

Pneumococcal vaccination decreases atherosclerotic lesion formation: molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL

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During the progression of atherosclerosis, autoantibodies are induced to epitopes of oxidized low-density lipoprotein (oxLDL) and active immunization of hypercholesterolemic mice with oxLDL ameliorates atherogenesis. We unexpectedly found that many autoantibodies to oxLDL derived from 'naive' atherosclerotic mice share complete genetic and structural identity with antibodies from the classic anti-phosphorylcholine B-cell clone, T15, which protect against common infectious pathogens, including pneumococci. To investigate whether *in vivo* exposure to pneumococci can affect atherogenesis, we immunized *Ldlr*^{-/-} mice with *Streptococcus pneumoniae*. This induced high circulating levels of oxLDL-specific IgM and a persistent expansion of oxLDL-specific T15 IgM-secreting B cells primarily in the spleen, which were cross-reactive with pneumococcal determinants. Pneumococcal immunization decreased the extent of atherosclerosis, and plasma from these mice had an enhanced capacity to block the binding of oxLDL to macrophages. These studies show molecular mimicry between epitopes of oxLDL and *S. pneumoniae* and indicate that these immune responses can have beneficial effects.

Atherosclerosis is a complex disease whose etiology is multifactorial, but clearly involves elevated low-density lipoprotein (LDL) levels¹. There is now evidence that atherosclerosis becomes a chronic inflammatory process and that the risk for its clinical sequelae is associated with elevation of markers of inflammation². Moreover, growing evidence suggests that both adaptive and innate immune mechanisms can modulate the progression of atherosclerosis³⁻⁵. Among the antigens identified in atherosclerotic lesions, oxLDL has a prominent role^{3,6}.

OxLDL contains a variety of oxidation-specific neopeptides on both the lipid and protein moieties⁶. For example, reactive decomposition products of phospholipid oxidation, such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC)⁷, can covalently modify protein and lipid moieties of LDL to form adducts that retain the intact phosphorylcholine headgroup⁸. Modification with POVPC and other decomposition products resulting from lipid peroxidation, such as malondialdehyde (MDA), leads to formation of neo-self epitopes that are recognized by innate and adaptive immunity^{9,10}.

Interactions of oxLDL with the innate immune system involve pattern-recognition scavenger receptors of macrophages, such as scavenger receptor A and CD36, which bind oxidation-specific ligands, including phosphorylcholine-containing oxidized phospholipids, and promote unregulated uptake of oxLDL³. The acute-phase reactant C-reactive protein, a primitive component of innate immunity and marker of atherosclerosis-related clinical events, also binds phosphorylcholine of oxidized phospholipids of oxLDL¹¹.

We previously documented that extensive atherosclerosis in apolipoprotein E-deficient (*ApoE*^{-/-}) mice is associated with robust antibody titers to oxLDL, enabling us to generate splenic B-cell lines from these 'naive' mice, which produced monoclonal IgM autoantibodies to oxLDL¹⁰, termed EO antibodies. Several different antibodies selected on the basis of binding to oxLDL were shown to recognize oxidized phospholipids containing the phosphorylcholine headgroup, such as POVPC, either present as an isolated lipid or covalently bound to apoB^{8,12}. These EO antibodies did not bind to native, unoxidized phospholipids even though they contained the same phosphorylcholine moiety^{8,12,13}. They also bound to the phosphorylcholine moiety of oxidized phospholipids in apoptotic cells, suggesting that LDL and viable cells contain a cryptic epitope, phosphorylcholine, that is revealed by oxidation or when cells undergo apoptosis^{10,13,14}. The EO antibodies to oxLDL blocked the binding and degradation of oxLDL by macrophages *in vitro*⁸. The genes encoding the antigen binding site of these EO antibodies (EO6, for example) were shown to be genetically and structurally indistinguishable from antibodies produced by the previously described B-1 cell clone, T15, which is known to be specific for phosphorylcholine¹³. T15 clonospesific natural antibodies have been studied for over 30 years and confer optimal protection to mice against lethal infection with *S. pneumoniae*^{15,16}, in which the same phosphorylcholine moiety is a prominent constituent of (lipo)teichoic acid components of the cell-wall polysaccharide¹⁷. In most mouse strains, the *in vivo* response to pneumococci is dominated by T15 antibodies. *In vitro* binding assays confirmed that the classic T15 antibody

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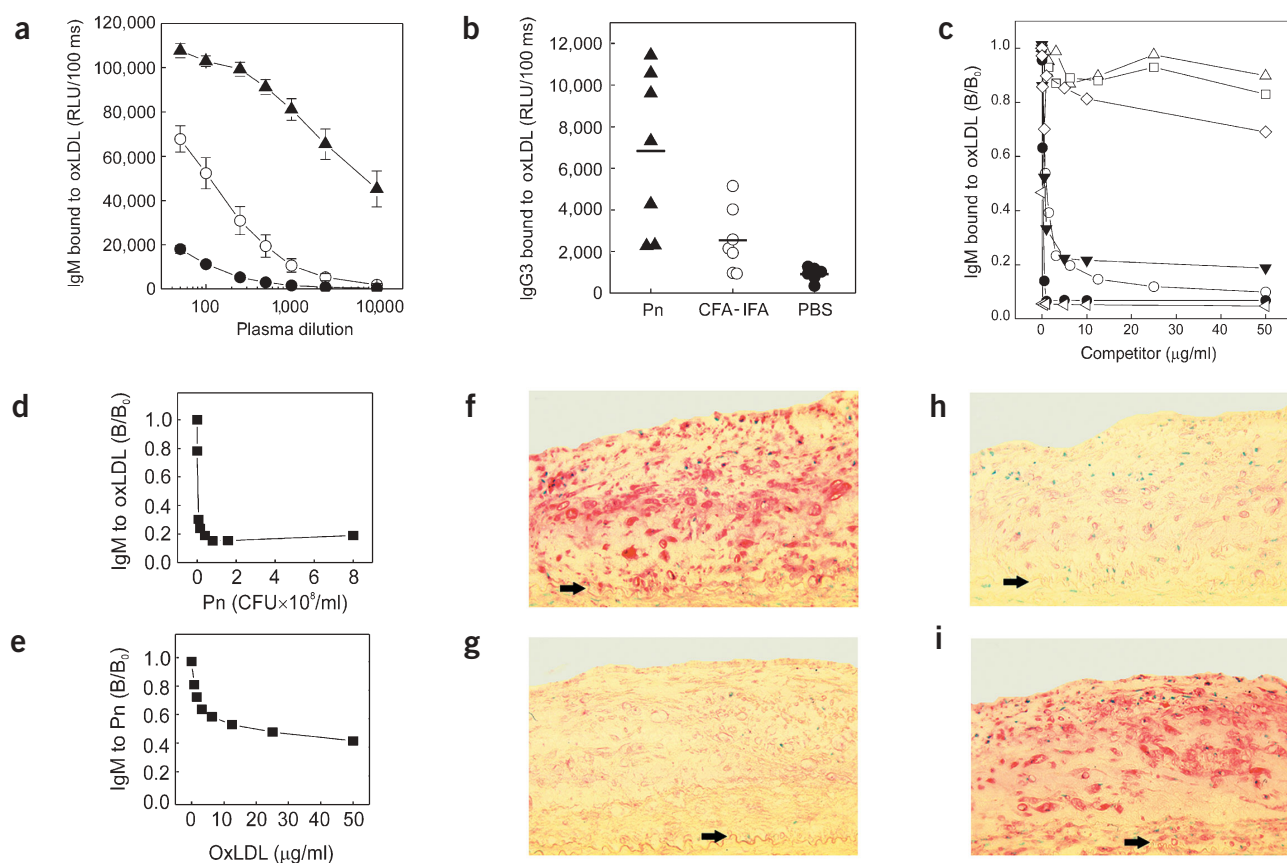


Figure 1 Pneumococcal immunization induces oxLDL-specific IgM. (a) Dilution curve of IgM binding to oxLDL in plasma of mice that received pneumococcal extract emulsified in Freund adjuvant (Pn; ▲; $n = 7$), PBS in Freund adjuvant (CFA-IFA; ○; $n = 7$) or PBS alone (●; $n = 6$). RLU, relative light units. Values represent mean \pm s.e.m. (b) Titers of IgG3 antibodies to oxLDL in plasma diluted 1:50. Results are from individual mice; horizontal bar represents mean for the group. (c) Competition immunoassay of pooled sera for binding of plasma IgM to oxLDL with increasing concentrations of native LDL (Δ), oxLDL (\circ), MDA-LDL (\square), T15-clonosppecific antibody AB1-2 (\blacktriangledown), phosphorylcholine-HCl (\bullet), phosphorylcholine-KLH (\blacktriangleleft), or KLH (\diamond). Data are expressed as a ratio of binding in the presence of competitor (B) divided by binding in the absence of competitor (B₀) and represent the mean of triplicate determinations. Results with MDA-LDL as competitor were obtained in a different experiment, using a representative plasma sample of the study. (d) Competition for binding of IgM to oxLDL by pneumococcal extract (Pn). (e) Competition for binding of IgM to pneumococcal extract (Pn) by oxLDL. (f–i) Sections of atherosclerotic aortas from balloon-catheterized, cholesterol-fed New Zealand white rabbits were stained with antisera from pneumococci immunized mice or the monoclonal antibody EO6. Epitopes recognized are indicated by red color; nuclei are counterstained with methyl green. Arrow indicates the internal elastic lamina. Sections were immunostained with pooled postimmune sera (f), pooled postimmune sera preabsorbed with excess pneumococcal antigen (g), pooled preimmune sera (h) or monoclonal antibody EO6 (i).

(IgA) specifically bound to oxLDL and POVPC, while the EO antibodies, such as the prototypic EO6, also bound to the phosphorylcholine-containing antigen, pneumococcal cell-wall polysaccharide (CPS)¹³.

These *in vitro* studies suggested molecular mimicry between immunodominant phosphorylcholine epitopes of oxidized phospholipids of oxLDL and the phosphorylcholine moiety of common microbial pathogens. We therefore hypothesized that *in vivo* exposure to *S. pneumoniae* could mimic the immune response to oxLDL present in lesions and, more importantly, that this might actively modulate atherogenesis. To test this hypothesis, we examined the immune response of LDL receptor negative (*Ldlr*^{-/-}) mice to a standard pneumococcal immunogen that has previously been extensively evaluated in studies of anti-bacterial defense.

RESULTS

Pneumococcal immunization expands IgM antibodies to oxLDL

Immunization of chow-fed adult *Ldlr*^{-/-} mice with pneumococcal extracts emulsified in Freund's adjuvant resulted in a strong induction

of IgM titers to oxLDL, which was not observed in mice immunized PBS alone (Fig. 1a). Induced antibodies to oxLDL were almost entirely IgM, with only weak IgG3 responses (Fig. 1b), consistent with previous reports that pneumococcal immunizations induce a thymus-independent type-2 response that is highly specific for phosphorylcholine. Notably, even five sequential immunizations did not induce oxLDL-specific IgG1 and IgG2a antibody responses (data not shown).

Using competition immunoassay studies, we showed that the binding of IgM to oxLDL of pooled antisera from immunized mice was completely inhibited by oxLDL (Fig. 1c). The pneumococcal immunogen also efficiently inhibited the binding of the antisera to oxLDL (Fig. 1d). Phosphorylcholine, the immunodominant determinant of pneumococcal cell-wall polysaccharide, also competed very effectively, either as a simple phosphorylcholine salt or as a conjugate of phosphorylcholine and keyhole limpet hemocyanine (phosphorylcholine-KLH; Fig. 1c). Neither native (nonoxidized) LDL, nor MDA-LDL nor KLH (Fig. 1c) inhibited binding. The T15-clonosppecific antibody, AB1-2, which identifies a determinant requiring coexpression of the

canonical T15-V_H and T15-V_L regions¹⁸, almost completely inhibited IgM binding from immune sera to oxLDL (Fig. 1c). Finally, in studies with a reciprocal design, oxLDL competed up to 60% of the binding of the induced antisera to the pneumococcal immunogen (Fig. 1e). These findings confirm that IgM antibodies to oxLDL induced *in vivo* by pneumococcal immunization are predominantly T15-clonotypic.

Mice immunized with complete Freund adjuvant and incomplete Freund adjuvant (CFA-IFA) alone had a more modest, but demonstrable, anti-oxLDL response (Fig. 1a). However, in competition immunoassays with pooled sera from the CFA-IFA group, this binding to oxLDL was effectively competed by both oxLDL and MDA-LDL, but neither phosphorylcholine-KLH nor AB1-2 showed a strong inhibition (Supplementary Fig. 1 online). Thus, the anti-oxLDL antibodies induced by immunization with CFA/IFA have different binding specificities than the predominant T15-expressing antibodies induced by pneumococcal immunization.

To further establish the molecular mimicry between epitopes of oxLDL and pneumococcal antigens, we immunized normocholesterolemic C57BL/6 mice, which do not develop atherosclerotic lesions, with oxLDL. This led to an increase in cell-wall polysaccharide-specific IgM, which was predominantly T15-clonotypic as shown by competition assays with AB1-2 (data not shown).

Antisera to pneumococci bind to atherosclerotic lesions

The antisera induced by pneumococcal immunization specifically recognized epitopes in atherosclerotic lesions (Fig. 1f); this recognition was effectively abolished by preincubation of the antisera with excess pneumococcal immunogen (Fig. 1g). Preimmune sera yielded no specific immunohistochemical staining (Fig. 1h). Finally, immunostaining with EO6 (Fig. 1i) resulted in a pattern closely resembling that obtained with sera from mice immunized with pneumococci.

Splenic cells of immunized mice secrete T15-IgM

To characterize the cellular origins of the induced humoral responses, we determined the frequency and anatomic distribution of IgM-secreting cells at sacrifice, more than 3 months after the last immunization. In the three treatment groups, the overall frequencies of total IgM-secreting cells in the spleen did not differ significantly (Fig. 2a). Pneumococcal immunization greatly increased the frequency of cells secreting cell-wall polysaccharide-specific IgM (Fig. 2b), with equivalent induction in the frequency of cells secreting oxLDL-specific IgM (Fig. 2c). Furthermore, using antibody AB1-2, we showed increased frequencies of T15-clonotypic IgM-secreting cells in the spleens of mice immunized with pneumococci (Fig. 2d), which was independently confirmed with two other T15-clonotypic markers, the V_HT15-specific antibody Tc68 and the V_LT15-specific antibody T139.2^{19,20} (Fig. 2e,f). Equivalent patterns were also shown in the bone marrow of immunized mice, but the frequencies of induced IgM-secreting cells were 50% lower than in the spleen (Supplementary Fig. 2 online).

Pneumococcal immunization reduces atherogenesis

To evaluate the effect of pneumococcal immunization on atherogenesis, *Ldlr*^{-/-} mice were immunized with pneumococci emulsified in Freund adjuvant, PBS in Freund adjuvant (CFA-IFA) or PBS alone, and then fed an atherogenic diet for 24 weeks (Fig. 3). Only the group immunized with pneumococci exhibited an IgM response specific for cell-wall polysaccharide (Fig. 3a), which was paralleled by the induction of IgM titers to oxLDL (Fig. 3b). Controls immunized with CFA-IFA or PBS alone initially exhibited only a modest or no response to oxLDL, respectively (Fig. 3b). On the other hand, IgM titers to the

unrelated model epitope MDA-LDL rose initially in both groups exposed to adjuvant, independent of the cholesterol feeding, and remained slightly higher throughout the study compared with the PBS group (Fig. 3c). Only very low titers of specific IgG antibodies were found in all groups (data not shown). Although hypercholesterolemia *per se* induced a low-titered anti-oxLDL response, it is clear that throughout the study pneumococcal immunization led to significantly ($P < 0.01$) higher levels of predominantly IgM antibodies to oxLDL.

Quantification of the time-averaged plasma cholesterol exposure for each group indicated that both adjuvant-treated groups had significantly lower plasma cholesterol than the PBS-treated group (Fig. 3d and Table 1). The levels were not different between the two adjuvant-treated groups, however. Triglyceride levels rose over time in all three groups, but were significantly lower only in the pneumococcal group compared with the PBS group ($P = 0.02$). Lipoprotein profiles by fast performance liquid chromatography (FPLC) of pooled plasma from

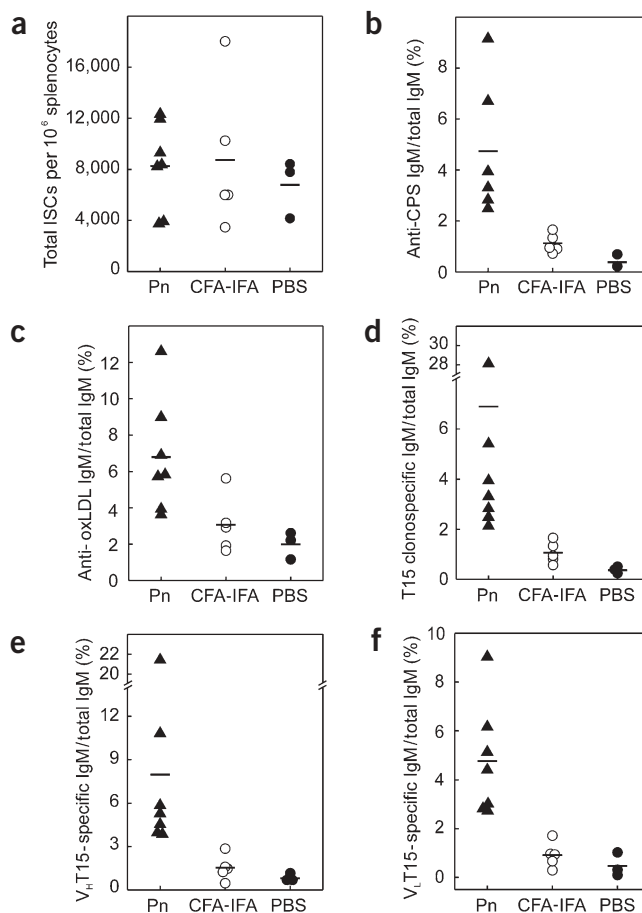


Figure 2 ELISpot assay of frequencies of immunoglobulin-secreting cells (ISCs) in the spleens of the three groups of immunized mice. Frequencies of specific ISCs in the spleens were determined by binding to wells coated with an antibody to mouse IgM (a), cell-wall polysaccharide (CPS; b), oxLDL (c), T15-clonotypic antibody (AB1-2; d), V_HT15 specific antibody (Tc68; e) or V_LT15 specific antibody (T139.2; f). In a, values are depicted for frequencies of total IgM-secreting cells, whereas values in other panels represent the number of IgM-secreting cells to the indicated antigen as a percent of total IgM-secreting cells. Results are from individual mice; horizontal bar represents mean for the group. Values were determined at time of sacrifice, more than three months after the last immunization. Pn, pneumococcal immunogen.

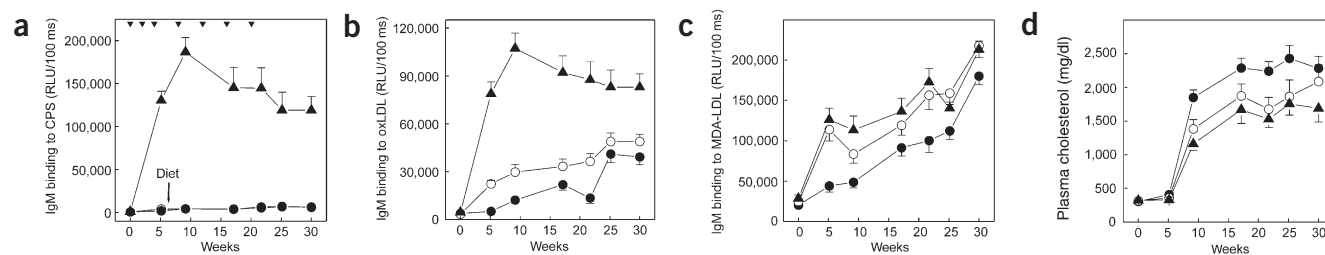


Figure 3 First pneumococcal intervention study (24 weeks of atherogenic diet). **(a)** Time course of IgM binding to cell-wall polysaccharide of plasma from mice immunized with pneumococci in Freund adjuvant (\blacktriangle ; $n = 9$), PBS in Freund adjuvant (\circ ; $n = 10$) or PBS alone (\bullet ; $n = 12$). The symbols in **b–d** are the same as in **a**. Six weeks after the primary immunization, mice were put on an atherogenic diet (black arrow). The time points for immunizations are indicated as black arrowheads at the top of the panel. Plasma samples were obtained before the initial immunization (0 time point) and at indicated times. The final point was obtained at sacrifice. Results are expressed as relative light units (RLU) per 100 ms. **(b)** Binding of IgM to oxLDL. **(c)** Binding of IgM to MDA-LDL. **(d)** Time course of total plasma cholesterol levels. All values represent mean \pm s.e.m.

each group showed a marked reduction in the very low-density lipoprotein, intermediate-density lipoprotein and LDL fractions in the pneumococcal group and a similar, but lesser, reduction in the CFA-IFA group (data not shown).

To explain the decreased levels of apoB lipoproteins, we postulated that large amounts of IgM formed immune complexes with minimally oxidized lipoproteins in plasma and effected enhanced plasma clearance. We measured IgM-apoB immune complexes in the plasma of these mice (Supplementary Fig. 3 online). There were significantly higher levels of immune complexes in the pneumococcal-immunized mice during most of the study ($P < 0.001$). At the end of the study, however, the levels of immune complexes in the pneumococcal-immunized mice decreased, despite high titers of oxLDL-specific antibodies, presumably reflecting a decreased level of oxLDL in the plasma at that time. Mice immunized with CFA-IFA also had higher levels of immune complexes when compared with the PBS group ($P < 0.05$), which is consistent with the demonstrated increase in IgM against MDA-LDL and oxLDL.

Immunization with pneumococci reduced the extent of atherosclerosis after 24 weeks of cholesterol diet (Table 1). Both the percentage of aortic surface covered by Sudan IV-positive lesions in *en face* preparations ($P < 0.01$) and the area of atherosclerotic lesions in the aortic origin ($P < 0.05$) were smaller compared with the PBS group. Surprisingly, in the CFA-IFA group, atherosclerosis was decreased at the aortic origin ($P < 0.05$), but not in the entire aorta, compared with the PBS group. Thus, there were no significant differences in lesion formation between the two groups that received adjuvant (Table 1).

Because the unexpected effects of Freund adjuvant on the extent of atherosclerosis confounded the interpretation of the impact of pneumococcal immunization, we initiated a second intervention study in which Freund adjuvant was not used. We immunized *Ldlr*^{-/-} mice with either pneumococci in PBS or with PBS alone, using a similar protocol (Fig. 4a). Even without adjuvant, mice immunized with pneumococci exhibited a strong IgM response to cell-wall polysaccharide (Fig. 4a) and oxLDL (Fig. 4b). In an analysis simultaneously comparing antibody responses from both experiments, equivalent titers of free antibodies to oxLDL were seen in mice receiving pneumococcal immunization in both the first and second intervention studies (data not shown). In contrast, the PBS group displayed only a minimal rise in oxLDL-specific IgM (Fig. 4b). In both groups, IgM titers to MDA-LDL rose in parallel, indicating that the pneumococcal immunization did not influence the development of these antibody responses (Fig. 4c). Again, pneumococcal immunization induced predominantly IgM responses, and minimal IgG titers to cell-wall polysaccharide were induced (Supplementary Fig. 4 online).

In the second intervention study, mice were sacrificed after 16 weeks of cholesterol feeding. All mice gained weight equally, and time-averaged plasma cholesterol and triglyceride levels were similar (Fig. 4d and Table 1). In this setting, the increased levels of immune complexes noted in the first intervention study were not observed (data not shown). Mice immunized with pneumococci had 21.5% less atherosclerosis in the aortic origin compared with the control group (0.249 mm²/section versus 0.317 mm²/section, $P < 0.05$; Table 1). The extent of atherosclerosis in the entire aorta was also decreased by 8.7%, but

Table 1 Overview of experimental groups from both intervention studies

Groups (number)	Weight (g) (% gain)	Plasma lipids		Atherosclerosis	
		TC (mg/dl)	TG (mg/dl)	<i>En face</i> ^a (% reduction)	Aortic origin ^b (% reduction)
Study 1					
PBS (12)	48.6 \pm 1.8 (180)	2,064 \pm 127	773 \pm 71	10.6 \pm 0.8	0.630 \pm 0.039
CFA-IFA (10)	48.2 \pm 1.7 (171)	1,634 \pm 150 ^c	622 \pm 89	8.6 \pm 1.0 (19.0)	0.519 \pm 0.025 ^c (17.6)
Pneumococci (9)	42.7 \pm 2.6 (156 ^c)	1,446 \pm 75 ^d	505 \pm 68 ^c	7.2 \pm 0.6 ^d (32.0)	0.494 \pm 0.032 ^c (21.7)
Study 2					
PBS (15)	45.0 \pm 1.3 (156)	1,747 \pm 65	627 \pm 52	8.4 \pm 0.6	0.317 \pm 0.025
Pneumococci (13)	42.5 \pm 1.8 (148)	1,532 \pm 132	552 \pm 65	7.7 \pm 0.6 (8.7)	0.249 \pm 0.018 ^c (21.5)

TC, total plasma cholesterol; TG, triglycerides (measured as the area under the cholesterol curve over time divided by days of cholesterol feeding).

^a*En face* measurements are given in percent lesions of the aorta. ^bAtherosclerosis in the aortic origin was analyzed by cross-sections through the aortic origin and values represent the average mm²/section; percent reduction in comparison with the respective PBS group. ^{c,d}Values that are statistically different from the respective PBS group (^c $P < 0.05$ and ^d $P < 0.01$). Data are mean \pm s.e.m.

this did not reach significance. Thus, pneumococcal immunization preferentially decreased atherosclerosis in older, more established lesions of the aortic origin. Notably, this reduction occurred despite hypercholesterolemia of $\approx 1,600$ mg/dl, an amount that in other models has overcome the impact of total immune deficiency (for example, lack of T and B cells)^{21,22}.

Plasma from immunized mice blocks oxLDL uptake

Monoclonal T15 and EO6 IgM antibodies block the binding and uptake of oxLDL by macrophages⁸. This prompted us to evaluate the capacity of plasma from different treatment groups to inhibit the binding of oxLDL to macrophages. Pooled plasma from mice immunized with pneumococci was considerably more effective in blocking binding of oxLDL to macrophages, compared with the plasma from control mice (Fig. 5). Similar results were seen in the first intervention study (data not shown).

Human sera contain IgM that react with CPS and oxLDL

To investigate whether epitope equivalence in the immune responses to pneumococci and oxLDL can also be observed in humans, we measured antibody binding to cell-wall polysaccharide and oxLDL of sera obtained from patients recently diagnosed with pneumococcal pneumonia. Whereas IgG titers to the two antigens did not correlate, IgM binding showed a significant correlation (Supplementary Fig. 5 online). In addition, sera from hypercholesterolemic patients displayed a significant correlation between IgM titers to oxLDL and cell-wall polysaccharide (Supplementary Fig. 5 online). Thus, these limited results indicate the potential for similar molecular mimicry between immune responses to cell-wall polysaccharide and oxLDL in humans.

DISCUSSION

Because our previous *in vitro* studies had shown molecular mimicry among the phosphorylcholine moieties of microbial cell-wall polysaccharide, oxLDL and apoptotic cells^{8,12,13}, we tested the hypothesis that immunization with pneumococci would elicit a high titer of T15-clonospesific antibodies to oxLDL (EO6, for example), which in turn would modulate the progression of atherosclerosis. In the current studies, immunization of cholesterol-fed *Ldlr*^{-/-} mice with a standard pneumococcal vaccine preparation induced a high titer of T15-clonospesific, oxLDL-specific IgM antibodies, which in turn reduced progression of atherosclerosis. These induced antibodies, which could be inhibited by the pneumococcal immunogen, specifically recognized determinants on oxLDL (Fig. 1), on apoptotic cells (data not shown) and in atherosclerotic lesions (Fig. 1), as previously described for the

'natural' T15 antibodies (that is, those arising without immunization)^{8,13,14}.

Mice that received Freund adjuvant alone also showed a moderate rise in oxLDL-specific IgM (Figs. 1 and 3) and a mild atheroprotective effect (Table 1), which confounded the interpretation of these results. In a second intervention study in which Freund adjuvant was omitted, immunization with pneumococci also induced significantly ($P < 0.001$) elevated antibody titers to oxLDL (Fig. 4). In contrast, the mice receiving buffer alone showed only a very modest rise in oxLDL-specific antibody titers. The fact that atherosclerosis in the aortic origin of these mice was significantly decreased in the absence of adjuvant, despite massive hypercholesterolemia, establishes a protective effect of pneumococcal immunization. The pneumococcal-induced antibodies to oxLDL were almost exclusively T15 IgM (Figs. 1, 2 and Supplementary Fig. 2 online), indicating that the expansion of IgM antibodies specific for a single epitope had a significant impact on lesion formation. Thus, our studies identify phosphorylcholine as a key epitope in the protective immune response to oxLDL.

Surprisingly, immunization with Freund adjuvant alone induced a mild atheroprotective effect. These mice had an unexpectedly high titer of antibodies to MDA-LDL and a modest increase in antibodies to oxLDL, which were demonstrable even before the high cholesterol diet was initiated. We postulate that immunization with this lipid-containing adjuvant induced a local inflammatory reaction, which led to lipid peroxidation and the generation of MDA (and other lipid peroxidation products), which in turn generated immunogenic adducts with local proteins, including LDL. We also found that CFA itself contains MDA epitopes, as demonstrated by the binding of monoclonal antibodies MDA2 and EO14, both specific for MDA-lysine^{9,10} (data not shown). Thus, it seems likely that CFA, by several mechanisms, provides the oxidation-specific epitope MDA that serves as an immunogen. This is highly relevant, as we and others have shown that immunization with MDA-LDL leads to a reduction in atherogenesis^{3,4,23,24}. While our studies were in progress, another group reported that Freund adjuvant alone decreased lesion formation in *Apoe*^{-/-} mice²⁵.

Evidence that T15 IgM binds to oxidized-phospholipid epitopes on oxLDL and prevents macrophage uptake of oxLDL^{8,13} suggests a mechanism that may contribute to the decreased progression of atherosclerosis in the pneumococcal-immunized mice⁸. Indeed, the T15 IgM enriched plasma from the pneumococcal-immunized mice had an enhanced capacity to inhibit the binding of oxLDL by macrophages *in vitro*. Because the uptake of oxLDL by scavenger receptors (such as scavenger receptor A or CD36) leads to foam-cell

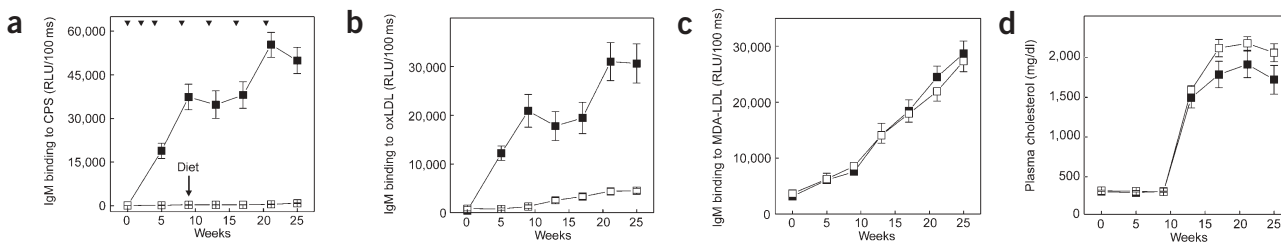


Figure 4 Second pneumococcal intervention study (16 weeks of atherogenic diet). **(a)** Time course of IgM binding to cell-wall polysaccharide (CPS) of plasma from mice immunized with pneumococci (■; $n = 13$) or PBS alone (□; $n = 15$). The symbols in **b–d** are the same as in **a**. Nine weeks after the primary immunization, mice were put on an atherogenic diet (black arrow). The time points for immunizations are indicated as black arrowheads at the top of the panel. Plasma samples were obtained before the initial immunization (0 time point) and at indicated times. The final point was obtained at sacrifice. IgM binding was determined as described in the legend of Fig. 3. Note that these measurements were done with a different luminometer that gives approximately four times lower relative light unit (RLU) values than the one used in Fig. 3. **(b)** Binding of IgM to oxLDL. **(c)** Binding of IgM to MDA-LDL. **(d)** Time course of total plasma cholesterol levels. All values represent mean \pm s.e.m.

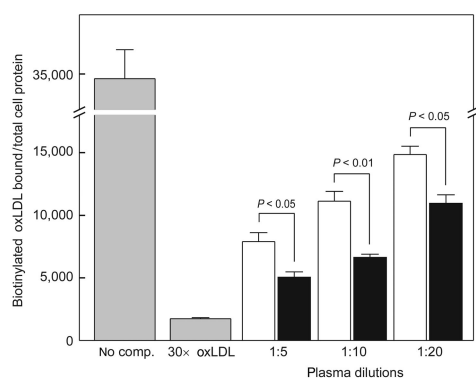


Figure 5 Plasma from pneumococcal-immunized mice inhibits oxLDL binding by macrophages. The specific binding of biotinylated oxLDL to mouse macrophages was shown by incubation in the absence and presence of 30-fold excess unconjugated oxLDL (■). To determine the capacity of the antisera to inhibit oxLDL binding, three dilutions of pooled plasma (1:5, 1:10 and 1:20) from mice immunized with pneumococci (■) or PBS (□) were added, together with biotinylated oxLDL, to macrophages. The extent of oxLDL binding is expressed as relative light units (RLU) per 100 ms per μ g total cell protein.

formation, such inhibition would be expected to impede atherogenesis¹. Under ordinary circumstances, IgM are primarily intravascular molecules. However, immunoglobulins, including IgM, are abundantly present in atherosclerotic lesions³ and we have explicitly shown the presence of T15 antibodies in atherosclerotic lesions of *Ldlr*^{-/-} mice¹³. Thus, once atherosclerotic lesions form, T15 antibodies can gain access to the subintimal space and potentially inhibit macrophage uptake of oxidized lipoproteins.

The spleen is a major anatomic source of oxLDL-specific IgM in nonimmunized atherosclerotic mice¹⁰ and in mice immunized with pneumococcal extracts (Fig. 2). Recently, the spleen has been recognized as the major source of natural protective antimicrobial antibodies, like those from the T15 B-cell clone^{26,27}. The spleen is also important in the maintenance of the B-1 cell pool in general, and splenectomized mice do not develop anti-phosphorylcholine responses²⁸. Thus, our data provide a mechanistic basis to explain in part the recent observations that splenectomy of *Apoe*^{-/-} mice enhances atherosclerosis and that this can be rescued by passive splenic B-cell transfer from *Apoe*^{-/-} donors²⁹.

These data support our hypothesis that oxidation-specific neo-self epitopes have a special relationship with recurrently arising clones that are part of a primitive tier of the host immune system, the B-1 cell pool^{30,31}, which has been selected during evolution for its beneficial roles in host defense and probably for protection from stressed self-structures (such as oxLDL and apoptotic cells)^{3,27,32}. As such, B-1 cells, typified by the T15 clone, are a major source of 'natural' antibodies. In several cases, these natural antibodies are important for initial antimicrobial (for example, *S. pneumoniae*) and viral defense^{33–36}. Our findings show that immunization with either oxLDL or pneumococci boosts specific anti-phosphorylcholine titers, which leads to a reduction in atherogenesis. These data provide direct evidence of the house-keeping functions that have been previously postulated for natural antibodies.

One can envisage that there has been evolutionary pressure to conserve the T15 clonospesific antibodies both for host defense against oxidatively modified self-structures and for protection against infections with specific phosphorylcholine-bearing pathogens³². Thus,

phosphorylcholine exposure generates a pathogen-associated molecular pattern that is recognized by pattern recognition receptors of highly conserved innate responses, which includes not only natural immunoglobulins, but also scavenger receptors of macrophages, such as CD36 and SR-B1^{37,38}, as well as C-reactive protein¹¹. The finding that immunization with pneumococcal antigen increases the titer of EO6-like antibodies and significantly reduces atherogenesis indicates the possibility that exposure to pathogens such as pneumococci could substantially influence the course of atherogenesis.

Much less is known about the human response. There are reports documenting that humans possess antibodies to phosphorylcholine with similar structural and genetic features^{39–41}. We have also shown, in limited data, a correlation between IgM to oxLDL and cell-wall polysaccharide in human subjects (Supplementary Fig. 5 online). Because infections with *S. pneumoniae* are common and pneumococcal vaccines are being increasingly used, it will be important to determine the potential impact of induced anti-phosphorylcholine responses on atherogenesis in humans. Although the results outlined in this report suggest a protective effect, this could primarily derive from the fact that the dominant antibody response in mice is IgM. The human immune response is more complex, and available pneumococcal vaccines have not been developed to optimize the IgM responses to cell-wall polysaccharide⁴⁰. Moreover, although the development of IgM responses may be beneficial, T-cell-dependent IgG responses to phosphorylcholine may lead to other, possibly adverse, effects (such as increased foam-cell formation after uptake of IgG-oxLDL immune complexes by Fc- γ receptors). In conclusion, we believe that our observation on anti-phosphorylcholine responses warrants further study to assess the influence of pneumococcal infections on atherosclerosis, as phosphorylcholine-based immunization strategies may have a therapeutic potential for ameliorating atherogenesis as well as other inflammatory diseases in which oxidized phospholipids are generated.

METHODS

Immunogens and antigens. Pronase-treated, heat-killed, R36a *S. pneumoniae*¹⁵, oxLDL and MDA-LDL⁹ were prepared as described. Phosphorylcholine-chloride salt and keyhole limpet hemocyanine (KLH) were from Sigma; phosphorylcholine-KLH was from Biosearch Technologies Inc.

Mice, immunizations and diets. *Ldlr*^{-/-} mice (tenth generation C57BL/6) were obtained from The Jackson Laboratories. For the initial immunization study, male and female mice, aged 12–15 weeks and fed nonatherogenic chow (Harlan Teklad W860), were divided into three groups. Group 1 ($n = 7$) was immunized with 10^8 CFU of pneumococcal immunogen–emulsified in CFA for the primary subcutaneous immunization or IFA for four subsequent intraperitoneal booster immunizations over 14–16 weeks. Group 2 ($n = 7$) was immunized with PBS in CFA or IFA (CFA-IFA). Group 3 ($n = 6$) received PBS alone.

For the first intervention study (with Freund adjuvant), 36 male mice, aged 15–19 weeks, were divided into three groups matched for body weight, age and plasma cholesterol levels. Mice were immunized with 10^8 CFU of pneumococcal immunogen in CFA-IFA ($n = 12$), CFA-IFA alone ($n = 12$) or PBS alone ($n = 12$; Fig. 4a). All mice were initially fed nonatherogenic chow for 6 weeks and then switched to an atherogenic diet containing 21.2% milkfat and 1.25% cholesterol (TD96121; Harlan Teklad) for 24 more weeks. During the study, four mice (two each in the pneumococcal immunogen and CFA-IFA group) died as a result of anesthesia and other causes and one mouse (pneumococcal immunogen group) was excluded from the final analysis due to lack of any weight gain.

For the second intervention study (without Freund adjuvant), 30 male mice, aged 15–16 weeks, were divided into equal groups matched for body weight, age and plasma cholesterol. Mice in the pneumococcal immunogen group ($n = 15$) were injected with 10^8 bacterial CFU in 200 μ l sterile PBS; the PBS group ($n = 15$) received PBS only. Mice were immunized as shown (Fig. 5). They were initially fed rodent chow for 9 weeks and then the atherogenic diet for 16 more weeks.

During the study, one mouse died of anesthesia overdose and another was excluded because of the formation of extensive aneurysms in the aortic origin.

Plasma aliquots obtained at baseline and 8 d after each immunization were stored at -20°C . Plasma cholesterol and triglyceride levels were determined using an automated enzymatic assay (Boehringer Mannheim). The experimental protocol was approved by the Animal Subjects Committee of the University of California San Diego.

Immunoassays. Antibody titers were determined by chemiluminescent enzyme immunoassays as described⁴². To show specificity, antisera from individual mice immunized with pneumococcal immunogen ($n = 7$) were serially diluted in PBS containing 0.27 mM EDTA and 2% bovine serum albumin to define the respective dilutions of each antiserum associated with similar binding activity to oxLDL or pneumococci. Equal volumes of each diluted antiserum were then pooled for the subsequent competition assay, in which increasing amounts of competitors were incubated overnight at 4°C with a fixed and limiting dilution of the pooled antisera, and then the binding to oxLDL- or pneumococcal-coated wells was determined by chemiluminescent immunoassay.

Enzyme-linked immunospot assays. The frequencies of immunoglobulin-, antigen- and clonospic-secreting splenocytes and bone marrow cells were quantitated by enzyme-linked immunospot (ELISpot) assay, using described methods⁴³. Microtiter wells were coated in parallel with 5 or 10 $\mu\text{g}/\text{ml}$ of either goat affinity-purified mouse-specific IgM (Jackson), oxLDL, MDA-LDL, cell-wall polysaccharide (Statens Serum Institut), AB1-2 (a mouse IgG1; provided by J.F. Kearney, University of Alabama)¹⁸, the V_LT15-specific antibody T139.2, the V_HT15-specific antibody Tc68 (rat IgG2a; provided by M. Scharff, Albert Einstein College of Medicine)¹⁹ or isotype controls.

Immunohistochemistry. Immunostaining of formal sucrose-fixed, paraffin-embedded sections of atherosclerotic lesions was performed as described²³, using a 1:1,000 dilution of pre- and post-immune sera from pneumococci-immunized mice as well as monoclonal IgM EO6⁸. Competitive immunostaining was done by 1 h preincubation of the antisera with 6×10^8 CFU of the pneumococcal extract.

Macrophage binding assay. Binding of biotinylated oxLDL to thioglycollate-elicited peritoneal macrophages from C57BL/6 mice plated in microtiter wells was assessed by a chemiluminescent binding assay as described⁴⁴. The binding of biotinylated-oxLDL (2 $\mu\text{g}/\text{ml}$) was determined in the absence or presence of diluted pooled plasma from the immunization groups. In parallel experiments, the specificity of the binding of biotinylated-oxLDL to macrophages was determined in the absence and presence of 30-fold unconjugated oxLDL. The binding is expressed as oxLDL bound in relative light units per μg total cell protein.

Evaluation of atherosclerosis. The extent of atherosclerosis was determined in a blinded fashion in *en face* preparations of the entire aortic tree, as well as in cross sections through the aortic origin, by computer-assisted image analysis as described⁴⁵.

Statistical analysis. Data are presented as mean \pm s.e.m. Results were analyzed by one-way analysis of variance and Student's unpaired *t* test.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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- Glass, C.K. & Witztum, J.L. Atherosclerosis. The road ahead. *Cell* **104**, 503–516 (2001).
- Libby, P., Ridker, P.M. & Maseri, A. Inflammation and atherosclerosis. *Circulation* **105**, 1135–1143 (2002).
- Binder, C.J. *et al.* Innate and acquired immunity in atherogenesis. *Nat. Med.* **8**, 1218–1226 (2002).
- Hansson, G.K., Libby, P., Schonbeck, U. & Yan, Z.Q. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ. Res.* **91**, 281–291 (2002).
- Hansson, G.K. Immune mechanisms in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **21**, 1876–1890 (2001).
- Hörkkö, S. *et al.* Immunological responses to oxidized LDL. *Free Radic. Biol. Med.* **28**, 1771–1779 (2000).
- Watson, A.D. *et al.* Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence *in vivo*. *J. Biol. Chem.* **272**, 13597–13607 (1997).
- Hörkkö, S. *et al.* Monoclonal autoantibodies specific for oxidized phospholipids or oxidized phospholipid-protein adducts inhibit macrophage uptake of oxidized low density lipoproteins. *J. Clin. Invest.* **103**, 117–128 (1999).
- Palinski, W. *et al.* Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis* **10**, 325–335 (1990).
- Palinski, W. *et al.* Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J. Clin. Invest.* **98**, 800–814 (1996).
- Chang, M.K., Binder, C.J., Torzewski, M. & Witztum, J.L. C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: Phosphorylcholine of oxidized phospholipids. *Proc. Natl. Acad. Sci. USA* **99**, 13043–13048 (2002).
- Friedman, P., Hörkkö, S., Steinberg, D., Witztum, J.L. & Dennis, E.A. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids. Importance of Schiff base formation and aldehyde concentration. *J. Biol. Chem.* **277**, 7010–7020 (2002).
- Shaw, P.X. *et al.* Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J. Clin. Invest.* **105**, 1731–1740 (2000).
- Chang, M.K. *et al.* Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc. Natl. Acad. Sci. USA* **96**, 6353–6358 (1999).
- Briles, D.E., Forman, C., Hudak, S. & Clafin, J.L. Anti-phosphorylcholine antibodies of the T15 idiotype are optimally protective against *Streptococcus pneumoniae*. *J. Exp. Med.* **156**, 1177–1185 (1982).
- Mi, Q.S. *et al.* Highly reduced protection against *Streptococcus pneumoniae* after deletion of a single heavy chain gene in mouse. *Proc. Natl. Acad. Sci. USA* **97**, 6031–6036 (2000).
- Snapper, C.M. *et al.* Distinct types of T-cell help for the induction of a humoral immune response to *Streptococcus pneumoniae*. *Trends Immunol.* **22**, 308–311 (2001).
- Kearney, J.F., Barletta, R., Quan, Z.S. & Quintans, J. Monoclonal vs. heterogeneous anti-H-8 antibodies in the analysis of the anti-phosphorylcholine response in BALB/c mice. *Eur. J. Immunol.* **11**, 877–883 (1981).
- Kenny, J.J. *et al.* Antigen binding and idiotype analysis of antibodies obtained after electroporation of heavy and light chain genes encoding phosphocholine-specific antibodies: a model for T15-idiotype dominance. *J. Exp. Med.* **176**, 1637–1643 (1992).
- Desaynard, C., Giusti, A.M. & Scharff, M.D. Rat anti-T15 monoclonal antibodies with specificity for VH- and VH-VL epitopes. *Mol. Immunol.* **21**, 961–967 (1984).
- Daugherty, A. *et al.* The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E $^{-/-}$ mice. *J. Clin. Invest.* **100**, 1575–1580 (1997).
- Dansky, H.M., Charlton, S.A., Harper, M.M. & Smith, J.D. T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. *Proc. Natl. Acad. Sci. USA* **94**, 4642–4646 (1997).
- Palinski, W., Miller, E. & Witztum, J.L. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc. Natl. Acad. Sci. USA* **92**, 821–825 (1995).
- Freigang, S., Hörkkö, S., Miller, E., Witztum, J.L. & Palinski, W. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neopeptides. *Arterioscler. Thromb. Vasc. Biol.* **18**, 1972–1982 (1998).
- Hansen, P.R. *et al.* Freund's adjuvant alone is antiatherogenic in apoE-deficient mice and specific immunization against TNF α confers no additional benefit. *Atherosclerosis* **158**, 87–94 (2001).
- Silverman, G.J. *et al.* A B cell superantigen-induced persistent "Hole" in the B-1 repertoire. *J. Exp. Med.* **192**, 87–98 (2000).
- Bendelac, A., Bonneville, M. & Kearney, J.F. Autoreactivity by design: innate B and T lymphocytes. *Nat. Rev. Immunol.* **1**, 177–186 (2001).
- Wardemann, H., Boehm, T., Dear, N. & Carsetti, R. B-1a B cells that link the innate and adaptive immune responses are lacking in the absence of the spleen. *J. Exp.*

- Med.* **195**, 771–780 (2002).
29. Caligiuri, G., Nicoletti, A., Poirier, B. & Hansson, G.K. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J. Clin. Invest.* **109**, 745–753 (2002).
 30. Masmoudi, H., Mota-Santos, T., Huetz, F., Coutinho, A. & Cazenave, P.A. All T15 Id-positive antibodies (but not the majority of V_HT15⁺ antibodies) are produced by peritoneal CD5⁺ B lymphocytes. *Int. Immunol.* **2**, 515–520 (1990).
 31. Berland, R. & Wortis, H.H. Origins and functions of B-1 cells with notes on the role of CD5. *Annu. Rev. Immunol.* **20**, 253–300 (2002).
 32. Silverman, G.J. *et al.* Neo-self antigens and the expansion of B-1 cells: lessons from atherosclerosis-prone mice. *Curr. Top. Microbiol. Immunol.* **252**, 189–200 (2000).
 33. Macpherson, A.J. *et al.* A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. *Science* **288**, 2222–2226 (2000).
 34. Ochsenbein, A.F. *et al.* Control of early viral and bacterial distribution and disease by natural antibodies. *Science* **286**, 2156–2159 (1999).
 35. Martin, F., Oliver, A.M. & Kearney, J.F. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* **14**, 617–629 (2001).
 36. Reid, R.R. *et al.* Endotoxin shock in antibody-deficient mice: unraveling the role of natural antibody and complement in the clearance of lipopolysaccharide. *J. Immunol.* **159**, 970–975 (1997).
 37. Gillette-Taylor, K., Boullier, A., Witztum, J.L., Steinberg, D. & Quehenberger, O. Scavenger receptor class B type I as a receptor for oxidized low density lipoprotein. *J. Lipid Res.* **42**, 1474–1482 (2001).
 38. Boullier, A. *et al.* The binding of oxidized low density lipoprotein to mouse CD36 is mediated in part by oxidized phospholipids that are associated with both the lipid and protein moieties of the lipoprotein. *J. Biol. Chem.* **275**, 9163–9169 (2000).
 39. Halpern, R., Kaveri, S.V. & Kohler, H. Human anti-phosphorylcholine antibodies share idiotopes and are self-binding. *J. Clin. Invest.* **88**, 476–482 (1991).
 40. Brown, M., Schiffman, G. & Rittenberg, M.B. Subpopulations of antibodies to phosphocholine in human serum. *J. Immunol.* **132**, 1323–1328 (1984).
 41. Schenkein, H.A. *et al.* Phosphorylcholine-dependent cross-reactivity between dental plaque bacteria and oxidized low-density lipoproteins. *Infect. Immun.* **69**, 6612–6617 (2001).
 42. Hörkkö, S., Miller, E., Branch, D.W., Palinski, W. & Witztum, J.L. The epitopes for some antiphospholipid antibodies are adducts of oxidized phospholipid and β 2 glycoprotein 1 (and other proteins). *Proc. Natl. Acad. Sci. USA* **94**, 10356–10361 (1997).
 43. Silverman, G.J. *et al.* The dual phases of the response to neonatal exposure to a VH family-restricted staphylococcal B cell superantigen. *J. Immunol.* **161**, 5720–5732 (1998).
 44. Miller, Y.I. *et al.* Minimally modified LDL binds to CD14, induces macrophage spreading via TLR4/MD-2 and inhibits phagocytosis of apoptotic cells. *J. Biol. Chem.* **278**, 1561–1568 (2003).
 45. Tangirala, R.K., Rubin, E.M. & Palinski, W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J. Lipid Res.* **36**, 2320–2328 (1995).