been reported that between 32% and 64% of hybridomas obtained from aged Apoe$^{−/−}$ mice on a hybrid C57BL/129 background specifically recognize OxLDL, and that some of these secrete antibodies of the T15-id. Therefore, it was possible that loss of the V$\mu$S107.1.42 encoded T15-id repertoire may impact B-cell development in the context of an Apoe$^{−/−}$ background even though, as described above, this V$\mu$ gene is not highly expressed. Therefore, we analyzed early B-cell development in V$\mu$S107.1.42-deficient mice through fluorescence-activated cell sorter analysis of the bone marrow from 4- to 6-week-old Apoe$^{−/−}$ V$\mu$S107.1.42$^{−/−}$, Apoe$^{−/−}$ V$\mu$S107.1.42$^{+/−}$, and
Atherosclerosis in the Absence of the V1 Gene

We found no significant differences in the ratios or absolute cell numbers of any B-cell examined stages in the 3 cohorts of mice. Therefore, we conclude that loss of a single or both copies of the VHS107.1.42 gene does not affect early B-cell development in the bone marrow.

Figure 1. Spleen B-cell development in the absence of the VHS107.1.42 gene. A, Schematic representation of the immunoglobulin heavy chain with selected V\textsubscript{H} genes annotated and the region around V\textsubscript{H}S107.1.42 enlarged. Previously published mRNA-seq reads are indicated and displayed logarithmically as reads per million (RPM). The V\textsubscript{H}S107.1.42 gene is outlined with a red box. B, Expression levels in RPM of V\textsubscript{H}S107.1.42 and V\textsubscript{H}11.2.53 genes from spleen B1 and follicular B cells. C, Polymerase chain reaction amplification from genomic DNA of the V\textsubscript{H}S107.3.62, V\textsubscript{H}S107.1.42, V\textsubscript{H}S107.2pg.43, and V\textsubscript{H}Q52.13.40 genes from Apoe\textsuperscript{−/−} V\textsubscript{H}S107.1.42\textsuperscript{−/+} control and Apoe\textsuperscript{−/−} V\textsubscript{H}S107.1.42\textsuperscript{−/−} experimental mice as indicated. D, Fluorescence-activated cell sorter analysis of bone marrow for B-cell differentiation in Apoe\textsuperscript{−/−} V\textsubscript{H}S107.1.42\textsuperscript{−/+}, Apoe\textsuperscript{−/−} V\textsubscript{H}S107.1.42\textsuperscript{−/−}, and Apoe\textsuperscript{−/−} V\textsubscript{H}S107.1.42\textsuperscript{−/−} mice aged 4 to 6 wk. Cells were gated to calculate absolute cell numbers for B1 (CD19\textsuperscript{−}B220\textsuperscript{−}), B-2 (CD19\textsuperscript{−}B220\textsuperscript{−}), pro-B (CD19\textsuperscript{−}B220\textsuperscript{−}c-Kit\textsuperscript{−}CD25\textsuperscript{−}IgM\textsuperscript{−}), pre-B (CD19\textsuperscript{−}B220\textsuperscript{−}c-Kit\textsuperscript{−}CD25\textsuperscript{−}IgM\textsuperscript{−}), immature B (CD19\textsuperscript{−}B220loIgM\textsuperscript{+}), and recirculating (CD19\textsuperscript{−}B220\textsuperscript{+}IgM\textsuperscript{+}) populations as shown in the graphs on the right. Each point represents an individual mouse, and error bars represent SEM.

Role of the V\textsubscript{H}S107.1.42 Gene in the Formation of Mature B-Cell Compartments and the Response to Phosphocholine-KLH Immunization

Mature B-1 cells can be readily isolated in the spleen and peritoneal cavity of the mouse. Therefore, we quantified the total number of B-1 (CD19\textsuperscript{−}B220lo) and B-2 (CD19\textsuperscript{−}B220\textsuperscript{−}) cells...
Figure 2. The dependence of peripheral B-cell development and immune responses on the VHS107.1.42 gene. **A**, Representative fluorescence-activated cell sorter (FACS) analysis of splenic B-cell populations in Apoe<sup>-/-</sup>S107.1.42<sup>+/+</sup>, Apoe<sup>-/-</sup>S107.1.42<sup>+/−</sup>, and Apoe<sup>-/-</sup>S107.1.42<sup>−/−</sup> mice aged 10 to 14 wk shown in the left. Cells were gated to calculate absolute cell numbers for B-1 (Continued)
at these 2 sites. Fluorescence-activated cell sorter analysis of the spleen from 10- to 14-week-old Apoe−/− VHS107.1.42+/−, Apoe−/− VHS107.1.42+/-, and Apoe−/− VpS107.1.42−/− mice demonstrated consistent numbers of B-1 and B-2 lymphocytes across all 3 genotypes (Figure 2A), strongly arguing against a critical function of the VpS107.1.42 gene in the generation of the peripheral B-1 cell population. Furthermore, these CD19−B220lo B-1 cells were uniformly CD43+ and could be further subdivide into CD5+(B-1a) and CD5− (B-1b) populations. We also repeated this analysis on the peritoneal cavity of 10- to 14-week-old mice (Figure 2B), and again we could not detect any significant differences in the mature B-1 and B-2 cell populations regardless of the presence or absence of the VpS107.1.42−/− immunoglobulin heavy chain gene.

It has been previously reported that VpS107.1.42−/− mice have strongly reduced primary immune responses to the hapten phosphocholine. We repeated this analysis using control Apoe−/− VpS107.1.42−/− and Apoe−/− VHS107.1.42−/− experimental mice immunized with phosphocholine-KLH (phosphocholine coupled to keyhole limpet hemocyanin) with Alum adjuvant. Both control and experimental mice robustly formed germinal centers and produced plasma cells 6 days after primary immunization (Figure 2C). However, we observed a significant 88% reduction in IgM antibodies recognizing phosphocholine in experimental mice consistent with the original description of the VpS107.1.42−/− mouse strain (Figure 2D). Finally, we performed competition ELISA assays on control mice using the AB1.2 clone that recognizes the T15-id as a capturing agent, and AB1.2, but not BSA, efficiently competed binding indicating that this primary antiphosphocholine response consists of the T15-id. Taken together, control Apoe−/− VpS107.1.42−/− and Apoe−/− VpS107.1.42−/− experimental mice have indistinguishable B-cell compartments under steady state conditions; however, the Apoe−/− VpS107.1.42−/− strain manifests a highly specific defect in the primary immune response to the phosphocholine hapten.

**Contribution of the VpS107.1.42 Encoded Antibodies to Humoral Immune Responses Against Common Atherosclerosis Antigens**

We next wished to determine how the loss of the VpS107.1.42 gene segment would affect the titers of selected immunoglobulins by using ELISA assays on plasma from male (Figure 3A) and female (Figure 3B) mice aged 10 or 20 weeks. Total plasma IgM levels in control Apoe−/− VpS107.1.42−/− and Apoe−/− VpS107.1.42−/− experimental mice were similar. Surprisingly, however, reactivity of plasma IgM with the AB1-2 monoclonal antibody, which specifically recognizes the heavy and light chain of T15-id,32 was unaffected by the loss of the VpS107.1.42 gene. Multiple antibody specificities, including the VpS107.1.42 encoded T15-id, react against phosphocholine (phosphocholine) and to antigens present in Cu2+ OxLDL. We next measured plasma IgM antibodies reacting against these antigens as well as another model of OxLDL, malondialdehyde modified LDL (MDA-LDL). Similar levels of reactivity against phosphocholine, Cu2+ OxLDL, and malondialdehyde modified LDL were present in both control Apoe−/− VpS107.1.42−/− and Apoe−/− VHS107.1.42−/− experimental mice. We next repeated these experiments using Ldlr−/− VpS107.1.42−/− and Ldlr−/− VHS107.1.42−/− mice and again found similar responses to these antigens (Figure 3C). In summary, the loss of the VHS107.1.42−/− gene does not reduce the plasma IgM titers against common atherosclerosis-associated antigens.

**Normal Development of Atherosclerosis in the Absence of VpS107.1.42 Encoded Immunoglobulins**

To gain insight into the role of the VpS107.1.42 gene in atherosclerosis development, we examined the extent of atherosclerotic lesion formation in our cohort of control Apoe−/− VpS107.1.42−/− and Apoe−/− VpS107.1.42+/- mice. We measured plaque development by en face staining of aortas in male and female mice at 10 and 20 weeks fed a normal chow diet. We found no significant difference in lesion formation between control and experimental mice at either 10 (Figure 4A) or 20 (Figure 4B) weeks regardless of sex. To evaluate whether VpS107.1.42 could help protect against atherosclerosis in the context of a Western diet, 10-week-old control male Apoe−/− VpS107.1.42−/− and Apoe−/− VpS107.1.42+/- mice were placed on a Western diet for 10 to 12 weeks. Analysis of the aortas by en face staining again revealed no significant difference in atherosclerosis plaque formation (Figure 4C). Finally, we determined atherosclerotic plaque formation in a separate strain of atherosclerotic prone mice. It has previously been shown that Ldlr−/− mice develop atherosclerosis in a diet-dependent manner.33 Therefore, we analyzed Ldlr−/− VpS107.1.42−/− and Ldlr−/− VHS107.1.42−/− mice to determine whether the VHS107.1.42 gene protects against atherosclerosis in a strain-dependent manner. Male mice aged 12 weeks were placed on a Western diet for a further 8 weeks before analysis. Similar to the Apoe−/− strain, induction of atherosclerosis in Ldlr−/− mice through Western diet was not affected by the loss of the VHS107.1.42 gene (Figure 4D). In summary, mice deficient for VHS107.1.42 develop atherosclerotic plaques similar to control mice independently of age, sex, diet, or genetic background.  

**Figure 2 Continued.** (CD19−B220lo) and B-2 (CD19−B220+) populations. Each point represents an individual mouse, and error bars represent SEM. Spleen B-1 and B-2 cells from 6-week-old mice were further analyzed for CD5 and CD43 expression as shown in the histograms. B, FACS analysis of the peritoneal cavity from 10- to 14-week-old mice as described in A. C, Spleen analysis from Apoe−/− VHS107.1.42−/− control and Apoe−/− VpS107.1.42−/− experimental mice aged 10 to 12 wk injected with phosphocholine (PC)-KLH/alum and analyzed after 6 d. Top, Germline center B cells (CD19−B220−IgD−CD95−GL7+) and the bottom plasma cells (CD19−B220loCD138−CD28+). Absolute cell numbers for the germline center (GC) and PC cell populations calculated from these gates are depicted in the graphs in the right. Control mice are depicted by white bars and experimental in gray. D, ELISA measurements of plasma antiphosphocholine IgM responses in plasma 6 d after PC-KLH/alum immunization from Apoe−/− VpS107.1.42−/− control and Apoe−/− VHS107.1.42−/− experimental mice. E, Competition ELISA with pooled plasma from Apoe−/− VpS107.1.42−/− mice captured with AB1.2 (anti-T15-id) and competed as indicated.
Function of V₄ᵥS107.1.42 Antibodies on Plasma Lipids

T15-id antibodies have been shown to form circulating immune complexes with ApoB100-carrying particles and have been suggested to function in preventing the uptake of OxLDL by macrophages. Therefore, we determined the plasma concentrations of cholesterol and triglycerides in control Apoε⁻/⁻ V₄ᵥS107.1.42⁺/⁻ and Apoε⁻/⁻ V₄ᵥS107.1.42⁻/⁻ experimental mice at 20 weeks on both a normal and a Western diet, as well as Ldlr⁻/⁻ V₄ᵥS107.1.42⁺/⁻ and Ldlr⁻/⁻ V₄ᵥS107.1.42⁻/⁻ mice maintained on a Western diet. Although plasma cholesterol levels were not altered in control Apoε⁻/⁻ V₄ᵥS107.1.42⁺/⁻ and Apoε⁻/⁻ V₄ᵥS107.1.42⁻/⁻ experimental mice on either normal or Western diet, we did observe a significant

Figure 3. The loss of the VHS107.1.42 gene and humoral immune responses in atherosclerosis. ELISA analysis of 10- and 20-wk-old (A) male and (B) female Apoε⁻/⁻ V₄ᵥS107.1.42⁺/⁻ control and Apoε⁻/⁻ V₄ᵥS107.1.42⁻/⁻ experimental mice. Indicated are the total plasma IgM levels, titers of plasma IgM recognized by the AB1-2 antibody normalized to total IgM levels and plasma IgM levels against the common atherosclerosis-associated antigens phosphocholine (PO)-BSA, Cu²⁺ oxidized low-density lipoprotein (CuOx-LDL) and malondialdehyde modified (MDA)-LDL all normalized to total plasma IgM levels. Each point represents an individual mouse, and error bars represent SEM. Control mice are depicted by white bars, and experimental are by gray. (C), Analysis of male Ldlr⁻/⁻ V₄ᵥS107.1.42⁺/⁻ control and Ldlr⁻/⁻ V₄ᵥS107.1.42⁻/⁻ experimental mice.
Atherosclerosis in the Absence of the \( V_1 \) Gene

Centa et al

A decrease (26%) in plasma cholesterol levels in experimental \( \text{Ldlr}^{-/-} \) \( V_{H} S107.1.42^{+/+} \) versus \( \text{Ldlr}^{-/-} \) \( V_{H} S107.1.42^{-/-} \) control mice (Figure 5A). This pattern was repeated when plasma triglycerides were measured, with no effect seen on loss of the \( V_{H} S107.1.42 \) gene on the \( \text{Apoe}^{-/-} \) background, but a significant 47% drop in experimental \( \text{Ldlr}^{-/-} \) \( V_{H} S107.1.42^{-/-} \) versus \( \text{Ldlr}^{-/-} \) \( V_{H} S107.1.42^{+/+} \) control mice (Figure 5B). We next determined the relative association of cholesterol with different lipoproteins by plasma high-performance liquid chromatography fractionation. Analysis of pooled serum revealed a small increase in cholesterol associated with high-density lipoprotein in experimental \( \text{Ldlr}^{-/-} \) \( V_{H} S107.1.42^{-/-} \) versus \( \text{Ldlr}^{-/-} \) \( V_{H} S107.1.42^{+/+} \) control mice (Figure 5C). Interestingly, loss of the \( V_{H} S107.1.42 \) gene correlated with small but significant decreases in body weight on both the \( \text{Apoe}^{-/-} \) and \( \text{Ldlr}^{-/-} \) genetic backgrounds (Figure 5D). These experiments would indicate that any humoral immune-mediated effects on lowering blood cholesterol and triglyceride levels are likely to be independent of the \( V_{H} S107.1.42 \) T15-id antibody specificity.

Atherosclerotic Plaque Formation in the Aortic Root Is Independent of \( V_{H} S107.1.42 \)

Atherosclerotic plaque formation in the aortic root is associated with deposition of IgM that has previously been shown to contain the T15-id. Therefore, we quantify the extent of

Figure 4. The role of the \( V_{H} S107.1.42 \) gene in the initiation and maintenance of aortic atherosclerosis plaque formation. A, Sudan IV en face staining of control \( \text{Apoe}^{-/-} V_{H} S107.1.42^{+/+} \) and \( \text{Apoe}^{-/-} V_{H} S107.1.42^{-/-} \) experimental mice at 10 wk fed on conventional chow diet. Quantification of the total percentage lesion area in the aorta for male and female mice at 10 wk are shown with a representative picture from female mice depicted in the right. Each data point represents an individual mouse, and error bars indicate SEM. B, As above, but with mice at 20 wk on conventional chow diet. C, Atherosclerosis in male \( \text{Apoe}^{-/-} V_{H} S107.1.42^{+/+} \) control and \( \text{Apoe}^{-/-} V_{H} S107.1.42^{-/-} \) experimental mice fed a Western diet for 10 to 12 wk. D, Aortic lesion development in male \( \text{Ldlr}^{-/-} \) \( V_{H} S107.1.42^{+/+} \) control and \( \text{Ldlr}^{-/-} \) \( V_{H} S107.1.42^{-/-} \) experimental mice fed a Western diet for 8 wk.
**Figure 5.** Body weight and plasma lipid levels in the absence of the VHS107.1.42 gene. 

A. Plasma from 20-wk-old male Apoe−/−VHS107.1.42+/− control and Apoe−/−VHS107.1.42−/− experimental mice (left and middle) or Ldlr−/− VHS107.1.42+/+ control and Ldlr−/−VHS107.1.42−/− experimental mice (right) were assayed to quantify total blood cholesterol levels. Apoe−/− mice were either maintained on a conventional diet throughout or were first placed on conventional diet until 10 wk followed by 10 to 12 wk on Western diet. The Ldlr−/− strain was placed on a conventional diet for 12 wk followed by 8 wk on a Western diet. Each point represents an individual mouse, and error bars show SEM. Control mice are depicted by white bars, and experimental are by gray. *P<0.05 vs control. 

B. Plasma triglycerides levels from mice cohorts as described above. **P<0.01 vs control. 

C. High-performance liquid chromatography fractionation of plasma lipoproteins to determine relative cholesterol content from Ldlr−/−VHS107.1.42+/+ control and Ldlr−/−VHS107.1.42−/− experimental mice. CR indicates chylomicron remnant; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NC, normal chow; and VLDL, very low-density lipoprotein. 

D. Body weight from mice maintained on a Western diet as described above. ****P<0.0001 vs control.
plaque formation in the absence of the $V_\mu S107.1.42$ encoded T15-id. Serial aortic root sections from female control $\text{Apoe}^{-/-}$ $V_\mu S107.1.42^{-/-}$ and $\text{Apoe}^{-/-} V_\nu S107.1.42^{-/-}$ experimental mice aged 20 weeks maintained on normal chow diet were stained with Oil Red O, and the total lesion area was quantified. We did not detect any significant differences in plaque formation between control and experimental mice similar to the results from the en face analysis of the aorta. Female $\text{Apoe}^{-/-} V_\nu S107.1.42^{-/-}$ had a similar extent of atherosclerosis development in the aortic root as $\text{Apoe}^{-/-} V_\mu S107.1.42^{-/-}$ and $\text{Apoe}^{-/-} V_\nu S107.1.42^{-/-}$ mice, indicating that plaque formation proceeds similarly regardless of the status of the $V_\nu S107.1.42$ gene (Figure 6A). In addition, subjecting male mice to 8 weeks of Western diet approximately doubled the extent of atherosclerosis but again quantification revealed no difference between control and experimental mice (Figure 6B). Finally, using immunofluorescence, we were also able to robustly observe widespread

Figure 6. Aortic root atherosclerosis and IgM deposition in VHS107.1.42-deficient mice. A, Oil-red-O–stained aortic roots of 20-wk female $\text{Apoe}^{-/-}$ $V_\nu S107.1.42^{-/-}$, $\text{Apoe}^{-/-} V_\nu S107.1.42^{-/-}$, and $\text{Apoe}^{-/-} V_\nu S107.1.42^{-/-}$ mice maintained on a conventional chow diet. Quantification of the mice is shown on the right. The areas under the curves did not differ significantly between experimental and control groups. B, Male $\text{Apoe}^{-/-} V_\mu S107.1.42^{-/-}$ and $\text{Apoe}^{-/-} V_\nu S107.1.42^{-/-}$ mice fed conventional diet (top) or a Western diet for 8 wk (bottom). C, Confocal microscopy of total IgM (green) staining in aortic root sections from 20-wk-old female $\text{Apoe}^{-/-} V_\nu S107.1.42^{-/-}$ control and $\text{Apoe}^{-/-} V_\nu S107.1.42^{-/-}$ experimental mice on normal chow (DAPI stained in blue). NC indicates normal chow.
IgM deposition in the atherosclerotic plaque of 20-week-old female mice maintained on a normal chow diet even in the absence of the \( V_\mu S107.1.42 \) gene encoded T15-id antibodies (Figure 6C).

**Discussion**

Our current understanding of the pathology of both atherosclerotic plaque formation and protection from *S. pneumoniae* infection strongly implicates a protective function for T15-id natural antibodies secreted by the B-1 subset of mature B cells. In this study, we have crossed mice that are deleted for the \( V_\mu S107.1.42 \) gene, which was thought to be required to produce the T15-id, with well-characterized atherosclerosis-prone mouse strains to determine the in vivo function of this humoral entity on plaque formation. In contrast to the protection from lethal infection from *S. pneumoniae* that the \( V_\mu S107.1.42 \) confers, the development of atherosclerosis seems to be unaffected by the loss of the \( V_\mu S107.1.42 \) gene.

The 2 major \( V_\mu \) genes expressed in mature B-1 cells are the \( V_\mu 11.2.53 \) and \( V_\mu 12.1.78 \) segments. Interestingly, these genes when combined with the correct \( \kappa \) light chain are known to bind phosphatidylcholine, but their function in atherosclerosis has not been determined. In contrast, \( V_\mu S107.1.42 \) is only expressed in a minor fraction of B-1 cells, and unsurprisingly deletion of \( V_\mu S107.1.42 \) did not elicit a detectable defect in B-1 lymphopoiesis.

We were unable to observe any differences in the humoral responses to common atherosclerosis antigens after crossing \( V_\mu S107.1.42^{-/-} \) mice with either the \( \text{Apoe}^{-/-} \) or the \( Ldlr^{-/-} \) strains. This is in agreement with previous experiments that revealed normal titers of antibodies against modified LDL in the \( V_\mu S107.1.42^{-/-} \) strain when compared with control mice. Furthermore, loss of \( V_\mu S107.1.42 \) still permitted humoral responses to phosphocholine in otherwise nonhyperlipidemic mice. However, we did not observe the expected decrease in T15-id reactivity in our hyperlipidemic strains. Although \( V_\mu S107.1.42 \) is thought to be an essential component of the T15-id, many studies have shown that alternative immunoglobulin heavy chains can participate in a T15-id+ positive V(D)J rearrangement. Notably this seems to be strain specific. In the BALB/c strain, >99% of the T15-id incorporates \( V_\mu S107.1.42 \), whereas in BALB/cxCB6 F1 hybrids, this ratio drops to 32%. Interestingly, the majority of peritoneal B-1a cells from the BALB/c strain that react against both phosphocholine and AB1-2 seem to use a \( V_\mu 11 \) immunoglobulin heavy chain gene. Crucially, however, it is the canonical T15-id incorporating \( V_\mu S107.1.42 \) that provides a protective immune response against *S. pneumoniae*. In this regard, it is noticeable that the BALB/c strain is the most resistant to lethal *S. pneumoniae* infection, and it could be speculated that its almost total incorporation of \( V_\mu S107.1.42 \) in its T15-id may confer this serological protection. The BALB/c strain is also among the most resistant to diet-induced atherosclerosis, and the relative contribution of \( V_\mu \) genes to its atheroprotective humoral immune repertoire, not least within the T15-id, may in part contribute to atherosclerosis resistance. Notably, the \( \text{Apoe}^{-/-} V_\mu S107.1.42^{-/-} \) experimental mice used in this study still had a strongly impaired acute response to phosphocholine, as measured by antiphosphocholine IgM titers in the primary immune response to phosphocholine-KLH immunization. This is consistent with the original description of \( V_\mu S107.1.42^{-/-} \) mice, which also showed a highly impaired primary immune response, whereas secondary immune responses were less affected because of the production of heteroclitic antibodies. Similarly, we demonstrate that in aged mice, the antibody titers against phosphocholine remained constant in the absence of \( V_\mu S107.1.42 \). Therefore, we conclude that the acute \( V_\mu S107.1.42^{-/-} \)-dependent antiphosphocholine IgM primary immune response, which is critical against *S. pneumoniae* infection, is dispensable for the protection against atherosclerosis. Alternatively, hypercholesterolemia in atherosclerosis-prone mice fails to robustly trigger this primary response in marked contrast to the vigorous reaction after immunization or bacterial infection. This suggests that hypercholesterolemia affects T15-id IgM responses differently or to a lesser extent. However, this may not be the case for other T15-id responses, produced independently of \( V_\mu S107.1.42 \). In line with a protective role of T15-id IgM responses on immunization, expansion of T15-id IgM by pneumococcal immunization has been shown to decrease atherosclerotic lesion formation.

In addition, multiple antigenic epitopes on OxLDL are important in the immune response to atherosclerosis and likely these function in a redundant manner or elicit a large number of minor responses that are not easily distinguished. Notably, we observed normal deposition of IgM within aortic root plaques even in the absence of \( V_\mu S107.1.42 \)-containing T15-id antibodies.

In summary, we have used 2 different mouse strains that are susceptible to atherosclerosis and rendered these strains unable to produce \( V_\mu S107.1.42 \)-containing antibodies. Under all the conditions we tested, we were unable to detect an effect on atherosclerosis, regardless of sex, diet, age, or anatomic location of the plaque. Therefore, we conclude that the \( V_\mu S107.1.42 \) gene is dispensable for atherosclerosis initiation and maintenance, which is in strong contrast to the protection against *S. pneumoniae* that this immunoglobulin heavy chain gene segment confers. We further speculate that protection against atherosclerosis mediated by IgM is likely to be mediated by many different \( V_\mu \) genes with multiple specificities, for example, for epitopes of OxLDL.

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M. Centa performed most experiments using the \( \text{Apoe}^{-/-} \). S. Gruber performed the experiments using the \( Ldlr^{-/-} \) strain and performed ELISA. D.K. Johansson contributed to the initial atherosclerosis analysis. D. Nilsson prepared aortas together with M. Centa. M. Centa, S. Gruber, G.K. Hansson, C.J. Binder, and S. Malin devised experiments and interpreted results. S. Malin and C.J. Binder initiated the study. S. Malin wrote the article with contributions from M. Centa, S. Gruber, G.K. Hansson, D.F.J. Ketelhuth, and C.J. Binder.

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Disclosures

None.

References


**Significance**

It is thought that humoral immune responses in the form of natural IgM protects against atherosclerosis. T15 idiotype antibodies have been implicated in the recognition and clearing of oxidized low-density lipoprotein and being essential for protection from Streptococcus pneumoniae infection. Therefore, it has been hypothesized that there is a common genetic determinant in the protection against both atherosclerosis and S pneumoniae infection. We have crossed mice deficient for the VHS107.1.24 gene with both the Apoe−/− and Ldlr−/− strains. We find no evidence that VHS107.1.24 protects against atherosclerosis or is critical to maintaining normal plasma titers of antibodies against antigens implicated in atherosclerosis. Our results indicate that antibody-mediated protection against atherosclerosis by B lymphocytes is likely to require multiple immunoglobulin heavy chain genes.
Atherosclerosis Susceptibility in Mice Is Independent of the \( V_1 \) Immunoglobulin Heavy Chain Gene


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RNA-seq analysis of the Immunoglobulin heavy chain
Previously published data sets from spleen B-1 and follicular B cells were analysed with DESeq2 and visualised in the IgB browser.

Mice strains and genotyping
V_{H}S107.1.42^-/(V_{H}1) mice were obtained from the National Institutes of Health and National Institute of Aging. Mice were backcrossed 10 times onto the C57BL/6 background. Apoe^-/^- mice were originally obtained from Taconic. LDL receptor-deficient mice (Ldlr^-/-) on a C57BL/6 background were purchased originally from The Jackson Laboratories (Bar Harbor, Maine, USA). Mice were bred under standard housing conditions. Adult mice received chow diet R70 (1254 kJ/100g, 14% protein, 4,5% fat; Lantmännen, Sweden). In some instances Apoe^-/^- mice were also put on Western diet R638 (15,6 MJ/kg, 17,2% protein, 21% fat; Lantmännen, Sweden). Ldlr^-/^- mice were placed a Western diet for 8 weeks consisting of 21% milk fat, 0.2% cholesterol (Ssniff Spezialdiäten, Soest, Germany, TD88137). PCR genotyping on ear or tail biopsies was performed. The V_{H}S107.1.42 gene was amplified with the primers: 5´AGTGATTTCTACATGGAGTGGGT-3´ and 5´TCG GTTCATCGTCTCCAGAG-3´ (136bp). The V_{H}S107.3.62 gene was amplified with the following primers: 5´-TCCAGTGTGAGGTGAAGCTG-3´ and, 5´-GGAAGGCACTTATGGTTG-3´ (152bp). V_{H}Q52.13.40 was amplified with 5´-CCTGTCCATCATTTGACTTG-3´ and TTGGTATGCTACGTCTTG-3´ (163bp) and V_{H}S107.2pg.43 with primers 5´-TCCAGTGTGAGGTGAAGCTG-3´ and 5´-CCGACCCCTTCATAGTGAC-3´ (215bp).

The Apoe gene was amplified with the following primers: primer 1 (common), 5´-GCCTAGCCGAGGGAGAGCCG-3´, primer 2, 5´-TGGAATCTTGGAGCTCTGGCAG-3´ and primer 3, 5´-GCGGCCCCACTGCTAC-3´. Primers 1 and 2 amplified a 155 bp DNA fragment and primers 1 and 3 amplified a 245 bp DNA fragment. The Ldlr gene was amplified using following primers: Ldlr-forward: 5´-ACCCCAAGACGTGCTCCCAGGATG-3´; Ldlr-reverse: 5´-CGCAGTGCTCCTCATGACTTTG-3´; neo cassette primer: 5´-AGGTGAGATGACAGAGAAGAAG-3´. Primers 1 and 2 (forward and reverse) amplify a 384 bp Ldlr wild-type fragment and Primers 1 and 3 (forward and neo cassette primer) amplify an 800 bp DNA fragment for the identification of the knock-out.

All mouse experiments were approved by the Stockholm Regional Board for Animal Ethics, permit numbers N164/14 and N111/11 and by the institutional animal experimentation committee and the Austrian Ministry of Science.

FACS analysis of immune cell populations
Bone marrow, spleens and peritoneal cavity were analysed by flow cytometry. During all steps of the staining cells were kept in FACS buffer (0.5% BSA, 0.5% 1M EDTA in PBS). Tissues were fractioned to single cell suspension and filtered with 100 µm sterile cell strainers. Erythrocyes were lysed for 3 min with 1 ml of ACK buffer on ice. Cells were seeded at 100*10^6 cells/ml and Fc blocking was carried out for 30 min. Staining was performed on ice for 40 min against mouse B220 (APC-Cy7 RA3-6B2), CD19 (APC 1D3), CD25 (PE-Cy7 PC61.5), c-kit (PE 2B8), CD5 (PE 53-7.3), CD43 (FITC S7), GL7 (APC GL-7), CD95 (PE-Cy7 Jo2), CD138 (APC 281-2), CD28 (FITC E18), IgD (PerCP 11-26c.2a) and IgM (Pacific Blue Il/41). Immune cell populations were defined as follows: B-1 (CD19+B220lo), B-2 (CD19+B220+), Pro-B (CD19+B220+IgM-c-Kit+CD25-), Pre-B (CD19+B220+IgM-c-Kit+CD25+), Immature B (CD19+B220intIgM+), recirculating B (CD19+B220hilgM+), germinal B (CD19+B220+IgD-GL7+CD95+), plasma cells (CD19loB220loCD138+CD28+).

Samples were analysed with a Dako CyAn (Beckman Coulter) flow cytometer.
ELISA
PC-BSA and PC-KLH (keyhole limpet hemocyanin) was obtained from Biosearch Technologies Inc. CuOx-LDL and MDA-LDL was prepared as described previously. For immunisation with PC-KLH, 100 μg of PC-KLH was precipitated on Alum and injected ip. 6 days after immunisation the spleen was analysed by FACS. Specific IgM responses against PC was determined by ELISA using plates coated with PC-BSA using alkaline phosphatase coupled goat anti-mouse IgM (Southern Biotechnology Associates) and alkaline phosphatase yellow (pNPP) Liquid Substrate (Sigma-Aldrich). Other antigen-specific antibody titers in plasma were determined by chemiluminescent ELISA as previously described. Purified rat anti-mouse IgM (Sigma-Aldrich) was used as capture Ab to measure total IgM levels. AP-labelled goat anti-mouse IgM (μ-chain specific; Sigma-Aldrich) was used for detection. For the detection of T15-clone-specific antibodies (e.g. E06) in plasma were quantified using a chemiluminescent based capture assay. In brief, the monoclonal anti-idiotypic antibody AB1-2 (provided by J. Kearney (University of Alabama at Birmingham, Birmingham, Alabama, USA), a mouse IgG1 which identifies a determinant requiring co-expression of the canonical T15 VH/T15 VL regions) was used as capture antibody. T15-clone-specific antibodies were detected using an AP-labelled goat anti-mouse IgM (μ-chain specific; Sigma-Aldrich). Purified E06 (a clone-specific IgM monoclonal antibody) was used as a positive control. For competition assays, AB1-2 was used for coating at 1 μg/ml and AP-labelled goat anti-mouse IgM (μ-chain specific; Sigma-Aldrich) was used for detection in the presence of inhibitors as indicated.

Cholesterol and triglycerides measurements
Cholesterol and triglyceride levels in mouse plasma were quantified using an enzymatic colorimetric method (Randox Laboratories) following the manufacturer’s protocol. Cholesterol lipoprotein profiles in plasma were determined by size-exclusion chromatography using HR10/30 Superose 6 column (GE Healthcare) and a Discovery BIO GFC-500 precolumn (5 cm × 7.8 i.d.; Supelco; Sigma-Aldrich) coupled to Prominence UFLC system (Shimadzu). The system was equilibrated with Tris-buffered saline, pH 7.4, and fractions were collected using Foxy Jr. fraction collector (Teledyne Isco, Inc.). Total cholesterol in each fraction was determined as described (Randox Laboratory, Ltd.).

Tissue preparation
Experimental Apoe<sup>−/−</sup> V<sub>H</sub>S107.1.42<sup>−/−</sup> and littermate Apoe<sup>−/−</sup> V<sub>H</sub>S107.1.42<sup>+/−</sup> control mice were sacrificed following inhalation of carbon dioxide. Blood was collected from the heart, prior to perfusion performed with 5 ml of PBS. For en face analysis of lesion, aortas were fixed in 4% para-formaldehyde (PFA) for 24 hours. For histological analysis, hearts were mounted in OCT (TissueTek SAKURA) and stored at -80°C for cryosectioning. Sections of proximal aorta were collected every 10 μm for a total of 800 μm starting from the aortic root, that were fixed in 4% formaldehyde (for Oil Red O staining) or acetone (for immunofluorescence).

Analysis of atherosclerotic lesion in en face preparations of the aorta
En face staining of dissected aortas with Sudan IV (5 g Sudan IV Sigma, 500 ml 70% ethanol, 500 ml 100% acetone) was performed as follows. Aortas were rinsed in 70% ethanol for 5 min, and stained in Sudan IV for 7-8 min. Following a washing step in 80% ethanol for 30 sec, aortas were rinsed in PBS and pictures were taken using a light microscope. Lesion quantification was performed with ImageJ software.

Histology of aortic root sections
Oil Red O working solution was prepared from a stock solution (1g oil red O, Sigma, in 100 ml isopropanol) by diluting 150 ml of stock in 100 ml H2O. Aortic sections were incubated in filtered Oil Red O working solution for 20 min and rinsed in water for 5 min. Hematoxylin (Mayers HTX, Histolab) staining was carried out and rinsed in tepid H2O. Lesion quantification was performed with ImageJ software in 8 consecutive sections, each 100 µm apart from each other. Ldlr<sup>-/-</sup> mice were analysed as previously described.

**Immunofluorescence staining of aortic root sections**

Frozen sections of the aortic root were first blocked with an Avidin/Biotin Blocking kit (Vector Laboratories) followed by a protein blocking step with 5% normal horse serum diluted in 0.1% Tween in TBS. The sections were then stained for total IgM (biotinylated rat anti-mouse IgM, Southern Biotechnology, followed by DyLight 488 conjugated Streptavidin, VECTOR Laboratories). Total IgA staining (biotin-anti-IgA and DyLight 488 conjugated Streptavidin, VECTOR Laboratories) was used as negative control. To reduce auto-fluorescence, sections were blocked in 0.03% Sudan Black (Sigma) in 70% ethanol and counterstained with DAPI (Sigma).

**Statistical analysis**

The data were analysed using Graphpad Prism. Student’s t test was performed, and the significance level was set at P < 0.05.